Integrated Platform including Automated Bligh and Dyer Extraction and Dual-Column UHPLC-MS/MS Separations for Metabolomic Analyses of Tissues and Cells

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Overview

- Automated Bligh and Dyer extraction for metabolomic studies.
- Dual-column UHPLC setup for the analysis of the polar and lipidic fractions.
- Alternating acidic and basic mobile phase for the separation of the polar fraction.
- Identification of unknown compounds by SWATH HR MS^2 spectra acquisition.

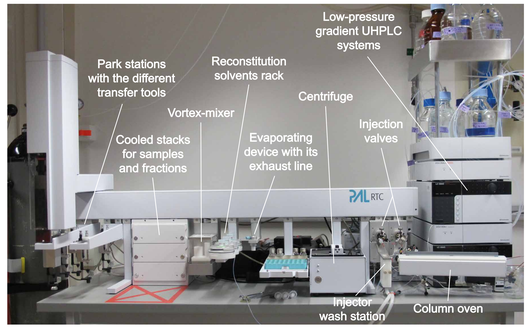
Introduction

Sample preparation workflows for metabolomic studies of tissues or cells require most of the time a Bligh and Dyer extraction or one of its variant (*e.g.* the Folch extraction). This step is cumbersome and generally performed manually in order to separate the aqueous fraction containing polar endogenous metabolites from the organic fraction containing apolar compounds like lipids. Proteins remain at the interface of the two solvents.

Here we propose to integrate an automated Bligh and Dyer extraction on a robotic system including a dual-column UHPLC-MS/MS platform for the metabolomic analysis of tissues or cells. The aqueous fraction is split and analysed sequentially at two different mobile phase pH values, whereas the lipidic fraction is analysed alternately with an extended gradient.

Instrumentation

Automated Sample Preparation Platform



The RTC robot (CTC Analytics) was equipped with several modules as shown on the picture above.

Dual-column UHPLC-MS Instrumentation

For chromatographic separation of the B&D fractions, two quaternary low-pressure Nexera LC30AD UHPLC pumps (Shimadzu) were used. The polar fractions were diluted on-line by the RTC robot and injected onto a 100 x 2.1mm XBridge BEH C18 XP column (Waters) running alternately with an acidic or a basic mobile phase. The organic fractions were evaporated to dryness, reconstituted and injected onto a 150 x 2.1 mm XBridge BEH C8 XP column. Both columns were kept at a temperature of 40 °C in a HotDog XL5090 oven (Prolab).

Mobile phases for C18 separations were: A) 5 mM NH_4FA + 0.1% FA (pH 3.0), B) ACN + 0.1% FA, C) 0.025% NH_4OH (pH 8.3 adj. w/ FA), D) ACN + 0.0125% NH_4OH.
Mobile phases for C8 separation were: A) 5 mM NH_4Ac + 0.1% AA (pH 4.2), B) ACN + 0.1% AA.
Gradient was linear from 0-100% in 10 min. for C18 separations (both pH) and in 15 min. for C8 separation with washing steps of 3 and 4.5 min., respectively.

The dual-column UHPLC platform was hyphenated to a TripleTOF 5600 MS (AB Sciex) by means of a Valco-VICI switching valve controlled by contact closure from the respective pumps. MS and MS/MS data were acquired in positive ESI polarity using a Turbo V ion source equipped with an APCI probe for automated calibration (CDS device, AB Sciex). SWATH MS/MS acquisition mode was performed with 24 variable Q_1 windows (15-50 u) and a TOF mass range of 50-800 u (polar fractions) and 50-950u (lipidic fractions) with accumulation time of 30 ms. Collision energy was ramped from 20 to 60 V. MS duty cycle was of ca. 0.85 s.

Software

The RTC robot was controlled by PAL Sample Control v. 2.1 (CTC Analytics).

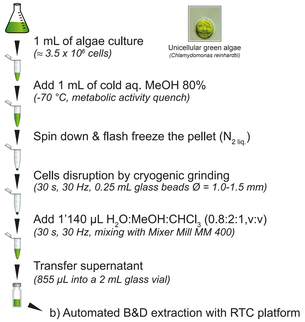
The two low-pressure gradient UHPLC systems were controlled by LabSolutions v. 5.6 SP2 (Shimadzu) by means of two independent hardware configurations.

The TripleTOF 5600 MS was controlled by Analyst TF 1.6 (AB Sciex) and data processing was done with PeakView v. 2.0 including the MasterView plug-in as well as MultiQuant v. 2.1 (AB Sciex).

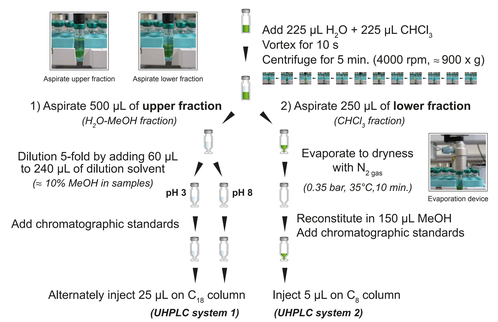
Plots were done with Tableau Desktop Professional v. 8.1 (Tableau Software) or Excel 2010 (Microsoft Office Professional Plus 2010).

1 Sample preparation workflow for Bligh and Dyer extraction

a) Manual off-line preliminary steps



b) Automated on-line sample preparation with RTC platform



2 Analysis of the upper H_2O-MeOH fraction *(UHPLC system 1)*

On-line sample dilution with the RTC robot

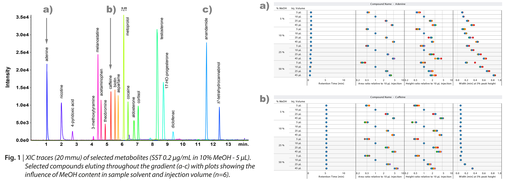


Fig. 1 | *XIC traces (20 mmu) of selected metabolites (SST 0.2 µg/mL in 10% MeOH - 5 µL). Selected compounds eluting throughout the gradient (a-c) with plots showing the influence of MeOH content in sample solvent and injection volume (n=6).*

For polar compounds (*e.g.* plot a), peak width increases when injecting more than 5 µL, but area ratio remains adequate up to 10% MeOH in the sample solvent even with an injection volume of 45 µL.

For lipophilic compounds (*e.g.* plot c), peak width remains at 0.1 min. with large injection (45 µL), however higher organic content in the sample solvent is required (solubility).

The water-methanol Bligh and Dyer fraction contains ≈ 50% MeOH, thus an **on-line dilution** is necessary to lower the organic content in the sample solvent. Different dilution factors, with their adapted injection volumes to keep the same amount injected on-column, were tested with the RTC robot.

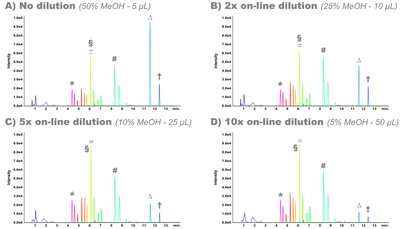


Fig. 2 | *XIC traces (20 mmu) of selected metabolites (SST 0.2 µg/mL in 50% MeOH). A) No dilution - 5 µL injected, B) 2-fold dilution - 10 µL injected, C) 5-fold dilution - 25 µL injected, D) 10-fold dilution - 50 µL injected. Plots on the right show the on-line dilution results for the selected compounds labeled on the XIC chromatograms (n=6).*

Alternating pH of mobile phases

The upper B&D fraction is analyzed at two different mobile phase pH (*i.e.* pH 3.0 and pH 8.3) to improve the retention of certain polar compounds (*e.g.* adenine, nicotine). When alternating the mobile phase pH, the column reconditioning time for the basic pH is critical to have a stable system. Thus, **injection scheme B** (*i.e.* alternating every injection) **was preferred**.

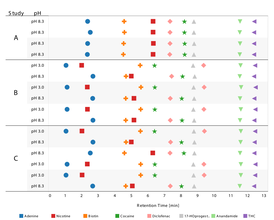
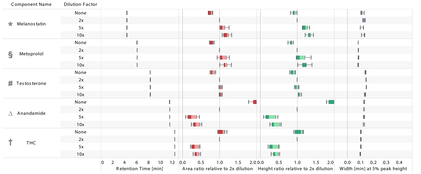


Fig. 3 | *Retention times of selected compounds (SST 0.2 µg/mL) for three injection schemes: A) Only pH 8.3 mobile phase, B) Mobile phase pH was alternated every injection, C) Mobile phase pH was alternated every second injection.*



For highly polar compounds (*e.g.* adenine, nicotine), these experimental conditions (*i.e.* high organic content or large injection volume) were detrimental to their peak shape.

For lipophilic compounds (*e.g.* Δ, †), losses were observed due to their poor solubility in mostly aqueous solvent (*i.e.* 5 or 10% MeOH).

For other compounds (*e.g.* *, §, #), consistent results were observed across the dilution factors.

3 Analysis of the lower organic fraction *(UHPLC system 2)*

Automated evaporation to dryness

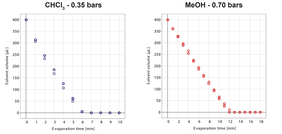


Fig. 4. | *Evaporation plots by a gentle N_2 flow at 35°C (n=3).*

The automated evaporation of 400 µL in 1.2 mL vase vials was achieved in **less than 7 min.** or 14 min. for **$CHCl_3$** or MeOH, respectively.

Distribution of the compounds after B&D extraction

After evaporation, different reconstitution volumes (*i.e.* 100, 150, 200 µL) of MeOH were tested for the organic fraction (injecting 5 µL on the C_8 column). 150 µL were found to sufficiently concentrate the sample and chosen for further experiments.

The automated B&D extraction lead to efficient separation of polar from unpolar metabolites (figure 5).

Only few compounds with amphiphilic properties were retrieved in both fractions (*e.g.* metoprolol). These results are in accordance with comparative analyses of manual extracted SST test mix.

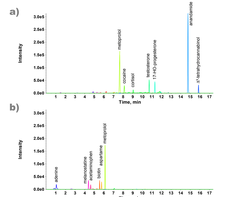


Fig. 5 | *Representative XIC traces (20 mmu) of selected metabolites (SST 0.2 µg/mL) separated by automated Bligh & Dyer extraction (n=3).*
a) Analysis of the organic fraction recon in 150 µL MeOH on C_8 column (inj. 5 µL)
b) Analysis of the 5x diluted aqueous fraction at pH 3 on C_{18} column (inj. 25 µL)

4 Comparison of automated *vs.* manual Bligh and Dyer extraction

To compare variation and repeatability of the automated Bligh & Dyer extraction via the RTC platform with the common manual procedure, n=5 extractions of *Chlamydomonas reinhardtii* algae were performed, respectively. From the analyses of aqueous (AQ) and organic (ORG) fractions, ca. 20 variables were selected randomly by means of Marker View and/or Peak View software (only monoisotopic peaks with S/N > 30 were considered) and coefficients of variation (CV) were calculated for the corresponding peak areas.

Lower variation (CV < 22%) and, thus, better repeatability was obtained with the automated approach throughout all experiments as shown in figure 6. This result was especially pronounced for the LC-MS runs of AQ fractions at pH 8 and of the ORG fractions.

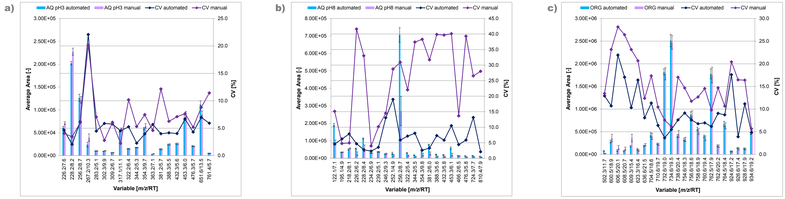


Fig. 6 | *Column diagrams showing the peak areas of selected variables (defined by m/z and retention time, RT) as well as their coefficients of variation obtained after analysis of the aqueous (AQ) and organic (ORG) B&D fractions from the automated or the manual extraction procedure (n=5): a) AQ fractions at pH 3 - C_{18} column, b) AQ fractions at pH 8 - C_{18} column, c) ORG fractions (pH 4) - C_8 column.*

5 Unknowns identification with data independent MS/MS acquisition

LC-MS/MS data from automated and manual B&D extractions of algae were acquired in SWATH mode enabling the generation of data independent MS/MS information due to dynamic Q1 windows.

The results could, thus, directly be subjected to a library search without the need of performing further targeted MS^2 experiments. Using both an in-house SWATH-based library and other commonly known spectral libraries (*e.g.* MassBank), certain unknowns could quickly be identified.

As an example figure 7 shows the experimental and theoretical LC-MS data of adenosine which was identified from analyses of AQ fractions at pH 8 (automated approach).

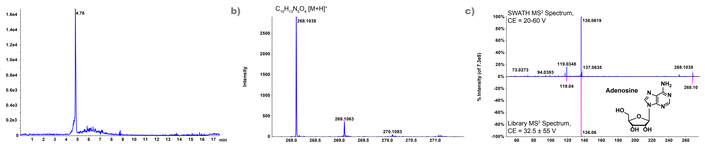


Fig. 7 | *Exemplary result after searching the in-house SWATH library for unknowns. Adenosine was identified in pH 8 analyses of the AQ algae fractions. In a) the corresponding XIC trace is shown. Validation of the isotopic pattern (Formula Finder) and obtained SWATH MS2 spectrum at 4.76 min. are depicted in b) and c), respectively.*

Conclusions

- Reducing the MeOH content in AQ fractions by on-line dilution enables better chromatographic resolution and peak shape.
- Alternating mobile phase pH is essential for improved retention and peak shape for more basic compounds.
- Automated Bligh & Dyer extraction shows similar efficiency, but improved repetability compared with the manual approach.
- Identification of unknowns is readily feasible due to SWATH-based acquisition following library search.