Novel Infrared Spectroscopic and Imaging Tools For Quality Control

Christian Huck
Institute of Analytical Chemistry and Radiochemistry, University Innsbruck
Austria
Motivation

Phytomics/Metabolomics

Step 1: Extraction
Step 2: Pretreatment
Step 3: Production
Step 4: Product
Step 5: Patient

Plant
Production
Patient
Approaches

Innovations

- Enrichment
- Desalting
- High-sample throughput

Near-infrared
Mid-Infrared
Imaging/mapping

Separation

Sample pretreatment

Screening

Spectroscopy

MALDI-TOF-MS/MS
Matrixfree-MALDI
MELDI-TOF
MALDI-imaging/mapping

LC, LC-MS/MS
μ-LC, μ-LC-MS/MS
CE, CE-MS
CEC
Motivation

- Non-invasive
- Simultaneous determination of several parameters
- Physical and chemical parameters
- Increase in selectivity, speed
- High-Throughput
- Alternative method

→ Fingerprint technique off-, on- and even in-line
Frederick William Herschel
(1738 - 1822)

W. Herschel, "Investigation of the powers of the prismatic colours to heat and illuminate objects", Phil. Trans. (1800) 255.
Herschel, W., "Experiments on the refrangibility of the invisible rays of the sun.", Phil Trans. (1800) 284.
Infrared spectroscopy

The Electromagnetic Spectrum

Overtones of Molecular vibrations
Electronic transitions
rotation vibrations stretch bend
Molecular bond
Valence electrons ionisation Inner shell electrons
HIGH PHOTON ENERGY
LOW PHOTON ENERGY

Infrared (IR) violet UV
stretch bend

Micro-wave Far IR Mid IR NIR Visible Ultra-violet Vacuum UV X-Ray

λ: 10^6 5000 2500 800 400 170 20 nm
v: 10^{-6} 200 4000 12500 25000 60000 5*10^5 cm^{-1}

Molecular bond vibrations stretch bend

Infrarot (IR) violet UV
stretch bend

Rotation vibrations stretch bend
Molecular bond
Valence electrons ionisation Inner shell electrons
HIGH PHOTON ENERGY
LOW PHOTON ENERGY
Model of the Oscillator

**Harmonic Oscillator**

Mid-Infrared

\[ E_{osc} = h\nu_{osc}\left(\nu + \frac{1}{2}\right) \]

\[ \Delta \nu = \pm 1 \]

Near-Infrared

\[ E_{osc} = h\nu_{osc}\left(\nu + \frac{1}{2}\right) - \chi h\nu_{osc}\left(\nu + \frac{1}{2}\right)^2 \]

\[ \chi = \text{anharmonicity constant} \]
Instrumentation

NIR spectroscopy

MIR/NIR chemical imaging

FTIR-ATR spectroscopy
The NIR spectrum is a fingerprint of the investigated material. It is characteristic of its chemical and physical properties.
### Spectral characteristics

Overview of prominent bands of complex biological samples (4000 cm\(^{-1}\) to 750 cm\(^{-1}\))

<table>
<thead>
<tr>
<th>Wavenumber [cm(^{-1})]</th>
<th>Assignation</th>
</tr>
</thead>
<tbody>
<tr>
<td>~3300</td>
<td>amide A, (\nu_{N-H}) of proteins</td>
</tr>
<tr>
<td>~3100</td>
<td>amide B, (\nu_{N-H}) with 1. overtone of the amide I band resonant (Fermi), proteins</td>
</tr>
<tr>
<td>~3010</td>
<td>(\nu_{C-H}), lipids, cholesterol, esters</td>
</tr>
<tr>
<td>~2920</td>
<td>(\nu_{C-H}) (&gt;CH(_2), Methyl) antisymmetric/symmetric, lipids, proteins, carbohydrates, esters</td>
</tr>
<tr>
<td>alternatively 2850</td>
<td></td>
</tr>
<tr>
<td>~2956</td>
<td>(\nu_{C-H}) (CH(_3), Methyl) antisymmetric/symmetric, lipids, proteins, carbohydrates, nucleic acids</td>
</tr>
<tr>
<td>alternatively 2872</td>
<td></td>
</tr>
<tr>
<td>~1745-1735</td>
<td>(\nu_{C=O}), esters and phospholipids</td>
</tr>
<tr>
<td>~1620-1695</td>
<td>amide I-band, proteins</td>
</tr>
<tr>
<td>~1550</td>
<td>amide II-band, proteins</td>
</tr>
<tr>
<td>~1400</td>
<td>(\nu_{C=O}) of COO(^{-})-group, fatty acids and amino acids</td>
</tr>
<tr>
<td>~1360-1260</td>
<td>amide III-band absorptions (predominantly C-N stretching) with significant contributions from (\nu_{CH_2}) of carbohydrate residues</td>
</tr>
<tr>
<td>~1350-1260</td>
<td>(\nu_{PO_2}^{-})</td>
</tr>
<tr>
<td>~1310-1240</td>
<td>amide III-band, proteins</td>
</tr>
<tr>
<td>~1250-1220</td>
<td>(\nu_{P=O}) symmetric of the&gt;PO(_2)-groups, phospholipids, nucleic acids</td>
</tr>
<tr>
<td>~1225</td>
<td>(\nu_{PO_2}^{-}) asymmetric of nucleic acids and phospholipids</td>
</tr>
<tr>
<td>~1185-1120</td>
<td>C-O ring vibrations of nucleic acid “sugars”</td>
</tr>
<tr>
<td>~1084</td>
<td>(\nu_{P=O}) symmetric of the &gt;PO(_2)-groups nucleic acids, phospholipids</td>
</tr>
</tbody>
</table>
Why can we see a signal?

**RAMAN**
\[
\frac{\partial \alpha}{\partial q} \neq 0
\]

**MID-INFRARED**
\[
\frac{\partial \mu}{\partial q} \neq 0
\]

**NEAR-INFRARED**
\[
\frac{\partial \mu}{\partial q} \neq 0 / \text{ANHARMONICITY } m<<M
\]

**polarizability**
**dipole moment**
**mechanical anharmonicity**

**HOMONUCLEAR**
- e.g. C=C

**POLAR**
- e.g. C=O

**FUNCTIONALITIES**
- e.g. C=O

**HIGH STRUCTURAL SELECTIVITY**

**LOW STRUCTURAL SELECTIVITY**

**OFTEN CHEMOMETRICS NECESSARY**
Measurement Principle

**Measurement Principle**

- Detector
- Diffuse reflection
- Transmission
- Monochromator
- Sample
- Spectra

**Physical Properties**
- Particle size, porosity, ...

**Chemical Properties**
- Surface modification, ...

**Analysis**
- Qualitative analysis
- Quantitative analysis
- Chemometrics / Multivariate data analysis
NIR – Sample presentation modes

TRANSMISSION

REFLECTANCE
or transflectance
0-45° optics or
integrating sphere

INTERACTANCE
Fibre optic probe
Sample preparation

- **MID-INFRARED**
  - NO SAMPLE PREPARATION
  - ONLY VIA ATR

- **NEAR-INFRARED**
  - NO SAMPLE PREPARATION

• NON-INVASIVE FOR HIGH SAMPLE THROUGHPUT
Instrumentation

Globar
Tungsten-Halogen Lamp

Grating
Interferometer
AOTF

MCT
InGaAs, InSb
PbS, Si, Ge
Diode-Array

Source → Sample → Monochromator → Sample → Detector

or
Optical Fiber Probe

- Source
- Mirrors
- Beam Splitter
- Detector
- Probe Head with Sapphire Window
- Sample
- Lightfiber-Bundle
Attenuated Total Reflection

Multiple Sample Interaction

M: Mirrors  S: Sample
\[ \alpha: \text{Angle of Incidence} \]
Chemometrics

NIR - Chemometrics

Univariate / Multivariate Analysis

Graph showing absorbance vs. wavelength for coarse and fine samples. PCA and PLS models are illustrated with scatter plots and regression lines. The graph includes metrics such as $R^2$, SEE, SEP, and BIAS.
NIR Strategy of Analysis

- Optimization of the reference method
  - HPLC
  - Content of the leading compound: 3',4',5'-trimethoxyflavone
  - Content of Flos Primulae veris
  - Content of other leading compounds
  - Content of other plants in Sinupret

Optimization of the NIR parameters
Choice of Sample Set

Frequent Distribution of Calibration Samples

- FREQUENCY
- CONCENTRATION

Preferential Distribution of Calibration Samples

- FREQUENCY
- CONCENTRATION
### NIR Strategy of Analysis

#### 3´,4´,5´-Trimethoxyflavone
Leading Compound

<table>
<thead>
<tr>
<th>Charge</th>
<th>Area (µV*sec)</th>
<th>Primula (g/100g)</th>
<th>Conc. LC (ng/µl)</th>
<th>NIR-MW (ng/µl)</th>
<th>Water (%)</th>
<th>EtOH (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>91102064 28.10.91</td>
<td>70490</td>
<td>0,493</td>
<td>0,219</td>
<td>0,219</td>
<td>80,38</td>
<td>15,7</td>
</tr>
<tr>
<td>91112182 05.11.91</td>
<td>71740</td>
<td>0,500</td>
<td>0,222</td>
<td>0,210</td>
<td>80,83</td>
<td>15,2</td>
</tr>
<tr>
<td>91112191 08.11.91</td>
<td>61346</td>
<td>0,442</td>
<td>0,197</td>
<td>0,230</td>
<td>80,25</td>
<td>15,7</td>
</tr>
<tr>
<td>91112302 22.11.91</td>
<td>66799</td>
<td>0,472</td>
<td>0,210</td>
<td>0,186</td>
<td>81,33</td>
<td>14,5</td>
</tr>
<tr>
<td>91112311 28.11.91</td>
<td>81551</td>
<td>0,555</td>
<td>0,245</td>
<td>0,193</td>
<td>79,69</td>
<td>15,7</td>
</tr>
</tbody>
</table>

#### Regression Analysis
- $R^2 = 0.9594$
- $SEE = 0.0057$
- $SEP = 0.0099$
- $BIAS = -0.0389$

#### Graphical Representation
- 1st derivative Normalisation

---

**Note:** The table and graph illustrate the analysis and prediction of compounds using NIRS (Near-Infrared Spectroscopy) compared to the true values obtained by HPLC (High-Performance Liquid Chromatography). The data shows a strong correlation ($R^2$) and low prediction errors (SEE, SEP, BIAS) indicating the effectiveness of the NIR strategy in analyzing the compounds of interest.
NIRS-Analysis of Water- and Ethanol Content

EtOH

Reference method: GC

Water

Reference method: Karl-Fischer
Determination of (a) naphthodianthrones and (b) phloroglucines. Conditions: Hypersil BDS-C18 (5 µm, 130 Å, 250 × 4 mm); mobile phase, A: 888.0 g buffer (880.0 g bidest, 2 ml 85% H₃PO₄, TEA (pH 2.80)), 80.0 g ACN, B: 49.64 g buffer (50.0 g Bidest, 1 ml 85% H₃PO₄, TEA (pH 6.10)), 85.04 g methanol, 275.28 g ACN; linear gradient; sample volume, 20 µl; peak assignment, PrPH, protopseudohypericin; PH, pseudohypericin; CPH, cyclopseudohypericin; PrH, protohypericin; H, hypericin; Hf, hyperforin; Ahf, adhyperforin.

NIRS of Naphthodianthrones and Phloroglucines in St. John’s Wort

NIR spectra
(a) Original spectra, (b) pretreated spectra

Predicted (NIRS) vs. True values (LC)
(a) Hypericin, (b) Hyperforin
(n=80). (a) \( R^2 = 0.99 \); SEP = 0.68; (b) \( R^2 = 0.99 \); SEP = 0.72
Quality Control of Achillea Species

Cluster Analysis

Loadings-plot (multiple compound model, MCM) of the four species of Achillea genus. A. millefolium (mil), A. clypeolata (cly), A. collina (col), A. nobilis (nob).

Quantitative Analysis

PLS regression lines of the SCM model for determining the n-decanoic acid content in the A. millefolium.

Cooperation with Prof. Guo Lanping, CACMS, Beijing and Prof. B. Kopp, University Vienna
NIRS Method for the Non-Invasive Determination of Total Flavonoid, Total Phenol Content and Antioxidative Potential in Herba

**Total Flavonoid Content**

*Achillea millefolium, Achillea absinthium, Artemisa annua, Fagopyrum esculentum, Genista tinctoria, Gynostemma pentaphyllum, Leonurus sibiricus, Mentha arvensis, Origanum dictamnus, Reseda luteola, Scutellaria baicalensis, Spilanthes acmella, Tanacetum parthenium*

**Total Phenolic Content**

Reference: Folin-Ciocalteau

**Antioxidative Potential**

Reference: DPPH
Preparative TLC of *Flos Primulae veris* and *Radix Primulae veris*, stationary phase: SiO$_2$ 60 F$_{254}$, 20x20, thin layer thickness: 1 mm; mobile phase: chloroform/acetone = 55 / 10; volume: 100 µl; detection: UV 365 nm 1 - 3 = *Flos Primulae* (butanol), 4 = DC-isolated substance, 5 - 7 = *Radex Primulae*, 8 - 9 = purified extract of *Flos Primulae*.
Scatter plot of predicted (NIRS) vs. true property (HPLC) for determination of baicalein (B) in radix scutellariae. B shows anti-inflammatory, antiviral (HIV), anti-tumor, antioxidant, free radical scavenging and anti-SARS coronavirus effects.

Principal components score image of the wild and cultivated radix scutellariae samples.
Surface enhanced infrared – Spectroscopy (SEIRS)

Signal Enhancement
- S/N Ratio
- Polarization

Gold Carrier
Surface enhanced infrared – Spectroscopy (SEIRS)

Bsp. Trypsine Absolute 1.5 µg

GOLD CARRIER

CaF$_2$

Amid I+II Bande
Tablet Production – On-line Analysis

PLS calibrations

<table>
<thead>
<tr>
<th></th>
<th>Laboratory probe</th>
<th>Process probe</th>
</tr>
</thead>
<tbody>
<tr>
<td>Assay</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Water</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acetone</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**NIR Predictions (%)**

**Reference values (%)**
Infrared Imaging Spectroscopy

16 element array views sample in blocks of 100 µm x 6.25 µm or 400 µm x 25 µm
Up to 5 steps per second and 80 spectra

Step The Stage To Generate Any Size Image…
A typical hyperspectral cube has up to several (ten)thousand spectra.

Conservative versus imaging spectroscopy

(A) Conservative Spectroscopy

The spectrum is representative for the total irradiated sample area.

(B) Imaging Spectroscopy

The spectra recorded by the individual detectors represent an image of the irradiated sample area.
Infrared Imaging Spectroscopy

3.9 x 3.9 mm²

260 x 260 μm²

50 x 50 μm²

1 : 1 Image
(Macro Chamber; Transmission, ATR)

61 μm res

15 x Objective
(Microscope; Transmission)

~ 10 μm res

20 x ATR-Objective
(Microscope)

~ 4 μm res

Detector

3.9 x 3.9 mm²
Workflow

1. Tissue
2. Sectioning
3. Tissue mounting on IR-Window
4. IR Microspectrometry and Imaging
5. IR Spectra
6. Spectral Diagnose
7. Image reassembling
Application – *Urtica dioica* root

IR spectra from 800 to 1800 cm\(^{-1}\) of a *Urtica sp.* root
IR spectroscopic 3D imaging

A) Optical microscopic image
B) amide I-band proteins
C) amide II-band proteins
D) carbohydrates, nucleic acids and phospholipids
IR spectroscopic 2 D - imaging

Total Scan

Ester (1770-1708 cm\(^{-1}\))

Cellulose OH (3350-3590 cm\(^{-1}\))

Protein Amide II (1563-1480 cm\(^{-1}\))

univariate
Cluster analysis

A) Optical microscopic image
B) Hierarchical cluster analysis
C) K-means clustering
D) Fuzzy C-means clustering

multivariate
IR spectroscopic 3 D – hyper space imaging
IR spectroscopic 3 D – hyper space imaging
Prostate tissue

(A) HE stained diagnostic slide:
1. cancer
2. stroma
3. benign glands

(B) nucleic acids, cholesterol, phospholipids and ester
(C) proteins
(D) lipids and carbohydrates

Principal component analysis (PCA)
Each colored data point represents one region of interest (ROI). The data sets were chosen from tumor- (red) and stroma- (green) tissue region, which are co-registered with the scanned H&E

Imaging Active Ingredients in Tablets
Acknowledgements

Prof. Dr. M. Popp, Prof. Dr. G. Abel

Prof. Yang Bin, China Academy of TCM, Beijing China

Prof. Dean Guo, Shanghai Institute of Materia Medica, China

Prof. Kopp, University Vienna, Austria
Prof. Bauer, University Graz, Austria
Prof. Stuppner, University Innsbruck, Austria
Questions?

I will be happy to answer!