**Wednesday** afternoon 16:30 – 18:00

Everybody go to sample preparation lab in D23 with Natalja

2 x plasma samples for each person (**Emilie, Zhara, Ece, Pablo, Pilipp, Djawed and Anne**)

1. Take 2 eppendorf tubes and name them 1 Enz + your name 2 Enz blank + your name
2. Add 0.5 mL of plasma to each tube
3. Add 1 mL of enzyme mixture to each tube
4. Add 1.5 µL of internal standards (13C3 -enterolactone and 13C3-enterodiol, conc. 10000 ng/mL)
5. Mix
6. Incubate in a mixer at 37 ˚C overnight

**Thursday** D 23 start 08:00 end 15:00

**8:30 – 9:30** for everybody lab 3277: Demonstration of extraction procedure for quantification of lignans in urine on plates (Kasper app. 1 hour) according to the protocol (urine sample preparation targeted metabolomics)

**After 9:30** two groups

**Group 1: Emilie, Zhara and Ece**

**Group 2: Pablo, Philipp, Djawed and Anne**

Group 1 goes to room 3292/LC-MS: Optimization of MS parameters for quantification of lignans (enterolactone/enterolactone IS, enterodiol/enterodiol IS, syringaresinol), takes app. 2 hours

Group 2 goes to the sample preparation lab 3277: Preparation of samples for lignan quantification, takes app. 2 hours

Each person has to prepare 8 plasma samples: 2 x enzyme hydrolyzed samples (prepared on Wednesday), 2 x - blank samples, 2 x spiked samples with High concentration of lignans and 2 x spiked samples with Low concentration of lignans.

1. Take 6 eppendorf tubes and name them: 1 blank + your name, 2 blank + your name, 1 High + your name, 2 High + your name, 1 Low + your name and 2 Low + your name
2. Add 0.5 mL of plasma
3. Add 1.5 µL of internal standards (13C -enterolactone and 13C-enterodiol conc. 10000 ng/mL) to all 6 samples
4. Add 7.8 µL of standard mix working solution with concentration of 100 ng/mL to samples 2 x Low
5. Add 62.5 µL of standard mix working solution with concentration of 400 ng/mL to samples 2 x High
6. Add 1.0 mL of 0.2 % formic acid in water to samples named: 1 blank + name, 2 blank + your name, 1 High + your name, 2 High + your name, 1 Low + your name and 2 Low + your name
7. Mix
8. Add 0.5 mL of 0.4 % formic acid in water to samples named: 1 Enz + your name and 2 Enz blank + your name
9. Mix
10. Centrifuge at 29703 g at 4 ˚C samples for 10 min all samples
11. Samples are ready for Solid Phase Extraction (SPE) according to the protocol (SPE for plasma targeted metabolomics)



At 13:00 Groups switch places

**15:30 – 16:30** for everybody lab 3282: Demonstration of Flow Injection Analyses (FIA) for optimization of source parameters

**16:30 - 18:00** two groups

Group 1: Ece, Djawed and Zhara go to room 3292/LC-MS with Natalja for optimization of MS parameters for quantification of vitamin D metabolites

Group 2: Emilie, Philipp and Pablo go to C20 with Mihai and Mette for metabolite identification of wheat and milk

**Detailed Description of samples for targeted metabolomics:**

We will measure 10 lignans: enterolactone, enterodiol, secoisolariciresinol, lariciresinol, isolariciresinol, hydroxymataresinol, syringaresinol, pinoresinol, medioresinol and mataresinol

**Standard curve**

Points of standard curve: 0.0244, 0.0488, 0.0977, 0.195, 0.39, 0.78, 1.56, 3.125, 6.25, 12.5, 25 and 100 ng/mL prepared in 25 % acetonitrile containing 25 ng/mL of 13C3-enterolactone and 13C3-enterodiol and 40 ng/mL of 13C1-glychocolic acid

**Working solutions**

Standard mix containing 10 lignans in 25 % acetonitrile in concentrations 400 ng/mL (1 mL) and 100 ng/mL (600 µL) used for spiking experiment. Internal standards 13C-enterolactone and 13C-enterdiol in 25 % acetonitrile in concentration 10000 ng/mL (1 mL). Enzymes β-glucuronidase/sulfatase in concentration 2 mg/mL in 0.05 M NaOAc pH 5. MilliQ (100 mL)containing 53.4 ng/mL 13C1-glychocolic acid.

 **Urine samples**

- 20 urine samples are collected from pigs fad two different diets, lignan rich and lignan low diets (description of diets see article “The effect of antibiotics and diet on enterolactone concentration and metabolome studied by targeted and non-targeted LC-MS metabolomics”). Because lignans are conjugated in plasma and urine with glucuronic acid and sulfate we use enzymes β-glucuronidase/sulfatase for deconjugation to be able to measure lignans as aglucones/free lignans.

- Pig 01, 02, 03, 04, 05, 16, 17, 18, 19, and 20 (Low lignan diet)

- Pig 06, 07, 08, 09, 10, 21, 22, 23, 24 and 25 (High lignan diet)

QC (Quality Control samples), 6 QC samples used per batch (3 x QC Low and 3 x QC High) + 3 x blank (MilliQ + enzyme mix and internal standards)

QC Low (mix of 16, 17, 18, 19), QC High (mix of 21, 22, 23, 24, 25)

Total number of samples: 29 (1 plate)

**Plasma samples**

Plasma samples are collected from pigs fad lignan low diet and therefore can be used for spiking experiments. For spiking experiments samples are not hydrolyzed to keep concentration of free lignans as low as possible. Spiking experiments are performed to calculate the recovery of lignans after the sample preparation procedure.

Total number of samples: 56 (1 plate per group)

**Solid Phase Extraction (SPE) for plasma samples of targeted metabolomics**





**Elution plate has to be sealed and centrifuged before LC-MS/MS analyses**

**Protocol (Urine samples for targeted metabolomics)**

Materials

* 300 µL urine (20 samples)
* 6 x QC samples (low and high)
* 3 x blank sample (MilliQ + enzymes + IS)
* 60 µL of 10000 ng/mL IS (13C-enterolactone and 13C-enterodiol) in 25 % ACN to each sample
* 1 mL of β-glucuronidase/sulfatase in concentration 2 mg/mL in 0.05 M NaOAc pH 5 to each sample
* 0.5 mL of 0.4 % formic acid to each sample
* 900 mL of 53.4 ng/mL of 13C-glychocolic acid in MilliQ to each sample
* 100 % Acetonitrile
* 25 % Acetonitril
* 20 % MeOH
* MilliQ
* Vacuum manifold and pump
* Sealing manifold
* C18 binding plate (25 mg material) and elution plate

Method

* To 300 µL of urine sample add 60 µL of IS and 1 mL of enzyme mix
* Incubate in a mixer overnight at 37 ˚C
* Add 0.5 mL of 0.4 % formic acid
* Centrifuge at 29703 g at 4 ˚C for 10 min
* Total volume of samples 1860 µL
* Transfer samples to the preparation plate and seal the plate for the demonstration of SPE procedure at the course
* Transfer 930 µL of sample from preparation plate to conditioned binding plate
* Samples elute slowly through the C18 material
* Wash samples with 1 mL of 20 % MeOH
* Dry the binding plate
* Elute lignans with 300 µL of 100 % acetonitrile to the elution plate
* Dilute samples with 900 µL of 53.4 ng/mL of 13C-glychocolic acid in MilliQ (samples are ready for the analyses of plant lignans
* For the analysis of enterolignans (enterolactone and enterodiol) samples has to be diluted 20 times, transfer 50 µL from elution plate of plant lignans to the new elution plate containing 950 µL of 25 % acetonitrile in MilliQ

**LC-MS/MS parameters**

LC is microLC 200 series from Eksigent/AB Sciex. The microLC is equipped with Ascentis® C18 column, 100 mm x 1.0 mm with 3.0 µm partical size. The LC conditions are following: eluent A consists of water with 0.1% formic acid and eluent B is acetonitrile with 0.1% formic acid. The column is equilibrated for 3 min. The gradient starts at 75% eluent A and 25% eluent B, is constant for 1 min, then increases to 85% eluent B during 3.3 min and is constant for 0.5 min. The total run time is 7.8 min.

* Auto-sampler racks 20 ˚C
* Column oven 30 ˚C
* Injection volume 5 µL

MS is QTrap 5500 mass spectrometer from AB Sciex. The mass spectrometer is equipped with an ESI source and operates in negative ionization mode.

**Source parameters**

* Curtain gas 20 psig
* Nebulizer gas (Gas1) 50 psig
* Heater gas (Gas2) 60 psig
* Temperature 300 ˚C
* Ionization spray -4500 eV

**MRM parameters**

**Compound-Dependent MS/MS Parameter, Declustering Potential (DP), Entrance Potential (EP), Collision Energy (CE) and Cell Exit Potential (CEP).**

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
|  | **Q1 mass (m/z)** | **Q3 mass (m/z)** | **DP****(V)** | **EP****(V)** | **CE****(eV)** | **CEP****(V)** |
| Enterolactone | 297.1 | 189.1 | -140 | -10 | -26 | -21 |
| Enterodiol | 301.1 | 253.1 | -140 | -10 | -32 | -19 |
| Matairesinol | 357.2 | 82.9 | -145 | -10 | -26 | -7 |
| Hydroxymataresinol | 373.1 | 217.1 | -115 | -10 | -32 | -13 |
| Secoisolariciresinol | 361.2 | 165.0 | -150 | -10 | -34 | -11 |
| Lariciresinol | 359.1 | 329.0 | -40 | -10 | -16 | -21 |
| Isolariciresinol | 359.2 | 344.0 | -165 | -10 | -26 | -31 |
| Pinoresinol | 357.2 | 151.0 | -155 | -10 | -24 | -11 |
| Syringaresinol | 417.1 | 181.0 | -170 | -10 | -26 | -13 |
| Medioresinol | 387.2 | 151.0 | -15 | -10 | -26 | -25 |
| Enterolactone 13C3 | 300.0 | 191.9 | -128 | -10 | -30 | -14 |
| Enterodiol 13C3 | 304.1 | 255.1 | -140 | -10 | -32 | -17 |
| Glychocolic acid 13C1 | 465.3 | 402.3 | -15 | -10 | -46 | -25 |