Electromembrane extraction and mass spectrometry for liver organoid drug metabolism studies

Frøydis Sved Skottvoll[a,b], Frederik Hansen[c], Sean Harrison[b], Ida Sneis Boger[a,b], Ago Mrsa[a,b], Magnus Saed Restan[c], Matthias Stein[d], Elsa Lundanes[a], Stig Pedersen-Bjergaard[c,e], Aleksandra Aizenshtadt[b], Stefan Krauss[b], Gareth Sullivan[b,f] , Inger Lise Bogen [g,h], Steven Ray Wilson[a,b*]

[a] Department of Chemistry, University of Oslo, P.O. Box 1033, Blindern, NO-0315 Oslo, Norway

[b] Hybrid Technology Hub-Centre of Excellence, Institute of Basic Medical Sciences, Faculty of Medicine, University of Oslo, P.O. Box 1112 Blindern, NO-0317 Oslo, Norway

[c] Department of Pharmacy, University of Oslo, P.O. Box 1068 Blindern, NO-0316 Oslo, Norway

[d] Institute of Medicinal and Pharmaceutical Chemistry, TU Braunschweig, Beethovenstr. 55, DE-38106 Braunschweig, Germany

[e] Department of Pharmacy, Faculty of Health and Medical Sciences, University of Copenhagen, Universitetsparken 2, 2100 Copenhagen, Denmark

[f] Department of Pediatric Research, Oslo University Hospital and University of Oslo, PO Box 1112 Blindern, 0317 Oslo, Norway

[g] Section for Drug Abuse Research, Department of Forensic Sciences, Oslo University Hospital, P.O. Box 4950 Nydalen, NO-0424 Oslo, Norway

[h] Institute of Basic Medical Sciences, Faculty of Medicine, University of Oslo, P.O. Box 1103 Blindern, NO-0317 Oslo, Norway

*Corresponding author: stevenw@kjemi.uio.no, +47 97010953. https://orcid.org/0000-0002-9755-1188

Abstract

Liver organoids (miniature, organ-like biomaterials derived from e.g. a patient´s stem cells) are emerging tools for precision drug development and toxicity screening. We demonstrate that electromembrane extraction (EME) is suited for collecting organoid-derived drug metabolites prior to mass spectrometry (MS)-based measurements. EME, which is essentially electrophoresis across an oil membrane, allowed drugs and drug metabolites to be separated from medium components (albumin, etc.) that could interfere with subsequent measurements. Multi-well EME (100 µL solutions) allowed for simple and repeatable monitoring of heroin metabolism kinetics. Organoid EME extracts were compatible with ultrahigh-pressure liquid chromatography (UHPLC) and capillary electrophoresis (CE), used to separate the analytes prior to detection. These initial efforts show that organoids are well-matched with various electrophoresis/chromatography techniques and MS measurements.

Keywords: Organoids; Drug metabolism; Electromembrane extraction; Liquid chromatography; Mass spectrometry

Introduction

Organoids are three-dimensional tissue models derived from e.g. primary tissues, embryonic stem cells or induced pluripotent stem cells (iPSC) [1-3]. These "mini" organs are emerging tools for studying human development and disease, serving as alternatives to e.g. animal models [4,5]. A wide variety of organoids are being developed and studied, e.g. brain, heart, tumor tissue and liver [6-9]. Liver organoids can be valuable models for studying drug metabolism and toxicity, perhaps even in a personalized fashion, as organoids can be derived from the cells of a patient. However, there are few studies dedicated to organoid drug metabolism using mass spectrometry [10] which is a central tool in small drug determination (in pharmacokinetics, forensics, etc.). Our goals have been to: develop and apply analytical approaches that are suited for liver organoids (**Figure 1, upper right**), minimize contaminations from e.g. medium (can contain 10 % fetal bovine serum), and assess the potential for advancement to online systems. For extracting drugs, and the metabolites produced by organoids, we have applied electromembrane extraction (EME, **Figure 1, left**). EME is essentially an electrophoretic separation across an oil membrane [11,12]. EME is highly successful separating small drugs from e.g. macromolecules, even in whole blood [13]. EME has recently advanced to the 96-well plate format [14-16] (**Figure 1, lower right**) and chip systems [17,18]. We demonstrate that EME is suited for studying drug metabolism in small organoid samples. We also show that EME organoid extracts are compatible with several variants of separation techniques and mass spectrometric measurements.

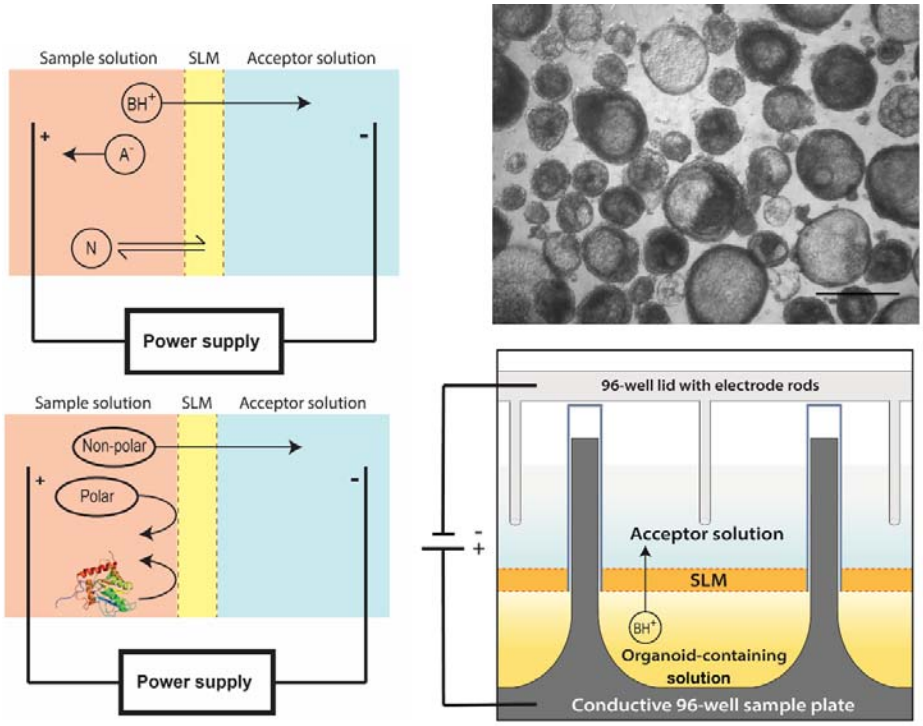

Figure 1. Left: EME principle. Charged analytes migrate from the sample solution across the supported liquid membrane (SLM) and into the acceptor solution. Extraction selectivity is obtained by voltage polarity and partitioning into and through the SLM. Polar molecules and macromolecules are effectively discriminated from extraction by the hydrophobic SLM. Upper right: Light microscope picture of iPSC derived liver organoids used in this study, scale bar 500 µm. Lower right: 96-well-format EME illustration.

Experimental

Chemicals and materials

2-Nitrophenyl octyl ether (NPOE), 2-nitrophenyl pentyl ether (NPPE), bis(2-ethylhexyl) hydrogen phosphite (DEHPi), bis(2-ethylhexyl) phosphate (DEHP), sodium hydroxide, ammonium formate (>99%), formic acid (FA, reagent grade 95%), were purchased from Sigma Aldrich (St. Louis, MO, USA). Ammonium hydroxide and acetonitrile (ACN, LC-MS grade) were purchased from VWR (Radnor, PA, US). Chromasolv methanol (LC-MS grade) was from Honeywell Riedel-de Haën (Seelze, Germany). Heroin HCl, 6-monoacetylmorphine (6-MAM) HCl and morphine were purchased from Lipomed AG (Arlesheim, Switzerland). Heroin-d9, 6-MAM-d6 and morphine-d3 were purchased from Cerilliant (Austin, TX, USA). Unless otherwise stated, the water used was type 1 water purified by a Direct-Q® water purification system from Millipore (Billerica, MA, USA).

106

107 **Organoids**

108 iPSC line AG27 was differentiated to form liver organoids as described by Harrison et
109 al. (manuscript in preparation). iPSC cell lines HPSI1213i-babk_2 and HPSI0114i-
110 vabj_3 were differentiated toward liver organoids using media from protocol by Ang
111 et al.[19], with modifications by Aizenshtadt et al. (manuscript in preparation). After 20
112 days differentiation, 50-60 organoids per well were treated with 10 or 50 µM heroin in
113 cell medium for 1, 3, 6, and 24 hours respectively (n=3), in separate 96-well plates.
114 Heroin HCl was first dissolved in 0.9% NaCl prior to dilution in cell medium.
115 Metabolism was stopped by adding FA to a final concentration of 0.11 M, and the
116 plates were frozen at -80 °C. In parallel, cell medium free from organoids (n=3) were
117 used as drug degradation control samples.

118

119 **EME-set-up**

120 The extraction setup and procedure have previously been described by Restan et al.
121 [16]. Prior to the extraction, 50 µL of the heroin-exposed liver organoid samples
122 (containing 0.11 M FA) was added to 40 µL water and 10 µL of the internal standard
123 solution containing morphine-d3, 6-MAM-d6 and heroin-d9, each at 1.5 µM. The
124 samples were then loaded into the wells of an in-house built 96-well stainless-steel
125 plate. Prior to this, 3 µL DEHP/NPOE (10/90, w/w) was immobilized into the
126 membrane pores (polyvinylidene fluoride, PVDF, 0.45 µm pore size) of a 96-well
127 MultiScreen-IP filter plate from Merck Millipore Ltd. (Carrigtwohill, Ireland). The steel
128 and filter plates were subsequently clamped together and 100 µL 10 mM ammonium
129 formate pH 3.1 was loaded into each well of the filter plate, and thus constituting the
130 acceptor solution. A conductive in-house built aluminum lid with 96 electrode rods
131 was placed onto the filter plate, and the whole construct was placed on a shaking
132 board. The steel plate holding the organoid solution was connected to the anode of
133 an external power supply, while the acceptor electrode lid was connected to the
134 cathode. Simultaneous extraction of all samples was performed for 15 minutes, at
135 900 rpm agitation, with 30 V applied for the first two minutes and 50 V applied for the
136 remaining extraction duration.

137

138

139

UHPLC-MS

Determination of heroin, 6-MAM and morphine was performed using UHPLC-MS based on a previously described method [20]. The sample extracts were analyzed using an Acquity™ UHPLC pump coupled to a Xevo TQ (triple quadrupole) MS with and electrospray ionization interface, all from Waters (Milford, MA, USA). Separation was achieved using the Acquity UPLC® HSS T3 C18 column (2.1 x 100 mm, 1.8 µm particles). Solvent A consisted of 10 mM ammonium formate buffer pH 3.1 and solvent B consisted of methanol. The sample injection volume was set to 7.5 µL, and the gradient elution was carried out at a flow rate of 0.5 mL/min at 65°C. MS-detection was performed in positive mode using multiple reaction monitoring. Data was acquired and processed using MassLynx 4.1 software (Waters).

Capillary electrophoresis

The CE separations with data handling were carried out using a 7100 CE instrument equipped with an on-column diode-array detector and a CE Chemstation software from Agilent Technologies (Waldbronn, Germany). Separations were performed using fused-silica capillaries from PolymicroTechnologies (Phoenix, AZ, USA), with L_{tot} = 59 cm and L_{eff} = 51 cm, 75 µm ID and 360 µm outer diameter (OD). The background electrolyte (BGE) consisted of 30 mM ammonium formate pH 8. Before each injection, the capillary was rinsed at a pressure of 1000 mbar with 0.1 M NaOH and water (0.5 min each), followed by the BGE (3 min). Injections were performed by stacking at 50 mbar: starting with diluted BGE (9 +1) for 3 s, followed by the sample injection (15 s, equivalent to around 107 nL) and the BGE (3 s). Separations and measurements were performed with an applied potential of +30 kV (25°C) and at an UV-absorbance of 214 nm.

Results and Discussion

In this study, analytical approaches were applied for liver organoid drug measurements. With the future objective of advancing to online analyses, EME was assessed for the clean-up of analytes from the organoid cell medium, a method previously shown to enable selective and fast extraction from complex matrices (and also on-chip) [21].

EME of heroin and metabolites

For testing the potential of EME-MS for analysis of liver organoids, heroin was chosen as a model substance, due to its familiar phase I metabolism to 6-MAM and morphine in the liver (**Figure 2**). Therefore, multi-well EME conditions focusing on these three compounds were assessed. Best recovery and repeatability for both standard solution and spiked cell medium samples were obtained using an EME system comprising 10 % (w/w) DEHP/NPOE as SLM, an extraction time of 15 minutes, and an extraction voltage of 50 V. With these parameters, the extraction current was <50 µA per well throughout the extraction. For increasing accuracy, correction for non-exhaustive extractions was done by spiking samples with isotopically labelled internal standards prior to extraction.

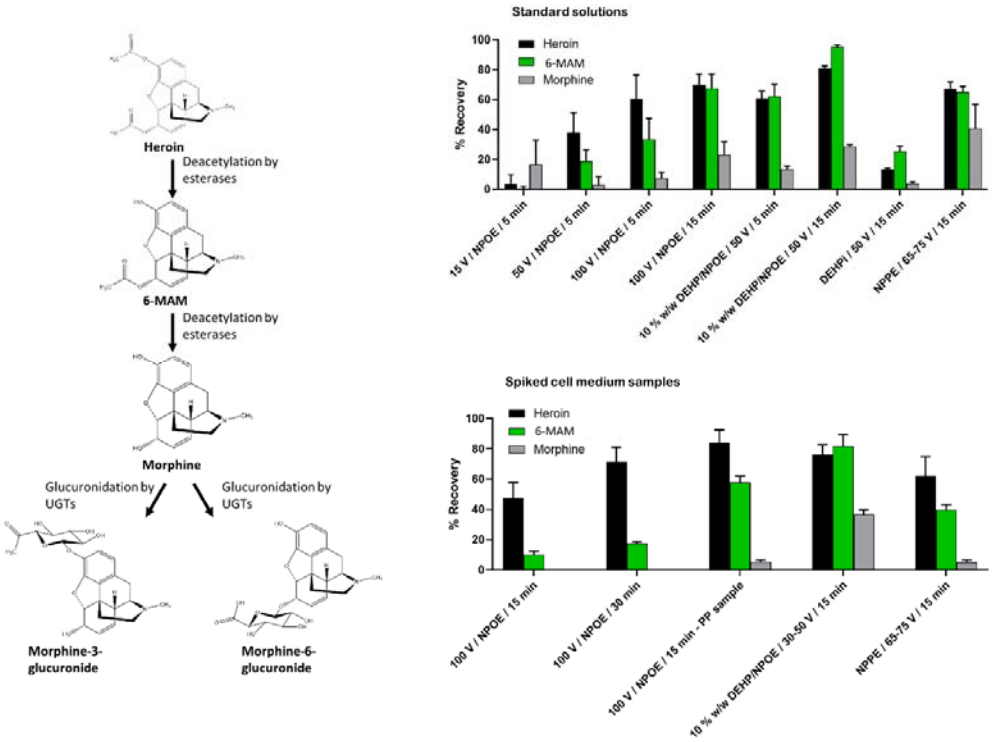

Figure 2. Left: Illustration of well-documented metabolites of heroin metabolism in the liver. In phase I metabolism, heroin is converted to 6-MAM and morphine, while familiar phase II metabolites are morphine glucuronides. Right: Analyte recovery (%) of multi-well EME under varying conditions, with standard solutions and spiked cell medium samples using CE-UV.

EME of organoids

Samples containing 50-60 liver organoids per well were exposed to 10 µM heroin. The extraction of analytes with EME was associated with satisfactory well-to-well

repeatability, see **Figure 3**. Heroin levels decreased with time to 6-MAM (both enzymatic and non-enzymatic), and with subsequent enzymatic metabolism to morphine, adding to the confirmation that the liver organoids had traits related to human livers (see **Figure S-1** for control samples). These trends were also similar to that observed with non-EME approaches, e.g. more manual sample preparation including centrifugation steps (tested with two organoid iPSC sources, **Figure S-2**). In accordance, using mass spectrometry-based proteomics, we could confirm a clear presence of the key liver enzymes human carboxyl esterases 1 and 2 (hCES1 and hCES2) in the organoids (see **Figure S-3**). However, the kinetics were (expectedly) substantially slower than that observed with e.g. microsomes and S9-fraction, see **Figure S-4**; it can therefore be speculated that direct measurements of drug metabolism can provide valuable insight when optimizing organoid development protocols. Although EME and MS are compatible with phase I metabolism monitoring, we were not able to observe phase II metabolites M3G/M6G. A key reason is a weakness of EME, that highly polar compounds have low recovery; this can in many cases be fine-tuned [22,23].

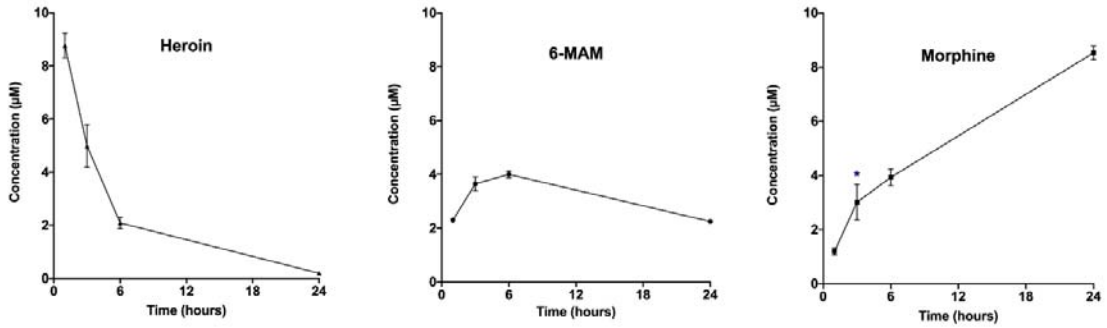

Figure 3. Liver organoid drug metabolism using multi-well EME and UHPLC-MS, studied with 50-60 organoids per well (n=3) for 1, 3, 6 and 24 hours. The asterisk indicates the removal of one data point due to poor internal standard signal.

Organoid EME extracts compatibility with various separation techniques

The organoid EME extracts were analyzed using UHPLC-MS instrumentation, which provided high resolution separations within 7 minutes. We have also investigated other separation approaches that can be compatible with small samples and online

action. NanoLC, a sensitive approach which has been mostly associated with proteomics in recent years, was seen to provide attogram detections some small molecule analytes such as heroin (results not shown). However, poor robustness was associated with nanoLC-MS analysis of more polar analytes, e.g. morphine. This was the case for both on-column injection and SPE-nanoLC. We also examined self-packed nano RPLC columns which were more compatible with highly aqueous mobile phases, but breakthrough/poor retention time repeatability was still an issue. Capillary electrophoresis, perhaps the most "chip-ready" of the techniques investigated, was capable of fast analysis of organoid EME extracts, here also demonstrated with simple UV detection (**Figure 4)**. However, organoid incubation in 50 µM heroin was needed in order to achieve detection with CE-UV.

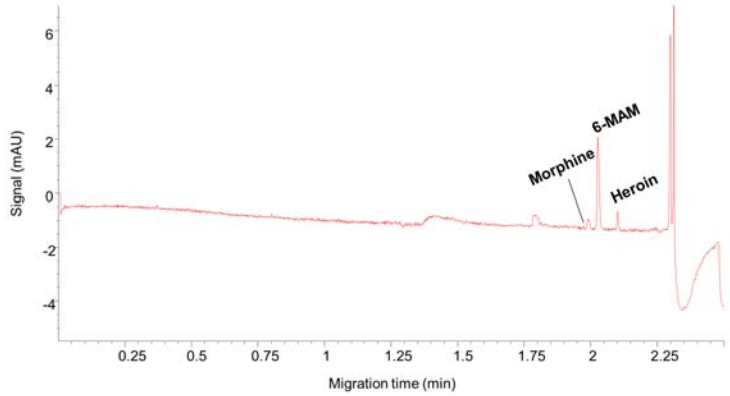

Figure 4. Electropherogram of the organoids´ analytes using simple CE-UV.

Conclusions

EME-MS is a promising concept for organoid analysis, here demonstrated for drug metabolism measurements. Following this proof-of-concept study, we will continue to develop EME and organoid analysis; a natural next step will be nanoliter-scale online EME-LC-MS of organoid samples. Related systems have been demonstrated with microsomes [18], but with larger separation columns, and arguably not suited for trace samples. Due to challenges with nanoLC, we will instead likely investigate the use of capillary LC or microbore LC, as a compromise between sensitivity and robustness. Disadvantages of the current EME settings are poor recovery of more polar metabolites. We are currently fine-tuning membrane solvents suited for such analytes.

Acknowledgements

This work was supported by the Research Council of Norway through its Centre of Excellence scheme, project number 262613. The work was also supported by the Olav Thon Foundation. Financial support from UiO:Life Science is also gratefully acknowledged. Technical assistance by Elisabeth Nerem (Department of Forensic Sciences, Oslo University Hospital, Oslo, Norway) was greatly appreciated.

References

(1) Lancaster, M. A.; Knoblich, J. A. *Science* **2014**, *345* (6194), 1247125.
(2) Clevers, H. *Cell* **2016**, *165* (7), 1586-1597.
(3) Rossi, G.; Manfrin, A.; Lutolf, M. P. *Nat. Rev. Genet.* **2018**, *19* (11), 671-687.
(4) Pampaloni, F.; Reynaud, E. G.; Stelzer, E. H. K. *Nat. Rev. Mol. Cell Biol.* **2007**, *8* (10), 839-845.
(5) Westmoreland, C.; Holmes, A. M. *Organogenesis* **2009**, *5* (2), 67-72.
(6) Lancaster, M. A.; Renner, M.; Martin, C.-A.; Wenzel, D.; Bicknell, L. S.; Hurles, M. E.; Homfray, T.; Penninger, J. M.; Jackson, A. P.; Knoblich, J. A. *Nature* **2013**, *501* (7467), 373-379.
(7) Stevens, K. R.; Kreutziger, K. L.; Dupras, S. K.; Korte, F. S.; Regnier, M.; Muskheli, V.; Nourse, M. B.; Bendixen, K.; Reinecke, H.; Murry, C. E. *Proc. Natl. Acad. Sci. U. S. A.* **2009**, *106* (39), 16568-16573.
(8) van de Wetering, M.; Francies, Hayley E.; Francis, Joshua M.; Bounova, G.; Iorio, F.; Pronk, A.; van Houdt, W.; van Gorp, J.; Taylor-Weiner, A.; Kester, L.; McLaren-Douglas, A.; Blokker, J.; Jaksani, S.; Bartfeld, S.; Volckman, R.; van Sluis, P.; Li, Vivian S. W.; Seepo, S.; Sekhar Pedamallu, C.; Cibulskis, K., et al. *Cell* **2015**, *161* (4), 933-945.
(9) Huch, M.; Dorrell, C.; Boj, S. F.; van Es, J. H.; Li, V. S. W.; van de Wetering, M.; Sato, T.; Hamer, K.; Sasaki, N.; Finegold, M. J.; Haft, A.; Vries, R. G.; Grompe, M.; Clevers, H. *Nature* **2013**, *494* (7436), 247-250.
(10) Lin, A.; Sved Skottvoll, F.; Rayner, S.; Pedersen-Bjergaard, S.; Sullivan, G.; Krauss, S.; Ray Wilson, S.; Harrison, S. *Electrophoresis* **2020**, *41* (1-2), 56-64.
(11) Pedersen-Bjergaard, S.; Rasmussen, K. E. *J. Chromatogr. A* **2006**, *1109* (2), 183-190.
(12) Drouin, N.; Kubáň, P.; Rudaz, S.; Pedersen-Bjergaard, S.; Schappler, J. *TrAC, Trends Anal. Chem.* **2019**, *113*, 357-363.
(13) Gjelstad, A.; Rasmussen, K. E.; Pedersen-Bjergaard, S. *Anal. Bioanal. Chem.* **2009**, *393* (3), 921-928.
(14) Eibak, L. E. E.; Rasmussen, K. E.; Øiestad, E. L.; Pedersen-Bjergaard, S.; Gjelstad, A. *Anal. Chim. Acta* **2014**, *828*, 46-52.
(15) Drouin, N.; Mandscheff, J.-F.; Rudaz, S.; Schappler, J. *Anal. Chem.* **2017**, *89* (12), 6346-6350.
(16) Restan, M. S.; Pedersen, M. E.; Jensen, H.; Pedersen-Bjergaard, S. *Anal. Chem.* **2019**, *91* (10), 6702-6708.
(17) Petersen, N. J.; Jensen, H.; Hansen, S. H.; Foss, S. T.; Snakenborg, D.; Pedersen-Bjergaard, S. *Microfluid. Nanofluid.* **2010**, *9* (4), 881-888.

(18) Hansen, F. A.; Sticker, D.; Kutter, J. P.; Petersen, N. J.; Pedersen-Bjergaard, S. *Anal. Chem.* **2018**, *90* (15), 9322-9329.
(19) Ang, Lay Teng, et al. *Cell Rep.* **2018**, *22* (8), 2190-2205.
(20) Karinen, R.; Andersen, J. M.; Ripel, Å.; Hasvold, I.; Hopen, A. B.; Mørland, J.; Christophersen, A. S. *J. Anal. Toxicol.* **2009**, *33* (7), 345-350.
(21) Pedersen-Bjergaard, S. *Anal. Bioanal. Chem.* **2019**, *411* (9), 1687-1693.
(22) Drouin, N.; Rudaz, S.; Schappler, J. *J. Pharm. Biomed. Anal.* **2018**, *159*, 53-59.
(23) Román-Hidalgo, C.; Martín-Valero, M. J.; Fernández-Torres, R.; Callejón-Mochón, M.; Bello-López, M. Á. *Talanta* **2017**, *162*, 32-37.
(24) Wilson, S. R.; Vehus, T.; Berg, H. S.; Lundanes, E. *Bioanalysis* **2015**, *7* (14), 1799-1815.