HPLC analysis methods used for analyzing sugars and sugar derivatives in IL media obtained from lignocellulosic biomass

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1-Ethyl-3-methylimidazolium chloride
EmimCl or \([\text{emim}]^+ [\text{Cl}]^-\)

Molar mass: 146.62 g/mol
Density: 1.1120 g/cm³ (at 80 °C)
Melting point: 77-80 °C ← impurities effect
Flash point: 186 °C
Viscosity: 47.4 mPas (at 80 °C)
Flame point: 515 °C
Solubility in water: ∞

Very hygroscopic

Information concerning melting point varies in literature
1-Ethyl-3-methylimidazolium acetate

EmimOAc or \([\text{emim}]^+ [\text{CH}_3\text{COO}]^-\)

- **Molar mass:** 170.21 g/mol
- **Density** 1.027 g/cm³ at 25 °C
- **Melting point** > 30 °C
- **Flash point:** 164 °C
- **Viscosity** 10 mPas (at 80 °C)
- **Solubility in water** ∞
SUGARS in trees

- **Cellulose**

- **Hemicellulloses**
  - (Galacto)glucomannans 20-25 % of softwood’s wood substance (water & alkali soluble)
  - Glucanes, galactanes & arabinogalactanes

- **Pectines**
  - Galacturonic acid units & some rhamnose units
  (→ Gives negative charge to fibers)
Saccharides & their derivatives

- Furfural
- 5-HMF
- Cellobiose
- Galactose
- Glucose
- Mannose
- Fructose
- Arabinose
- Xylose

Valuable platform chemicals

Monosaccharides

Galacturonic acid
Glucuronic acid

analyzed by:
- GC
- HPLC
- CE
**Focus/aim**

- **Norway spruce** (*Picea abies*)
- **Scots pine** (*Pinus sylvestris*)
- **Silver Birch** (*Betula pendula*)

- debarking
- sawing
- chipping
- screening
- freeze-drying

**IL**

**HEAT**

**SUGARS & THEIR DERIVATIVES**
Analysis of carbohydrates on extract

Without acid methanalysis

- calibration samples: STD sugars with xylitol in MeOH
- ISTD: xylitol instead of sorbitol
  → Evaporation (N₂) + vacuum oven →
- silylation:
  - pyridine, HMDS and TMCS
  → shaking (and ultrasonic bath)
HPLC & GC chromatograms vs. CE electropherogram
HPLC
High Pressure/Performance Liquid Chromatography
HPLC column

- heavy-walled stainless steel
- equipped with end-fitting containing porous frits
  - Frits filter incoming sample and support the compressed bed of HPLC packing wetted with the mobile phase.

 Görund column = protective column placed in-line between injector and main column

(Marvin C. McMaster, HPLC, a practical user’s guide, 2nd edition, Wiley 2007.)
HPLC Column Packings

- Silica and bonded-phase silica
  - Classic, Type II, and hybrid silica
- Polymer reverse phase
- Zirconium and MS bonded-phase
- Ion exchange: polymer, Zr, & Si
- Size separation: polymer and silica

(Marvin C. McMaster, HPLC, a practical user’s guide, 2nd edition, Wiley 2007.)
## Silica columns

<table>
<thead>
<tr>
<th>Column</th>
<th>Phase</th>
<th>Solvents</th>
<th>Application</th>
</tr>
</thead>
<tbody>
<tr>
<td>C18</td>
<td>Octyldecyl</td>
<td>AN, MeOH, H₂O</td>
<td>General nonpolars</td>
</tr>
<tr>
<td>C8</td>
<td>Octyl</td>
<td>AN, MeOH, H₂O</td>
<td>General nonpolars</td>
</tr>
<tr>
<td>Phenyl</td>
<td>Styryl</td>
<td>AN, MeOH, H₂O</td>
<td>Fatty acids, double bonds</td>
</tr>
<tr>
<td>Cyano</td>
<td>Cyanopropyl</td>
<td>AN, MeOH, THF, H₂O</td>
<td>Ketone, aldehydes</td>
</tr>
<tr>
<td>Amino</td>
<td>Aminopropyl</td>
<td>H₂O, AN, MeOH, THF, CHCl₃, CH₂Cl₂</td>
<td>Sugar, anions</td>
</tr>
<tr>
<td>Diol</td>
<td>Dihydroxyhexyl</td>
<td>AN, MeOH, THF, H₂O</td>
<td>Proteins</td>
</tr>
<tr>
<td>SAX</td>
<td>Aromatic</td>
<td>Salt buffers</td>
<td>Anions</td>
</tr>
<tr>
<td>SCX</td>
<td>Aromatic</td>
<td>Salt buffers</td>
<td>Cations</td>
</tr>
<tr>
<td>DEAE</td>
<td>Alkyl ether</td>
<td>Salt buffers</td>
<td>Proteins, anions</td>
</tr>
<tr>
<td>CM</td>
<td>Alkyl ether</td>
<td>Salt buffers</td>
<td>Proteins, cations</td>
</tr>
<tr>
<td>SI</td>
<td>(none)</td>
<td>AN, MeOH, H₂O</td>
<td>Polar organics, positional isomers</td>
</tr>
</tbody>
</table>

(Marvin C. McMaster, HPLC, a practical user's guide, 2nd edition, Wiley 2007.)
How to select solvents/eluents

• Select solvents with polarity opposite the column polarity.

(Marvin C. McMaster, HPLC, a practical user’s guide, 2nd edition, Wiley 2007.)
...HPLC solvents

some criteria for selection

• UV cutoffs
  → for wavelength selection

• Molar mass
  → for MS contaminations

• Boiling point
  → for high temperature work

(Marvin C. McMaster, HPLC, a practical user's guide, 2nd edition, Wiley 2007.)
### HPLC solvents

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Formula</th>
<th>MW (Da)</th>
<th>Boiling Point (°C)</th>
<th>UV Cutoff (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetonitrile</td>
<td>CH₃CN</td>
<td>41.05</td>
<td>81.6</td>
<td>190</td>
</tr>
<tr>
<td>Chloroform</td>
<td>CHCl₃</td>
<td>119.38</td>
<td>61.7</td>
<td>245</td>
</tr>
<tr>
<td>Dichloromethane</td>
<td>CH₂Cl₂</td>
<td>84.93</td>
<td>40.0</td>
<td>235</td>
</tr>
<tr>
<td>Ethanol</td>
<td>CH₃CH₂OH</td>
<td>46.08</td>
<td>78.5</td>
<td>210</td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>CH₃CO₂CH₂CH₃</td>
<td>88.12</td>
<td>77.1</td>
<td>260</td>
</tr>
<tr>
<td>Diethyl ether</td>
<td>(CH₃CH₂)₂O</td>
<td>74.12</td>
<td>34.5</td>
<td>220</td>
</tr>
<tr>
<td>Heptane</td>
<td>CH₃(CH₂)₅CH₃</td>
<td>100.21</td>
<td>98.4</td>
<td>200</td>
</tr>
<tr>
<td>Hexane</td>
<td>CH₃(CH₂)₄CH₃</td>
<td>86.18</td>
<td>69</td>
<td>200</td>
</tr>
<tr>
<td>Isopropanol</td>
<td>CH₃CH(OH)CH₃</td>
<td>60.11</td>
<td>82.4</td>
<td>210</td>
</tr>
<tr>
<td><strong>Methanol</strong></td>
<td>CH₃OH</td>
<td>32.04</td>
<td>65</td>
<td>205</td>
</tr>
<tr>
<td>n-Propanol</td>
<td>CH₃CH₂CH₂OH</td>
<td>60.11</td>
<td>97.4</td>
<td>210</td>
</tr>
<tr>
<td>Tetrahydrofuran</td>
<td>C₄H₈O</td>
<td>72.12</td>
<td>66</td>
<td>215</td>
</tr>
<tr>
<td>Toluene</td>
<td>C₆H₅(CH₃)</td>
<td>92.15</td>
<td>110.6</td>
<td>285</td>
</tr>
<tr>
<td><strong>Water</strong></td>
<td>H₂O</td>
<td>18.02</td>
<td>100</td>
<td>none</td>
</tr>
</tbody>
</table>

(Marvin C. McMaster, HPLC, a practical user’s guide, 2nd edition, Wiley 2007.)
HPLC, High Performance Liquid Chromatography

Hewlett Packard 1100 series HPLC

- refractive index detector
- diode array UV detector
- HPX-87K carbohydrate column
Analysis conditions for **RP-C8** HPLC column

- **Flow**: 0.8 and 0.4 ml/min
- **Temperature**: 25 °C
- **Injection volume**: 1.00 µl
- **Detectors**: RI and UV
- **Eluents**: water (H₂O/ACN, H₂O/MeOH)

Also Tried:
- Flow: (0.1-1)
- Temperature: (25–60 °C)
- Injection volume: (1-5)
Reversed phase & normal phase → Difference

For normal phase elution order is that the polar solutes elute later than non-polar lipophilic ones.

For reversed phase elution order is that the polar solutes elute before non-polar ones.
Reverse-Phase Chromatography

- Polar (hydrophilic) compounds elute before nonpolar (lipophilic) compounds
- Ionization of compounds disturbs analysis ➜ often prevented by adding buffer
e.g. phosphate, acetate or carbonate
- Nonpolar columns require polar solvents
- Water/Acetonitrile or water/methanol
  (when neutral compounds are analyzed)

(Robert Franzén, KEM-4350 Kromatografia ja Massaspektroskopia, Kromatografia – peruskäsitteet, menetelmät & laitteet, lecture material, TTY/Kemia, 2005.)
(Marvin C. McMaster, HPLC, a practical user’s guide, 2nd edition, Wiley 2007.)
**Separation model**

**Isocratic elution**
– Constant mobile phase composition

**Gradient elution**
– mobile phase composition is changed stepwise or continuously (reproducible)

(Marvin C. McMaster, HPLC, a practical user’s guide, 2nd edition, Wiley 2007.)
Retention times are different for different chemical compounds but they also depend on the following method parameters:

- eluents/solvent mixture (+ gradient or isocratic)
- flow in the column
- temperature
- injection volume

→ new **calibration curves*** must be done when any method parameter is changed

*) In this research needed for **ILs and sugars**
Resolution

\[ R = \frac{1}{4} \left( \frac{\alpha - 1}{\alpha} \right) \sqrt{N} \frac{K'}{1 + K'} \]

\[ K' = \text{Retention factor} = \frac{V_A - V_0}{V_0} \]

\[ \text{Alpha} = \text{Sep. factor} = \frac{(V_A - V_0)}{V_B - V_0} \]

\[ N = \text{Retention factor} = 16 \left( \frac{V_A}{V_B} \right) \]

Resolution (\( R \))—A measure of the completeness of a separation. Influenced by \( K' \) (solvent polarity), \( N \) (column efficiency, and \( a \) (system chemistry.

(Marvin C. McMaster, HPLC, a practical user’s guide, 2nd edition, Wiley 2007.)
HPLC analysis
Challenges in monosaccharide analysis (HPLC)

- anomer mutarotation $\rightarrow$ wide & split peaks
- loss of reducing sugars (T)
- formation of Schiff bases ($R^1R^2C=NR^3$)
- shortened column lifetime
- long analysis time
- salt interferences

http://www.sepscience.com/Sectors/Enviro/Articles/521-/Overcoming-Challenges-in-Carbohydrate-Separations
Problems with HPLC

- 70 % are column problems.
- 80 % are due to bad water.

Trouble-shooting

- Column QA with standards, (4-std test mixture)
- Treating Column killers.
- Reverse order system diagnosis.
- System Pacification

(Marvin C. McMaster, HPLC, a practical user’s guide, 2nd edition, Wiley 2007.)
HPLC Column Killers

- Bonded Phase Loss - low pH, temp.
- End Voids - packing dissolved
- Adhering organic compounds
- Pressure increases
- Center voids - channeling

(Marvin C. McMaster, HPLC, a practical user’s guide, 2nd edition, Wiley 2007.)
Loss of Bonded-phase

(Marvin C. McMaster, HPLC, a practical user's guide, 2nd edition, Wiley 2007.)

Effect of Bonded Phase Loss

Caused by hydrolysis of Si-O-Si bonds → Avoid by keeping pH 2.5-7.5!
Dissolving Packing

(Marvin C. McMaster, HPLC, a practical user’s guide, 2nd edition, Wiley 2007.)

Effect of Dissolved Packing - End Void

Packing dissolves at $\text{pH} > 8$ or at high salt conc. $\rightarrow$ open & repack column head!
Bound Non-polar Material

(Marvin C. McMaster, HPLC, a practical user’s guide, 2nd edition, Wiley 2007.)

Effect of Bound Non-Polar Material

Late peak disappear. → Treatment: wash with strong solvent, use clean water.
Center Void Formation

(Marvin C. McMaster, HPLC, a practical user’s guide, 2nd edition, Wiley 2007.)

Effect of Channeling - Center Void

Avoid shocking or reversing the column. → Save shock & column reversal for treatment, run reversed.

![Graphs showing the effect of channeling on center void formation.](image)
RI-detector:

Sucrose 0.5 %

Tailing: tailing area can be ignored 🔄 (not 100 % purity)
Thank You!