Author: Laan, T. van der
Title: High-throughput quantification and unambiguous identification for metabolomics
Issue Date: 2020-12-14

Chapter 6

Conclusions and perspectives

CONCLUSIONS

The field of metabolomics is increasingly being implemented in various disciplines. This popularity results into an increased demand for metabolic profiles, which pushes the qualitative and quantitative performance of current analytical techniques to its limits. Mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectroscopy have shown to be very useful in acquiring metabolic profiles. These techniques have been used to answer the two most important questions in metabolomics: what is the identity and quantity of a metabolite in a mixture. Although technological advances have led to an unsurpassed performance of MS and NMR in the field of metabolomics, the potential of these techniques has not been fully exploited due to several analytical challenges. The presence of severe matrix effects hampers the fast quantification of metabolites in complex mixtures by mass spectrometry. In addition, the analysis complex mixtures will also result in complex NMR and MS/MS spectra which complicates the unambiguous identification of unknown metabolites. The aim of this thesis was to tackle these challenges by the development and application of innovative fractionation approaches and state-of-the-art MS and NMR analyses. We have used fractionation to facilitate the quantification and identification of metabolites in complex mixtures. Fractionation has shown to decrease the complexity of biological samples in a high-throughput fashion, which allowed for the fast quantification of metabolites using MS. Fractionation has also shown to be useful for in-depth purification and concentration of unknown features prior to NMR analysis. In this chapter, we will provide an overall conclusion with regards to the conducted research on fractionation. We will discuss the technical modifications needed to further increase the throughput of our fractionation platforms. We will also discuss a new data independent acquisition (DIA) technique and an advanced NMR coil, which could increase the selectivity and sensitivity of our fractionation platforms, respectively. We will finalize this chapter with a general conclusion on the power of metabolomics platforms in life sciences.

Throughput in metabolomics

Metabolomics is becoming increasingly important in several disciplines, ranging from the food industry to drug research and healthcare. Many metabolites have been identified that might have a diagnostic or prognostic value. Biomarker discovery in the field of metabolomics is mostly conducted by correlating metabolite levels with a certain physiological state, e.g. therapy response or disease progression. However, the underlying causal relationship is still often unknown and only a limited number of metabolites have been validated as clinical biomarkers. In order to achieve more mechanistic insights into metabolite biomarkers and validate more potential biomarkers, large-scale metabolomics studies are needed.[1,2] When dynamic biomarkers are applied in the clinic, multiple time points have to be included in order to properly monitor disease progression or drug response. When these dynamic biomarkers are used for common diseases, it drastically increases the demand for metabolic profiles. Ideally, these studies are analyzed by high-throughput platforms. This accelerates the biomarker discovery and application process and will most likely also reduce its costs because less instrument time and lab personnel is needed.

MS offers great possibilities for fast metabolic profiling. MS allows for a wide metabolic coverage and quantitative MS data can be obtained in principle in less than a second.[3] However, the presence of matrix effect is currently limiting the speed of MS analyses. Severe matrix effects are especially occurring during the analysis of biological samples because of the complexity of this sample type. To minimize matrix effect, MS is often coupled to a separation technique, e.g. liquid chromatography, in order to decrease the complexity of a sample before the ionization source.[4] However, performing this separation step is at the cost of analysis time.

Separation-free platforms, e.g. flow injection analysis (FIA), have been developed in order to improve the throughput of MS analyses. In these platforms, matrix effect is often tackled by a dilute-and-shoot approach or by using fast sample preparation methodologies. However, the cleanup efficiency of these methods is limited. The dilution of samples also dilutes the analytes while highly abundant matrix components can still cause suppression. In addition, commonly used sample preparation techniques, i.e. solid-phase extraction (SPE) and liquid-liquid extraction (LLE), usually result in two fractions. SPE allows for a retained and not retained fraction, whereas LLE allows for a polar and apolar fraction. These fractions are obtained either off-line in parallel or on-line and, when combined with FIA-MS, introduced into the MS without further separation. This allows for little cleanup efficiency and a high chance of severe ion suppression.

In this thesis, we have improved cleanup efficiency in a high-throughput fashion by the development of more extended fractionation approaches. We have employed high performance columns, which allow for within-fraction separation, and coupled multiple columns in series increasing the number of fractions. This makes the cleanup efficiency of our fractionation approaches higher than conventional sample preparation techniques, which only result in two fractions and do not allow for within-fraction separation. Our fractionation approaches are faster than conventional chromatography because time-consuming gradients are replaced by fast solvent switches. We have used shorter columns than conventionally applied to decrease the time needed for column flushing and equilibration. Therefore, fractionation provides an ideal balance between cleanup efficiency and throughput as is demonstrated in **chapter 3** and **4**.

Quantification in metabolomics

The quantitative analysis of metabolites in biological samples is essential in biomarker discovery and clinical decision-making.[5] Quantitative data is needed in order to find potential biomarkers that categorize patients and/or healthy volunteers into different clinical groups, e.g. good drug responder versus bad drug responder or healthy versus diseased. Eventually, a validated biomarker has to be quantified in patients and aid clinical decision-making. The accuracy of quantitation is of high importance to set clear boundaries between different clinical groups and to assign new patients to these groups. Inaccurate quantification can lead to the mislabeling of patients and all the associated consequences. Therefore, good quantitation is essential for a proper biomarker discovery and application pipeline. Quantitation can be impaired by the presence of matrix effect when samples consist of varying matrix composition, e.g. urine and blood samples taken at different times of the day. Variability in matrix composition will vary ion suppression/enhancement and, therefore, decrease the precision and repeatability of the analysis. Therefore, matrix effect should be properly controlled and ideally not present at all.

Fractionation can be used to obtain quantitative data in a high-throughput fashion. In **chapter 2**, we have accomplished this by employing fast reversed phase chromatography. We shifted the aim of general liquid chromatography from separating analytes to trapping known ion suppressors. Literature demonstrated that salts and phospholipids are notorious ion suppressors in blood samples.[6,7] We evaluated the effect of these ion suppressors in a flow injection analysis (FIA) coupled to MS. In this study, phospholipids suppressed the MS signal to a greater extent than salts and therefore, we developed a fast LC-MS analysis that enabled the removal of these matrix components from the elution region of the analytes.

The metabolomics application of **chapter 2** realized the quantification of gut metabolites that are involved in the trimethylamine-N-oxide (TMAO) metabolism. Since these gut metabolites have been linked to an increased risk of cardiovascular disease (CVD), there is an increased interest to get quantitative data on these potential biomarkers.[8] Since these gut metabolites are highly polar and phospholipids are apolar, the phospholipids are easily trapped by a conventional reversed phase column, leaving the gut metabolites in a cleaned flow-through. We could drastically decrease the LC gradient time because the analytes did not experience any retention on the column and the (phospho)lipids were eluted from the column by switching the mobile phase almost directly to 100% organic. The eluting lipids were directed to waste, resulting in less contamination of the MS. The fast LC-MS method successfully removed all ion suppression caused by the phospholipids in an analysis time of only three minutes.

The platform in **chapter 2** allowed for the quantification of five metabolites. The aim of **chapter 3** was to extend the fractionation approach in order to quantify 50 chemically diverse metabolite biomarkers. Although **chapter 2** demonstrated that phospholipids were the major source of ion suppression, salts also caused a considerable amount of ion suppression during the analysis of a blood plasma sample. The matrix effect profile of endogenous levels of phospholipids and salts together already quite closely resembled the matrix effect experienced in analysis of a non-fractionated plasma sample. Therefore, it is hypothesized that the majority of signal suppression is caused by these two matrix components. To overcome the ion suppression of both phospholipids and salts, we have extended the fractionation approach of **chapter 2** by the addition of two extra columns in **chapter 3**. The additional columns included a cation and anion exchange column in order to trap positive and negative salts, respectively. The combination of reversed phase and ion exchange resulted in a fractionation that was based on hydrophobic interactions and charge. This allowed for a very comprehensive fractionation of the metabolome and, therefore, we were able to quantify fifty known prognostic and/or diagnostic biomarker metabolites from very diverse chemical classes, i.e. amino acids, amines, acylcarnitine, sugars, purines, organic acids and fatty acids. To target a very broad chemical range of metabolites, the approach in **chapter 3** used three serially coupled columns to allocate known ion suppressors, such as phosphoplipids and positive and negative ions from salts, over different fractions, minimizing their adverse effects during electrospray ionization.

The throughput of the comprehensive fractionation methodology in **chapter 3** was maintained by the use of fast solvent switches and short columns (≤1 cm). This allowed for an analysis time of only three minutes per MS polarity. Moreover, the use of high performance (particle size ≤ 5 μm) SPE columns allowed for a within-fraction separation. This resulted in the separation of phospholipids from acylcarnitines and fatty acids within the same fraction. The beneficial effect of the fractionation platform was demonstrated by a comparison with FIA. The fractionation decreased the ion suppression from 89% to 25% and allowed for a sensitivity that was sufficient to analyze endogenous concentrations of a wide range of metabolites in plasma. Although the fractionation method only took 3 minutes per MS polarity, it demonstrated a similar sensitivity in comparison with conventional LC-MS. LC-MS has a considerably lower throughput because of the increased gradient time (3-22 minutes per sample).[9,10,11,12,13,14] Therefore, we have shown that by efficiently fractionating metabolites and known ion suppressors, it is possible to achieve a similar performance in comparison with LC-MS in a high-throughput fashion.

A challenge in a fractionation-based separation is the lack of separation of isomers. Isomers have the same *m/z* value, which means they cannot be distinguished on an MS^1 level. However, structural isomers are structurally different which means that the fragmentation of these isomers could lead to the production of unique product ions. When unique product ions are found, it possible to distinguish structural isomers. In **chapter 5**, we have evaluated different types of DIA techniques in order to selectively quantify unique product ions of structural isomers. We have shown that variable sequential window acquisition of all theoretical mass spectra (SWATH) in combination with hydrophilic interaction chromatography (HILIC) allowed for the selective quantification of five structural isomer pairs.

The use of fixed SWATH windows resulted in several inaccurate quantifications, which demonstrated that the customization of SWATH windows is of utmost importance in the prevention of product ion overlap. In MS^{ALL}, in which the Q1 filter covered the whole mass range of interest, peak integration was hampered by fluctuating baselines and the linearity and repeatability were drastically impaired in comparison with SWATH. The combination of DIA and fractionation resulted, in general, in low quality MS/MS scans. Even the use of variable SWATH windows could not prevent product ion overlap. This was most likely due to the relatively small peak widths that were a result of the high-throughput character of the separation. Smaller peaks allow for less fragmentation time, which means that the size of the SWATH windows has to increase. This results in the fragmentation of more precursor ions per SWATH window, which leads to more product ion overlap. The MS/MS performance is even further pushed by the presence of severe coelution which is often a problem in high-throughput platforms. Nevertheless, our results demonstrate that a conventional separation in combination with variable SWATH results in the accurate quantification of structural isomers.

Identification in metabolomics

In many metabolomics applications, the identity of the analytes of interest is not known in advance. An untargeted metabolic screening is used in these applications to find new interesting metabolic features that could potentially be used as valuable biomarkers. In order to translate an unknown metabolic feature to a structurally annotated metabolite, an identification step is necessary. However, the unambiguous identification of metabolites is considered as one of the most challenging aspects of present-day metabolomics. Methodologies used for metabolite identification are often sensitive to errors or very labour-intensive. MS and NMR are often combined to elucidate the structure of unknown metabolic features, because of their unique advantages and their complementary nature.

MS-based identification strategies are performed on the basis of high-quality MS/MS and MS^n scans. MS/MS scans are needed in order to obtain structural information about certain *m/z* and retention time features. The MS/MS scan can be deciphered by the user or searched in MS/MS libraries and eventually lead to a potential match. However, the acquisition of these MS/MS scans for unknown features remains challenging. In data dependent acquisition, the coverage of MS/MS scans is limited because precursor ions are selected based on, for example, intensity and an inclusion list. DIA provides a more comprehensive coverage since all precursor ions in the selected mass range are fragmented. However, the mass range of the first quadrupole (Q1) often has to be increased in order to achieve this coverage. This leads to the formation of complex MS/MS spectra, which decreases the power of these scans substantially.

In **chapter 4**, we have used SWATH in order to obtain selective DIA fragmentation data. We have shown that by using a HILIC separation in combination with SWATH, the top hit from a NIST 2017 library search matched the known identity of 20 test features. The use of MS^{ALL} scans resulted in MS/MS scans that were too complex for metabolite identification. The combination of the fractionation platform of **chapter 3** and DIA also resulted in MS/MS spectra that were unsuitable for identification purposes. The data analysis software was unable to recognize specific fragmentation patterns from the spectral library in the crowded DIA MS/MS data. This shows that an extensive separation and selective fragmentation are essential in the acquisition of untargeted MS/MS spectra.

The certainty of metabolite identifications drastically improves when structural annotations are confirmed by two complementary techniques.[15] In addition, when spectral matching does not result in a library hit (e.g. compound has not been identified before or does not fragment), NMR is crucial for de-novo structure elucidation. Therefore, we have developed an identification platform for complex mixtures including MS and NMR in **chapter 6**. We have used this platform to identify five taste-related unknown retention time and *m/z* features in soy sauce. Soy sauce is a complex mixture and the abundance of compounds of interest can be low, which makes structural elucidation by NMR a challenging task. For this, we developed a fractionation strategy for the purification of unknown features prior to NMR analysis. The fractionation employed a directed two-dimensional chromatography method, which was adaptable to differences in orthogonality of features and impurities. There are several chromatography phases known that offer orthogonal separations. Our results demonstrated that the combination of low and high pH reversed phase chromatography yielded the cleanest fractions for most unknown features. However, one feature highly benefited from the combination of reversed phase chromatography and HILIC. Fractions were collected off-line in order to concentrate the unknown features prior to the NMR analysis. The purified features were clean and concentrated enough for the acquisition of one and two-dimensional NMR spectra, which lead to the structural confirmation of the unknown features.

Although the use of one-dimensional chromatography and NMR is common practice, we have demonstrated that there are still highly abundant impurities present in first dimension fractions. These impurities might cause peak overlap with the unknown features preventing the recognition of typical spin patterns. Therefore, the use of a second dimension chromatography offers great potential in reducing the complexity further. However, the usefulness of the second dimension chromatography is highly dependent on its orthogonality to the first dimension. This orthogonality is in turn highly dependent on the chemical properties of the unknown feature and the remaining impurities. Therefore, it is important to critically assess the composition of the first dimension fractions. This determines which second dimension chromatography results in the most efficient separation of the unknown feature from the remaining impurities.

FUTURE PERSPECTIVES

High-throughput quantification

In **chapter 2**, we demonstrated an unconventional use of high performance chromatography columns by trapping ion suppressors instead of employing a time-consuming separation of the analytes. In **chapter 3,** we extended this approach by adding multiple high performance columns in series, which enabled the allocation of ion suppressors and allowed for the analysis of a wide variety of chemical classes. We have shown that this approach decreased ion suppression drastically and that the sensitivity was sufficient for the analysis of a wide range of endogenous metabolites in biological samples.

The analysis time of the fractionation method in **chapter 3** was substantially lower in comparison with general LC-MS. However, in comparison with other high-throughput platforms, the analysis time is still relatively high. This can be explained by the hardware that is used for the fractionation method. A conventional LC-MS system was extended with an additional pump and two six-port valves. LC-MS systems include gradient pumps, which are used for the loading and extraction of LC columns. These pumps make use of a mixer with a relatively large dead-volume and are optimized for reliable gradient formation instead of fast solvent switches. Since run times are generally long, the autosampler of an LC-MS system is also not designed to deliver a high sample throughput. Moreover, the connections of three guard columns, six-port valves, pumps and an MS resulted in a relatively large dead-volume, which was disadvantageous for the throughput of the fractionation.

An example of a dedicated automated SPE device is the RapidFire system, which is manufactured by Agilent Technologies.[16] In this system, the sample and solvent handling, tubing and positions of modules are all designed in a way that is most beneficial in terms of sample throughput. The analysis time for this method is dramatically lower than our proposed method. The analysis has been shown to be feasible within 8.5 s, whereas three minutes are needed in our fractionation platform. Where we perform three SPE procedures instead of one per analysis and achieve a significant reduction in matrix effect, the RapidFire is considerably faster in the execution of an SPE.

The coverage and cleanup efficiency of dedicated automated SPE devices are limited because only one SPE column is used at a time. Therefore, these systems would highly benefit from the inclusion of serially coupled high performance columns. When different sorbent chemistries are connected through six-port valves, a more comprehensive fractionation can be realized as is demonstrated in our fractionation platform. By extending already existing automated SPE devices with our proposed methodology, an optimized solvent handling and sample injection can be combined with a fractionation that is based on both polarity and charge. This would result in a fractionation platform that excels in both coverage and reduction of ion suppression as well as throughput.

Next generation data independent acquisition

In **chapter 5**, we demonstrated a promising global profiling method with a good qualitative and quantitative performance. The combination of variable SWATH and HILIC allowed for correct identifications and accurate quantifications. However, with increased Q1 mass windows, the occurrence of product ion overlap or complex MS/MS spectra remains a risk factor. When multiple compounds fall within the same SWATH window and have identical retention times, the association between precursor ions and product ions is lost. This makes it more difficult to deconvolute MS/MS spectra that are obtained with larger Q1 window sizes. Therefore, the selectivity of analytical methodologies using SWATH analysis should always be carefully examined. It might be possible that the performance of our variable SWATH and HILIC method only holds for the particular metabolomics application used in **chapter 5**. A new performance assessment has to be made when, for example, a different biological matrix is analyzed. Differences in sample types can substantially change the complexity and composition of the metabolites and it is possible that additional coeluting compounds result in more product ion overlap or complex MS/MS spectra. This could eventually lead to a lack of quantitative and qualitative performance. Moreover, SWATH was not selective enough for our high-throughput application due to the severe coelution of metabolites and the relatively large SWATH windows, which were a result of small peak widths.

Recently, Sciex introduced a new technique, which increases the selectivity of SWATH analysis: scanningSWATH. In scanningSWATH, the Q1 scans over the mass range of interest in steps of 0.1 Dalton per 0.1 ms within each MS cycle.[17] The time-of-flight (TOF) pulses are synchronized with the Q1 scanning steps, which gives the MS data an extra dimension (Q1 *m/z*) next to the exact mass and retention time. This allows the user to plot the product ion intensity against Q1 *m/z*, which makes it possible to identify product ion overlap from other precursor ions. The extra dimension allows for a better association between product and precursor ions and, therefore, results in a more selective DIA type of fragmentation. The Q1 window size is in a similar range as conventional SWATH, which means that Q1 windows encompassing the precursor ion of interest can be summed in order to increase the sensitivity of the acquired TOF scans.

In **chapter 5**, some DIA platforms demonstrated complex MS/MS spectra and product ion overlap. The lack of selectivity in current DIA techniques could be overcome by the use of scanningSWATH. Concerning the chromatography of the fractionation platform, the mass range of **chapter 5** is easily fragmented using the scanning speed of scanningSWATH. By introducing the extra Q1 m/z dimension, the product ions could more easily being traced back to their corresponding precursor ion, improving the selectivity of the MS/MS spectra. This results in cleaner MS/MS spectra for identification and a more accurate quantification of analytes. Eventually, this might lead to a high-throughput global profiling method, in which unknown features can be both identified as well as quantified.

The breath of processing options for scanningSWATH data makes it challenging to develop data analysis software that exploits the full potential of scanningSWATH.[18] When one wants to aim for the highest selectivity, it is key to sum TOF pulses that do not demonstrate product ion overlap with other coeluting precursor ions. However, when sensitivity becomes critical, it might be more important to sum all the data containing the concerned analyte. Eventually a compromise between sensitivity and selectivity should lead to data analysis software that allows for the implementation of scanningSWATH into the field of metabolomics.

Sensitive and affordable NMR analysis

In **chapter 6**, we demonstrated a comprehensive fractionation platform for the purification of unknown features in complex samples, resulting in purified features, which were clean and concentrated enough for the acquisition of one and two-dimensional NMR data. However, in order to have an adequate sensitivity during the NMR analysis, a sufficient amount of starting material is needed. Due to column capacity, ten consecutive injections were needed to realize this, which made the fractionation procedure relatively time-consuming. The amount of starting material could be reduced by the use of more sensitive NMR systems, e.g. higher field systems or cryogenically cooled probes. However, this increase in sensitivity comes at the cost of substantially higher instrument costs.

A cost-effective way of improving the sensitivity of NMR is the use of microcoils.[19] The active volume of these particular coils is in the microliter range, which allows for a more sensitive and cheaper coil design. Moreover, the coil design makes it possible to position the sample closer to the detection coil, which in turn also increases the sensitivity. Olson *et al.* have shown that in comparison with a conventional NMR probe, the mass sensitivity of a microcoil probe is approximately tenfold higher.[20] Because the sensitivity improvement is based on mass, the use of a microcoil is highly advantageous for mass-limited or pre-concentrated samples.

Our directed two-dimensional chromatography method could highly benefit from the use of microcoil NMR. The number of injections on the HPLC system could be reduced when the mass sensitivity in a microcoil is substantially higher. This will dramatically improve the throughput of the identification platform. Although there are clear advantages of the use of microcoil NMR, the sample introduction into the microcoil is less straightforward. Fractions have to be concentrated into a few microliters and subsequently positioned into the microcoil without dilution and diffusion, which is a challenging task. However, there are several analytical techniques which could make this possible. First of all, machine vision-controlled droplet evaporation has been shown to allow for solvent exchange and pre-concentration of samples into a submicroliter droplet.[21] In this pre-concentration technique, samples are infused into a hanging droplet at a constant flow rate. The heating rates are adjusted by machine vision in order to maintain a stable droplet. The system allows for up to 90% solvent exchange, which can be used to replace the sample solvent by deuterated solvents for NMR analysis. Moreover, concentrating a sample into a droplet makes the sample volume and microcoil volume more compatible.

The transfer of concentrated microliter samples can be established by using flow NMR in combination with segmented flow analysis. When concentrated samples are injected into an immiscible carrier fluid, diffusion is prevented, which allows for the transfer of samples over long distances without causing dilutions. This is beneficial for flow NMR analysis, because the samples often have to be transferred over a few meters of tubing because the sampler is positioned outside the magnet’s fringe field. Fluorocarbon fluid appeared to be a promising immiscible carrier fluid and it allowed for the transfer of microliter samples to a microcoil probe.[22] The sample can be positioned and analyzed in the microcoil by a stop-flow analysis.

FINAL REMARKS

The impact of metabolomics in life sciences is becoming increasingly clear. At the heart of these developments are good analytical platforms, which allow for metabolite identification and quantification. MS and NMR have shown to be very powerful at providing qualitative and quantitative data. However, the potential of these techniques has not been fully exploited due to several analytical challenges. We have shown that these challenges can be overcome by using a fractionation approach. Fractionation can be applied in all shapes in sizes, which makes it highly flexible and adaptable. We have shown its applicability for purification prior to NMR analysis and high-throughput MS analyses. These methodologies demonstrated that, for the development of an analytical platform, knowledge about your matrix components is as important as knowledge about your analytes. This knowledge allowed us to achieve an in-depth purification of metabolites from complex mixtures, which is essential for NMR identification. On-line fractionation realized a fast and comprehensive analysis of the metabolome while maintaining a performance that was comparable with conventional LC-MS. Therefore, we have made a big step in bringing large-scale metabolomics closer to reality. This progress can be continued by the implementation of some recent technological advances, which could highly benefit a fractionation-based analysis. We expect that fractionation coupled to a state-of-the-art analyzer will realize a global metabolic profiling method that allows for both selective identification and accurate quantification in a high-throughput fashion. A fast and global metabolic profiling method will facilitate the analysis of large-scale metabolomics studies and strengthen the impact of metabolomics in life sciences.

REFERENCES

1. Miggiels, P., Wouters, B., Westen, G. J. Van, Dubbelman, A. & Hankemeier, T. Novel technologies for metabolomics: more for less. TrAC - Trends Anal. Chem. 2018, 1–9 (2018).
2. Zampieri, M., Sekar, K., Zamboni, N. & Sauer, U. Frontiers of high-throughput metabolomics. Curr. Opin. Chem. Biol. 36, 15–23 (2017).
3. Sinclair, I. et al. Novel Acoustic Loading of a Mass Spectrometer: Toward Next-Generation High-Throughput MS Screening. J. Lab. Autom. 21, 19–26 (2016).
4. Gowda, G. A. N. & Djukovic, D. Overview of Mass Spectrometry-Based Metabolomics: Opportunities and Challenges. Methods Mol Biol 1198, 3–12 (2014).
5. Zhou, J. & Yin, Y. Strategies for large-scale targeted metabolomics quantification by liquid chromatography-mass spectrometry. Analyst 141, 6362–6373 (2016).
6. Trufelli, H., Palma, P., Famiglini, G. & Cappiello, G. An overview of matrix effects in liquid chromatography-mass spectrometry. Indian J. Exp. Biol. 30, 491– 509 (2011).
7. Ismaiel, O. A., Zhang, T., Jenkins, R. G. & Karnes, H. T. Investigation of endogenous blood plasma phospholipids, cholesterol and glycerides that contribute to matrix effects in bioanalysis by liquid chromatography/mass spectrometry. J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 878, 3303–3316 (2010).
8. Wang, Z. et al. Gut flora metabolism of phosphatidylcholine promotes cardiovascular disease. Nature 472, 57–63 (2011).
9. Trabado, S. et al. The human plasma-metabolome: Reference values in 800 French healthy volunteers; Impact of cholesterol, gender and age. PLoS One 12, 1–17 (2017).
10. Chuang, C. K. et al. A method for lactate and pyruvate determination in filter-paper dried blood spots. J. Chromatogr. A 1216, 8947–8952 (2009).
11. Giesbertz, P., Ecker, J., Haag, A., Spanier, B. & Daniel, H. An LC-MS/MS method to quantify acylcarnitine species including isomeric and odd-numbered forms in plasma and tissues. J. Lipid Res. 56, 2029–2039 (2015).
12. Matsunami, R. K., Angelides, K. & Engler, D. A. Development and validation of a rapid 13C6-Glucose Isotope Dilution UPLC-MRM Mass Spectrometry Method for Use in determining system accuracy and performance of blood glucose monitoring devices. J. Diabetes Sci. Technol. 9, 1051–1060 (2015).
13. TAKAHASHI, H. et al. Long-Chain Free Fatty Acid Profiling Analysis by Liquid Chromatography–Mass Spectrometry in Mouse Treated with Peroxisome Proliferator-Activated Receptor α Agonist. Biosci. Biotechnol. Biochem. 77, 2288–2293 (2013).
14. Liu, J. et al. Simultaneous targeted analysis of trimethylamine-N-oxide, choline, betaine, and carnitine by high performance liquid chromatography tandem mass spectrometry. J.

Chromatogr. B Anal. Technol. Biomed. Life Sci. 1035, 42–48 (2016).

15. Pan, Z. & Raftery, D. Comparing and combining NMR spectroscopy and mass spectrometry in metabolomics. Anal. Bioanal. Chem. 387, 525–527 (2007).
16. Zhang, X. et al. SPE-IMS-MS: An automated platform for sub-sixty second surveillance of endogenous metabolites and xenobiotics in biofluids. Clin. Mass Spectrom. 2, 1–10 (2016).
17. Ivosev, G., Cox, D. M., Bloomfield, N., Wasim, F. & Leblanc, Y. Scanning SWATH ® Acquisition Method for Improved Compound Screening. ASMS (2018).
18. Messner, C. et al. ScanningSWATH enables ultra-fast proteomics using high-flow chromatography and minute-scale gradients. bioRxiv 656793 (2019). doi:10.1101/656793
19. Grimes, J. H. & O'Connell, T. M. The application of micro-coil NMR probe technology to metabolomics of urine and serum. J. Biomol. NMR 49, 297–305 (2011).
20. Olson, D. L. et al. Microflow NMR: Concepts and capabilities. Anal. Chem. 76, 2966–2974 (2004).
21. Schoonen, J. W. et al. Solvent exchange module for LC-NMR hyphenation using machine vision-controlled droplet evaporation. Anal. Chem. 85, 5734–5739 (2013).
22. Kautz, R. A., Goetzinger, W. K. & Karger, B. L. High-throughput microcoil NMR of compound libraries using zero-dispersion segmented flow analysis. J. Comb. Chem. 7, 14–20 (2005).