Automation Arrives:
Fully integrated automated Bligh and Dyer extraction and dual-column analysis for metabolomics analyses of tissues and cells
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Metabolomic studies provide us with key insights into biological systems and can help improve our understanding of the basis of a disease, elucidate the mechanism of action of a drug, and inform the development of relevant biomarkers. In practice, for metabolites to be studied, they need to be effectively extracted from a sample, a process that can be challenging due to their broad physico-chemical diversity and the potentially large spectrum of concentrations of different metabolites in a single sample.

Liquid-liquid extraction is often used to extract metabolites. However the solvents employed as well as the experimental practice must be carefully considered in order to mitigate any risk affecting the quality of the resulting extract. Bligh and Dyer extraction, or one of its variants, such as Folch extraction, has been used for many years and is generally considered an efficient method for the extraction of lipids and polar endogenous metabolites from tissues and cells. However, it is still performed manually, and is a laborious task that is becoming increasingly out of place as laboratories move to automated analysis and handle large sets of samples.

In this article the authors describe recent work that details a fully automated Bligh and Dyer extraction and dual-column UHPLC-MS/MS separation for metabolomic analyses of tissues and cells, and compares the new procedure to a traditional manual method.

Advancing processes

Laboratories are constantly challenged to push the limits of analysis. New and improved measurement technology drives detection limits and time to result down. As a consequence, bottlenecks and variability stemming from sample prep have come under the spotlight. Robotic approaches to sample prep are now routine but ‘smart sample prep’ where robotics interfaces directly to an analyzer, and software integration is also achieved, are much less common. In many laboratory settings automation has proven effective in increasing efficiency and improving repeatability. Streamlining workflow in this way not only increases consistency but allows scientists to devote more time to operations that require their unique skills and experiences.

Full integration

Sample preparation workflows for metabolomic studies of tissues and cells often require a liquid-liquid extraction (LLE), which takes advantage of differing distribution coefficients to enrich metabolites and to separate them from undesired compounds. Now, an automated Bligh and Dyer extraction on a robotic system has been fully integrated with a dual-column ultra high-pressure liquid chromatography tandem mass spectrometer (UHPLC-MS/MS) platform for the metabolomics analysis of tissues and/or cells (Figure 1).
Instrumentation and software set-up

For the LC-separation of the Bligh and Dyer fractions, two quaternary low-pressure Nexera LC30AD UHPLC pumps (Shimadzu) were used. A PAL RTC robot (CTC Analytics) performed the sample prep (as outlined in figure 2), and injected the resulting aqueous fractions onto a 100 x 2.1 mm XBridge BEH C18 XP column (Waters) running alternately with an acidic or a basic mobile phase. The organic (CHCl$_3$) fractions were evaporated to dryness, reconstituted and injected onto a 150 x 2.1 mm XBridge BEH C8 XP column. Both columns were maintained at 40°C.

The dual-column UHPLC platform was hyphenated to a TripleTOF 5600 MS (AB Sciex). Mass spectrometry and tandem mass spectrometry data were acquired in positive electrospray ionization (ESI) mode using a Turbo V ion source equipped with an APCI probe. To complement the hardware configuration and allow for true integration the PAL RTC robot was controlled by PAL Sample Control v 2.1 software (CTC Analytics). This interface was linked with the LabSolutions v 5.6 SP2 software (Shimadzu) and used to control the UHPLC system.

Sample preparation

Figure 2 shows the sample preparation workflow for automated Bligh and Dyer extraction. The method proved to be far more streamlined than conventional sample preparation. Importantly, scientists only had to perform a few manual steps before loading samples onto the PAL RTC robot: initially 1 mL of cold MeOH was added to the raw samples, which were then spun ahead of flash freezing and cryogenic grinding. After the addition of a solution of H$_2$O:MeOH:CHCl$_3$ the sample was then transferred to the PAL RTC robot where it underwent the automated Bligh and Dyer extraction.

The RTC platform performed all the necessary extraction steps, splitting the fractions and adding the correct volumes and concentrations of the appropriate reagents and chromatographic standards as needed. The method proved significantly less labor intensive and reduced the overall sample preparation time.

Analysis of the upper H$_2$O:MeOH fractions was then conducted on UHPLC system 1 with on-line dilution and standard addition by the RTC robot and alternating the pH of the mobile phases. It was found that for polar compounds the peak width increased for injections more than 5 µL. However area ratio remained adequate with up to 10% MeOH in the sample solvent, even with an injection volume of 45 µL. For lipophilic compounds peak width remained at 0.1 min with large injections of 45 µL, but higher organic content in the sample solvent was required.

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**a) Manual off-line preliminary steps**

1 mL of algae culture

Add 1 mL of cold aq. MeOH 80%

Spin down & flash freeze the pellet (N$_2$ gas)

Cells disruption by cryogenic grinding

Add 1‘140 µL H$_2$O:MeOH:CHCl$_3$ (0.8:2:1, v:v)

Transfer supernatant

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**b) Automated on-line sample preparation with RTC platform**

Aspirate upper fraction

Aspirate lower fraction

Add 225 µL H$_2$O + 225 µL CHCl$_3$

Vortex for 10 s

Centrifuge for 5 min. (4000 rpm, $= 900 \times g$)

1) Aspirate 500 µL of upper fraction

Dilution 5-fold by adding 60 µL to 240 µL of dilution solvent

Add chromatographic standards

Alternate injection 25 µL on C$_{18}$ column

(2) Aspirate 250 µL of lower fraction

(2) Aspirate 250 µL of lower fraction

Evaporate to dryness with N$_2$ gas

(0.35 bars, 35°C, 10 min.)

Reconstitute in 150 µL MeOH

Add chromatographic standards

Inject 5 µL on C$_8$ column

(UHPLC system 1)

(UHPLC system 2)

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Figure 2: Automated sample preparation workflow for Bligh and Dyer extraction
An on-line dilution was necessary to lower the organic content in the sample solvent as the \( \text{H}_2\text{O-MeOH} \) Bligh and Dyer fraction contains 50% MeOH. Different dilution factors, with their adapted injection volumes kept the same amount injected on-column, were tested with the PAL RTC robot.

The upper (aqueous) fraction was analyzed with two different mobile phases (pH 3.0 and pH 8.3) to improve the retention of certain polar compounds, such as adenine and nicotine. When the mobile phases were alternated, the column reconditioning time for the basic pH was critical to ensure the system remained stable. Therefore injection scheme B, as shown in Figure 4 was preferred as each injection was alternated.

For highly polar compounds, high organic content or a large injection volume were detrimental to their peak shape. Conversely, for lipophilic compounds, losses were observed due to poor solubility in mostly aqueous solvent, such as those including only 5 or 10% methanol. However for other compounds, consistent results were observed across the dilution factors.

The lower organic fraction was analyzed on UHPLC system 2. With the automated system, evaporation of 400 µL in 1.2 mL vial vials was achieved in less than seven minutes for \( \text{CHCl}_3 \), and less than 14 minutes for MeOH. After evaporation reconstituted volumes of 100, 150 and 200 µL of MeOH were tested for the organic fraction by injecting 5 µL on the column. It was found that 150 µL were sufficiently concentrated so this sample was chosen for further testing.

Only few compounds with amphiphilic properties, such as metoprolol, were retrieved in both fractions. These results are in agreement with comparative analyses of manually extracted samples. In order to assess the repeatability of the automated Bligh and Dyer extraction process with the RTC platform manual procedures were performed, which also allowed for the comparison of any variation between the two methods. It was found that lower variation and therefore better repeatability was achieved with the automated method throughout all experiments conducted, as shown in Figure 5.

By acquiring LC-MS/MS data in SWATH mode, the results could be directly subjected to a library search without the need of performing further targeted experiments, allowing for the fast identification and quantification of certain unknowns. This significantly reduced experimental time and resulted in faster processing of results.
Figure 4: 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 alternating every injection, C) Mobile phase pH alternating every second injection.

<table>
<thead>
<tr>
<th>Compound Name</th>
<th>Formula</th>
<th>MW (g/mol)</th>
<th>LogP</th>
<th>LogD</th>
<th>pKa (Acid) GALAS</th>
<th>pKa (Base) GALAS</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td>(pH=3.0)</td>
<td>(pH=8.3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td>pKa</td>
<td>Reliability</td>
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<td>Acetaminophen</td>
<td>C₈H₁₀NO₂</td>
<td>151.2</td>
<td>0.40</td>
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<td>Adenine</td>
<td>C₅H₅N₅</td>
<td>135.1</td>
<td>0.43</td>
<td>-1.10</td>
<td>9.9</td>
<td>0.4</td>
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<tr>
<td>Aldosterone</td>
<td>C₂₁H₃₀O₅</td>
<td>360.4</td>
<td>1.07</td>
<td>1.07</td>
<td>13.7</td>
<td>15.6</td>
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<td>Aspartame</td>
<td>C₉H₁₄N₄O₅</td>
<td>294.3</td>
<td>0.93</td>
<td>-1.73</td>
<td>2.4</td>
<td>11.1</td>
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<tr>
<td>Biotin</td>
<td>C₈H₁₀N₂O₂</td>
<td>194.2</td>
<td>0.28</td>
<td>0.28</td>
<td>0.28</td>
<td>0.28</td>
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<tr>
<td>Caffeine</td>
<td>C₈H₁₀N₂O₂</td>
<td>194.2</td>
<td>0.28</td>
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<td>0.28</td>
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<td>Cocaine</td>
<td>C₁₀H₁₄N₂</td>
<td>162.2</td>
<td>0.82</td>
<td>-2.92</td>
<td>0.37</td>
<td>0.37</td>
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<td>Cortisol</td>
<td>C₈H₁₀N₂O₂</td>
<td>320.5</td>
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<td>13.2</td>
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<td>Diclofenac</td>
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<td>17-Hydroxyprogesterone</td>
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<td>13.9</td>
<td>0.9</td>
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<td>Melanostatin</td>
<td>C₁₃H₁₄N₂O₃</td>
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<td>triacetic acid</td>
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<td>Metoprolol</td>
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<td>Nicotin</td>
<td>C₁₀H₁₄N₂</td>
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<td>0.82</td>
<td>-2.92</td>
<td>0.37</td>
<td>0.37</td>
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<tr>
<td>4-Pyridoxic acid</td>
<td>C₈H₁₀NO₂</td>
<td>183.2</td>
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<td>Testosterone</td>
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<td>Theobromine</td>
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<td>180.2</td>
<td>-0.34</td>
<td>0.34</td>
<td>9.9</td>
<td>0.4</td>
</tr>
</tbody>
</table>

Table 1: Calculated logP, logD and pKa for the SST compounds.
Calculated with ACD/Labs Percepta software Release 2012
In conclusion

As laboratories are striving to uncover more «unknowns» and increase our understanding of biological processes there is a drive for procedures to become more efficient and repeatable. This is also true for extraction procedures which when performed manually can be time intensive and cumbersome, taking the valuable time of scientists. The automated Bligh and Dyer extraction described here was found to not only be more time efficient, but also to improve repeatability and quality of extraction and separation when compared to the standard manual approach.

References

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