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Liquid-phase metabolomics analysis: from preparation to injection

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We are still developing and optimizing this protocol

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Abstract

The purpose of this notice is to define two types of injection (RPLC and HILIC) on an LC-HRMS instrument for liquid-phase metabolomics analysis.

The steps cover the sample preparation, the quality control and the analytical condition.

Guidelines

Recommandations:

- Perform several blank injections at the start of analysis to check for contamination;
- Perform two injections of "Mix metabo 2" at the beginning and end of the sequence;
- Check that there has been no drift in retention times, that intensity is good and that mass accuracy is correct;
- Injection of 6 QC at start of sequence;
- Injection of a QC every 5 samples;
- Inject diluted QC (1/2, by 4 and by 8) and check that the molecules follow this decrease in concentration.

Materials

Solvents :

- Methanol (LC-MS, Honeywell) - CAS 67-56-1
- Chloroform (ANALAR Normapur)- CAS 67-66-3
- Water (LC-MS, ThermoFisher Scientific) - CAS 7732-18-5
- MTBE - CAS 1634-04-4
- Acetonitrile (LC-MS, Carlo Erba) - CAS 75-05-8
- Ammonium acetate (Supelco) - CAS 631-61-8

Calibrants :

- Thermo Solution: ESI+ (Reference: 88322)
- Thermo Solution: ESI- (Reference : 88324)

Safety warnings

EPI

Before start

- To avoid any degradation of samples, the use of plastic materials should be strictly limited. A temperature close to zero must be maintained throughout the process;
- The protocols are presented for guidance only, and it will be necessary, in the context of a new project, to carry out pre-tests in order to assess the proportions of matrix and solvents. Similarly, MS and gradient parameters can be adjusted according to matrix richness;
- Ready-to-use column: reconditioning of the column after each injection sequence.

I. Sample preparation

1 **For serum, cell culture or plasma**

1.1 **Attention**: The use of filter cones is essential for matrix sampling

21m 20s

Triphasic preparation: Blight and Dyer

- Thaw samples previously stored at -20 °C or -80 °C on ice
- After thawing, vortex each sample for 00:00:20
- Remove 30 µL of matrix and transfer to a 5 mL glass tube
- Add 190 µL of cold methanol (LC-MS) and vortex for 00:00:20
- Add 390 µL of cold chloroform solution (ANALAR Normapur) and vortex for 00:00:20
- Add 120 µL water (LC-MS) and vortex for 00:00:20
- Centrifuge tubes for 00:20:00 at 450 x g, 4°C
- After centrifugation, remove 190 µL of the methanolic phase and transfer to an HPLC vial
- Evaporate vials under nitrogen flow at 25 °C
- Store vials at -20 °C

1.2 *Triphasic preparation: MTBE*

29m 20s

- Thaw samples previously stored at -20 °C or -80 °C on ice
- After thawing, vortex each sample for 00:00:20
- Place 30 µL of matrix and 220 µL of cold methanol (LC-MS) in a 5 mL glass tube
- Vortex the tube for 00:00:20
- Add 750 µL of MTBE (LC-MS) and vortex for 00:00:20
- Place samples at 4 °C and vortex every 00:02:00 for 00:06:00
- Add 188 µL of water (LC-MS), vortex for 00:00:20
- Centrifuge tubes for 00:20:00 at 450 x g
- Collect 190 µL of aqueous phase. Evaporate vials to dryness under nitrogen flow at 25 °C

- Store vials at -20 °C

1.3 *Methanol extraction*

20m 35s

- Thaw samples previously stored at -20 °C or -80 °C on ice
- After thawing, vortex each sample for 00:00:20
- 400 µL matrix + 1400 µL MeOH in an eppendorf tube
- Vortex for 00:00:15
- Centrifuge at room temperature at 4.5 rpm for 00:20:00
- Collect 370 µL of the methanolic fraction and place in a vial without insert
- Evaporate to dryness under nitrogen at 25 °C

2 **For urine matrix**

2.1 *Mesuring pH and urine density*

- Thaw in ice 0 °C samples previously stored at -20 °C or On ice
- Take an aliquot of 1 mL in a 5 mL glass tube
- Vortexing
- Centrifuge at 750 x g, 4°C , at least 5 min
- The pH is measured using pH paper. The pH value can provide information to explain why a sample may be atypical
- The urine specific gravity value is used to normalize the sample. It is measured using a refractometer. Zero measurement is carried out before each use and every 30 samples measured. Milli-Q water is placed on the refractometer's measuring cell, the "Zero" button is pressed and the value of 1,000 is displayed.
  Between each measurement, rinse the cell with Milli-Q water, then wipe clean with paper. To obtain good measurements, make sure the cell is sufficiently covered with liquid (approximative 200 µL of liquid are required to cover the cell).

2.2 *Normalization*

The normalization presented here is a urinary density normalization. The Excel model "normalization by urine density" can be used to perform all normalization calculations. The dilution rate depends on the type of sample analyzed (human urine, pig urine, etc.)
In the Excel "Urine Density Normalization", in order to evaluate the dilution ratio, a standard curve is created by measuring the urine density of the urine with the highest urine density at

different dilution ratios. Normalization is then performed for each sample. Randomly check the new urine specific gravity value of a few samples.
The excel file is available on LABERCA's "GED".

CITATION

Jacob CC, Dervilly-Pinel G, Deceuninck Y, Gicquiau A, Chevillon P, Bonneau M, Le Bizec B (2017). Urinary signature of pig carcasses with boar taint by liquid chromatography-high-resolution mass spectrometry..
LINK
https://doi.org/10.1080/19440049.2016.1265152

2.3 *Filtration and centrifugation* 30m

- Collect 500 µL of urine and place in an Eppendorf tube fitted with a 10kDa filter
- Centrifuge at 13000 x g for about 00:30:00 at about 5 °C
- Remove the filter and store the filtrate at 4 °C

3 Preparing samples for injection

3.1 *For RPLC analysis* 10m 20s

- If samples have been frozen, thaw in melting ice
- Transfer samples to 100 µL of LC-MS-grade **H2O-ACN (95:5)** and vortex for 00:00:20
- If necessary, centrifuge samples for 00:10:00 at 450 x g and 4 °C
- Transfer to a vial with insert

3.2 *For HILIC analysis* 10m 20s

- If samples have been frozen, thaw on ice
- Transfer samples to 100 µL **H2O/ACN mixture (10:90)** and vortex for 00:00:20
- If necessary, centrifuge samples for 00:10:00 at 450 x g and 4 °C
- Transfer to a vial with insert

II. Quality control

4 Preparing the "Mix Metabo 2"

4.1 A solution of standard compounds used in metabolomics, called "Mix Metabo 2" at 1, 5 or 10 ng/µL. This solution can be used as an internal standard, but also as a standard at the start of a sequence to validate instrumental performance.
This mix contains the following 4 standard compounds, available from Sigma-Aldrich and Cluzeau Info Labo (CIL): L-leucine-5,5,5-d3 (CAS: 87828-86-2), L-tryptophan-2,3,3-d3 (CAS: 133519-78-5), Indole-2,4,5,6,7-d5-3-acetic acid (CAS: 76937-78-5), 1,14-tetradecanedioic-d24 acid (CAS: 130348-88-8).

In a 15mL amber vial, add the following volumes of each standard at 1mg/mL to obtain the metabo 2 mix solution at 10 ng/µL:

Name	Solvent	Volume introduce
L-tryptophan-2,3,3-d3	H2O:EtOH (25:75), 1 mg/mL	100 µL
L-leucine-5,5,5-d3	H2O:EtOH (75:25), 1 mg/mL	100 µL
Indole-2,4,5,6,7-d5-3-acetic acid	H2O:EtOH (25:75), 1 mg/mL	100 µL
1,14-tetradecanedioic-d24 acid	H2O:EtOH (25:75), 1 mg/mL	100 µL

Preparation of stock solutions

Make up to 10 mL with LCMS water/HPLC ethanol solution (25:75, (v:v)).

Preparing Mix Metabo 2 as the standard for the start of the sequence:

In a vial with insert, take 100 µL of the mix, evaporate under nitrogen at 25 °C. Then transfer to 100 µL of LC-MS-grade Acetonitrile (ACN)/Water (5:95, (v:v)) for reverse-phase analysis, or 100 µL of LC-MS-grade ACN/H2O (90:10) for HILIC analysis.

5 QC

5.1 The QC corresponds to the sample pool, and can be carried out at different stages of the manipulation. If the matrix is in liquid form, it is preferable to carry out the QC before handling, and to treat the QC pool as a sample.

In the case of a solid matrix, QC pooling can be carried out when the organic phase is sampled.

Caution: Allow for a sufficiently large volume of QC to be injected throughout the sequence, and plan for possible re-injections.

6 **Blank**

6.1 A "handling blank" can be carried out to assess the contamination caused by the handling. The handling blank follows the same protocol as that for the samples, the only difference being that the matrix is replaced by water.

7 **Preparing the mobile phase (PM)**

7.1 Preparation of mobiles phases:

- For RPLC analysis: H2O/ACN (95/5)
- For HILIC analysis: H2O/ACN (10/90)

III. Analytical conditions

8 **Instrument set-up**

Create a randomized injection sequence incorporating QC injection every five samples.

Typical sequence :

. 3*Phase Mobile (PM)
. 2* Mix metabo 2
. White handling
. 6* QC
. 5* samples
. 1 QC
. 5* samples
...
. QC
. Metabo 2 mix
. 3* PM

Instruments used :

- LC-HRMS : Mass spectrometer QExactive - Orbitrap - Thermo Fisher
- LC-HRMS : Mass spectrometer Exactive - Orbitrap - Thermo Fisher
- LC-QTOF : Mass spectrometer Synapt Acquity - TOF - Waters

9 LC-HRMS instrument : LC-QExactive

9.1 *RPLC analysis*

Solvents:

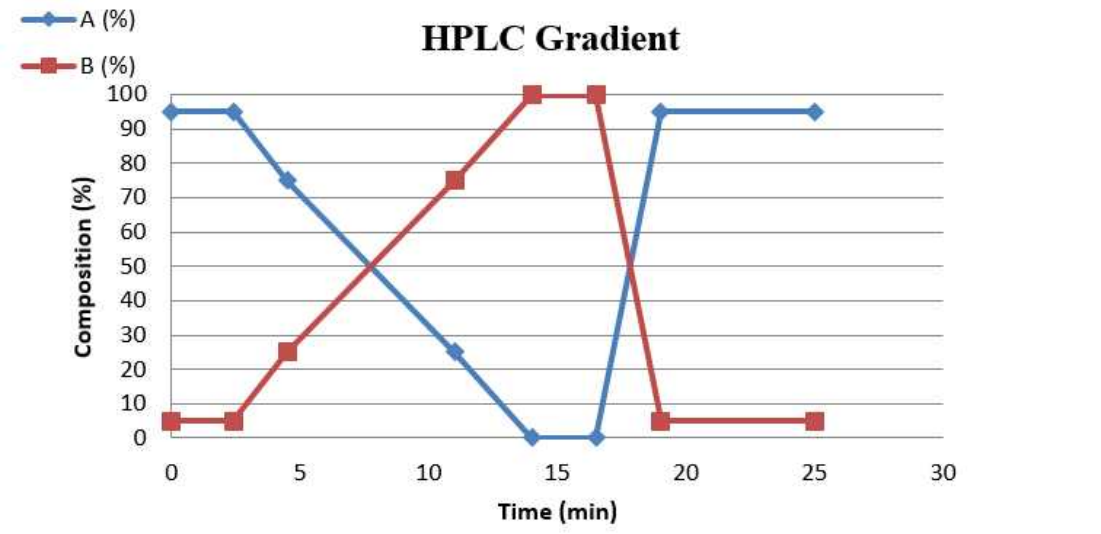
- Solvent A: 1 L H2O, 1 mL glacial acetic acid
- Solvent B: 1 L ACN, 1 mL glacial acetic acid
- Washing solutions depending on equipment type

Gradient:

This is a typical gradient, which can be re-evaluated depending on the type of analysis performed.

	Time (min)	A (%)	B (%)
	0	95	5
	2.4	95	5
	4.5	75	25
	11	25	75
	14	0	100
	16.5	0	100
	19	95	5
	25	95	5

Elution gradient



Wash solution:

Rinse bottle N°1 H2O LC-MS; rinse bottle N°2 H2O-MeOH or H2O-ACN (20 :80).
Q-Exactive: H2O-MeOH or H2O-ACN (95:5). ACN and methanol solvents are HPLC grade, Milli-Q grade water and LC-MS grade isopropanol

Column:

The column used is a **Hypersil Gold C18 (1.9µm, 100mm*2.1mm)**, Thermo Scientific. This column is used with a pre-column whose filter is changed regularly. Flow rate is 0.4 mL/min, oven temperature 35 °C .
The Hypersil Gold C18 column is reconditioned before storage in ACN/H2O (80:20).

Tune parameters:

These are typical parameters, which can be re-evaluated according to the type of analysis performed.

	A	B
	Sheath gas (L/h)	55
	Auxiliary gas (L/h)	10
	Sweep gas (L/h)	0
	Capillary temperature (°C)	350
	Capillary voltage (V)	(+): 30 ; (-): -30
	Tube lens voltage (V)	(+): 100 ; (-): -80

Injection on Thermo brand instruments, for both ionization modes

9.2 *HILIC analysis*

Solvents:

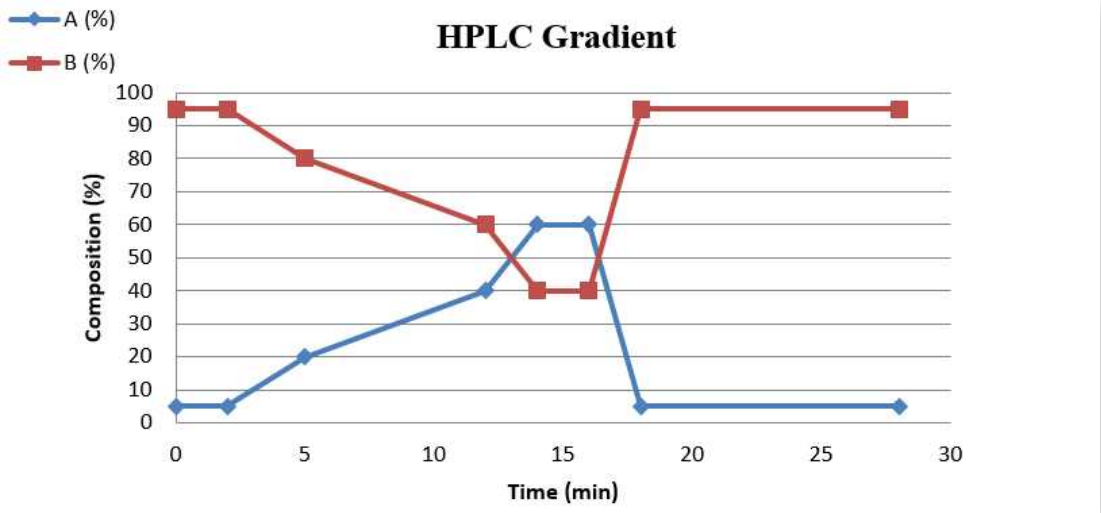
- Solvent A: 990 mL H2O, 10 mL 1000 mM ammonium acetate
- Solvent B: 990 mL ACN, 10 mL 1000 mM ammonium acetate
- Washing solutions depending on equipment type

Gradient:

This is an example of a gradient, which can be re-evaluated depending on the type of analysis performed.

	Time (min)	A (%)	B (%)
	0	5	95
	2	5	95
	5	20	80
	12	40	60
	14	60	40
	16	60	40
	18	5	95
	28	5	95

Elution gradient



Wash solution:

Rinse bottle N°1 H2O LC-MS; rinse bottle N°2 H2O-MeOH or H2O-ACN (20 :80).
Q-Exactive: H2O-MeOH or H2O-ACN (95:5). ACN and methanol solvents are HPLC grade, Milli-Q grade water and LC-MS grade isopropanol

Column:

The column used is a **SeQuant ZIC HILIC (5µm, 100mm*2.1mm)**, Merck KGaA. This column is used with a pre-column whose filter is changed regularly. Flow rate is 0.25 mL/min, oven temperature 25°C.
The ZIC HILIC column is reconditioned before storage in ACN-H2O (80:20).

Tune parameters:

These are typical parameters, which can be re-evaluated according to the type of analysis performed.

	A	B
	Sheath gas (L/h)	55
	Auxiliary gas (L/h)	10
	Sweep gas (L/h)	0
	Capillary temperature (°C)	350
	Capillary voltage (V)	(+): 30 ; (-): -30
	Tube lens voltage (V)	(+): 100 ; (-): -80

Injection on Thermo brand instruments, for both ionization modes

	A	B
	Source temperature (°C)	120 to 150
	Sampling cone (V)	30 to 40
	Source offset (V)	80
	Source gas flow (mL/min)	50
	Desolvatation temperature (°C)	650
	Cone gas flow (L/h)	400
	Desolvatation gas flow (L/h)	800

Injection on Synapt (Waters) type instruments, in positive ionization mode

Remark:

The use of solvents containing salts can lead to clogging of the tubing. To avoid this effect, perform several injections at the end of the sequence without heating the probe (HESI = 50°C). In addition, recondition the column with salt-free solvents, taking care that the reconditioning solvents also pass into the source and not into the waste garbage can.

10 **Calibration**

10.1 Thermo Solution: ESI+ (Reference : 88322) et ESI- (Reference : 88324)
Calibration to be carried out each time you start using the project
Cone and capillary cleaning according to instrument

11 **Acquisition software**

11.1
- QExactive: Xcalibur (version 4.4) and processing: Chromeleon
- Exactive: Xcalibur (version) and processing: Aria

Citations

Step 2.2

Jacob CC, Dervilly-Pinel G, Deceuninck Y, Gicquiau A, Chevillon P, Bonneau M, Le Bizec B. Urinary signature of pig carcasses with boar taint by liquid chromatography-high-resolution mass spectrometry.
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