9 June 2026

Expanded Evaluation of a Robust On-Line Sample Cleanup-LC-MS Platform Suited for New Approach Methodologies (NAM)

Marcus Kamande[1], Julie Bekkhus[1], Aysan Safavi[2], Mads T. S. Gisle Johnsen[1], Patrik Appelblad[3], Steven Ray Wilson[1], Hanne Røberg-Larsen[1]

1. Department of Chemistry, University of Oslo
2. School of Engineering and Natural Sciences, University of Iceland
3. Discovery Solu5ons, Merck Life Science AS

Abstract

Sample preparation in bioanalysis can require significant numbers of (manual) steps and consumables. Such procedures can be bottlenecks regarding cost, throughput, and greenness. This is also the case for analysis of New Approach Methodologies (NAMs) such as organoids and organ-on-a-chip systems. In this context, approaches tailored for measuring drugs in NAM-related cell culture media (CCM) are being developed. For mass spectrometry-based analysis, automated filtration/filter backflush solid-phase extraction liquid chromatography AFFL-SPE-LC is utilized. Case studies have shown that the "AFFL" platform allows for vast reductions in sample preparation efforts, as the "self-cleaning" filtration features and SPE conditions remove potentially clogging/contaminating materials from samples of biological origin, e.g. the salt- and protein content in CCM. Here, broader demonstrations of the AFFL platform's traits for CCM analysis are provided, employing an extended panel of small-molecule drugs with varying polarity. The investigated AFFL platform shows reproducible chromatographic performance, as well as fit-for-purpose inter-matrix and inter-column robustness. Hundred-scale injections can be performed with satisfactory repeatability (e.g. retention time RSDs <1%), without interference from the various CCM matrices investigated. Practical NAM applications are also demonstrated.The approach has clear advantages in greenness, obtaining an AGREE prep score of 0.71, contrasting the <0.55 scores of more conventional/commercial approaches. The plastic consumable usage of our approach is 28 g/100 samples, a nearly 20-fold improvement over a previously established benchmark (500 g/100 samples).Taken together, these results demonstrate that AFFL provides a robust, low-waste, and scalable LC–MS based workflow for chemical analysis of NAM-derived samples.

Keywords

Cell Culture Medium, Inline, Automation, NAM, LC-MS, SPE

Expanded Evaluation of a Robust On-Line Sample Cleanup-LC-MS Platform suited for New Approach Methodologies (NAM)

Marcus Kamande[1], Julie Bekkhus[1], Stian Kogler[1], Aysan Safavi[2], Mads T. S. Gisle Johnsen[1], Patrik Appelblad[3], Steven Ray Wilson[1*] and Hanne Røberg-Larsen[1]

[1]Section of Chemical Life Science, Department of Chemistry, University of Oslo.

[2] School of Engineering and Natural Sciences, University of Iceland, VR-II, Hjardarhaga 6, 107, Reykjavik, Iceland

[3] Discovery Solutions, Merck Life Science AS, Drammensveien 123, 0277 Oslo, Norway.

* **Corresponding author**. Steven Ray Wilson, Section for Chemical Life Sciences, Department of Chemistry, Faculty of Mathematics and Natural Sciences, University of Oslo, P.O. Box 1033 Blindern, 0315 Oslo, Norway. E-mail: stevenw@kjemi.uio.no

Abstract

Sample preparation in bioanalysis can require significant numbers of (manual) steps and consumables. Such procedures can be bottlenecks regarding cost, throughput, and greenness. This is also the case for analysis of New Approach Methodologies (NAMs) such as organoids and organ-on-a-chip systems. In this context, approaches tailored for measuring drugs in NAM-related cell culture media (CCM) are being developed. For mass spectrometry-based analysis, automated filtration/filter backflush solid-phase extraction liquid chromatography AFFL-SPE-LC is utilized. Case studies have shown that the "AFFL" platform allows for vast reductions in sample preparation efforts, as the "self-cleaning" filtration features and SPE conditions remove potentially clogging/contaminating materials from samples of biological origin, e.g. the salt- and protein content in CCM. Here, broader demonstrations of the AFFL platform's traits for CCM analysis are provided, employing an extended panel of small-molecule drugs with varying polarity. The investigated AFFL platform shows reproducible chromatographic performance, as well as fit-for-purpose inter-matrix and inter-column robustness. Hundred-scale injections can be performed with satisfactory repeatability (e.g. retention time RSDs <1%), without interference from the various CCM matrices investigated. Practical NAM applications are also demonstrated.

The approach has clear advantages in greenness, obtaining an AGREE prep score of 0.71, contrasting the <0.55 scores of more conventional/commercial approaches. The plastic consumable usage of our approach is 28 g/100 samples, a nearly 20-fold improvement over a previously established benchmark (500 g/100 samples).

Taken together, these results demonstrate that AFFL provides a robust, low-waste, and scalable LC–MS based workflow for chemical analysis of NAM-derived samples.

Keywords: Automation; Cell Culture Medium; Inline; LC-MS, NAM; Solid Phase Extraction

Statements and Declarations: PA is employed by Merck Life Science AS in Norway and holds a role within the global Merck Life Science Chemistry R&D organization.

Introduction

An emerging focus is being directed toward developing technologies and methodologies for the chemical analysis of organoids and organ-on-a-chip systems (1-3). These laboratory-grown miniature models of organ functions (typically <1mm in size per organoid) are increasingly being used in drug discovery and disease modelling (4). Such New Approach Methodologies (NAMs) are considered promising alternatives to animal models, which involve many millions of animal sacrifices annually and often show limited predictability regarding clinical outcomes (5). Consequently, the development and implementation of NAMs are being encouraged by regulatory bodies (6) and are increasingly explored and utilized from basic research to big pharma.

Despite substantial progress in the biological complexity of organ models (such as liver, gut, brain, and retina systems), their broader adoption remains limited. Although miniaturization and multi-chip capabilities of NAMs hold promise (and expectancies) of high throughput in drug development, key challenges include standardization, automation, and reproducible output of these minute but complex samples. Addressing these challenges includes bioanalytical strategies tailored to NAMs, for example metabolomics/lipidomics, proteomics, and drug analysis (1).

In this study, we have focused on developing automated and inline analytical workflows for investigating drug uptake and metabolism in liver-based models (7–9). In such studies, the primary sample matrix is often cell culture medium (CCM), which functions both as the delivery vehicle for drugs and as a reservoir for excreted metabolites. CCM is a complex matrix containing salts, small molecules, proteins, and/or blood-derived components such as fetal bovine serum (FBS). The CCM composition can vary depending on manufacturer/recipe and application. The combination of protein-rich matrices, small sample volumes, and increasing throughput demands make conventional off-line sample preparation workflows less suited for NAM-based studies.

However, inline systems such as solid phase extraction-liquid chromatography (SPE-LC) (10-12) can be challenged by robustness issues, due to matrix precipitation and clogging (10,13). As a result, analysts often pre-clean samples prior to injecting onto inline systems (offline filtration or offline miniaturized SPE, so-called “zip-tipping”). To minimize potentially erroneous handling steps, it is both elegant and feasible to proceed with an alternative automated filtration and filter backflush approach, online trapping or solid-phase extraction LC approach (AFFL-SPE-LC; hereafter referred to as AFFL) (7,14). In this configuration (**Figure 1**), samples pass through a filter unit prior to reaching an analyte trapping column. This setup prevents particulate matter from reaching the trapping column which is packed with 3 µm silica particles (having small pore size and with octadecyl (C18) functionalization). Applying appropriate loading conditions (3% organic modifier) removes salts and most of the albumin present in typical CCMs (7). During elution of retained analytes to the analytical column, the

AFFL filter is simultaneously backflushed, resisting pressure build-up prior to the next injection. It has previously been shown that this modified SPE-LC approach significantly improves robustness compared to conventional configurations (13) and has undergone method validation for NAM metabolism of the potassium channel blocker tolbutamide (7). Building on these findings and motivated by challenges in reproducibility of analytical workflows for NAMs, we here expand the performance investigation of AFFL for CCM analysis. We critically assess and exemplify the system with various drugs from a small molecule-panel spanning a broad LogP range. We compare environmental and operational aspects ("greenness") of AFFL with more conventional and commercial approaches to sample cleanup (15), documenting substantial advantages of our inline approach. Together, our results and applications point to AFFL serving as a robust platform for CCM analysis in NAM research, requiring a minimum of pre-injection handling and consumables.

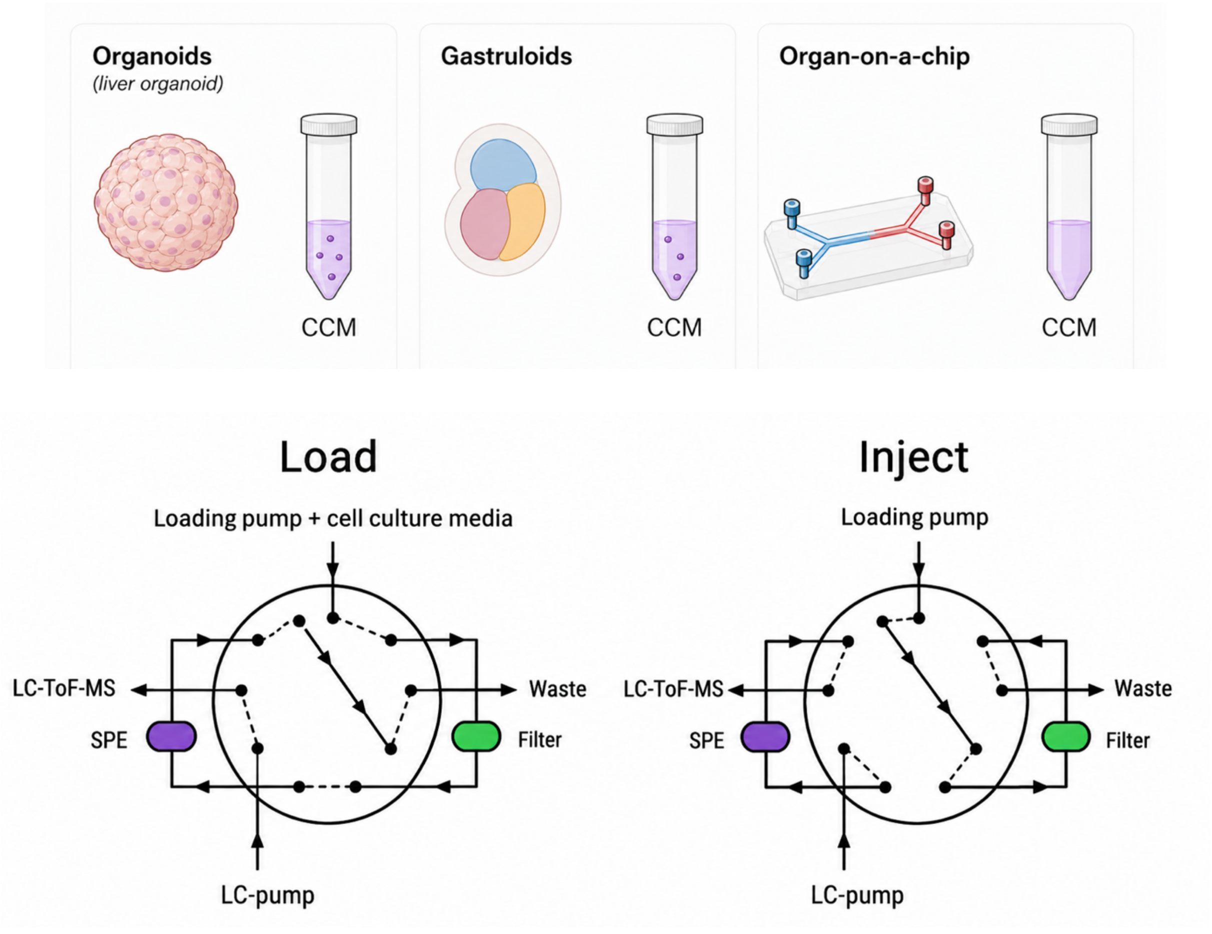


Figure 1. Top: New Approach Methodologies (NAMs) include organoids, gastruloids, and organ-on-a-chip systems, serve as models of organ functionality/early development and are emerging tools for e.g. drug discovery and toxicology studies. **Bottom:** AFFL-SPE-LC-ToF-MS platform. This modified SPE-LC system allows for highly robust sample clean-up, which reduces the number of pre-injection steps.

Materials and Methods

Chemicals and Reagents

Fluoxetine hydrochloride (CAS: 56296-78-7) was from Thermo Fisher Scientific. TeSR™-E5/E6 Basal Medium, TeSR™-E6 20X Supplements were from StemCell technologies. Type 1 water was purified with a Milli-Q integral ultrapure water purification system from Merck Life Science (Darmstadt, Germany). 90 different drugs and endogenous metabolites were provided from Norsk Medisinaldepot AS (NMD, Oslo, Norway) and Merck Life Science (Darmstadt, Germany). The full list is provided in the **Supporting Information SI1**.

Solutions

Four cell culture media (CCMA-D) consisting of various compositions of salts, metabolites, and protein were prepared and provided by the Hybrid Technology Hub (UiO, Oslo, Norway) and were stored at 4 °C. see **Supporting Information SI2** for details.

Analytes were dissolved in methanol, DMSO or water to final 90-drug stock solutions of 5 µg/mL per drug (stored at -28 °C). The CCM were spiked with the 90-drug stock solution for final concentrations of 10 and 100 ng/mL.

Three different mobile phases were prepared, and their compositions are shown in **Table 1**.

Table 1. The three mobile phases used and their individual composition.

Name	Mobile phase type	Composition
A	Aqueous LC phase	Water containing 0.1% (v/v) formic acid
B	Organic LC phase	Methanol containing 0.1% (v/v) formic acid
C	Loading phase	Water/methanol (97:3, v/v) containing 0.1% (v/v) formic acid

Consumables and Equipment

96-well Microplate (U-bottom) with cell-repellent surface was from Greiner Bio-One (Kremsmünster, Austria). Pipettes used were Finnpipette ® Thermo Fisher Scientific (Waltham, MA, US). Low binding pipette tips (PCR performance) and pipette tips (PCR performance) were from Sarstedt (Nümbrecht, Germany). Protein LoBind Tube 1.5 mL and Safe-Lock Tubes 1.5 mL were from Eppendorf (Hamburg, Germany). 1.5 mL amber glass autosampler vials and polypropylene screwcaps were from VWR.

AFFL-SPE-LC-MS Platform

A MaXis II ETD Time of Flight (ToF) mass spectrometer from Bruker (Billerica, Massachusetts, USA) was used for most of the study. A TSQ Vantage from Thermo Scientific (Waltum, MA) was used for carry-over studies. For the Maxis II ETD instrument, the ESI nebulizer was set to 200 °C with the dry gas flowrate set to 4 L/min and the capillary set to 3.5 kV. The endplate offset was set to 500 V and the instrument was operated in full scan mode (50-1500 *m/z*). For the

TSQ Vantage, the spray voltage was set to 3 kV and the temperature to 200 °C. The instrument was operated in SRM mode, see supporting information **SI3** for transitions.

With both MS instrument, a Dionex UltiMate 3000 system from Thermo Scientific was used for LC autosampling, loading, column heating and chromatography. The analytical column oven was set to 40° C and the autosampler storage was set to 4 °C. The injection volume was 5 µL. The LC flow rate was 0.1 mL/min and the gradient program is shown in **Table 2**.

Table 2. Applied LC solvent program.

Time (min)	%A	%B	Flow (mL/min)	%C (Load pump)	Valve
0	80	20	0.1	100	Load
1-2	80→5	20→95	0.1	100	Inject
2-8	5	95	0.1	100	Inject
8.00-8.01	5→80	95→20	0.1	100	Inject
8.01-9	80	20	0.1	100	Inject
9-10	80	20	0.1	100	Load

The AFFL loading pump was set to 0.1 mL/min flowrate with a constant 100% mobile phase C. The AFFL valve system switches from the load position to the inject position at minute 1.00. The system switches back to the load position at minute 9.00.

The analytical columns used were 50 x 1.0 mm Purospher® STAR endcapped RP-18 columns (50111-U) with 2 µm particle size (100 Å pore size) and the SPE trapping columns were 5.0 x 1.0 mm I.D. Purospher® STAR endcapped RP-18 (3 µm/100 Å pores), both provided by Merck Life Science. Viper SST Fingertight Fittings were from Thermo scientific.

Software

Chromeleon chromatography data system (version 7.1) from Thermo Fisher Scientific was used for controlling the LC system and AFFL pump. Compass HyStar from Bruker (Billerica, Massachusetts, USA) was used for connecting the LC system to the computer and Compass DataAnalysis from Bruker was used for data analysis together with the open-source windows client application skyline by MacCoss Lab. LogP values were primarily calculated using Chemicalize software (Chemaxon, Budapest, Hungary) and may vary due to pH conditions, ionization state, and calculation method.

Results and discussion

General chromatographic performance of the platform

To assess a wide-ranging LogP drug panel (ca. –4 to 6) in a relatively short timeframe, we employed a steep solvent gradient (20-95% B from 1 to 2 minutes) with a subsequent 95% plateau until 8 minutes. Time-of-flight MS enabled simultaneous full-scan detection of all compounds in the ~90-drug panel within a single analysis and was used to e.g. study 2D plots of retention time vs. *m/z* throughout the study (**Figure 2**; drug panel solved in CCMA, see **SI2** for details).

The AFFL platform enabled detection of a broad range of the injected analytes, illustrated with antipyrin (LogP 1.2), denatonium (LogP 2.2) and meclizine (LogP 6.4) in **Figure 2** (bottom). The extracted ion chromatograms are overlays of 120 injections, demonstrating a high repeatability (RSD <1%) when injecting the matrix-rich CCM samples (containing salts, polar nutrients, drugs, proteins) without pre-injection sample preparation steps. Expectedly, compounds of more polar character would generally have poor/no retention on the reverse phase trapping column. However, for the majority of the drug panel, ample signals were generated within the timeframe.

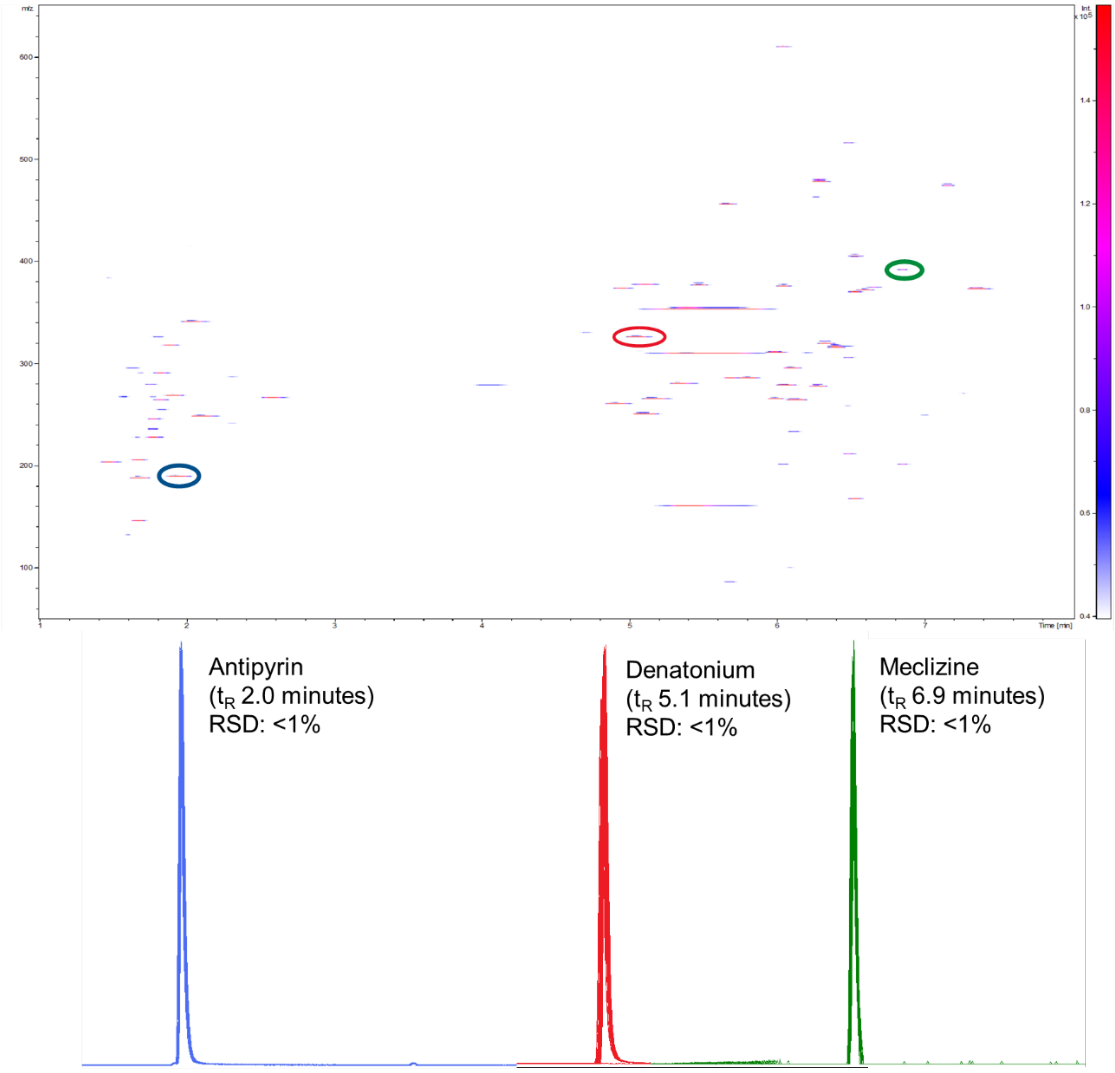
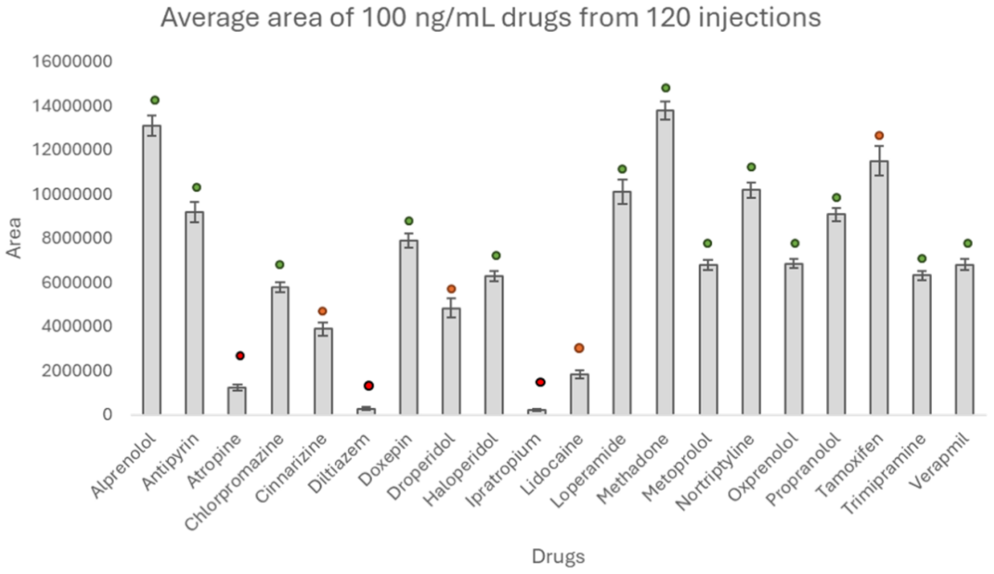


Figure 2: Top: t_R versus *m/z* plots of an aqueous 90-drug mixture in CCM. Bottom: Overlay of 120 extracted ion chromatograms of antipyrin, denatonium, and meclizine in CCM.

Similar performance across various cell culture media

Although playing similar roles, CCM from various vendors/recipes for drug experiments can vary in composition (e.g. amounts of supplements such as albumin/FBS) and potentially have differing degrees of matrix effects. Four types of CCM (CCMA-D, see **SI2** for details) spiked with the drug panel were used to evaluate analytical traits of the AFFL platform. **Figure 3** shows that the between-matrix retention times and peak areas of selected compounds were generally satisfactory (<5% RSD). Compared to purely aqueous samples, the signal intensities in the various CCMs did not have obvious variations, implying few suppression/enhancement effects. However, compounds, cocaine in particular, would notably degrade in CCM during a week of injections. Taken together, the retention time and peak area evaluations suggested an overall inter-CCM robustness.



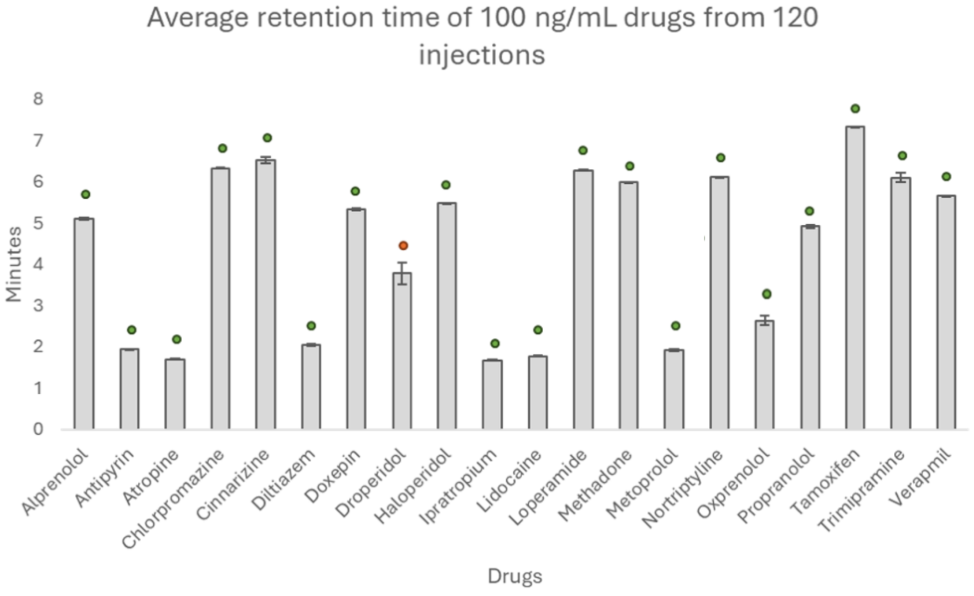


Figure 3. Retention time and peak area of 20 selected drugs (spanning polar ipratropium to hydrophobic tamoxifen), from the 90-drug mixture in 4 different CCM (CMA-D; see Materials and Methods for details). The area and retention time of the drugs are shown in average of a combined 120 injections in 4 varying CCM. RSD values between 5–10% are indicated in orange, while values 10-20% are indicated in red. A tendency toward higher deviation for early-eluting compounds was observed, while more hydrophobic late-eluting compounds generally displayed lower variability.

Two-dimensional plotting shows few MS-interferences

Using UV-based approaches, we have previously documented an improved removal of protein with our AFFL platform, for CCM samples containing albumin or FBS (7). We here extended the analysis of potential contaminants entering the HRMS using 2D plots similar to Figure 2. An absence of protein bands (i.e. spread of multiple charged peaks) in the four different CCM solutions was established (**Figure 4)**. In accordance with this observation, routine ESI source cleaning requirements were minimal; biosample-based source fouling was a more prominent issue in earlier non-AFFL platforms used in our lab for CCM samples.

We and others (15,16) have recently documented contaminants (curved, repeating units with mass differences of 44 Da) in samples from polymer-based containers used in sample preparation. Such contaminants can be a significant source of interferences but were not detected in the plots shown in Figure 4. In summary, we find no obvious sources of familiar contaminants from several CCM using the AFFL platform.

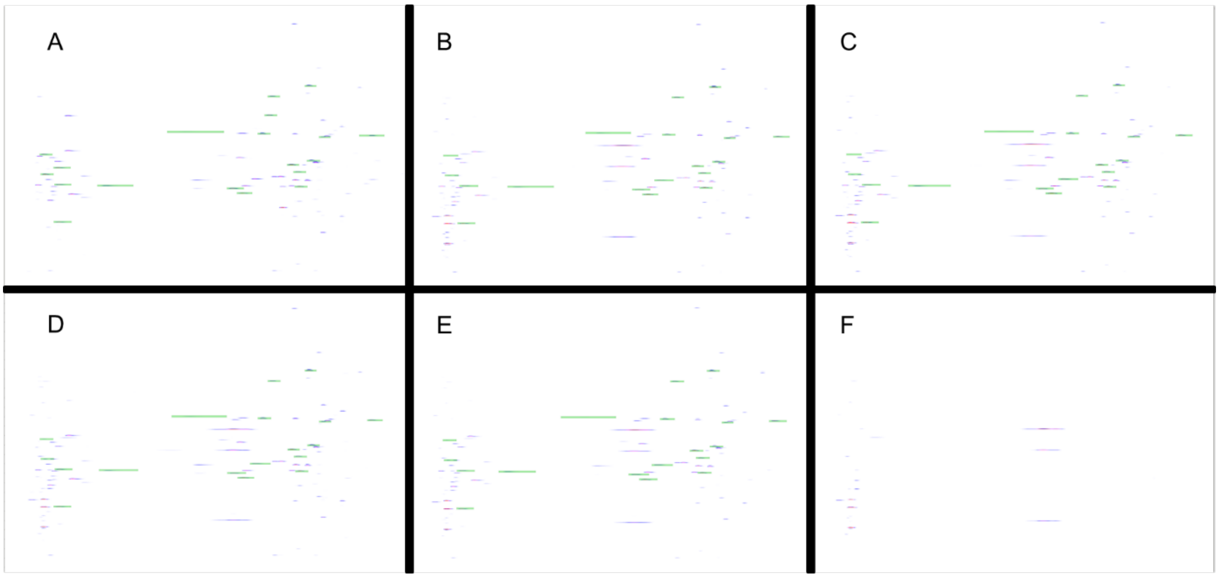


Figure 4. t_R (X-axis, 0-10 min) versus *m/z* (Y-axis, 50-650) plots of a 90-drug mixture in aqueous solution (A) and four different cell culture media (B–E). A cell culture medium blank is included (F). Signal intensity is represented as a color gradient (red = $6x10^5$, magenta = $4x10^5$, blue = $2x10^5$, white = $<1x10^5$). The figures demonstrate a low degree of interfering compounds in the CCM, and an absence of (bio) polymer contaminants.

Sample Preparation Ruggedness - cartridge to cartridge reproducibility

We have established that the AFFL platform is capable of >1000 injections without need for cartridge replacement (7); such performance was also observed during the course of this study. However, in the event of a replacement need, we recommend five sample conditioning/saturation injections with a new SPE are sufficient to obtain the same performance as with the previous unit (tested with four SPEs, **Figure 5)**. After initial conditioning injections, the inter-SPE RSD for retention time for this study was between 0.1% and 0.2% (same sample injected 20 times across four SPEs, for a total of 80 injections).

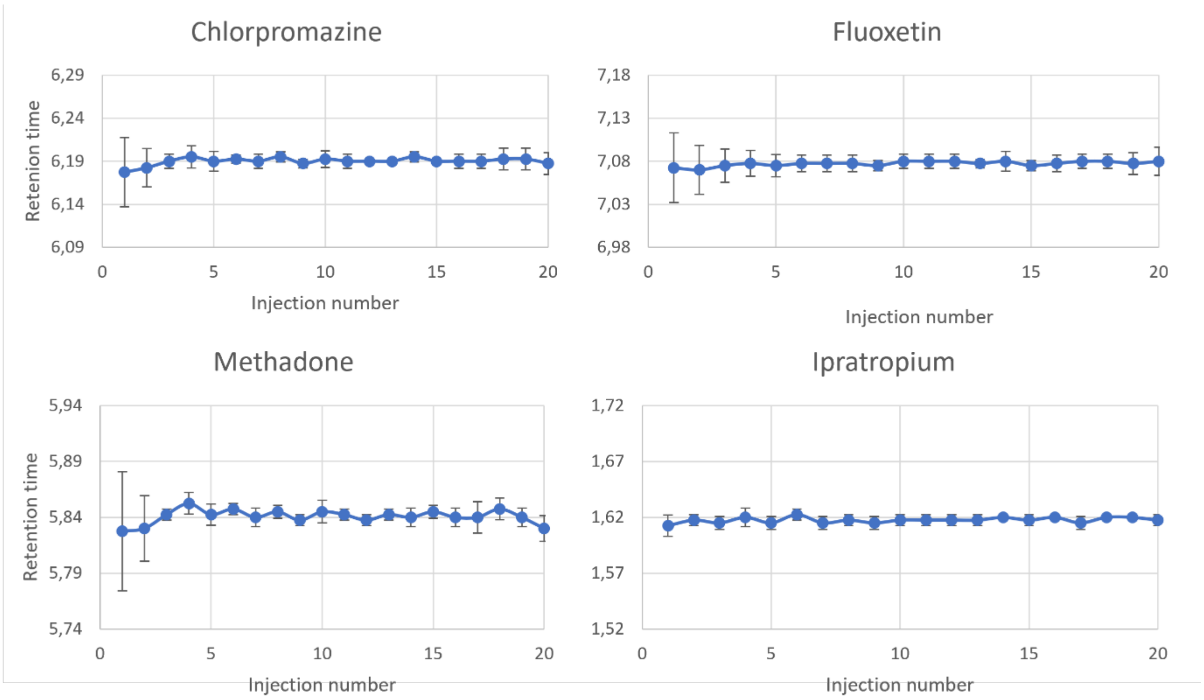


Figure 5. Top: Four different drugs used to illustrate the SPE cartridge to cartridge reproducibility and performance (n=4). On the Y-axis is retention time and on the X-axis is the injection number, ranging from 1-20. Each injection number consists of 4 injections, one from each SPE.

Carry-over

Considering the completeness and demonstration of system automation and the varied matrix of CCM, a carry-over assessment was carried out. Carry-over for measured peak area was between 0.3-0.4% for representative drugs (here monitored with sensitive triple quadrupole-MS), see **Figure 6**. We consider these values satisfactory, as hundreds of ng/mL are common top concentrations in e.g. drug metabolism studies using NAM models.

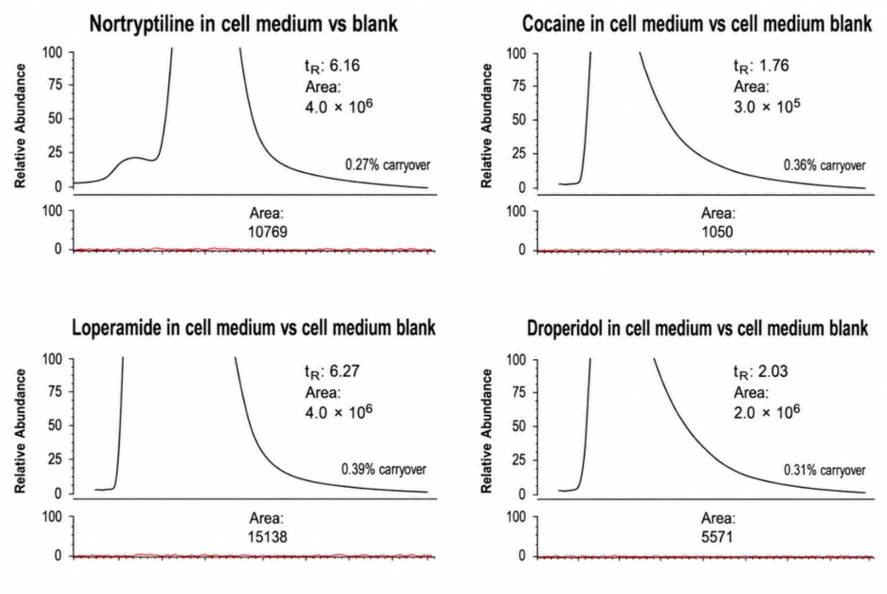
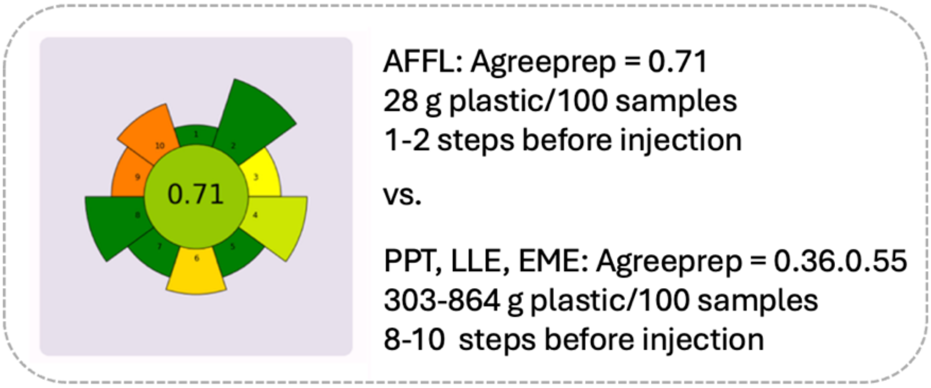


Figure 6. Four different drugs and their chromatograms are displayed compared to the subsequent blank injection. The area of each drug is measured in a 100 ng/ml mixture and cell culture medium blank.

Significantly higher greenness compared to conventional approaches

Greenness and sustainability have increasing focus, and life sciences contribute to several million tons of plastic per year (17). To calculate and benchmark the greenness of the approach, the AGREEprep scoring system (18) was employed. On a scale from 0 to 1, where 1 is a perfect score, our method was calculated to 0.71 (see also **SI4**). For context, we have recently assessed various established/commercial approaches for bioanalysis (protein precipitation, liquid-liquid extraction, electromembrane extraction), calculating scores as being 0.36-0.55 (15). Hence, the AFFL platform can be considered as having a significantly higher greenness score, with key reasons including low reagent consumption and in-line action. The latter allows for a substantial reduction in plastic consumables: with our method, only one plastic pipette per sample is used, i.e. for transfer from organoid container/MPS to autosampler vial. In contrast to this one-step (or two-step, if internal standard addition is required), conventional/commercial approaches can require ca. ten handling steps. With 50 µL tips, and when including an internal standard, the total plastic consumption of the AFFL platform is 28 g plastic/100 samples. In comparison, the PPT, LLE, and EME approaches use 511 g (suggested benchmark), 864 g, and 303 g, respectively (15). Thus, a striking ca. 20-fold reduction of plastics is achieved compared to the PPT benchmark (see **Figure 7** for summary).



AFFL: Agreeprep = 0.71
28 g plastic/100 samples
1-2 steps before injection

vs.

PPT, LLE, EME: Agreeprep = 0.36.0.55
303-864 g plastic/100 samples
8-10 steps before injection

Figure 7. Overview of sustainability and steps of AFFL compared to earlier studies approaches (15). See also SI3 for details on calculations.

Practical application: Understanding variation issues with a multi-well plate system for gastruloid studies

We are currently applying the described platform for drug uptake/metabolism studies with various NAMs. In an ongoing study of effects of anti-depressants on early development (fluoxetin and human gastruloids), we could observe unsatisfactory variations in replicate treatment wells. Cell culture media samples from the system wells were directly transferred to sample vials and analyzed with AFFL. After 24 hours, we could observe that outer wells had significantly higher concentrations of the drug (**Figure 8**), which could be related to evaporation in the open wells, and mitigating steps could be taken. This serves as a simple example of the utilization of our minimal-handling workflow, in this case addressing sources of variability in costly experiments using NAM platforms.

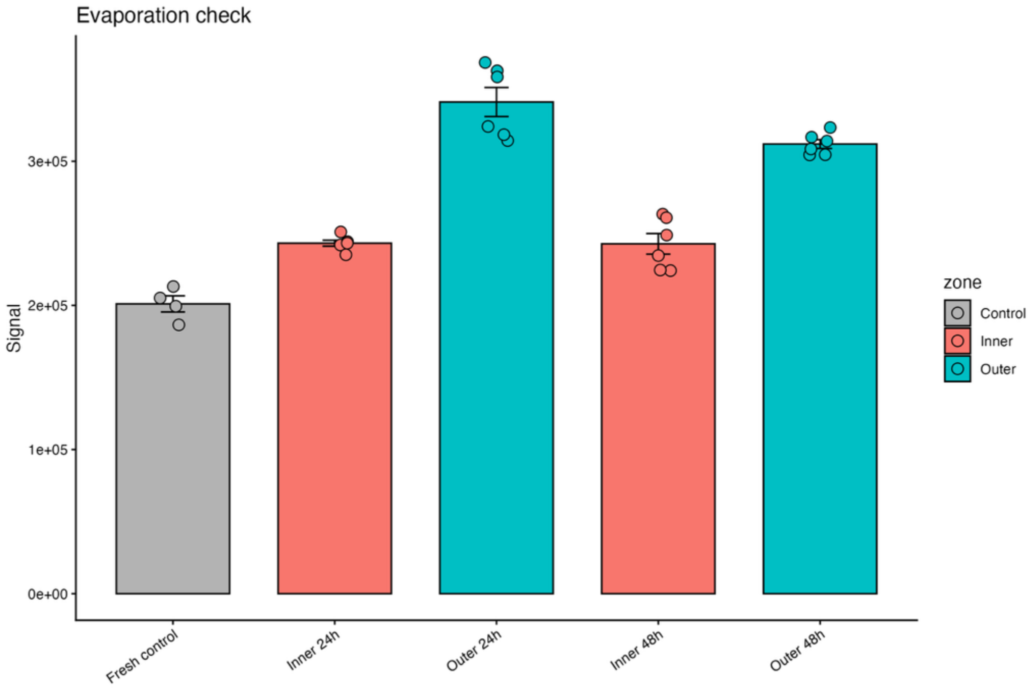


Figure 8. Illustration of relative levels of fluoxetine in gastruloid wells, illustrating elevated levels in outer wells, pointing to proneness to evaporation.

Limitations and options

In this study, we have relatively long analysis times of 10 minutes (loading, analysis, re-equilibration) to assess the wide LogP ranging drug panel, but this can be reduced/tailored when focusing on fewer analytes. Peak widths can likely be further reduced through reductions in dead volumes/choice of tubing, as pressure increases/clogging were absent throughout the study. The described settings have especially limitations regarding hydrophilic drugs, and it might be possible to use other stationary phase chemistries that are more tuned for polar substances, but with potential compatibility issues between trapping and analytical separation conditions. For example, hydrophilic interaction liquid chromatography (HILIC) may be useful for the separation of polar and hydrophilic compounds but this separation mode has limitations, due to the need for injection solutions with high ACN content (19). In a preliminary study on the use of HILIC for CCM samples, we found that there was limited compatibility of the current setup with the addition of ACN to CCM samples, as the resulting protein precipitations could clog the otherwise robust platform and reduce chromatographic efficiency. The described performance assessments are here performed with aqueous cell culture media used for small-molecule drug studies but may have limitations for variants containing substantial amounts of organic solvents and/or serum, e.g. DMSO-containing freezing media. Regarding the precision of green scoring, we have previously seen that AGREEprep scores can have a between-person variation of roughly 10 % (15).

Conclusions

Expanded evaluations of the AFFL-SPE-LC platform for small-molecule drug analysis in cell culture media have been undertaken. We find that the system demonstrates robust chromatographic performance over an expanded panel of analytes, and robustness to various CCM matrices. Notably, the system has substantial advantages regarding number of steps/automation and greenness, benchmarked to conventional/commercial approaches. The system will continue to be fine-tuned for chromatographic performance, speed and down-scaling; Preliminary data suggests that a 0.3 mm ID separation column can also be suited for the instrumental setup and sample type, which will allow for a further reduction in solvent- and sample consumption, as well as potentially enhancing sensitivity through reduced radial dilution. The latter can provide a marked advantage in e.g. single organoid analysis (20). Taken together, these results support a shift toward fully inline analytical workflows for complex biological samples in NAM-based research.

Acknowledgements

We thank the Norwegian Research Council for providing funding (project 105303). We also thank Dr. Aleksandra Aizenshtadt for providing the cell culture media used in this study, and Dr. Frederik Andre Hansen for donating the drug mixture.

References

1. Kogler S, Kømurcu KS, Olsen C, Shoji J ya, Skottvoll FS, Krauss S, mfl. Organoids, organ-on-a-chip, separation science and mass spectrometry: An update. TrAC Trends Anal Chem. 2023;161:116996. doi:10.1016/j.trac.2023.116996

2. Leung CM, de Haan P, Ronaldson-Bouchard K, Kim GA, Ko J, Rho HS, mfl. A guide to the organ-on-a-chip. Nat Rev Methods Primer. 2022;2(1):33. doi:10.1038/s43586-022-00118-6

3. Zhao Z, Chen X, Dowbaj AM, Sljukic A, Bratlie K, Lin L, mfl. Organoids. Nat Rev Methods Primer. 2022;2(1):94. doi:10.1038/s43586-022-00174-y

4. Wang D, Villenave R, Stokar-Regenscheit N, Clevers H. Human organoids as 3D in vitro platforms for drug discovery: opportunities and challenges. Nat Rev Drug Discov. 2026;25(3):204–26. doi:10.1038/s41573-025-01317-y

5. Hartung T. The (misleading) role of animal models in drug development. Front Drug Discov. 2024;4. doi:10.3389/fddsv.2024.1355044

6. FDA pushes to replace animal testing. Nat Biotechnol. 2025;43(5):655–655. doi:10.1038/s41587-025-02690-0

7. Kogler S, Pedersen GM, Martínez-Ramírez F, Aizenshtadt A, Busek M, Krauss SJK, mfl. An FDA-Validated, Self-Cleaning Liquid Chromatography–Mass Spectrometry System for Determining Small-Molecule Drugs and Metabolites in Organoid/Organ-on-Chip Medium. Anal Chem. 2024;96(29):12129–38. doi:10.1021/acs.analchem.4c02246

8. Kogler S, Skottvoll FS, Hrušková H, Rise F, Aizenshtadt A, Krauss S, mfl. Electromembrane Extraction Provides Unprecedented Selectivity for Drugs in Cell Culture Media Used in Organoid and Organ-on-Chip Systems. Anal Chem. 2025;97(9):4923–31. doi:10.1021/acs.analchem.4c04994

9. Harrison SP, Siller R, Tanaka Y, Chollet ME, de la Morena-Barrio ME, Xiang Y, mfl. Scalable production of tissue-like vascularized liver organoids from human PSCs. Exp Mol Med. 2023;55(9):2005–24. doi:10.1038/s12276-023-01074-1

10. Rogeberg M, Malerod H, Roberg-Larsen H, Aass C, Wilson SR. On-line solid phase extraction–liquid chromatography, with emphasis on modern bioanalysis and miniaturized systems. J Pharm Biomed Anal. 2014;Review Papers on Pharmaceutical and Biomedical Analysis 201387:120–9. doi:10.1016/j.jpba.2013.05.006

11. de la Serna Calleja MÁ, Bolado S, Jiménez JJ, López-Serna R. Performance critical comparison of offline SPE, online SPE, and direct injection for the determination of CECs in complex liquid environmental matrices. Microchem J. 2023;187:108395. doi:10.1016/j.microc.2023.108395

12. Chen L, Yan X, Zhou X, Peng P, Sun Q, Zhao F. Advances in the on-line solid-phase extraction-liquid chromatography-mass spectrometry analysis of emerging organic

contaminants. TrAC Trends Anal Chem. 2023;160:116976. doi:10.1016/j.trac.2023.116976

13. Brandtzaeg OK, Johnsen E, Roberg-Larsen H, Seip KF, MacLean EL, Gesquiere LR, mfl. Proteomics tools reveal startlingly high amounts of oxytocin in plasma and serum. Sci Rep. 2016;6(1):31693. doi:10.1038/srep31693

14. Svendsen KO, Larsen HR, Pedersen SA, Brenna I, Lundanes E, Wilson SR. Automatic filtration and filter flush for robust online solid-phase extraction liquid chromatography. J Sep Sci. 2011;34(21):3020–2. doi:10.1002/jssc.201100553

15. Noreng L, Øiestad ÅML, Hansen FA, Røberg-Larsen H, Wilson SR, Øiestad EL. Toward Sustainable Clinical Analysis: Benchmarking Plastic Use in LC–MS Sample Preparation – Exemplified by Ketamine Analogs in Whole Blood. Anal. Chem. 2026; 98, 16, 11899–11909. doi.org/10.1021/acs.analchem.5c08225

16. Canez CR, Li L. Studies of Labware Contamination during Lipid Extraction in Mass Spectrometry-Based Lipidome Analysis. Anal Chem. 2024;96(8):3544–52. doi:10.1021/acs.analchem.3c05431

17. Urbina MA, Watts AJR, Reardon EE. Labs should cut plastic waste too. Nature. 2015;528(7583):479–479. doi:10.1038/528479c

18. Wojnowski W, Tobiszewski M, Pena-Pereira F, Psillakis E. AGREEprep – Analytical greenness metric for sample preparation. TrAC Trends Anal Chem. 2022;149:116553. doi:10.1016/j.trac.2022.116553

19. Taylor MR, Kawakami J, McCalley DV. Managing sample introduction problems in hydrophilic interaction liquid chromatography. J Chromatogr A. 2023;1700:464006. doi:10.1016/j.chroma.2023.464006

20. Kømurcu KS, Zawadzka ME, Meszka I, Aizenshtadt A, Hrušková H, Aakervik LE, mfl. A Validated Mass Spectrometry Platform for Oxysterol Analysis of Single Human Gastruloids and Liver Organoids. Anal Chem. 2026;98(9):6792–804. doi:10.1021/acs.analchem.5c07140

Supporting Information for:

Expanded Evaluation of a Robust On-Line Sample Cleanup-LC-MS Platform suited for New Approach Methodologies (NAM)

Marcus Kamande[1], Julie Bekkhus[1], Stian Kogler[1], Aysan Safavi[2], Mads T. S. Gisle Johnsen[1], Patrik Appelblad[3], Steven Ray Wilson[1*] and Hanne Røberg-Larsen[1]

[1]Section of Chemical Life Science, Department of Chemistry, University of Oslo.

[2] School of Engineering and Natural Sciences, University of Iceland, VR-II, Hjardarhaga 6, 107, Reykjavik, Iceland

[3] Discovery Solutions, Merck Life Science AS, Drammensveien 123, 0277 Oslo, Norway.

* **Corresponding author**. Steven Ray Wilson, Section for Chemical Life Sciences, Department of Chemistry, Faculty of Mathematics and Natural Sciences, University of Oslo, P.O. Box 1033 Blindern, 0315 Oslo, Norway. E-mail: stevenw@kjemi.uio.no

SI1: Overview over the 90 drugs used in the study, including their molar mass, pKa values, logP (calculations mostly from Chemicalize software, Chemaxon, Budapest, Hungary), and producers. The same panel (analytical grade compounds) has been used in earlier studies, e.g. Kogler et al (2025). Values can be treated as approximate, particularly for highly ionizable compounds.

Name	Molar mass	Acidic pKa	Basic pKa	logP	Producer
6-di-tert-butyl-4-(dimethylaminomethyl)phenol	263.425	10.75	8.8	4.602	Merck Life Sciences (Darmstadt, Germany)
6-MAM	327.38	10.19	9.08	1.307	Merck Life Sciences (Darmstadt, Germany)
Acetyl choline	146.209			-4.22	Merck Life Sciences (Darmstadt, Germany)
Adenine	135.13	10.29	3.66	-0.53	Merck Life Sciences (Darmstadt, Germany)
Alprenolol	249.354	14.09	9.67	2.693	Merck Life Sciences (Darmstadt, Germany)
Amantidine	151.253		10.71	1.466	Merck Life Sciences (Darmstadt, Germany)
Amiodarone	645.32		9.08	7.635	Merck Life Sciences (Darmstadt, Germany)
Amitriptyline	277.411		9.76	4.81	Merck Life Sciences (Darmstadt, Germany)
Antipyrin	188.23		0.49	1.219	Merck Life Sciences (Darmstadt, Germany)
Atenolol	266.341	14.08	9.67	0.425	Merck Life Sciences (Darmstadt, Germany)
Atropine	289.375	9.39	15.15	1.571	Merck Life Sciences (Darmstadt, Germany)
Benzamidine	120.155	11.53		0.894	Merck Life Sciences (Darmstadt, Germany)
Bumetanide	364.42	3.69	2.7	2.423	Merck Life Sciences (Darmstadt, Germany)
Butylhydrazine	88.154	NaN	8.49	0.514	Merck Life Sciences (Darmstadt, Germany)
Chlorpromazine	318.86	NaN	9.2	4.535	Merck Life Sciences (Darmstadt, Germany)
Chlorprothixene	315.86	NaN	9.76	5.066	Merck Life Sciences (Darmstadt, Germany)
Cimetidine	252.34	10.13	6.53	-0.109	Merck Life Sciences (Darmstadt, Germany)
Cinnarizine	368.524	NaN	8.1	5.88	Merck Life Sciences (Darmstadt, Germany)
Clofazimine	473.4		5.89	7.304	Merck Life Sciences (Darmstadt, Germany)
Clomipramine	314.86	NaN	9.2	4.883	Merck Life Sciences (Darmstadt, Germany)
Clotrimazole	344.84		6.26	5.839	Merck Life Sciences (Darmstadt, Germany)

Cocaine	303.358	NaN	8.85	2.282	Norsk Medisinaldepot AS (NMD, Oslo, Norway)
Denatonium	325.475	NaN		2.2	Merck Life Sciences (Darmstadt, Germany)
Diltiazem	414.52	8.18	12.86	2.727	Merck Life Sciences (Darmstadt, Germany)
Dopamine	153.181	9.27	10.01	0.03	Merck Life Sciences (Darmstadt, Germany)
Doxepin	279.383	9.76		3.84	Merck Life Sciences (Darmstadt, Germany)
Droperidol	379.435	6.75	12.72	3.014	Merck Life Sciences (Darmstadt, Germany)
Enalapril	376.453	5.2	3.67	0.588	Merck Life Sciences (Darmstadt, Germany)
Ephedrine	165.236	9.52	13.89	1.318	Merck Life Sciences (Darmstadt, Germany)
Epinephrine	183.207	8.91	9.69	-0.43	Merck Life Sciences (Darmstadt, Germany)
Famotidine	337.44	7.97	10.03	-1.95	Merck Life Sciences (Darmstadt, Germany)
Fluoxetine	309.332	9.8		4.173	Merck Life Sciences (Darmstadt, Germany)
Halofantrine	500.43	14.47	10.2	8.057	Merck Life Sciences (Darmstadt, Germany)
Haloperidol	375.87	13.96	8.2	3.661	Merck Life Sciences (Darmstadt, Germany)
Hydralazine	160.18		3.9	0.751	Merck Life Sciences (Darmstadt, Germany)
Hydroxyzine	374.91	15.12	7.45	3.413	Merck Life Sciences (Darmstadt, Germany)
Ipratropium	332.463	15.15		-1.81	Merck Life Sciences (Darmstadt, Germany)
Isoniazid	137.142	13.61	3.35	-0.69	Merck Life Sciences (Darmstadt, Germany)
Lidocaine	234.343	13.78	7.75	2.843	Merck Life Sciences (Darmstadt, Germany)
Loperamide	477.05	13.96	9.56	4.771	Merck Life Sciences (Darmstadt, Germany)
Luminol	177.163	8.61	1.61	-0.064	Merck Life Sciences (Darmstadt, Germany)
Meclizine	390.96		7.71	6.388	Merck Life Sciences (Darmstadt, Germany)
Mepiquat	114.211			-3.12	Merck Life Sciences (Darmstadt, Germany)
Metaraminol	167.208	9.03	9.68	-0.04	Merck Life Sciences (Darmstadt, Germany)
Metformin	129.167	15.14	12.29	-0.918	Merck Life Sciences (Darmstadt, Germany)
Methadone	309.453		9.12	5.007	Norsk Medisinaldepot AS (NMD, Oslo, Norway)

Metoprolol	267.369	14.09	9.67	1.759	Merck Life Sciences (Darmstadt, Germany)
Mianserin	264.372		6.92	3.831	Merck Life Sciences (Darmstadt, Germany)
N-acetylputrescine	130.191		9.9	-1.026	Merck Life Sciences (Darmstadt, Germany)
N-guanylurea	102.097	13.6	9.79	-2.03	Merck Life Sciences (Darmstadt, Germany)
Nicotinamide	122.127	13.39	3.63	-0.39	Merck Life Sciences (Darmstadt, Germany)
Nortriptyline	263.384		10.47	4.426	Merck Life Sciences (Darmstadt, Germany)
Noscapine	413.426		7.14	2.581	Merck Life Sciences (Darmstadt, Germany)
O-desmethylvenlafaxine	263.381	10.13	9.01	2.274	Merck Life Sciences (Darmstadt, Germany)
Oxprenolol	265.353	14.09	9.67	2.168	European Pharmacopoeia Reference Standard
Papaverine	339.391		6.03	3.08	Merck Life Sciences (Darmstadt, Germany)
Perphenazine	403.97	15.59	7.81	3.692	Merck Life Sciences (Darmstadt, Germany)
Pethidine	247.338		8.16	2.456	Norsk Medisinaldepot AS (NMD, Oslo, Norway)
Pimozide	461.557	12.9	8.38	5.826	Merck Life Sciences (Darmstadt, Germany)
Piperazine	86.138		9.56	-0.72	Merck Life Sciences (Darmstadt, Germany)
Practolol	266.341	14.03	9.67	0.832	Merck Life Sciences (Darmstadt, Germany)
Procaine	236.315		8.96	1.88	Merck Life Sciences (Darmstadt, Germany)
Prochlorperazine	373.94		7.99	4.382	Merck Life Sciences (Darmstadt, Germany)
Promazine	284.42		9.2	3.931	Merck Life Sciences (Darmstadt, Germany)
Promethazine	284.42		9.05	4.288	Merck Life Sciences (Darmstadt, Germany)
Propranolol	259.349	14.09	9.67	2.584	Merck Life Sciences (Darmstadt, Germany)
Pyridoxine	169.18	9.4	5.58	-0.95	Merck Life Sciences (Darmstadt, Germany)
Pyrilamine	285.391		8.76	3.044	Merck Life Sciences (Darmstadt, Germany)
Quinine	324.424	13.89	9.05	2.513	Merck Life Sciences (Darmstadt, Germany)
Raloxifene	473.59	9	8.42	5.465	Merck Life Sciences (Darmstadt, Germany)
Ranitidine	314.4	7.8		0.991	Merck Life Sciences (Darmstadt, Germany)

Reserpine	608.688	7.02		3.531	Merck Life Sciences (Darmstadt, Germany)
Salbutamol	239.315	9.4	10.12	0.344	Merck Life Sciences (Darmstadt, Germany)
Serotonin	176.219	10	9.31	0.482	Merck Life Sciences (Darmstadt, Germany)
Sotalol	272.36	9.43	10.07	-0.395	Merck Life Sciences (Darmstadt, Germany)
Sulfadiazine	250.28	2.01	6.99	0.387	Merck Life Sciences (Darmstadt, Germany)
Sulfamethazine	278.33	2	6.99	0.65	Merck Life Sciences (Darmstadt, Germany)
Sulfamethoxalol	253.28	1.97	6.16	0.791	Merck Life Sciences (Darmstadt, Germany)
Tamoxifen	371.524		8.76	6.351	Merck Life Sciences (Darmstadt, Germany)
Telmisartan	514.629	3.62	5.86	6.13	Merck Life Sciences (Darmstadt, Germany)
Thiamine	265.35	5.54	15.5	-3.09	Merck Life Sciences (Darmstadt, Germany)
Thioridazine	370.57	8.93		5.469	Merck Life Sciences (Darmstadt, Germany)
Timolol	316.42	9.76	14.08	1.336	Merck Life Sciences (Darmstadt, Germany)
Triclabendazole	359.65	10.31	4.39	5.884	Merck Life Sciences (Darmstadt, Germany)
Triisopropanolamine	191.271	9.28	14.81	-0.63	Merck Life Sciences (Darmstadt, Germany)
Trimipramine	294.442	9.42		4.758	Merck Life Sciences (Darmstadt, Germany)
Tyramine	137.182	9.66	10.41	0.68	Merck Life Sciences (Darmstadt, Germany)
Tyrosine methyl ester	195.218	6.99	9.51	0.92	Merck Life Sciences (Darmstadt, Germany)
Venlafaxine	277.408	9.06	14.42	2.739	Merck Life Sciences (Darmstadt, Germany)
Verapamil	454.611	9.68		5.043	Merck Life Sciences (Darmstadt, Germany)

SI2: The various media used and their individual components. CCM A is WE basal + knockout, CCMB is PreS, CCMC is WE basal + BSA and CCMD is WE basal with FBS.

Components			Media variants						
Vendor	**Cat.N**	**Name**	**WE**	**WE basal**	**WE basal +Knockout**	**WE basal + 1% FBS**	**WE basal + 0,5 % BSA**	**PreS**	**PostS**
Thermo Fisher Scientific	A1217601	**William's E Medium, no phenol red**	100%	98%	97%	97%	97%	97%	97%
Life Technologies AS	41400045	**Insulin-Transferrin-Selenium (ITS -G) (100X)**	n/a	0.10%	0.10%	0.10%	0.10%	0.10%	0.10%
Life Technologies AS	35050061	**GlutaMAX™ Supplement**	n/a	1%	1%	1%	1%	1%	1%
Life Technologies AS	11140050	**MEM Non-Essential Amino Acids Solution (100X)**	n/a	0.10%	0.10%	0.10%	0.10%	0.10%	0.10%
Merck Life Sciences	P4333-100ML	**Penicillin-Streptomycin**	n/a	1%	1%	1%	1%	1%	1%
Thermo Fisher	11360070	**Sodium Pyruvate (100 mM)**	n/a	1 mM	1 mM	1 mM	1 mM	1 mM	1 mM
Thermo Fisher	10828028	**KnockOut™ Serum Replacement**	n/a	n/a	1%	n/a	n/a	1%	1%
VWR	1035-30-100ML	**Bovine serum albumin (BSA)**	n/a	n/a	n/a	n/a	1%	n/a	n/a
Fisher Scientific	10309433	**Cytiva HyClone™ Fetal Bovine Serum, South American Origin**	n/a	n/a	n/a	1%	n/a	n/a	n/a
Sigma Aldrich	E8875-1G	**Merck Life Sciences**	n/a	n/a	n/a	n/a	n/a	1 nM	0.1 nM
R&D Systems	5925-FS-010/CF	**Recombinant Human FSH alpha/beta Protein**	n/a	n/a	n/a	n/a	n/a	50 mIU/mL	5 mIU/mL

SI3: MRM transition table

Overview of the 10 compounds and their corresponding precursor ions, product ions, fragmentation voltages, collision energy (CE) voltages, ESI spray voltages, retention times, retention windows, and ESI ionization modes.

Name	Prec. ion	Prod. ion	Frag (V)	CE (V)	Ret. time (min)	Ret. window	Polarity
Atropine	290.41	124.1	166	25	3.2	0.5	Positive
Atropine	290.41	93.0	166	33	3.2	0.5	Positive
Cinnarizine	369.51	167.1	166	21	6.2	1	Positive
Cinnarizine	369.51	152.1	166	50	6.2	1	Positive
Cocaine	304.2	182.1	166	21	3.8	0.5	Positive
Cocaine	304.2	105.0	166	37	3.8	0.5	Positive
Doxepin	280.2	107.0	166	25	4.8	0.5	Positive
Doxepin	280.2	91.0	166	49	4.8	0.5	Positive
Droperidol	380.2	194.1	166	13	4.4	0.5	Positive
Droperidol	380.2	165.1	166	29	4.4	0.5	Positive
Loperamide	478.11	267.2	166	25	5.9	0.5	Positive
Loperamide	478.11	210.1	166	50	5.9	0.5	Positive
Methadone	310.2	265.2	166	13	5.4	0.5	Positive
Methadone	310.2	105.0	166	33	5.4	0.5	Positive
Nortriptyline	264.41	105.0	166	21	5.3	0.5	Positive
Nortriptyline	264.41	91.0	166	25	5.3	0.5	Positive
Quinine	163.1	189.1	166	13	3.3	0.5	Positive
Quinine	163.1	117.0	166	41	3.1	0.5	Positive
Salbutamol	240.31	222.1	166	5	1.5	1	Positive
Salbutamol	240.31	148.1	166	17	1.5	1	Positive

SI4: AGREEprep calculation score input

Comments to criterion #: 1. Inline sample preparation; 2. 3% MeOH and 0.1% FA of 0.1 mL = 30 µL hazardous materials per loading if pump is on during the 10-minute run. 3. Inline SPEs are used for hundreds of injections. 4. Amount waste used for SPE loading is 1 mL if pump is on during the 10-minute run. 5. Injection volume is 5 µL, max 50 µL used in sample vial, depending on experiment/number of replicates run. 6. Six samples per hour with the described setup (can be reduced for less comprehensive analyses). 7. Fully automated system with 2 or less steps. 8. Approximate value set to ca. 5 Wh. 9. LC-MS is end-platform. 10. Low amounts of MeOH and FA, Automated HPLC pump for loading (i.e. sample preparation).