Seminar 5
Protocol For NMR Based Metabolomics. Tips And Tricks

Dr. Dimitra Benaki
School of Pharmacy, Dept. Pharmaceutical Chemistry
Systems Biology

Systems Biology


Regulatory mechanisms
- Postranslational modifications
  - Conformation
  - Degradation
  - Secretion
- Activation

Enzyme kinetics
- Degradation
- Inter-conversion
- Transport
- Secretion
- Accumulation
- Exogenous intake

Metabolome

Transcriptome
- Alternative splicing
  - Micro RNAs
  - RNA editing
  - RNA stability
  - RNA structure

Proteome

Genome
- Epigenetics
  - Insertion-deletions
  - Allelic variants
  - Pseudogenes

What will or may happen

Complexity

Systems biology

happened

is happening
Applications of Metabolomics

- Human diseases
  - Diagnosis
  - Determination of disease state
  - Prevention
  - Biomarker discovery
  - Risk determination

- Pharmacology
  - Drug discovery
  - Treatment
  - Doses evaluation

- Toxicology
  - Toxicity assessment
  - Toxic effects of drugs

- Food technology
  - Food safety, quality
  - Nutrigenomics

- Microbial biotechnology
  - Microbial improvement
  - Fermentation
  - Biotechnological compounds

- Plant biotechnology
  - Crop improvement
  - Transgenic breeding
  - Plant breeding
  - Improve stress tolerance

- Systems biology
  - Dynamics in biological systems
  - Explore metabolic networks

- Enzyme discovery
  - Discovery of biochemical pathways
  - Link changes in metabolite levels to catalytic activity
  - Improve catalytic efficiency of enzymes

4th Workshop on Holistic Analytical Methods, 17-19 April 2016
Metabolomics’ Methods

- NMR
- GC,
- HPLC,
- UPLC,
- HPTLC
- CE (capillary electrophoresis)

Hyphenated
- LC-MS,
- GC-MS
- GC-MS/MS
- UHPLC-MS
NMR in Metabolomics

- Minimal sample preparation (biofluids)
- No extra steps, i.e. separation
- Measures multiple metabolites simultaneously
- Non-destructive
- Fast analysis
- High reproducibility
- Highly quantitative
- High throughput
- Steadily increasing sensitivity
Metabolomics Workflow

- Collection & Storage
- Sample Preparation
- Data Acquisition
- Multivariate Statistical Analysis
- Biomarkers identification
- Validation
Metabolomics Workflow

- Experimental design
- Collection & Storage
- Sample Preparation
- Data Acquisition
- Multivariate Statistical Analysis
- Biomarkers identification
- Validation

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In the absence of a proper design it is essentially impossible to distinguish biological variation from technical variation. When these two sources of variation are confounded, there is no way of knowing which source is driving the observed results.
Experimental Design

✓ Avoid the fridge temptation
Experimental Design

✓ Avoid the fridge temptation
Metabolomics Workflow

Collection & Storage

Experimental design

Sample Preparation

Data Acquisition

Multivariate Statistical Analysis

Biomarkers identification

Validation
Collection & Storage

- A single person should collect/harvest the initial material
- Stop enzymatic processes

Transfer in dry ice

Store at -80 °C
Metabolomics Workflow

1. Collection & Storage
2. Sample Preparation
   - Experimental design
   - Collection & Storage
3. Data Acquisition
4. Multivariate Statistical Analysis
5. Biomarkers identification
6. Validation

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NMR in Metabolomics
Metabolomics sample preparation

*metabolites found within:*

- **Biofluid**
  - Urine,
  - Blood (serum, plasma),
  - Saliva,
  - Breath,
  - CSF,
  - Amniotic, etc

- **Cell**
  - Cytosolic metabolites
  - Released metabolites

- **Tissue, Organ**
  - Mammals: liver, kidney, heart, tumour, muscle, brain, fat tissue, etc.

- **Plants**
  - Leaves, roots, fruits, etc.

- **Whole organism**
  - Insects (Flies, etc)
  - Marine organisms
  - Worms, etc.

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Metabolomics sample preparation

metabolites found within:

- **Biofluid**
  - Urine,
  - Blood (serum, plasma),
  - Saliva,
  - Breath,
  - CSF,
  - Amniotic, etc

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- **Plants**
  - Leaves, roots, fruits, etc.

- **Whole organism**
  - Insects (Flies, etc)
  - Marine organisms
  - Worms, etc.
Metabolomics sample preparation

**Standardized Protocols**

- **pH adjustment:** phosphate buffer pH 7.4; NaN₃ to eliminate bacterial growth
- **axis calibration:** Internal Standard (TSP, DSS; 0.01%)
  & “QUALITY CONTROL”
  # Blood samples
- **field lock:** deuterated solvent (10% D₂O in Urine; 50% in plasma)

- Centrifuge (+4 °C) and transfer 550 μL in NMR tube

- **Plasma samples:** gentle handling, no vortex, no centrifuge, remove protein particles with a needle

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Metabolomics sample preparation

metabolites found within:

- **Biofluid**
  - Urine,
  - Blood (serum, plasma),
  - Saliva,
  - Breath,
  - CSF,
  - Amniotic, etc

- **Cell**
  - Cytosolic metabolites
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- **Tissue, Organ**
  - Mammals: liver, kidney, heart, tumour, muscle, brain

- **Plants**
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- **Whole organism**
  - Insects (Flies, etc)
  - Marine organisms
  - Worms, etc.

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Metabolomics sample preparation

Standardized Protocols

- **Frozen sample**

- **Homogenization** in liquid N\(_2\) (manually), high throughput tissue homogenizer with beads → weight (<100 mg) and store at -80 °C till extraction homogenizer probes (in extraction solvent; 1\(^{st}\) step extract.)

- **Extraction** 3 solvent system MeOH – CHCl\(_3\) – dH\(_2\)O (-20 °C)

  WORK ON ICE

  2 phase system; collection; repeat

- **Lyophilisation; store at -80 °C**

- **Reconstitution** axis calibration (Internal Standard; TSP, DSS; 0.01%)

  field lock (100% D\(_2\)O buffered; pH 7.4; NaN\(_3\))

  centrifuge and transfer 550 μL in NMR tube
Metabolomics sample preparation

**Standardized Protocols**

- **Frozen sample**
- **Homogenization** in liquid N\(_2\) (manually), high throughput tissue homogenizer with beads → weight (<100 mg) and store at -80 °C till extraction homogenizer probes (in extraction solvent; 1\(^{st}\) step extract.)

**Extraction**

- **MeOH – CHCl\(_3\) – dH\(_2\)O** (−20 °C)
- WORK ON ICE
- 2 phase system; collection; repeat

- **Lyophilisation; store at -80 °C**

- **Reconstitution**

  - axis calibration (Internal Standard; TSP, DSS, 0.01%)
  - field lock (100% D\(_2\)O buffered; pH 7.4; NaN\(_3\))
  - centrifuge and transfer 550 μL into NMR tube

- **INCLUDE BLANK SAMPLES (reconstitution buffer)**

**CHECK SOLVENTS BEFORE EXTRACTION**

**CHECK SOLVENTS BEFORE RECONSTITUTION**

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Metabolomics sample preparation

Solvent check before NMR sample preparation

D₂O + 10% Urine Buffer

D₂O
Metabolomics sample preparation

Solvent check before extraction

MeOH lyophilized, reconstituted in 100% CD$_3$OD (new bottle)
NMR Reproducibility

PC3 cell line
NMR Reproducibility

PC3 cell line

Glutathione (GSH)

DMA

Glu

Ace
NMR Reproducibility

PC3 cell line from 2 different persons

- Glutathione (GSH)
- DMA
- Control 1
- Control 2
- Glu
- Ace

Graph showing NMR spectra with different compounds identified.
NMR Reproducibility

Urine samples recorded in 600 MHz Bruker AVANCE III, Athens

Buffer A (PBS pH=7.4): original from Bruker
Buffer B (PBS pH=7.4): local preparation

Urine NMR samples
Sample 1-5 person E buffer A 1EA – 5EA
Sample 1-5 person E buffer B 1EB – 5EB
Sample 1-5 person S buffer A 1SA – 5SA
Sample 1-5 person S buffer B 1SB – 5SB
NMR Reproducibility
NMR Reproducibility

PCA
NMR Reproducibility
NMR Reproducibility

✓ accepted sample preparation
Basic analytical issues - NMR

✓ Run tube and buffer blanks
✓ Use the same tube type for a project
✓ Take care = consistent sample preparation
✓ Sample presentation – give the spectrometer a chance!

![Diagram showing correct and incorrect sample presentation]
Metabolomics Workflow

1. Experimental Design
2. Collection & Storage
3. Sample Preparation
4. Data Acquisition
5. Multivariate Statistical Analysis
6. Biomarkers Identification
7. Validation

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Nuclear Magnetic Resonance

- Reproducible results on concentration critical / crucial parameters:
Nuclear Magnetic Resonance

- Reproducible results on concentration critical / crucial parameters:
  
  **Acquisition**
  
  ✓ relaxation delay,
  
  ![Diagram of Recycle Delay (d1) and Pulse Width (pw) with Acquisition Time (at)]
Nuclear Magnetic Resonance

- Reproducible results on concentration critical / crucial parameters:

**Acquisition**

- relaxation delay, $D[1] = 4.00000000$
- pulse width, $P[1] = 11.63$

---

![Diagram showing pulse width (pw) and recycle delay (d1) with acquisition time (at)](image)

- Pulse Width (pw)
- Recycle Delay (d1)
- Acquisition Time (at)
Nuclear Magnetic Resonance

- Reproducible results on concentration critical / crucial parameters:

**Acquisition**

- ✓ relaxation delay, $D[1] = 4.0000000$
- ✓ pulse width, $P[1] = 11.63$
- ✓ acquisition time, $AQ [sec] = 2.6563926$
Nuclear Magnetic Resonance

- Reproducible results on concentration critical / crucial parameters:

**Acquisition**

- relaxation delay, $D[1] = 4.00000000$
- pulse width, $P[1] = 11.63$
- acquisition time, $AQ [sec] = 2.6563926$
- spectrum analysis, $TD = 65536$
- number of scans, $NS = 192$
- receiver gain...

![Diagram of pulse width and recycle delay](image)
Nuclear Magnetic Resonance

- Reproducible results on concentration critical / crucial parameters:

**Acquisition**

- RELAXATION DELAY, $D[1] = 4.0000000$

- pulse width, $P[1] = 11.63$

- acquisition time, $AQ[sec] = 2.6563926$  
  - Acquisition time

- spectrum analysis, $TD = 65536$  
  - Size of fid

- number of scans, $NS = 192$  
  - Number of scans

- receiver gain... $RG = 90.5$  
  - Receiver gain

---

![Diagram](image-url)

- Recycle Delay ($d1$)
- Acquisition Time ($at$)
- Pulse Width ($pw$)
Acquisition Parameters

**Experiment**
- **Name**: [Field]
- **ID**: [Field]

**Parameters**
- **PIL/PROG**: [Field]
- **ACQ/PROG**: [Field]
- **Title**: [Field]
- **File**: [Field]

**Spectrum**
- **Ver**: [Field]
- **AW**: [Field]
- **TV**: [Field]
- **BW**: [Field]
- **GR**: [Field]
- **DW**: [Field]
- **DE**: [Field]
- **DE[µsec]**: [Field]
- **HPP/In**: [Field]

**Receiver**
- **Gain**: [Field]
- **Denv**: [Field]
- **Denv[µsec]**: [Field]
- **DOR**: [Field]
- **DC[µsec]**: [Field]
- **HR[In]**: [Field]

**Integration**
- **Pulse increment**: [Field]
- **Intergrating pulse**: [Field]
- **Oversampling duration**: [Field]
- **GIP pulse length**: [Field]

**Auxiliary**
- **Power mode**: [Field]
- **Program parameters**: [Field]
- **Loop counter**: [Field]
- **Correction angle phase**: [Field]
- **GIP position**: [Field]
- **Rotation frequency sample**: [Field]
Acquisition Parameters

Processing Parameters
NMR Standard Operation Procedure

makes science easier

- Water suppression test: 2M M Sucrose with 0.5mM DSS, 2 mM NaN₃ in 10% D₂O and 90% H₂O
- Temperature long term stability
- Temperature calibration: CD₃OD 99.8%
- ¹H resolution test: 1% CH Cl₃ in Acetone-d6
- Sensitivity test: 0.1% Ethylbenzene in CDCl₃
- Field drift (CDCl₃)

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NMR Standard Operation Procedure

makes science easier

- Sensitivity test: 0.1% Ethylbenzene in CDCl₃

\[ \text{SINO} = 650.9 \]

noise from 5.99 to 3.99 ppm

\[ \text{SINO} = 776.2:1 \]

noise from 4.47 to 4.07 ppm

Good sensitivity can be obtained with good resolution and good lineshape only. The splitting between the two central lines of the methylene quartet should go lower than 15% (using a lb of 1 Hz)

Automated S/N calculation is performed using sinocal
NMR Standard Operation Procedure

makes science easier

- $^1$H resolution test: 1% CDCl$_3$ in Acetone-$d_6$

The AU [hwcal](#) program determines the resolution at the half height of the chloroform line.
NMR Standard Operation Procedure

*makes science easier*

- 2 mM Sucrose with 0.5 mM DSS, 2 mM NaN₃ in 10% D₂O and 90% H₂O

Water line width at 50% and 10% of DSS, S/N, and resolution calculations by typing `suppcal`
NMR Standard Operation Procedure

makes science easier

- 2 mM Sucrose with 0.5 mM DSS, 2 mM NaN₃ in 10% D₂O and 90% H₂O

\[ \text{SINO} = 154.2:1 \]

Water line width at 50% and 10% of DSS, S/N, and resolution calculations by typing \textit{suppcal}
NMR Standard Operation Procedure

✓ Temperature Calibration

edte window
NMR Standard Operation Procedure

✓ Temperature Calibration

Temperature:
- 300 K
- 310 K

Biological Samples:
- Plasma

CD$_3$OD, 99.8% (Bruker std)

$^1$H 1D; ns 1; ds 0; zg30

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Source: Bruker Instruments, Inc. VT-Calibration Manual

100% Methanol: \( T = -23.832 \Delta^2 - 29.46 \Delta + 403.0 \)

(\( \Delta \) is the shift difference (ppm) between CH\(_3\) & OH peaks)
2-Apr-2016, After the filling and before TASCAMAR setup.

Which sample?
- 90% Glycerol in DMSO-d6 (G)
- Glycerol pure (GP)
- 4% Methanol in Methanol-d4 (M)
- Methanol pure (MP)
- 99.9% Methanol-d4 (D)

OK Cancel
2 April 2016, After He Filling and before TASCAMAR setup
300.07 Hz
-1299.95 Hz K.4x

Sample: 99.8% Methanol-d4
chemical shift difference = 1.526 ppm
actual temperature = 298.97 K
WEEKLY
Instrument Performance

✓ Long Term Stability Test

CD$_3$OD, 99.8% (Bruker std)
$^1$H 1D; ns 1; ds 0; zg2D
Profiling Optimization

- temperature stabilization unit
- robotic sample changer of 60 sample positions (B-ACS 60)
- and supporting automation software

- 1st sample transfer
- Temperature equilibration; 5 min
- Tuning and matching
- Pulse calibration
- Shimming (rsh; tsg)
- Lock
- O1 optimization
- TSP line width
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manual tuning-matching optimization
1D SHIMMING

Parameters
maximum order = 5
probehead = Z814601_0044
solvent = CDC13
shim nucleus = 2H
nuclei optimized for = 1H
offset (from lock) = 7.24 ppm
optimization parameters = 1s
line width 1H = 0.10 Hz
envelope shape / strictness = 2.00 / 200

Results:
initial B0 stdDev = 4.89 Hz
sample size = 2.12 cm, position = -0.04 cm
final B0 stdDev = 0.21 Hz; Improvement = 23.0
envelope width = 0.43 Hz
shim changes:
Z +215
Z2 +18
Z3 +62
Z4 +259
Z5 +630
duration = 1 min 42 sec
completed successfully
finished Sat Apr 02 13:10 24 2016
Start acquisition on: dbStdTests 21 1 C:\Bruker\TOPSPIN2.pi6 AGROCOS
No acquisition running

GammaB at 15.0 dB: 6954.1 Hz
90C pulse at 15.0 dB: 39.97 us
90C pulse at 8.0 dB: 10.04 us
**Probe's name**: 5 mm PABBI 1H/D-88 Z-GRD 2814601/0044 [33]

**Solvent(s)**: MeOD_Ag

**Nucleus**: 1H

---

<table>
<thead>
<tr>
<th>Description</th>
<th>pulse [usec]</th>
<th>power level</th>
</tr>
</thead>
<tbody>
<tr>
<td>90 deg. transmitter</td>
<td>10</td>
<td>3</td>
</tr>
<tr>
<td>cprd</td>
<td>82</td>
<td>21.28</td>
</tr>
<tr>
<td>Tossy spin lock</td>
<td>0.06</td>
<td>11.94</td>
</tr>
<tr>
<td>ROE</td>
<td>0.25</td>
<td>22.08</td>
</tr>
<tr>
<td>CW irradiation</td>
<td>5.00</td>
<td>76.98</td>
</tr>
</tbody>
</table>

**Logical channel**: F1 + F2

---

<table>
<thead>
<tr>
<th>Description</th>
<th>pulse [usec]</th>
<th>power level</th>
</tr>
</thead>
<tbody>
<tr>
<td>90 deg. decoupler</td>
<td>10</td>
<td>3</td>
</tr>
<tr>
<td>cprd</td>
<td>82</td>
<td>21.28</td>
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<tr>
<td>Tossy spin lock</td>
<td>0.06</td>
<td>13.71</td>
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<tr>
<td>ROE</td>
<td>0.2</td>
<td>23.67</td>
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<tr>
<td>CW irradiation</td>
<td>0.30</td>
<td>101.42</td>
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</table>

**Logical channel**: F3

---

<table>
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<th>Description</th>
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<th>power level</th>
</tr>
</thead>
<tbody>
<tr>
<td>2nd cprd (pow. gated)</td>
<td>19.16</td>
<td></td>
</tr>
<tr>
<td>Tossy spin lock</td>
<td>0.30</td>
<td>101.42</td>
</tr>
<tr>
<td>ROE</td>
<td>0.30</td>
<td>101.42</td>
</tr>
<tr>
<td>CW irradiation</td>
<td>0.30</td>
<td>101.42</td>
</tr>
</tbody>
</table>

---

**Logical channel**: Global

---

**Standard hard pulses**

---

**Standard shape pulses**

---

**Save**  **Copy to probe**  **Copy to solvent**  **Print screen**  **Exit**
*AU program for 1D data acquisition.*

*Description/Usage:* The lock phase is optimized, a pulse calibration is performed.

*Author(s):* Ulrich Braumann

*Organization:* Bruker BioSpin GmbH

*Email:* ulrich.braumann@brucker-biospin.de

*Name:* au_prof1d

*Date:* 2007/08/20

*x לך* 070716 save parameters

---

**GETCURDATA**

// optimize lockphase

AUTOPHASE;

// determine 90deg pulse automatically, no display of results

// ATTENTION: pulse calibration starts with PROSOL values

current values are IGNORED

XCMD("pulsedcal fast quiet");

XCMD("saveprofile");

run experiment
```plaintext
/* au_prof 03.04.2007 */
/***************************************************************************/
/* Short Description: */
/* AU program for profiler data acquisition. */
/***************************************************************************/
/* Keywords: */
/* rg_profiling */
/***************************************************************************/
/* Description/Usage: */
/* Gets PI/P1 from experiment number below current dataset */
/* RG is also taken, works only for JRES and COSY */
/***************************************************************************/
/* Author(s): */
/* Name: Ulrich Braumann */
/* Organisation: Bruker BioSpin */
/* Email: ulrich.braumann@Bruker-biospin.de */
/***************************************************************************/
/* Name Date Modification: */
/* cur 070305 created */
/* rfx 070403 separation into external AU */
/***************************************************************************/
/* */
/***************************************************************************/
GETCURDIA
XODMB "getprofpar";
ERRORABORT
// run experiment, no RQA ??
Z0 ERRORABORT
QUIT
```
`jresgpprqf`
Metabolic Profiling Automation

[Image: A screenshot showing the ICON NMR interface with options for Routine Spectroscopy, Automation, Toolbox, and Configuration.]
### Composite Experiments

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>HOESY</td>
<td>1d 1H and HOESY with gradients</td>
</tr>
<tr>
<td>HomoNuclear_PRESaturation</td>
<td>ProtonPR; COSYPR; HOESYPR; TOCSYPR;</td>
</tr>
<tr>
<td>HomoNuclear</td>
<td>COSY; COSY1R; HOESY;</td>
</tr>
<tr>
<td>Full Set</td>
<td>proton; cosy; cosy1r; qc-dept; bc;</td>
</tr>
<tr>
<td>HOESY2DPPR</td>
<td>HOESY with preset and 2 gradients; by mmmsu</td>
</tr>
<tr>
<td>HOESY2DDPH</td>
<td>HOESY phase sensitive with 2 gradients; by mmmsu</td>
</tr>
<tr>
<td>WURIE</td>
<td>urine 1H, Jres and SQUEID</td>
</tr>
<tr>
<td>Natural_Products</td>
<td>routine</td>
</tr>
<tr>
<td>WURIE_set</td>
<td>urine 1H and Jres</td>
</tr>
<tr>
<td>HOESY2DDPH.sar</td>
<td>Rainer</td>
</tr>
<tr>
<td>TEPA_noesygppr1d.comp</td>
<td>noesy for serum</td>
</tr>
<tr>
<td>Serum 2D</td>
<td>full set of serum experiments</td>
</tr>
<tr>
<td>COSY45S5W</td>
<td>sw opt. COSY45 (magn. mode)</td>
</tr>
<tr>
<td>COSY90S5W</td>
<td>sw opt. COSY90 (magn. mode)</td>
</tr>
<tr>
<td>COSY45PSW</td>
<td>sw opt. COSY with gradients (magn. mode)</td>
</tr>
<tr>
<td>COSY90PSW</td>
<td>sw opt. COSY with de filter (Sttare_4DPR)</td>
</tr>
</tbody>
</table>

### Component Experiment Viewer

#### Experiments

<table>
<thead>
<tr>
<th>Experiments</th>
<th>F2 Reference</th>
<th>F1 Reference</th>
</tr>
</thead>
</table>

#### Commands

- Save
- Default
- Revert

### Status Line

- Icon: Configuration
- File
- Help

### Automation

- User Settings
  - User Manager
  - Composite Experiments
  - Additional Users
  - Originator Items

- Automation
  - Master Switches
  - Automation Window
  - Lock/Shim Options
  - Solvent/Probe
  - Dependencies
  - Tuning/Matching
  - Temperature Handling
  - LC-NMR Options
  - Sample/Track Options
  - Fail Safe / Error Handling
  - Web Interface

- General Options
- ToolBox Setup
- Accounting
<table>
<thead>
<tr>
<th>No.</th>
<th>Solvent</th>
<th>Comment</th>
<th>Locked</th>
<th>Locked</th>
<th>r التقاط (Türkçe)</th>
<th>File Name</th>
<th>Type</th>
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</thead>
<tbody>
<tr>
<td>9</td>
<td>CH3CN+D2O</td>
<td>CH3CN+D2O</td>
<td></td>
<td></td>
<td></td>
<td>dbCell+D2O</td>
<td>TOPSHIM_WITH_AUT</td>
</tr>
<tr>
<td>10</td>
<td>CH3OH+D2O</td>
<td>HPLC Solvent (Acetonitrile/D2O)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>11</td>
<td>CH3OH+D2O</td>
<td>HPLC Solvent (Methanol/D2O)</td>
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<td></td>
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<td>12</td>
<td>D2O</td>
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<tr>
<td>13</td>
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<td>14</td>
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<td>Dimethylformamide-d7</td>
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Resulting 1D NOESY spectra of urine

- Calibrated to TSP signal at $\delta$ 0.0 ppm
- Phase and Baseline corrected
Assignment process
2D spectra
Literature and web databases
(HMDB, BMRB)
Chenomx

600MHz 1H-13C DEPT135-HSQC

600MHz 2D TOCSY
Batch Effects

Cladobotryum genus species
Batch Effects

Cladobotryum genus species
Batch Effects

Cladobotryum genus species
Batch Effects

Cladobotryum genus species

bt02TI_set1&2tries_M20 (PCA-X)

Colored according to Obs ID (species)

- C. apic
- C. dend
- C. fungi
- C. myco
- C. rubr
- C. rubr-t
- C. vari
- C. verti

R2X[1] = 0.426144  R2X[2] = 0.198183  Ellipse: Hotelling T2 (0.95)
Batch Effects
Figure 2. Alignment of NMR spectra is critical for the comparison of the data. (A) Collection of 1D NMR spectra corresponding to a set of urine samples; (B) same set of NMR spectra after the application of speaq [20]. The application of this bioinformatics tool translates into a better alignment of the spectra, thus overcoming the impact of chemical and physical variations on the chemical shifts of the metabolites present in those samples.
Peak Alignment

CSF, MS patients
$M_{DA_1}$ Intercepts: $R_2 = (0.0, 0.186), Q_2 = (0.0, -0.303)$
100 permutations 3 components

Vivaldi, leucine and isoleucine biosynthesis
Phenylalanine, tyrosine and tryptophan biosynthesis
Glycine, serine and threonine metabolism
Tryptophan metabolism
Phenylalanine metabolism
Taurine, hypotaurine metabolism
Nicotinate and nicotinamide metabolism
Glycine, serine and threonine metabolism
Alanine, aspartate and glutamate metabolism
Purine metabolism

Overview of Pathway Analysis
Heterocovariance based metabolomics

a powerful tool accelerating bioactive natural products identification

Morus alba case
Heterocovariance based metabolomics

✓ Tyrosinase Inhibition Activity

✓ $^1$H NMR profile of *Morus alba* fractions 1-30

30 fractions from *Morus alba* by CPC
Heterocovariance based metabolomics
1H NMR spectrum of purified 2,4,3′-trihydroxydihydrostilbene

SHY plot
correlation of mass at m/z 229.086
at RT = 7.89 min and NMR

STOCSY
NMR peak correlation

HETCA
covariance of biological activity
with corresponding NMR data
$^1$H NMR spectrum of purified oxyresveratrol

SHY plot correlation of mass at m/z 243.066 at RT = 7.70 min and NMR

STOCSY NMR peak correlation

HETCA covariance of biological activity with corresponding NMR data
Heterocovariance based metabolomics

oxyresveratrol

2,4,3′-trihydroxydihydrostilbene <1%

total extract
Heterocovariance based metabolomics as a powerful tool accelerating bioactive natural product identification
Nektarios Aligiannis†, Maria Halabalaki†, Eliza Chaita, Eirini Kouloura, Aikaterini Argyropoulou, Dimitra Benaki, Eleftherios Kalpoutzakis, Apostolis Angelis, Konstantina Stathopoulou, Stavroula Antoniou, Maria Sani, Oliver Werz, Verena Krauth, Birk Schütz, Hartmut Schäfer, Manfred Spraul, Emmanuel Mikros* Leandros A. Skaltsounis.

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Bruker BioSpin, Rheinstetten, Germany
Dept. of Pharm. Med. Chemistry, Inst. of Pharmacy, Friedrich-Schiller-University Jena, Germany
THANK YOU

for your attention