Effects of protolichesterinic acid isolated from *Cetraria islandica* on lipid composition in cultured cancer cells evaluated using HPLC-MS/MS

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Outline

• Introduction
• Lichens
• HPLC-MS/MS Validation and results
• D-optimal screening on UPLC-MS/MS
• Palmitic acid
• Conclusions
Metabolic changes in cancer

- Energy metabolism
- Lipid metabolism
- Warburg effect: glycolysis in favour of oxidative phosphorylation
- Autophagy
Therapeutic opportunities

- Cancer metabolism is a target for current drug development
- Fatty acid synthase (FAS) is highly expressed in most human carcinomas
  - Level of expression varies and determines sensitivity to FAS inhibitors
- Products of cyclooxygenase (COX) and lipoxygenases (LOX) pathways linked to carcinogenesis
  - Inhibitors suggested as chemopreventive
- Metformin
  - Reduced cancer risk and cancer-related mortality
Lichen compounds

• Several lichen species have been used in traditional medicine.
  – Treatment for tuberculosis and tumours
• Symbiosis between fungi and algae
• Metabolites derived from lichens shown to have anti-proliferative and cytotoxic effects
• Usnic acid
  – Proton shuttle (energy metabolism and autophagy)
• Protolichesterinic acid
  – 5- and 12-Lipoxygenase inhibitor
  – Possible FAS inhibitor
Cetraria islandica
Aims

• Development of a sample preparation technique and LC-MS/MS method for cultured cells
  – Palmitic acid
  – LTB₄
  – 12-HETE
  – 5-HETE

• Effects of protolichesterinic acid on lipid metabolism
  – Lipoxygenases and FAS
  – Extraction of protolichesterinic acid from *Cetraria islandia*
    • Purification with preparative high-pressure liquid chromatography
Methods

- Protein precipitation
  - Poor sensitivity
- Liquid-Liquid extraction
  - Low recovery
- Solid phase extraction
  - Good sensitivity

![Optimization of SPE graph]

- LTB4
- 12-HETE
- 5-HETE

% Organic (Methanol) vs. Peak Area

Peak Area

0 5000 10000 15000 20000 25000
HPLC-MS/MS

<table>
<thead>
<tr>
<th>Tandem Quadrupole</th>
<th>Quattro Ultima</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPLC</td>
<td>1525 Micro HPLC pump at 0.25 ml/min</td>
</tr>
<tr>
<td>Analytical Column</td>
<td>Waters Xbridge C18, 3.5µ, 2.1 x 50mm</td>
</tr>
<tr>
<td>Column Temperature</td>
<td>40°C</td>
</tr>
<tr>
<td>Mobile Phase</td>
<td>A: 0.1% formic acid : Acetonitrile (95:5 % v/v)</td>
</tr>
<tr>
<td></td>
<td>B: Acetonitrile (100 % v/v)</td>
</tr>
<tr>
<td>Injection volume</td>
<td>20 µL</td>
</tr>
<tr>
<td>Source temperature</td>
<td>120°C</td>
</tr>
<tr>
<td>Desolvation temperature</td>
<td>450°C</td>
</tr>
<tr>
<td>Polarity</td>
<td>ES-</td>
</tr>
</tbody>
</table>
Calibration Curves

**Compound: LTB₄**  \[ R^2 = 0.998375 \]
PBS buffer; 120 – 30.000pg/mL

**Compound: 5-HETE**  \[ R^2 = 0.994470 \]
PBS buffer; 120 – 30.000pg/mL
Detection of LOX products

LTB₄

12-HETE

5-HETE
LOQ and Blank matrix
Validation Results

<table>
<thead>
<tr>
<th>Validation Parameters</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LTB₄</td>
</tr>
<tr>
<td>Range</td>
<td>120 - 30,000 pg/mL</td>
</tr>
<tr>
<td>LOQ</td>
<td>%</td>
</tr>
<tr>
<td>Accuracy</td>
<td>9.3</td>
</tr>
<tr>
<td>Precision</td>
<td>5.4</td>
</tr>
<tr>
<td>Intra-Assay</td>
<td>%</td>
</tr>
<tr>
<td>Accuracy</td>
<td>-1.2 - 3.6</td>
</tr>
<tr>
<td>Precision</td>
<td>1.9 - 5.0</td>
</tr>
<tr>
<td>Recovery</td>
<td>64.9%</td>
</tr>
<tr>
<td>Stability</td>
<td>Stable for;</td>
</tr>
<tr>
<td>Auto sampler</td>
<td>At least 12 hr.</td>
</tr>
</tbody>
</table>
Quantification of LOX products in Capan 2 cancer cells

5-HETE

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>EtOH Control</th>
<th>2.5 µg/mL PA</th>
<th>5.0 µg/mL PA</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-HETE</td>
<td>274,7</td>
<td>261,0</td>
<td>226,3</td>
<td>250,5</td>
</tr>
</tbody>
</table>

LTB₄ and 12-HETE

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>EtOH Control</th>
<th>2.5 µg/mL PA</th>
<th>5.0 µg/mL PA</th>
</tr>
</thead>
<tbody>
<tr>
<td>LTB₄</td>
<td>20,0</td>
<td>29,4</td>
<td>48,5</td>
<td>27,1</td>
</tr>
<tr>
<td>12-HETE</td>
<td>29,7</td>
<td>34,7</td>
<td>17,8</td>
<td>32,2</td>
</tr>
</tbody>
</table>
Design of Experiments (DoE)

**Objective**
- Improve Sensitivity
- Lower Analysis Time

**Responses**
- Peak Area
- Peak height
- Retention time
D-optimal design

- Two qualitative factors
  - Type of column
  - Type of organic phase
- Five quantitative factors
- Modeling was performed with PLS (partial least squares)

Software: Modde 8.0, Umetrics AB
# Experimental Screening

<table>
<thead>
<tr>
<th>Factors</th>
<th>UPLC-MS/MS Ranges</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gradient slope</td>
<td>1.0 - 4.0 min</td>
</tr>
<tr>
<td>Organic start</td>
<td>5 - 40%</td>
</tr>
<tr>
<td>Flow</td>
<td>0.45 - 0.65 mL/min</td>
</tr>
<tr>
<td>Type of solvent</td>
<td>Acetonitrile or Methanol</td>
</tr>
<tr>
<td>Type of column</td>
<td>UPLC BEH C&lt;sub&gt;18&lt;/sub&gt;, 1.7 µm, 2.1x100mm</td>
</tr>
<tr>
<td></td>
<td>UPLC CSH C&lt;sub&gt;18&lt;/sub&gt;, 1.7 µm, 2.1x100mm</td>
</tr>
<tr>
<td>Capillary voltage</td>
<td>1 - 3.5 kV</td>
</tr>
<tr>
<td>Collision energy</td>
<td>10 - 20 eV</td>
</tr>
</tbody>
</table>
PLS components

![Bar chart showing R2 and Q2 values for different components.](chart.png)
Loading scatter plot

- Ar1~
- He1~
- Ar2~
- He2~
- Col
- Colum(CSH C18)
- Org
- OrgP(MeOH)
- Gra
- Cap
- Flow
- Flow*Cap
- Flow*Org
- Flow*Gra
- Flow*OrgP(MeOH)
- Gra*Cap
- Gra*Org
- Gra*OrgP(MeOH)
- Col*Cap
- Col*Org
- Col*OrgP(MeOH)
- OrgP(MeOH)*Cap
- OrgP(MeOH)*Col
- OrgP(MeOH)*Gra
- OrgP(MeOH)*Org
- OrgP(MeOH)*Gra
- OrgP(MeOH)*Cap
- OrgP(MeOH)*Flow
- OrgP(MeOH)*Flow*Cap
- OrgP(MeOH)*Flow*Org
- OrgP(MeOH)*Flow*Gra
Regression coefficients scaled and centered (peak area)

5-HETE

12-HETE
4-D contourplot (Area)

5-HETE

12-HETE

Organic phase = MeOH

Organic phase = AcCN

Mobile phase flow rate = 0.45
Gradient slope = 1
Detection of Palmitic acid
Conclusions

• HPLC-MS/MS method was developed and validated for quantification of LOX-pathway products at Pico-gram levels
• Quantification of 5-HETE was successful in capan 2 pancreatic cancer cells
• The DoE was useful for evaluating factors affecting responses of the method
• Palmitic acid not quantifiable because of contamination problems
Bioprospecting from Icelandic sea
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