Planar Chromatography

Application

Fully automatic application
Half automatic application

Chromatography

Gradient elution
Dipping
Spraying
Heating
Horizontal development

Derivatization

Evaluation

Fully automatic chromatography

Scanner UV/Vis/Fluor
Spectra 190-800 nm
Image documentation
Video densitometry

today
tomorrow
Overview of sample application

- Critical step in the TLC procedure
- How to do it best?
  ✓ Advantages of automated application
  ✓ Modes of application
  ✓ Contact or spray-on technique? Bands or spots?
  ✓ Advantages of bandwise application
  ✓ Influence and properties of the application solvent
  ✓ Devices and examples for application volumes
- Special cases
  ✓ Overspotting
  ✓ Application for preparative purposes
  ✓ Application of effluent from HPLC
- GLP conform, Instrument Validation, Operational Qualification
Advantages of automated application

- GLP conform software documentation
- Better reproducibility
  - No damage of layer
  - Exact volume applied
- Enables band application which improves separation
- More convenient
- Time-saving
- Standardized rinsing procedure (avoids cross over)
- Independent of personal variances
- A „must“ for quantitative HPTLC!
Modes of application
Contact or spray-on technique?

Nanomat contact application

Linomat spray-on technique

Note: Application solvent has great influence by contact application. Band application improves separation!
Influence of the application solvent

Note: Application solvent has a great influence by contact application. It should have as less elution power as possible!
Properties of the application solvent

Volatility
- Volatility enables evaporation - if the application solvent not completely evaporated it can influence chromatography (heading).
- The more volatile, the faster the application rate can be.

Elution power
- Elution power should be as low as possible, however sample should sufficiently be dissolved.
Bands or spots?

Spots

Bands

Note: Band application improves separation - especially by high sample volumes!
Advantages of bandwise application

✓ Better resolution (about 32 % according to Touchstone and Levin, J. Liqu. Chromatog. 3 (1980) 1853)

✓ Better S/N ratio because of evaluation of the homogeneous middle part (consequently better reproducibility, LOQ and LOD)

✓ Enabling a multi-level calibration by application of different volumes of the same standard solution via spray-on technique (less labor time and avoidance of dilution errors)
How to get a focussed start zone

- Choose suitable solvent for application
- Decrease rate of application
- Dilute the sample and apply higher volumes
- Concentration of 0.001% (10 ng/µL) to 0.1 % (1 µg/µL)
- Apply bandwise instead of spotwise

High volumes of matrix-rich samples can be applied as areas followed by a focussing pre-run with a polar solvent, e.g. methanol, upto the upper edge of the start zone area
Application devices

Fully automatic devices
• CAMAG Automatic TLC Sampler (ATS4)
• DESAGA AS 30 TLC Applicator and Sampler
• Zinsser Analytic GmbH Lizzy-TLC
• Baron TLS 100

Half automatic devices
• CAMAG Linomat 5

Manual devices
• CAMAG Nanomat 4
• OM Laboratory SA-101 Multiple Sample Applicator
• DESAGA TLC Spotter PS 01
• Romer TLC AutoSpotter
Examples for application volumes

ATS 4
Syringe
10, 25, 100 µL
100 nL – 1 mL

Linomat 5
Syringe
100, 500 µL
1 µL – 500 µL

Nanomat 4
Capillary
0.5, 1, 2, 5 µL

High sample volumes:
• Option with heated spray nozzle
• Spraying as rectangles/area

Note: The higher the application volume, the more volatile and unpolar the solvent for application should be - otherwise it should be applied slowly.
Overlapped application of bands

Shift of hR$_F$ value due to matrix interference or different compounds?
Overlapped application of standard and sample gives the right answer!

Application for preparative purposes

- With 500 µL syringe
- High volume of sample applied as streak, e.g. 18 cm band
- TLC layer thickness > 500 µm
- Devices
  - CAMAG Linomat 5: Half automatic device, PC controlled
  - Alltech TLC sample streaker: Manual device
Application of effluent from HPLC

- Special application device called DuoChrom
- Flow rate 100 µL/min for methanol (40 µL/min for methanol - water 3:7)
- Average cut time 1-2 min, delay time 2 - 600 s
- Application as rectangles/area
- Spray-on technique with heated spray nozzle allows higher flow rates
HPLC-AMD coupling

1. HPLC
   Reversed phase

2. HPTLC
   Normal phase
Iprodione in lettuce

U. Wippo, H.-J. Stan,
Deutsche Lebensmittel-Rundschau 5, 144-148 (1997)
Surface water spiked with 50 pesticides
Surface water spiked with 50 pesticides

Plate 1
Fraction 3

Plate 3
Fraction 25

klaus.burger@mail.isis.de
Benefits of HPLC-AMD

- Multi-method
- Enhanced separation power
- Peak purity tests
- Post-chromatographic derivatization
- Results by two independent methods
- Use as single devices
- Gain in flexibility and analytical quality
General application parameters
Sequence and layout
High performance mode of application

Sample application

Rinsing
Sample application

ATS 4 Instrument Validation

<table>
<thead>
<tr>
<th>Positioning test</th>
<th>Target</th>
<th>Detected</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table backlash</td>
<td>≤200µm</td>
<td>XOX</td>
</tr>
<tr>
<td>Table reproducibility</td>
<td>≤25µm</td>
<td>XOX</td>
</tr>
<tr>
<td>Table leeway</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Tower backlash</td>
<td>≤200µm</td>
<td>XOX</td>
</tr>
<tr>
<td>Tower reproducibility</td>
<td>≤50µm</td>
<td>XOX</td>
</tr>
<tr>
<td>Tower leeway</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Rack backlash</td>
<td>≤200µm</td>
<td>XOX</td>
</tr>
<tr>
<td>Rack reproducibility</td>
<td>≤50µm</td>
<td>XOX</td>
</tr>
<tr>
<td>Rack leeway</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Syringe backlash</td>
<td>≤100µm</td>
<td>XOX</td>
</tr>
<tr>
<td>Syringe reproducibility</td>
<td>≤10µm</td>
<td>XOX</td>
</tr>
<tr>
<td>Syringe leeway</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Lift backlash</td>
<td>≤200µm</td>
<td>XOX</td>
</tr>
<tr>
<td>Lift reproducibility</td>
<td>≤50µm</td>
<td>XOX</td>
</tr>
<tr>
<td>Lift leeway</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Punch move adjustment</td>
<td>≤200µm</td>
<td>XOX</td>
</tr>
<tr>
<td>Punch delay min</td>
<td>&gt;180ms</td>
<td>XOX</td>
</tr>
<tr>
<td>Punch delay max</td>
<td>&lt;300ms</td>
<td>XOX</td>
</tr>
<tr>
<td>Punch leeway</td>
<td>Yes</td>
<td>Yes</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Syringe test (Spray)</th>
<th>Target</th>
<th>Detected</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spray test</td>
<td>4-6</td>
<td>7</td>
</tr>
<tr>
<td>Spray test (repeat)</td>
<td>4-6</td>
<td>5</td>
</tr>
<tr>
<td>Leakage test 1</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Leakage test 2</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Rinsing</td>
<td>Yes</td>
<td>Yes</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Syringe test (Contact)</th>
<th>Target</th>
<th>Detected</th>
</tr>
</thead>
<tbody>
<tr>
<td>Contact test</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Leakage test 1</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Leakage test 2</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Rinsing</td>
<td>Yes</td>
<td>Yes</td>
</tr>
</tbody>
</table>

ATS 4 Operational Qualification

- Checksum of the installed software
- Cleaning of spray nozzle
- Check of state of the septum punch
- Manual confirmation of gas-tightness of the syringe and application pattern
- Reproducibility of phenacetin
  - by contact application:
    - volume error is \( \leq 1.5\% \)
    - (or the total error is \( \leq 2.1\% \))
  - by spray application:
    - volume error is \( \leq 1.5\% \)
    - (or the total error is \( \leq 1.8\% \))
Which TLC system?

- Stationary Phase
- Mobile Phase
- Detection
Overview of stationary phase

- Separation mechanisms
- Guidelines for the selection
- Impregnation
- Prewashing
- Activation
- TLC versus HPTLC versus UTLC
- Layer support and binder
- Fluorescence (= phosphorescence) indicator
- Manufacturer/batch dependence
- Declaration
- Detection reagents
Separation mechanisms

- Adsorption chromatography
- Partition chromatography
- Complex chromatography
- Ion exchange chromatography
Separation mechanisms

**Adsorption**
- Functional groups
- Polarity

**Partition**
- Chain length
- Lipophilicity

**Complex**
- Complex stability
- E.g. double bondings

- SiOH
- C18
- AgNO₃

- Hydrocarbons
- Ethers
- Esters
- Carbonyls
- Alcohols
- Acids
Separation mechanisms

Ion exchange

Stationary phase

Mobile phase

Analytes
<table>
<thead>
<tr>
<th>Stationary Phase</th>
<th>Suitable Classes of Compounds</th>
</tr>
</thead>
<tbody>
<tr>
<td>Silica gel</td>
<td>All classes of compounds</td>
</tr>
<tr>
<td>Aluminium oxide</td>
<td>Basic compounds (alkaloids, amines, etc.), steroids, terpenes, aromatic and aliphatic hydrocarbons</td>
</tr>
<tr>
<td>Amino phase</td>
<td>Sugars, carboxylic acids, sulfonic acids, phenols, purines, pyrimidines, nucleotides</td>
</tr>
<tr>
<td>Cyano phase</td>
<td>All classes of compounds, PHB esters</td>
</tr>
<tr>
<td>Diol phase</td>
<td>All classes of compounds, steroids, hormones</td>
</tr>
<tr>
<td>RP 2, 8, 18 phases</td>
<td>Polar substances, separation according to lipophilic properties and chain length, steroids, tetracyclins, phthalates, barbiturates, nucleo bases, aminophenols</td>
</tr>
<tr>
<td>Polyamide</td>
<td>Phenols, flavonoids, nitro compounds</td>
</tr>
<tr>
<td>Silica gel impregn.</td>
<td>PAHs (caffeine), number of diol groups (boric acid), number of isolated double bonds (silver nitrate)</td>
</tr>
<tr>
<td>Chiral phase</td>
<td>Enantiomers</td>
</tr>
</tbody>
</table>
Terminology and polarity

- Normal phase
  polar SP + non polar MP

- Reverse phase
  non polar SP + polar MP

- Polarity of the layer
  Si > NH$_2$ > CN/Diol > RP-2 > RP-8 > RP-18
Impregnation of the layer

# Impregnation of the layer

<table>
<thead>
<tr>
<th>Impregnation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>No impregnation</td>
<td></td>
</tr>
<tr>
<td>Impregnation in a 4% solution of sodium acetate for 2 s</td>
<td></td>
</tr>
<tr>
<td>Impregnation in a 10% solution of sodium acetate for 20 s</td>
<td></td>
</tr>
</tbody>
</table>

Separation of ginkgolides with toluene - ethyl acetate – acetone - methanol 20:10:10:1.2 derivatization with acetic anhydride, see CBS 91
# Impregnation of the layer

<table>
<thead>
<tr>
<th>Formation of complexes with</th>
<th>Concentration of impregnation solution</th>
<th>Fields of application</th>
</tr>
</thead>
<tbody>
<tr>
<td>EDTA</td>
<td>10%</td>
<td>Cephalosporins, tetracyclines, metal ions, phospholipids, phenols</td>
</tr>
<tr>
<td>Boric acid or borate</td>
<td>5%</td>
<td>Ascorbic acids derivatives, sugars, phosphatidylinositol, urethane derivatives, mono-/di-/triglycerides, stearic acid, lipids</td>
</tr>
<tr>
<td>Transition metals salts</td>
<td>5-20%</td>
<td>Amino acids, aromatic amines, sulfonamide, anilines, quinolines, phenol derivatives</td>
</tr>
<tr>
<td>Iron(III) salts</td>
<td>5-20%</td>
<td>Phenolic acids</td>
</tr>
<tr>
<td>Silver nitrate</td>
<td>3-20%</td>
<td>Interaction of Ag⁺ with π-electrons of double/triple bounds. Fatty acids, diglyceride/triglyceride, phospholipids, glycolypids, steroids</td>
</tr>
</tbody>
</table>
## Impregnation of the layer

### Formation of charge transfer complexes

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration</th>
<th>Complex Formed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caffeine</td>
<td>4%</td>
<td>Polycyclic aromatic hydrocarbons (PAH)</td>
</tr>
</tbody>
</table>

### Ion-pairing

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration</th>
<th>Complex Formed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quaternary ammonium salts</td>
<td>0.05 M</td>
<td>Sulfa drugs, penicillins</td>
</tr>
</tbody>
</table>

### Adjustment of pH-value

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration</th>
<th>Complex Formed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inorganic acids</td>
<td>0.1-0.5 N</td>
<td>Phenols, acids, aromatic amines</td>
</tr>
<tr>
<td>Potassium/sodium hydroxide</td>
<td>0.1-0.5 N</td>
<td>Alkaloids, amines, basic compounds</td>
</tr>
<tr>
<td>Buffer salts</td>
<td></td>
<td>Curcumin derivatives, sugars, heavy metals, phloroglucinols</td>
</tr>
</tbody>
</table>
# Impregnation of the layer

## Modification of partition coefficient

<table>
<thead>
<tr>
<th>Substance</th>
<th>Modifications</th>
</tr>
</thead>
<tbody>
<tr>
<td>Formamide</td>
<td>Local anesthetics, alkaloids, digitalis glycoside, nitrophenols</td>
</tr>
<tr>
<td>Ammonium sulfate</td>
<td>Lipids, phospholipids</td>
</tr>
<tr>
<td>Sodium nitrite</td>
<td>Phenols</td>
</tr>
<tr>
<td>Sodium bisulfite-citrate buffer</td>
<td>Sugars</td>
</tr>
<tr>
<td>Sodium sulfate</td>
<td>Sugars</td>
</tr>
<tr>
<td>Sodium acetate</td>
<td>0.1 M</td>
</tr>
<tr>
<td>Lithium/sodium/potassium salts</td>
<td>4-10 % Terpene lactones</td>
</tr>
<tr>
<td>Ammonium thiocyanate</td>
<td>Metal ions</td>
</tr>
<tr>
<td>Butylamine</td>
<td>Metal ions</td>
</tr>
</tbody>
</table>
Prewashing of the layer

- to get rid of impurities (lab atmosphere, packing material, i.e. shrink wrapping foil etc.)
- to get rid of binder components which can be eluted by polar solvents
- to get a better baseline
- to improve LOD and LOQ
- to improve reproducibility

Important...
- for old layers
- for ultra trace analysis (ppt range)
- if working range is near the LOD or LOQ
Prewashing of old layers

Pre-chromatographed with methanol, see CBS 91
Best way to do it?

According to Maxwell et al., JPC 12, 109-113 (1999)
- Two step cleaning method: with methanol first pre-development then immersion for 5 min, air-dry for 5 min, followed by heating at 80 °C for 15 min

According to Jork et al. (about 10 years old)
- immersion in iso-propanol over night or for at least 2 hours, followed by heating at 120°C for 30 min

According to CAMAG (current recommendation)
- pre-development with methanol followed by heating at 120°C for 20 to 30 min

According to Dr. Burger (current recommendation)
- in a clean bench for at least 8 hours, followed by heating at 30 min at 50 - 100 °C
  - neutral: with methanol
  - acidic: formic acid – methanol 1:100, then methanol or
  - basic (for acidic plates, e.g. Merck No. 15445): solution of 0,0001% sodium hydroxide (2 mL 0,1 M NaOH in 10 L methanol), then methanol
Best way to do it?

Note:

- Use very clean solvents for prewashing!

- Avoid any contamination again during drying!

- Cool down the active plate to room temperature in a dust and fume free environment (e.g. a large empty desiccator) and let it equilibrate with the relative humidity of the laboratory atmosphere!

- Be care of storage and declaration of prewashed plates!
Activation of the layer

- Silica gel: after 3 min 50 % of the max. water content is adsorbed
- Aluminum oxide: after 12 min 80 % ...

- Within a few minutes the humidity of the air is adsorbed
- Activation or storage in the desiccator - what happens during application?
- Breathing onto the layer can cause local de-activation

Reproducible humidity regulation by conditioning with definite dilutions of sulfuric acid or saturated salt solutions or use mid- or unpolar stationary phases
## Activation of the layer

<table>
<thead>
<tr>
<th>mass % ( \text{H}_2\text{SO}_4 )</th>
<th>% rel. humidity</th>
<th>saturated salt solution</th>
<th>% rel. humidity</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>96</td>
<td>( \text{Pb(NO}_3\text{)}_2 )</td>
<td>98</td>
</tr>
<tr>
<td>20</td>
<td>88</td>
<td>( \text{KBr} )</td>
<td>84</td>
</tr>
<tr>
<td>30</td>
<td>75</td>
<td>( \text{NaNO}_2 )</td>
<td>66</td>
</tr>
<tr>
<td>40</td>
<td>56</td>
<td>( \text{NaHSO}_4\cdot\text{H}_2\text{O} )</td>
<td>52</td>
</tr>
<tr>
<td>50</td>
<td>35</td>
<td>( \text{KF} )</td>
<td>31</td>
</tr>
<tr>
<td>60</td>
<td>16</td>
<td>( \text{HCOOK} )</td>
<td>21</td>
</tr>
<tr>
<td>70</td>
<td>3</td>
<td>( \text{ZnCl}_2\cdot1.5\ \text{H}_2\text{O} )</td>
<td>10</td>
</tr>
</tbody>
</table>
Activation of the layer

45% relative humidity  
32% relative humidity

Fingerprint (alkylamides) of Echinacea purpurea with toluene - ethyl acetate - cyclohexane - formic acid 24:6:3:0.9 derivatization with anisaldehyde, see CBS 91
# TLC versus HPTLC versus UTLC

<table>
<thead>
<tr>
<th></th>
<th>TLC</th>
<th>HPTLC</th>
<th>UTLC</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Silica gel</strong></td>
<td>irregular particles</td>
<td>irr./glob. particles</td>
<td>monolithic without binders</td>
</tr>
<tr>
<td><strong>Mesopores</strong></td>
<td>60 Å = 6 nm</td>
<td>60 Å = 6 nm</td>
<td>30-40 Å = 3-4 nm</td>
</tr>
<tr>
<td><strong>Mean particle size</strong></td>
<td>10 - 15 µm</td>
<td>5 - 7 µm</td>
<td>1 - 2 µm macrospores</td>
</tr>
<tr>
<td><strong>Particle distribution</strong></td>
<td>wide</td>
<td>narrow</td>
<td>narrow</td>
</tr>
<tr>
<td><strong>Layer thickness</strong></td>
<td>200, 250 µm</td>
<td>100, 200 µm</td>
<td>ca. 10 µm</td>
</tr>
<tr>
<td><strong>Number of samples</strong></td>
<td>max. 12 20 x 10 cm</td>
<td>36 – 72 20 x 10 cm</td>
<td>12 6 x 3,6 cm</td>
</tr>
<tr>
<td><strong>Migration distance</strong></td>
<td>100 - 150 mm</td>
<td>30 - 70 mm</td>
<td>10 - 30 mm</td>
</tr>
<tr>
<td><strong>Migration time</strong></td>
<td>15 - 200 min</td>
<td>5 - 30 min</td>
<td>1 - 6 min</td>
</tr>
<tr>
<td><strong>Solvent use</strong></td>
<td>50 - 100 mL</td>
<td>5 - 20 mL</td>
<td>1 - 4 mL</td>
</tr>
<tr>
<td><strong>Detection limit:</strong></td>
<td>Abs 100 - 1000 ng</td>
<td>10 - 100 ng</td>
<td>1 - 10 ng</td>
</tr>
<tr>
<td></td>
<td>Fluor 1 - 100 ng</td>
<td>0,1 - 10 ng</td>
<td>0,01 – 0,1 ng</td>
</tr>
</tbody>
</table>
60 mean pore size in Angström (= 6 nm)
F with fluorescent indicator
254 excitation wavelength of F
s acid stabile fluorescent indicator (blue)
R specially purified
RP 2, 8, 18 reversed phase with 2, 8, 18 hydrocarbon chain length
W water-tolerant layer
PSC preparative layer, thickness > 0,25 mm
(G gypsum as binder)
(H without foreign binders)
Which TLC system?

- Stationary Phase
- Mobile Phase
- Detection
Overview of mobile phase

- Chromatographic separation
- Classification of solvents (Trappe, Snyder)
- Optimization scheme
- Isotherms, peak asymmetry
- Polarity differences in mobile phase mixtures
- Vapor pressure of solvents
- Variations in temperature
- Stabilizers (manufacturer, batch)
- Diffusion (van Deemter)
- Viscosity (law of migration)
- Developing distance
- GLP recommendations
Chromatographic separation

Vapor phase

Stationary phase

Mobile phase
### Chromatographic separation

<table>
<thead>
<tr>
<th>Interactions</th>
<th>kJ/mol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Van der Waals forces</td>
<td>5 - 20</td>
</tr>
<tr>
<td>Dipole-induced dipole</td>
<td>8 - 25</td>
</tr>
<tr>
<td>Dipole-dipole</td>
<td>25 - 40</td>
</tr>
<tr>
<td>Hydrogen bonding</td>
<td>25 - 40</td>
</tr>
<tr>
<td>Ionic bonding</td>
<td>250 - 1050</td>
</tr>
<tr>
<td>Covalent bonding</td>
<td>670 - 3360</td>
</tr>
</tbody>
</table>
Chromatographic separation

- The mobile phase moves by capillary forces through the particle pores (6 – 10 nm).

- The substances are dissolved in the mobile phase and are transported over a certain migration distance.

- Different adsorption and/or partition equilibria cause different remaining times in the stationary phase.
According to Trappe

- eluotropic series listed according to increasing elution power
- elution power is defined as adsorption energy per unit surface area of sorbent
- dependent on the sorbent
- standardized on pentane
<table>
<thead>
<tr>
<th>Silica gel</th>
<th>Aluminum oxide</th>
<th>Polyamide</th>
</tr>
</thead>
<tbody>
<tr>
<td>n-Hexane</td>
<td>Pentane</td>
<td>Water</td>
</tr>
<tr>
<td>Pentane</td>
<td>n-Hexane</td>
<td>Methanol</td>
</tr>
<tr>
<td>Cyclohexane</td>
<td>Cyclohexane</td>
<td>Ethanol</td>
</tr>
<tr>
<td>Carbon tetrachloride</td>
<td>Carbon tetrachloride</td>
<td>Propanol</td>
</tr>
<tr>
<td>Toluene</td>
<td>Toluene</td>
<td>Acetalone</td>
</tr>
<tr>
<td>Chloroform</td>
<td>Diethylether</td>
<td>Ethylmethylketone</td>
</tr>
<tr>
<td>Dichloromethane</td>
<td>Chloroform</td>
<td>Acetone</td>
</tr>
<tr>
<td>Diethylether</td>
<td>Dichloormethane</td>
<td>Acetonitrile</td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>Acetone</td>
<td>Formamide</td>
</tr>
<tr>
<td>Acetone</td>
<td>Ethyl acetate</td>
<td>Dimethyl formamide</td>
</tr>
<tr>
<td>Ethanol</td>
<td>Pyridine</td>
<td>Dil. sodium hydroxide</td>
</tr>
<tr>
<td>Methanol</td>
<td>Ethanol</td>
<td></td>
</tr>
<tr>
<td>Pyridine</td>
<td>Methanol</td>
<td></td>
</tr>
<tr>
<td>Water</td>
<td>Water</td>
<td></td>
</tr>
</tbody>
</table>
According to Snyder

- solvent strength
- selectivity groups (selectivity triangle)
## Normal phases

<table>
<thead>
<tr>
<th>Group</th>
<th>Solvent</th>
<th>Solvent strength</th>
</tr>
</thead>
<tbody>
<tr>
<td>Decrease</td>
<td>n-Hexane</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>n-Butylether</td>
<td>2,1</td>
</tr>
<tr>
<td>I</td>
<td>Isopropylether</td>
<td>2,4</td>
</tr>
<tr>
<td></td>
<td>Methyl-t-butylether</td>
<td>2,7</td>
</tr>
<tr>
<td></td>
<td>Diethylether*</td>
<td>2,8</td>
</tr>
<tr>
<td></td>
<td>n-Butanol</td>
<td>3,9</td>
</tr>
<tr>
<td>II</td>
<td>2-Propanol*</td>
<td>3,9</td>
</tr>
<tr>
<td></td>
<td>1-Propanol</td>
<td>4,0</td>
</tr>
<tr>
<td></td>
<td>Ethanol*</td>
<td>4,3</td>
</tr>
<tr>
<td></td>
<td>Methanol</td>
<td>5,1</td>
</tr>
<tr>
<td>III</td>
<td>Tetrahydrofuran*</td>
<td>4,0</td>
</tr>
<tr>
<td></td>
<td>Pyridine</td>
<td>5,3</td>
</tr>
<tr>
<td></td>
<td>Methoxyethanol</td>
<td>5,5</td>
</tr>
<tr>
<td></td>
<td>Dimethylformamide</td>
<td>6,4</td>
</tr>
</tbody>
</table>
### Reverse phases

<table>
<thead>
<tr>
<th>Group</th>
<th>Solvent</th>
<th>Solvent strength</th>
</tr>
</thead>
<tbody>
<tr>
<td>Decrease</td>
<td>Water</td>
<td>0</td>
</tr>
<tr>
<td>II</td>
<td>Methanol*</td>
<td>2.6</td>
</tr>
<tr>
<td></td>
<td>Ethanol</td>
<td>3.9</td>
</tr>
<tr>
<td></td>
<td>2-Propanol</td>
<td>4.2</td>
</tr>
<tr>
<td>III</td>
<td>Tetrahydrofuran</td>
<td>4.5</td>
</tr>
<tr>
<td>VI</td>
<td>Acetonitrile*</td>
<td>3.2</td>
</tr>
</tbody>
</table>
Prisma model

Solvent strength

Irregular part

Regular part

Pedestal - modifier

Selectivity
Optimization of mobile phase

- Neat solvents
- Decreasing (Hex) or increasing (H$_2$O) of solvent strength
- Try mixtures, consider the addition of modifiers (acids, bases)
- Selection of optimal mixture
Neat solvents

HPTLC Vario Chamber

ATS 4
Partition and adsorption isotherms

\[ K = \frac{C_s}{C_m} \]

Selectivity \( \ast \) = \( \frac{K_1}{K_2} \)

- \( K = 0.2 \)
- \( K = 2 \)
Peak asymmetry

Partition isotherm

Concentration profile

Shape of the zone

- o.k.
- Tailing
- Heading

Convex

Concave
Overloading of the layer with substance
- reduce amount or (take plate with higher layer thickness)

Retarded desorption due to active sorbent
- use chamber saturation, preconditioning, modified layer

Reaction between substance and sorbent
- prewash, change or modify layer; mask interferences

Local gradient by polar solvent rests from application
- remove solvent rests

Convex partition/adsorption isotherm
- change system, reduce substance

Dissociation of weak acids or bases
- buffer layer or/and solvent, add acids or bases to solvent

Chemical change of substance
- modify layer, work in protected atmosphere
Wet start zone & weak mobile phase

- **dry start zone, stronger mobile phase**

Concave partition/adsorption isotherm

- **change system**
Mobile phase mixtures

- TLC - isocratic? No!
  - Solvent composition changes
  - Solvent migration rate changes

- HDCHDC

\[ \begin{align*}
\text{Front} & \quad \text{Front} & \quad \text{Front} & \quad \text{Front} \\
3 & & 2 & & 1 \quad 4
\end{align*} \]

- solvents at the bottom

- with wetted filter paper

- Chamber saturation

- Solvent migration rate changes
Stabilizers (manufacturer, purity grade)

Chloroform LiChrosolv stabilized with amylene

Chloroform p.A. stabilized with ethanol
Zone diffusion according to van Deemter

$$H = A + \frac{B}{v} + C \cdot v$$

A, C for TLC: $H \sim 30 \mu m$
B for HPTLC: $H \sim 12 \mu m$

A = Layer quality, Eddy diffusion
B = Diffusion term, longitudinal diffusion
C = Retardation term, local non-equilibrium

$$H = 2 l dp + \frac{2 \gamma D}{v} + \frac{w \cdot dp^2 \cdot v}{D}$$

H = Plate height
v = Velocity of solvent front
l = Function of layer packing
dp = Particle diameter
$\gamma$ = Labyrinth factor
D = Diffusion coefficient
w = Factor of packing structure
Law of migration

\[ V_F = k \frac{\gamma}{\eta \times 2 \times z_F} \]

- \( V_F \): velocity of solvent front
- \( \gamma \): surface tension
- \( \eta \): viscosity
- \( z_F \): migration distance
Developing distance - velocity

Note: Do not exceed a developing distance of 6 cm on HPTLC plates.

The higher the developing distance, the lower the velocity of mobile phase, the more influence of diffusion effects!
Developing distance - resolution

Influence of the developing distance and $R_F$ values

Note: Components of complex mixtures should be spread over the entire separation distance. The most critical substance pair should be maintained at $R_F$ 0.3 for best separation.
GLP recommendations

- Use multi component solvent systems only once - composition will change!
- Prepare solvents freshly!
- Don’t use the trough chamber as "shaker"!
- Consider volume contraction – measure separately!
- Prewash old layers!
- Don’t breathe onto the layer or blow fluffs off - you should condition in other modes!
- Note all relevant factors incl. humidity and temperature
- For chamber saturation use a filter paper wetted with solvent and let the vapor phase equilibrate for at least 30 min
- Use data pair method to reduce plate inhomogeneity
Use of solvents

Multiple use of solvents

1. run 2. run 3. run 4. run

Chloroform – methanol – ammonia 56:14:1

Do´nt re-use solvents!

Preparation of solvents

Ethyle acetate – formic acid – acidic acid – water 100:11:11:27

17 days old

Prepare solvents freshly!

Data pair method
One-dimensional development

Capillary technique

- simple
  - vertical
  - horizontal
  - antiparallel

- multiple
  - same step
  - descending steps
  - ascending steps

Multi-dimensional development

Forced flow technique

- OPLC
  - $\Delta p$
- RPC, HPPLC
  - $\Delta p$

AMD
- Step-gradient polar - unpolar
Examples of developing chambers

- Twin Trough Chamber
- Automatic Developing Chamber (ADC)
- Horizontal Developing Chamber (HDC)
- Automated Multiple Development (AMD)
Modes of the Twin Trough Chamber

unsaturated

pre-conditioned with mobile phase

pre-conditioned with different medium
Horizontal Developing Chamber (HDC)
Allergenic disperse dyes in textiles

A. Bonhoff et al., STR Testing & Inspection AG, Steinach, Switzerland, optimized at CAMAG Lab, see CBS 82
Allergenic disperse dyes in textiles

Confirmation

Sample 1  Sample 2  Sample 3  Sample 4  Sample 5

Orange 3  Orange 36/37

Blue 124

Red 1

Yellow 3

Blue 1

Blue 106
Automated Multiple Development (AMD)
Automated Multiple Development (AMD)

- Burger et al. (1984): polarity gradient by multiple development with different solvents
- Also possible: pH gradient
- Drying under vacuum improved precision and reliability
- Focusing to sharp zones
- Zone profile independent of migration distance
- Migration distance independent of matrix
- Automation
- Separation of substances differing in polarity to a high extent
- Separation number > 40 at a migration distance of 80 mm
AMD - stepwise development

Start zone
Run 1
Run 2
Run 3
Run 4
Run 5
Run ..
Run n

Focusing
AMD - focusing effect
1. Start with a universal gradient - examples:

<table>
<thead>
<tr>
<th>Increasing solvent</th>
<th>Base solvent</th>
<th>Decreasing solvent</th>
</tr>
</thead>
<tbody>
<tr>
<td>methanol</td>
<td>dichloromethane</td>
<td>n-hexane</td>
</tr>
<tr>
<td>methanol</td>
<td>t-butyl methyl ether</td>
<td>n-hexane</td>
</tr>
<tr>
<td>acetonitrile</td>
<td>dichloromethane</td>
<td>n-hexane</td>
</tr>
<tr>
<td>methanol/water</td>
<td>acetonitrile</td>
<td>dichloromethane</td>
</tr>
<tr>
<td>methanol/water</td>
<td>t-butyl methyl ether</td>
<td>dichloromethane</td>
</tr>
<tr>
<td>various solvents</td>
<td>ethyl acetate</td>
<td>various solvents</td>
</tr>
<tr>
<td>acetone</td>
<td>various solvents</td>
<td>various solvents</td>
</tr>
</tbody>
</table>
2. If necessary change pH of the universal gradient
   - Add small amounts (0.01-2 %) of NH$_3$, HCOOH, CH$_3$COOH etc. to the polar solvent
   - Fill the conditioning bottle with 0.1-4 N solution of acids or bases

3. Go on with the best universal gradient
   - leave out parts not used
   - spread parts where substances are close together
   - optimized shallower gradient results

4. If no sufficient separation was yield so far
   - take a base solvent of different selectivity, e.g. t-butyl methyl ether, acetonitrile etc.
   - change the stationary phase, e.g. diol, amino, cyano or RP18 W
Polarity gradients gentler than those given in the table below cannot be recommended:

<table>
<thead>
<tr>
<th>Polarity change over 10 steps</th>
<th>min. change of volume [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methanol to dichloromethane</td>
<td>5</td>
</tr>
<tr>
<td>Acetonitrile to dichloromethane</td>
<td>10</td>
</tr>
<tr>
<td>T-butyl methyl ether to n-hexane</td>
<td>15</td>
</tr>
<tr>
<td>Dichloromethane to n-hexane</td>
<td>30</td>
</tr>
</tbody>
</table>

To avoid increasing diffusion of peaks 5-10 steps are sufficient for isocratic development.
AMD - hints

If the time of the gradient is too long (e.g. a gradient with 25-steps on a 200 µm plate with 3 mm increments takes about 4 h)

- Use 100 µm layers: shorter developing and drying times (about 2.5 h)

- Leave out parts not used: reduction to 20 steps (about 2.5 h)

- Use shorter drying times if possible

- Use spherical silica gel plates – reduces time, also drying time, to about 50 %

✓ 20 step gradient on spherical silica gel in 1.5 h for 18 samples, i.e. 5 min per sample.
Identification of ball pen inks

- Product classification
- Determination of document age

F. Köhler, P. Seiler, Bundeskriminalamt, Wiesbaden, see CBS 74
Pesticides in drinking and surface water

Multi-wavelength scan
ISO/TS 11370

1 = Naphtalin-1-sulfonsäure
2 = Prochloraz
3 = Triazoxid
4 = Ethidimuron
5 = Simazin
6 = Bromazil
7 = Carbofuran
8 = Metribuzin
9 = Azinphos Methyl
10 = Coumaphos
11 = Prosulfocarb
12 = Dichlofuanid
13 = Parathion
14 = Fenthion
15 = Dinoseb
16 = Prothiofos
je 100 ng

klaus.burger@mail.isis.de
Carbohydrates in beer and wine

G. Lodi et al., University of Ferrara, Italy, see CBS 69