Multi-way Analysis of 2D Liquid Chromatographic Metabolomics Data

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Indole-3-Acetic Acid (IAA) in Plants

- Primary growth hormone responsible for cell division and elongation, flowering, root initiation, fruit ripening, promoting vascular tissue growth, controlling premature abscission of leaves and fruit

- Synthesized from tryptophan by a variety of pathways

- **Metabolic Profiling** – the identification and quantification of a selected group of metabolites in a biological system

- **Metabolomics** – unsupervised comparison of different biological samples to elucidate differences in metabolite levels

- **2DLC with diode array detection (DAD)** was used to compare mutant and wild type maize samples to 26 indolic metabolite standards

Fiehn, O. *Plant Mol. Biol.* **2002**, 48, 155-71
6 spectral components of 26 indolic standards

A: 19 compounds

B: 2 compounds

C: 2 compounds

D

E

Wavelength (nm)
2DLC Instrumentation Capable of Gradient Elution in Both Dimensions

- HP 1090 w/autosampler and Ternary Pump
- Eluent Pre-heater
- 1st Dimension Column
- HP 1090 Pump B (A Solvent)
- HP 1090 Pump C (B Solvent)
- Tee
- Eluent Pre-heater
- 2nd Dimension Column
- DA Detector
- Waste
2DLC Data Structure – Three Way Data

Data Dimensions:
1. 1\textsuperscript{st} Dimension Retention Time (min)
2. 2\textsuperscript{nd} Dimension Retention Time (sec)
3. Wavelength (nm)
Four-Way Quadrilinear Data

- 1st Dimension, Retention Time, minutes
- 2nd Dimension, Retention Time, seconds
- 3rd Dimension, Wavelength, nm
- 4th Dimension, Sample number

The instrument response of a pure component in all domains is unique, consistent, and independent of the presence of other species.

Description of Samples

- Mobile phase blank
- Standard mixture containing 26 indoles
- Duplicate wild type maize seedling samples
- Duplicate *orp* mutant maize seedling samples
  - Lacks gene for tryptophan synthase β
  - IAA is produced via tryptophan-independent pathway
Fixed Size Image Window – Evolving Factor Analysis

• FSIW-EFA uses sections of an image and performs factor analysis on a moving window

• Rank information is *local* – results estimate the complexity of an image for exploratory analysis

• Traditional EFA approaches would require unfolding of the three-way data set and loss of complex spatial structure

• Can be used to select sections of data for subsequent analysis

Summed Rankmap:
Standards, Wild Type, Mutant

First dimension retention time (min)
Second dimension retention time (sec)

6 components
1 component
Window Target Testing Factor Analysis

1. SVD: \[ \mathbf{D} = \mathbf{U} \cdot \mathbf{S} \cdot \mathbf{V}^T \]
2. Target test: \[ \mathbf{T} = (\mathbf{V}^T \cdot \mathbf{V})^{-1} \cdot \mathbf{V}^T \cdot \mathbf{L}^* \]
3. Rotate: \[ \hat{\mathbf{L}}^* = \mathbf{V}^T \cdot \mathbf{T} \]
4. Correlate: \[ \theta = \cos^{-1} \rho \]

Results of Qualitative Analysis

2nd Dimension Ret. Time (sec)

0 5 10 15 20 25 30

1st Dimension Retention Time (min)

0 5 10 15 20 25 30

Wild Type Maize

Mutant Maize
2D-Chromatograms – 220 nm

Standard
Wild-type
Mutant
Modeling with PARAFAC and fALS

The **four-way** PARAFAC model is represented mathematically as a sum over all of the elements of each mode (where c is the rank of the data)\(^a\):

\[
d_{hijk} = \sum_{f=1}^{c} w_{hf} x_{if} y_{jf} z_{kf} + e_{hijk}
\]

The PARAFAC model is solved using alternating least squares (ALS)

\[
\text{D} = \text{X} \cdot \text{Y}^T
\]

\[
\text{X} = (\text{Y}^T)\dagger \cdot \text{D}
\]

\[
\text{Y}^T = \text{D} \cdot (\text{X})\dagger
\]

**ALS with flexible constraints (fALS)**\(^b\) allows the selective application of the unimodality constraint to selected components

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\(^a\)Andersson, C. A.; Bro, R. *Chemom. Intell. Lab. Syst.* **2000**, *52*, 1-4

PARAFAC-ALS vs. fALS

- **PARAFAC-ALS**
  - Multilinearity required
  - Constraints provide LS solutions with guaranteed convergence
  - Constraints must be applied to all components

- **fALS**
  - Multilinearity optional
  - Constraints may be ad-hoc, but not LS optimal
  - Constraints can be applied to selected components

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Data Analysis Procedure

- Section data according to local complexity
- Rank determination in each section
- \text{PARAFAC-ALS}^a – SVD initiated, non-negativity
- \text{fALS}^b – initiated with previous, non-negativity
- \text{fALS}^b – initiated with previous, unimodality on non-background components
- \text{PARAFAC} – \text{ALS}^a – initiated with previous, non-negativity


PARAFAC Results for Selected Section

![Graph 1](image1.png)

![Graph 2](image2.png)

![Graph 3](image3.png)

![Graph 4](image4.png)
Reconstructed PARAFAC Results – Wt1

Reconstructed data with background components removed
Results of Analysis of Entire Data Set

![Graph showing retention times for different samples](image)

- **First Dimension Retention Time (min.)**
- **Second Dimension Retention Time (sec.)**

- **Colors of Data Points:**
  - Mutant only
  - WT only
  - Standard
  - Mutant & WT
  - Mutant & Std
  - WT & Std
  - All

- **Substances:**
  - Tryptophan
  - 5-hydroxy-L-tryptophan
  - Tryptamine
# Quantitative Results of PARAFAC Analysis

- 95 distinct chromatographic peaks were resolved
- Many peaks showed differential expression between wild type and mutant samples

<table>
<thead>
<tr>
<th></th>
<th>Mutant 1</th>
<th>Mutant 2</th>
<th>WT1</th>
<th>WT2</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-hydroxy-L-tryptophan</td>
<td>ND*</td>
<td>0.6</td>
<td>ND*</td>
<td>ND*</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>1.4</td>
<td>1.1</td>
<td>1.8</td>
<td>1.6</td>
</tr>
<tr>
<td>Indole-3-acetyl-L-alanine</td>
<td>ND*</td>
<td>ND*</td>
<td>0.8</td>
<td>0.3</td>
</tr>
<tr>
<td>Tryptamine</td>
<td>1.9</td>
<td>ND*</td>
<td>ND*</td>
<td>ND*</td>
</tr>
</tbody>
</table>

*ND – not detected

Quantitative results are in µg of indole per gram of plant material
Selectivity in Multi-way Analysis\textsuperscript{a}

- Messick, Kalivas, Lang (MKL)\textsuperscript{b}
  - PARAFAC
  - Appropriate when all components are calibrated
  - $SEL_n = \{[(X^TX)^*(Y^TY)]^{-1}\}_{nn}^{-1/2}$

- Ho, Christian, Davidson (HCD)\textsuperscript{c}
  - GRAM
  - Appropriate when only the target analyte is calibrated
  - $SEL_n = \{[(X^TX)^{-1}]_{nn}[(Y^TY)^{-1}]_{nn}\}^{-1/2}$

\textsuperscript{a}Olivieri, A. C., \textit{Anal. Chem.} 2005, 77, 4936-3946
\textsuperscript{b}Messick, N. J.; Kalivas, J. H.; Lang, P. M. \textit{Anal. Chem.} 1996, 68, 1572-1579
\textsuperscript{c}Ho, C.-N.; Christian, G. D.; Davidson, E. R., \textit{Anal. Chem.} 1980, 52, 1071-1079
Selectivity in Multi-way Analysis

• The selectivity calculations predict the relative decrease in precision that is observed relative to that observed for a pure sample.

• Olivieri observed that for some multi-way situations, neither selectivity formulation predicted the results of Monte Carlo calculations.
  – $SEL_{MKL}$ – upper limit of selectivity
  – $SEL_{HCD}$ – lower limit of selectivity

Calculation of Selectivity for 2DLC vs. 2D-LC-DAD

- **X, Y, and Z** simulated to approximate the real data
- **X and Y** – 1\textsuperscript{st} and 2\textsuperscript{nd} dimension retention profiles, using resolved retention times and simulated, Gaussian peaks
- **Z** – spectral profiles – as resolved by PARAFAC
- Background components omitted from the analysis
MKL Selectivity

2D-LC
2D-LC-DAD

Selectivity vs. Peak Number

VCU
MKL Selectivity – 2D-LC vs. 2D-LC-DAD

<table>
<thead>
<tr>
<th></th>
<th>Peak 1</th>
<th>Peak 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>2D-LC SEL</td>
<td>$1 \times 10^{-5}$</td>
<td>$1 \times 10^{-5}$</td>
</tr>
<tr>
<td>2D-LC-DAD SEL</td>
<td>$8 \times 10^{-3}$</td>
<td>$8 \times 10^{-3}$</td>
</tr>
</tbody>
</table>
MKL Selectivity – 2D-LC vs. 2D-LC-DAD

<table>
<thead>
<tr>
<th></th>
<th>Peak 1</th>
<th>Peak 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>2D-LC SEL</td>
<td>0.33</td>
<td>0.33</td>
</tr>
<tr>
<td>2D-LC-DAD SEL</td>
<td>0.89</td>
<td>0.91</td>
</tr>
</tbody>
</table>

![Graph showing first and second dimension retention times and relative absorbance vs. wavelength.](image)
# MKL Selectivity – 2D-LC vs. 2D-LC-DAD

<table>
<thead>
<tr>
<th></th>
<th>Peak 1</th>
<th>Peak 2</th>
<th>Peak 3</th>
<th>IAA-alanine</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>2D-LC SEL</strong></td>
<td>0.33</td>
<td>0.33</td>
<td>0.98</td>
<td>0.16</td>
</tr>
<tr>
<td><strong>2D-LC-DAD SEL</strong></td>
<td>0.89</td>
<td>0.91</td>
<td>0.98</td>
<td>0.48</td>
</tr>
</tbody>
</table>

The table above compares the selectivity of 2D-LC and 2D-LC-DAD for MKL. The retention times and absorbance values for different peaks are shown in the graphs. The second dimension retention time (sec.) and the first dimension retention time (min.) are plotted against the second dimension retention time, with relative absorbance shown against wavelength (nm).
# MKL Selectivity Comparisons

<table>
<thead>
<tr>
<th>Data Dimensions</th>
<th>Average selectivity per component</th>
<th>Peak Capacity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Column 1, single wavelength</td>
<td>0.15</td>
<td>50</td>
</tr>
<tr>
<td>Column 1 + DAD</td>
<td>0.36</td>
<td>n.a.</td>
</tr>
<tr>
<td>Column 2, single wavelength</td>
<td>0.05</td>
<td>17.4</td>
</tr>
<tr>
<td>Column 2 + DAD</td>
<td>0.25</td>
<td>n.a.</td>
</tr>
<tr>
<td>2D-LC, single wavelength</td>
<td>0.78</td>
<td>870</td>
</tr>
<tr>
<td>2D-LC-DAD</td>
<td>0.84</td>
<td>n.a.</td>
</tr>
</tbody>
</table>
Conclusions

1. Three chemometric methods (WTTFA, PARAFAC-ALS, and fALS) have been applied to four-way quadrilinear data generated by running multiple samples with 2D-LC-DAD.
2. These methods result in a great enhancement in S/N and background suppression.
3. Several indole conjugates have been identified in mutant and wild type maize samples.
4. The indole content of the wild type and mutants are clearly differentiated.
5. The quantitative capabilities of multi-way modeling have been demonstrated.
6. Multivariate selectivity has been shown to relate to chromatographic figures of merit.
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