Analytik von Enantiomeren mittels Massenspektrometrie
Eine Diskussion des Stands der Technik

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Analysis of Enantiomers

Concept:

Formation of diastereomers using a chiral reagent or reference compound – a so-called Chiral Selector System

\[ [(R) + (S)] + [R'] \rightleftharpoons \frac{K(R)}{K(S)} \rightarrow [R - R'] + [S - R'] \]

Diastereomeric Adducts/Complexes or Diastereomeric Products

Analysis Methods:

• Physical separation of diastereomers or enantiomers: LC, GC, SFC, CE, CEC, FAIMS

• Diastereomers are measured simultaneously: NMR, kinetic MS/MS

FAIMS-MS: Gas-phase High Field Asymmetric Waveform Ion Mobility Spectrometry-MS
Diastereomeric complexation from both sides

If \( K_{a,(R)} \neq K_{a,(S)} \) and/or \( K_{d,(R)} \neq K_{d,(S)} \), then (R)-SO is enantioselective for the SA enantiomers.
Model of Chiral Molecular Recognition in Chromatography
(contribution of solution phase phase neglected)

Important Facets:

If $K_{(S)} \uparrow K_{(R)}$, then $\Delta G_{(S)} \uparrow \Delta G_{(R)}$

Thus, $\Delta (\Delta G) = -RT \ln (\alpha)$

Modulation by:
- Medium (solvent)
- $pH$ (if ionic interactions)
- Tether, temperature

Reciprocity Principle:

“SO”

“SA”
Principles of Molecular Recognition

**MEDIUM CONDITIONS**
(Mobile phase, pH, ionic strength)

- **Electrostatic fit** – *functional* complementarity
  - Anion \(\leftrightarrow\) Cation
  - H-donor \(\leftrightarrow\) H-acceptor
  - \(\pi\)-acid (e- poor) \(\leftrightarrow\) \(\pi\)-base (e- rich)
  - Dipole (induced) \(\leftrightarrow\) Dipole (induced)
- **Hydrophobic fit** – *geometric* match of *hydrophobic* complementarity
- **Steric fit** – (partial) *size* and *shape* complementarity
  - binding pocket, cleft, bay area, *interaction sites*
### Noncovalent Interactions: Relative Strength

<table>
<thead>
<tr>
<th>Recognition forces</th>
<th>Relative strength</th>
<th>Lipophilicity</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Electrostatic interactions - Complementarity</strong></td>
<td></td>
<td>[kJ/mol]</td>
</tr>
<tr>
<td>Ionic interactions</td>
<td></td>
<td></td>
</tr>
<tr>
<td>via H-bond</td>
<td>40</td>
<td>Polar</td>
</tr>
<tr>
<td>without H-bond</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>Ion-dipole interactions</td>
<td>4 to 17</td>
<td></td>
</tr>
<tr>
<td>H-bonds</td>
<td>4 to 17</td>
<td></td>
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<tr>
<td>Van der Waals forces</td>
<td>4 to 17</td>
<td></td>
</tr>
<tr>
<td>Orientation forces</td>
<td>4 to 17</td>
<td></td>
</tr>
<tr>
<td>(permanent dipole -permanent dipole)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Induction forces</td>
<td>2 to 4</td>
<td></td>
</tr>
<tr>
<td>(permanent dipole -induced dipole)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dispersion forces</td>
<td>4 to 17</td>
<td></td>
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<tr>
<td>(induced dipole-instantaneous dipole)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aryl-aryl charge transfer (π-π-interactions)</td>
<td>4 to 17</td>
<td></td>
</tr>
<tr>
<td>face-to-face</td>
<td>4</td>
<td>Hydrophobic</td>
</tr>
<tr>
<td>face-to-edge</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Hydrophobic interactions - Similarity</strong></td>
<td>4</td>
<td></td>
</tr>
</tbody>
</table>

Compiled from, H. Kubinyi, QSAR: Hansch Analysis and Related Approaches, VCH, 1993
Modulation of Intermolecular Interactions by Medium Type of Solvent selectively influences SO-SA Complex

- Protic
- Non-protic
  - Polar
  - Non-polar

- Aliphatic
- Aromatic
  - E-rich
  - E-poor

AcOH, H2O, MeOH, Acetonitrile, DMSO, DMF, Chloroform, Toluene, Hexane, etc. (ordering by dielectric constants)
Intermolecular Interactions

- **In Solution Phase**
  - *hydrophobic interactions* can be reinforced (via polar environment, solvent)
  - *hydrogen bonding* can be triggered (via solvent type)
  - *electrostatic interactions* can be weakened (via pH adjustment and buffer salts)

- **In Gas Phase**
  - *hydrophobic interaction* will be weakened (diminish)
  - *hydrogen bonding* will be reinforced
  - *electrostatic interactions* will be reinforced

Can one compare Molecular Recognition in the Gas Phase versus Solution Phase?
Molecular Recognition in “Solution Phase” of Immobilized SOs

QN + DNB-Leu
\[ \alpha = 0.87 \ (1.15) \]
\[ \Delta \Delta G = -0.35 \text{ kJ.mol}^{-1} \]

(S) + DNB-Leu
\[ \alpha = 15.87 \]
\[ \Delta \Delta G = -6.85 \text{ kJ.mol}^{-1} \]

80 MeO-20 H₂O
20 mM NH₄Ac
pH 6.0

Separations of DNB-Leu

1. ionic
2. π-π
3. H-bond
4. steric

Diastereomeric Complex of Cinchona Type SO with SA

X-Ray Diffraction

van der Waals
π base – π acid

Coulombic attraction

hydrogen bonding

DNB-(S)-Leu

β-Cl-tBuCQN

represents more stable complex

How to Measure/Assess Binding and Molecular Recognition

• Classify measurements by system surroundings:
  – Solution Phase
  – Gas Phase
  – Other

Solution-Phase Toolbox
  • NMR ($^1$H, $^{13}$C, NOESY, etc.)
  • Microcalorimetry (DSC, ITC)
  • Separations (LC, CE, gel, etc.)
  • Other Spectroscopy (FTIR, UV/Vis, Fluorescence, CD, etc.)
  • Dialysis

Gas-Phase Toolbox
  • MS (ESI, GIB, ICR, etc.)
  • MS/MS (CID)

Other
  • Computations (MD, DFT, etc.)
  • X-Ray Diff. (solid phase)
Solution Phase Techniques
(NMR, FTIR, microcalorimetry, and others)

- **Advantages**
  - Commonly employed, well developed, and diverse
  - Effect of solution on binding
    - Competition, charge mediation (acid/base), solvophobicity
  - Mimic physiological conditions
  - Common reaction medium
  - Correlation possible with complementary techniques

- **Disadvantages**
  - Limited speed and throughput
  - Relatively large sample amounts
  - Interference by solvent
Gas Phase Techniques (MS & MS/MS)

• **Advantages**
  – Speed; high throughput
  – Low sample consumption
  – Well established methods for assessing noncovalent binding
    • Solution and gas-phase based measurements
  – Well developed instrumentation; multiple ionization sources
  – Removal of solvent interferences (MS/MS)
  – Stoichiometry through mass analysis

• **Disadvantages**
  – Limited correlation by complementary gas phase techniques
  – Molecule/complex response dependence on efficiency of ionization (especially for small molecules)
  – Solution to gas phase correlation is system dependent
    • Native states, specificity, Interaction strengths
MS Technology: A More Efficient Approach for Selector Screening and ee Analysis?

Can it be correlated to Solution Phase Observations?

- Soft ionization – MS
  - Preservation of diastereomeric complexes
  - ESI, MALDI, LSIMS, etc.

- ESI-MS
  - Polar or ionic molecules/complexes
  - Aqueous (hydroorganic) compatible
  - High sensitivity
  - High Speed

But, does it mimic solution phase situations?

- Less synthesis
- No immobilization
- No column packing
- Higher throughput
Electrospray Ionization (ESI)

- Soft ionization source (little to no fragmentation)
- Ionic noncovalent complexes (“adduct ions”) readily observed
  - Artifact of system (co-analytes)
  - Induced through selection of components (molecular recognition)

- Applicable to small and large molecules and complexes
- Barrage of solution and gas phase methods to evaluate molecular recognition and binding events
Mechanism of ESI Ion Generation

1. Capillary held at high potential.

2. The high electric field generates a mist of highly charged droplets.

3. The droplets reduce in size by evaporation of the solvent or by “Coulomb explosion” (droplet subdivision resulting from the high charge density).

4. Ultimately, fully desolvated ions result from complete evaporation of the solvent.
Partitioning of Complexes in ESI Droplets

HG⁺ ↔ H + G⁺

Responses Dependent Upon:
- Excess charge (conc.)
- Degree of HG⁺ complexation
- Surface Affinity
- Relative Partition Constants
- Solvation Energy

Droplet Surface Layer

Droplet Interior

H = Host molecule
G⁺ = Guest ion
X⁻ = Counterion
E⁺ = Electrolyte

Complications

• Simple equilibria expressions generally insufficient to explain the real picture
Example: AcArg + pLeu (0.06 mM, equimolar) in 50/50 ACN/H₂O
Screening SO-SA Interactions by ESI-MS

• Transfer from solution to the gas phase…

Diastereomeric Complexes

Condensed phase

Gas phase

Quantitative Transfer?

Binding equilibria
Solvation
Ionic Strength
pH

Shrinking Droplet
Equilibrium Partitioning
Relative Ionization Efficiency

Desolvation
Equilibrium Shifts
GP Thermodynamics
Collisions

Adduct (Complex) Ion Abundances

Ionization

A

m/z
Current State-of-the-Art (MS)

M. Sawada

Host-guest association with enantiomer labeling (FAB-MS)

Current State-of-the-Art (MS/MS)

R.G. Cooks

The Kinetic Method (CID of diastereomeric complexes)
Current State-of-the-Art (MS/MS)

R.G. Cooks

The Kinetic Method (CID of diastereomeric complexes)

Current State-of-the-Art

- Mainly, gas phase methods – tuning necessary
- Instrument modification
- Isotopic labeling
- Not optimal for automated screening of selector and selectand libraries

Our Approach:

Host – guest association between cinchona alkaloid-type selectors and model selectands

Basic Screening ($\alpha_{MS}$ vs. $\alpha_{HPLC}$)

Titration ($K_{d,MS}$ vs. $K_{d,ITC}$)
- solution vs. gas phase

Competitive binding
- screening libraries
- mechanistic aspects
All components (SO, SA, NaOAc) present at 10 µM
Experiments A & B performed sequentially

\( \alpha_{MS} = 13.1 \)
Screening by MS versus HPLC

<table>
<thead>
<tr>
<th>SO</th>
<th>$\alpha_{\text{HPLC}}$</th>
<th>(R) vs. (S)-SA</th>
<th>$\alpha_{\text{MS}}$</th>
<th>(R) vs. (S)-SA</th>
</tr>
</thead>
<tbody>
<tr>
<td>QN</td>
<td>1.2</td>
<td>(R)</td>
<td>1.22 ± 0.06</td>
<td>(R)</td>
</tr>
<tr>
<td>QD</td>
<td>1.3</td>
<td>(S)</td>
<td>1.04 ± 0.04</td>
<td>ND</td>
</tr>
<tr>
<td>DIPPCQN</td>
<td>3.5</td>
<td>(S)</td>
<td>1.56 ± 0.07</td>
<td>(S)</td>
</tr>
<tr>
<td>DIPPCQD</td>
<td>2.7</td>
<td>(R)</td>
<td>2.3 ± 0.1</td>
<td>(R)</td>
</tr>
<tr>
<td>tBuCQN</td>
<td>15.8</td>
<td>(S)</td>
<td>9.5 ± 0.4</td>
<td>(S)</td>
</tr>
<tr>
<td>tBuCQD</td>
<td>12.5</td>
<td>(R)</td>
<td>13.1 ± 0.7</td>
<td>(R)</td>
</tr>
</tbody>
</table>

- **Good empirical correlation…**
  - ✔ Relative ranking within pseudo-enantiomeric forms
  - ✔ Conformational selectivity reproduced
  - ✔ Highest magnitude of enantioselectivity

\[
\alpha_{\text{HPLC}} = \frac{k'_{(R)}}{k'_{(S)}},
\]
MS Titrations

- 1:1 binding model (from NMR, ITC)
- Fit data to extract binding constants

\[ \text{SO} + \text{SA} \xrightleftharpoons[\text{K}_d]{\text{K}_a} [\text{SO} \cdots \text{SA}] \]

- Assumptions
  - Measurement in limit of dilute solution
  - Measurement below saturation of ionization process
  - Response factor of free and complexed ions equal

\[ \text{K}_d = \frac{[\text{SA}]_{eq}[\text{SO}]_{eq}}{[\text{SO} \cdots \text{SA}]_{eq}} \quad \text{Dissociation constant} \]

\[ A = \frac{[\text{SO} \cdots \text{SA}]_{eq}}{[\text{SO} \cdots \text{SA}]_{eq} + [\text{SO}]_{eq}} \]

\[ \alpha'_{MS} = \frac{A_{(R)}}{A_{(S)}} \approx \frac{\text{K}_{d,(R)}}{\text{K}_{d,(S)}} \]

"Apparent Enantioselectivity"
• Preformed complexes from solution exist, but…
  – Saturation at higher concentration
  – Inconsistent response factor assumption
  – Limited by instrumental sensitivity

\[ [\text{SO}] \text{ and } [\text{NaOAc}] = 10 \, \mu\text{M}; [\text{SA}] = 0.1 \text{ to } 20 \, \mu\text{M} \]

\[ F^2 C_{0,SA} I^2 + F \left( C_{0,SA} - C_{0,SO} - K_d \right) - K_d = 0 \]

\[ K_d = 215.4 \, \mu\text{M} (\_R = 1.46) \]

\[ K_d = 24.2 \, \mu\text{M} (\_R = 0.31) \]

\[ \alpha'_{\text{MS}} = 8.9 \]
Relative ranking consistent with HPLC and ITC data
Equilibrium shift observed to *higher binding affinity* for tBuCQN/D SO systems with MS
  - Consistent with enthalpically-driven binding

<table>
<thead>
<tr>
<th>SO</th>
<th>SA (DNB-Leu)</th>
<th>$K_d$, MS (µM)</th>
<th>$\alpha'_{MS}$</th>
<th>$K_d$, ITC (µM)</th>
<th>$\alpha'_{ITC}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>QN</td>
<td>(R)</td>
<td>174 (4.03)</td>
<td>1.0</td>
<td>83</td>
<td>1.1</td>
</tr>
<tr>
<td></td>
<td>(S)</td>
<td>169 (4.20)</td>
<td></td>
<td>91</td>
<td></td>
</tr>
<tr>
<td>QD</td>
<td>(R)</td>
<td>122 (4.79)</td>
<td>1.0</td>
<td>77</td>
<td>1.3</td>
</tr>
<tr>
<td></td>
<td>(S)</td>
<td>123 (5.01)</td>
<td></td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>DIPPCQN</td>
<td>(R)</td>
<td>114 (3.40)</td>
<td>1.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(S)</td>
<td>76.4 (2.95)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DIPPCQD</td>
<td>(R)</td>
<td>78.0 (1.88)</td>
<td>2.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(S)</td>
<td>155 (2.30)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>tBuCQN</td>
<td>(R)</td>
<td>160 (4.07)</td>
<td>5.0</td>
<td>4550</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>(S)</td>
<td>32.0 (1.41)</td>
<td></td>
<td>270</td>
<td></td>
</tr>
<tr>
<td>tBuCQD</td>
<td>(R)</td>
<td>27.3 (1.32)</td>
<td>8.4</td>
<td>111</td>
<td>9.0</td>
</tr>
<tr>
<td></td>
<td>(S)</td>
<td>229 (3.35)</td>
<td></td>
<td>1000</td>
<td></td>
</tr>
</tbody>
</table>
MS Competition

- Screening SOs
- Incorporate a deuterated internal standard
  - DNB-(R)- or DNB-(S)-d_{10}-Leu

"pseudo-racemate"

\[
\begin{array}{c}
\text{tBuCQN (5 µM) + DNB-(R)-Leu (10 µM) + DNB-(S)-d_{10}-Leu (10 µM) [1:2:2]} \\
\end{array}
\]

\[
\alpha_{\text{MS}} = \frac{\text{SO+(S)SA}}{\text{SO+(R)SA}} = 11.1
\]
MS Competition

- $\alpha_{\text{MS}}$ values from one experiment
- Incorporation of I.S. for improved analysis

➤ Assume no deuterium isotope effect (DIE) for affinity and selectivity
- Common MS assumption (proteomics & enantioselective MR)

For a 1:1 Isotope Mix:

$[\text{SO}] = 10 \mu\text{M}$
$[\text{rac-SA}] = 5 \mu\text{M}$
$[\text{rac-d}_{10}-\text{SA}] = 5 \mu\text{M}$
Deuterium Isotope Effect

• During competition, nondeuterated SA binds significantly stronger than deuterated SA
• Severely limits screening approach without correction
Deuterium IEs – Theory & Expt.

- As a solution phase phenomena…
  - RP-HPLC (Tanaka and coworkers, among others)
  - Deuteration results in a decreased "VdW volume" of the molecular subunit compared to non-deuterated counterpart
  - In RP-HPLC, deuterated compounds are less retained.


**RP-HPLC of SAs**

- DNB-rac-d<sub>10</sub>-Leu
  - $k = 17.8$

- DNB-rac-Leu
  - $k = 18.6$

- DNB-d<sub>10</sub>-Leu
  - $k = 17.8$

- DNB-Leu
  - $k = 18.6$

Superspher 100 RP-18e

60:40:1 H<sub>2</sub>O/ACN/HOAc

Flow rate = 0.5 ml/min
Deuterium IEs – Theory & Expt.

- As a **gas phase** phenomena…
  - Kinetic Method determination of gas phase acidity
    - Cooks and coworkers, Gross and coworkers
    - Bracketing measurements with standards
  - Deuterated analytes are **stronger gas phase acids** than their non-deuterated counterparts


![CID-MS of Mixed Dimer](image)

Unequal distribution of offspring ion abundances
Deuterated analyte is a stronger gas phase acid
Sodiation and Alkali Metal Ion Effects

- Dominant diastereomeric ionic complex is observed in the sodiated form…

10 µM tBuCQN + 10µM DNB-(S)-Leu (in 50/50 MeOH/H₂O + 10 µM NaOAc)

- Addition of NaOAc (10 µM) to stabilize ion signal
  - Sodium ion present in all commercial solvents (≈ 10⁻⁵ – 10⁻⁶ M)
NaOAc Molar Excess and $\alpha_{MS}$ (ion abundance)

- Marked increase in enantioselectivity with large molar excess.
- Stabilization of high affinity pair/destabilization of low affinity pair
- Deuterium IEs are unaffected by change in molar excess.
Other Alkali Metal Cations

- All measurements based on [SO+SA+X]⁺ performed by competitive binding.
- $\alpha_{\text{MS}}$ / ion abundance effects mirror that for Na⁺.
- Stabilization of high affinity pair / destabilization of low affinity pair

1a = SO
Observations & Assumptions

Observations

- Profile differences between Na\(^+\) and other alkali cations at low molar excess due to higher Na\(^+\) background.
- Counterion effects (Ac\(^-\) versus Cl\(^-\)) tested but not observed

Assumptions

- Alkali effect is a combination between:
  1. Electrospray ionization efficiency as a function of ionic strength
  2. Condensed phase equilibrium shifts due to ionic strength and alkali cation type
- Only competitive binding screening employed; assume operation under linear response and linear binding conditions (?)
Interesting Aspect: Where is the Cation?

\[ \text{Coulombic attraction} \]

\[ \pi - \pi \]

\[ \text{hydrogen bonding} \]

\[ \text{van der Waals} \]

\[ \text{Na}^+ \]

1.9 Å
Selectand Variation (N-acetyl-Leu)

- Ac-Leu has no $\pi$-binding functionality
- Similar enantioselectivity enhancement, but different ion abundance profile
- Rules out cation – selectand $\pi$ interaction.
Current Status of the Project

- **Screening** – compared $\alpha_{MS}$ (ion abundances) with $\alpha_{HPLC}$ (retention factors) reflecting thermodynamic parameters
  - Good qualitative correlation for enantioselectivity
- **Titration** – extracted binding constants from data fit to modeled solution phase binding equilibrium
  - Shift in quantitative binding affinities with ESI
  - Good qualitative correlation for enantioselectivity


- **Competitive binding** – isotopic labeling of selectand
  - Reproduction of enantioselectivities, but…
    - *Deuterium isotope effects* observed
    - *Alkali metal salt concentration effects* observed
Summary – Solution Phase Studies

• **Plethora of techniques allows for in-depth study**
  - Excellent enantiomeric discrimination with chiral selectors in solution phase for simple enantiomeric probes
  - Quantitative thermodynamic information possible (Binding energies)
  - Good correlation between techniques available
  - Modes/mechanism of binding effectively uncovered
  - Contributions from ‘other’ tools (MD, X-Ray, etc.) further enhance understanding

• **Currently…**
  - Limited studies using diverse selectors with diverse enantiomer probes
  - Time consuming to use all tools
  - Automated parallel screening of diverse CSPs using diverse mobile phases becomes state of the art (miniaturized columns)

• To rapidly screen performance of selectors (and selectands), an alternative approach could be useful…
Summary of MS Experiments: Academic- or Application-oriented?

- **Merits:**
  - Automated, high sensitivity measurements
  - Little limit on system sizes
  - Relative stability constants and enantioselectivities
    - Small differences in $\alpha$ can be uncovered
    - Reciprocal screening of SO & SA enantioselectivities
  - Quantitative e.e. determination possible…

\[
R^2 = 0.9993
\]

$R^2 \approx 0.9993$
Summary of MS Experiments: Academic- or Application-oriented?

- **Limitations:**
  - Inherent dependence of ESI on ionization efficiencies
    - Tied to structural aspects of free and complexed ions
    - DIEs
      - Address with internal standardization/correction factors?
  - Analyses susceptible to complication by multiple equilibria
    - Ion suppression
    - Isobaric influences (mass resolution)
    - Limit complexity in cassette dosing experiments
  - Dependences on solution phase composition
    - e.g., alkali acetate concentration and metal type
  - Limited quantitative capability (e.e.) relative to traditional liquid phase separation strategies.