Chapter 7

Conclusions and Perspectives, Samenvatting

CONCLUSIONS AND PERSPECTIVES

With the persistent efforts to improve healthcare while the costs are ever-increasing, there is a growing demand for healthcare innovations including faster and cheaper analytical methods to support clinical decisions. The in-depth phenotyping of a biological system, such as comprehensive small-molecule profiling, will be key for breakthroughs in biomedical and pharmacological research towards the development of personalized medicine. However, there is often an inverse relation between sensitivity (coverage of the number and type of small-molecules) and the speed and costs of the assay. Although classical small-molecule assays such as microscopy- and enzyme-linked immunosorbent assays predominantly rely on single analyte readouts at a possible high-throughput manner, there is a need for more in-depth small-molecule assays while maintaining reasonable throughput. Yet the comprehensive, high-throughput profiling of a wide variety of small molecules is a major challenge, especially in the field of metabolomics.

In this thesis the development of new methods towards high-throughput, mass spectrometry-based profiling of small molecules in complex biological samples is presented. One option for high-throughput mass spectrometric profiling is direct infusion in which no separation techniques are employed prior to MS detection. With the pre-analytical part being a major bottleneck in a typical direct-infusion MS-based analytical workflow, the focus was set on the development of new sample pretreatment and sampling procedures, including miniaturized and automated concepts with the potential for high-throughput application.

In **Chapter 2** the current state-of-the-art in sample pretreatment techniques for mass spectrometry-based metabolomics has been reviewed. Although to date no universal sample pretreatment technique is available for the comprehensive analysis of the metabolome, the combination of protein and lipid removal is often reported to be an effective strategy to improve metabolite coverage and reproducibility. It has been described that the recent innovations in automated offline well-plate extraction (including protein precipitation (PPT), liquid-liquid extraction (LLE) and solid-phase extraction (SPE) have allowed fast sample cleanup and partly removed the bottlenecks associated with sample pretreatment. It has been suggested that in the future sample pretreatment procedures should not only be automated and fast, but also quantitative and standardized so that metabolomics data of different studies are comparable within and between labs. This is especially important as some metabolites can be partly bound to proteins or other factors in blood, and are partly free in solution.

In **Chapter 3** the rapid phenotyping of early zebrafish embryogenesis using nanoESI high resolution (HR) direct infusion mass spectrometry (DI-MS) has been described. It has been demonstrated that with limited but efficient sample pretreatment in-depth profiles could be obtained which could distinguish five developmental stages of early embryogenesis with a time resolution of 1 hour. It has been shown that with thorough data cleanup, 102 molecular features proved relevant which reflected the onset of (earlier reported) gene expression and the increase in energy requirement. It was concluded that effective and preferably fast sample pretreatment procedures are desirable to obtain more reproducible in-depth information about metabolites at lower concentrations.

By using limited sample preparation and proper data processing, in-depth metabolic profiles of complex samples can be obtained using nanoESI-DI-MS. Still, in order to exploit the full potential of DI-MS, effective sample pretreatment/fractionation methods should be developed (as proposed in **Chapter 4** and **5**) which preferably includes the extraction of small polar molecules and its separation from interfering (endogenous) salts, as proposed in **Chapter 2.**

In **Chapter 4**, three-phase electroextraction (3-phase EE) into a two microliter droplet, which was hanging from a conductive pipette tip, has been described. By the electromigration of analytes from a larger volume of aqueous sample, through an immiscible organic filter phase, into a small-volume of aqueous acceptor phase, a fast analyte enrichment of metabolites has been achieved. The organic filter phase composition imposed the selectivity of 3-phase EE, a feature which is useful in tuning the extraction into the analyte window of interest, and thus preventing the migration of proteins into the acceptor phase. Therefore, 3-phase EE has great versatility as analyte enrichment and purification (deproteinization) can be integrated in one single method. Proof of principle towards the online integration in an automated nanoESI-DI-MS platform has been realized which shows the potential of 3-phase EE in HTS.
It is expected that the range of analytes compatible with 3-phase EE may be extended by the optimization of the organic filter phase composition which may make 3-phase more suitable for comprehensive profiling purposes. In addition, though not demonstrated in this thesis, 3-phase EE might have desalting properties caused by the organic filter phase. Furthermore, by the optimization of 3-phase EE geometries, enrichment could be further improved. In future, by using microfluidic-chip technologies, 3-phase EE could be further downscaled and the potential of massive parallelization in the context of high-throughput screening (HTS) could be explored.
In **Chapter 5** a miniaturized automated LLE has been developed in a 384 well-plate based on gas pressure-assisted mixing followed by passive phase separation. Our method enabled the whole extraction procedure being executed and integrated in an automated nanoESI-DI-MS robot. Through varying the gas pressure and its duration, the effectiveness of the mixing procedure in terms of recovery has been optimized. It has been demonstrated that for drugs in human plasma, this new platform proved excellent analytical characteristics in terms of precision and linearity compared to e.g. a conventional LLE procedure. These experiments show the potential of this new fully-automated platform for the analysis of drugs in complex (volume-limited) samples such as plasma and dried blood spots.
As demonstrated in this thesis, the dichloromethane phase can be successfully analyzed after the LLE procedure, which makes this method very suitable for the analysis of relatively apolar, small molecules such as drugs but possibly also lipids. In principle, both phases could be infused as the analysis of the (cleaned-up) aqueous phase might also be useful for measuring the more polar molecules. However, caution must be exercised when the aqueous phase contains high salt concentrations and dilution might be an extra step which should be taken into account.
In **Chapter 6** a miniaturized sampling method using a microneedle has been described which enabled monitoring drug uptake in the (small-volume) yolk of zebrafish larvae using MS analysis. It has been demonstrated that the current methods based on embryo lysis did not take into account the possible adherence of drugs to the skin, resulting in false-positive uptake readouts. It has been shown that our new method offers a great possibility to monitor how any novel compound behaves within the zebrafish system, as to date no single physicochemical property has been identified to accurately predict compound uptake. With our new method, the uptake data of two commonly used anti-tuberculars, together with 15 preclinical compounds, have been monitored and correlated to high-throughput screening data from M. marinum infected zebrafish larvae as well as to other conventional *in vivo* and *in vitro* models. It has been proposed that our improved zebrafish drug screening platform could obtain new insights into interpretation and prediction of drug efficacy.
It is expected that this method opens a doors for the use of zebrafish embryos for in-depth

pharmacological assessment of activity (such as PK modelling) and therefore enables a wider acceptance for the zebrafish in the field of drug discovery and development. Moreover, next to the targeted drug analysis, a comprehensive profiling of small molecules could be performed in order to gain extra phenotypic information such as in the field of metabolomics and systems pharmacology.

High-throughput small molecule profiling requires new approaches in sample preparation to allow fast DI-MS analysis. In summary, in this thesis first steps of new methods have been presented which are promising in small-molecule analysis using DI-MS. In **Chapter 4** and **5** new concepts regarding sample pretreatment have been developed. In general, the potential of both 3-phase EE and the described micro-LLE method might be improved when a derivatization method can be integrated prior to extraction. By altering the physico-chemical properties of the relatively polar target molecules it might enable effective desalting and improved ionization efficiencies.

Most of the methods presented in this thesis were integrated with direct infusion MS, as it is potentially the most suitable MS-based platform for comprehensive, high-throughput profiling of small molecules. However, as described in **Chapter 6**, the small-volume yolk samples were analysed with LC-MS. Future research could be directed towards its coupling to DI-MS for which 3-phase EE might be a promising candidate since analytes can be purified and enriched in a small volume as described in **Chapter 4**.

When the presented high-throughput concepts are further optimized, it is expected that these methods can be used in biomedical and pharmacological screening which eventually may result in the development of personalized medicine.