Summary
Metabolites are small molecules (usually < 1 kDa) that form the molecular fundament of life and are the result of biological systems interacting with their environment. The (poly)carboxylic acid intermediates of the Krebs cycle serve as a good example as they are essential to the cellular respiratory system and regulate energy metabolism. Metabolomics is defined as ‘the scientific study of the metabolome, or set of metabolites within an organism, cell or tissue’. In the last decade, metabolomics has emerged as an important field next to the other ‘-omics’ technologies. By being able to directly investigate metabolites participating in biochemical processes, disciplines such as systems biology greatly benefit. Also, metabolites might serve as biomarkers of disease progression and prediction, to monitor and diagnose, respectively. Basically, two different approaches can be taken when metabolites are to be measured. It is important to determine whether a defined set of compounds or as many metabolites as possible are of interest/to be analysed. When earlier research or theory is ground for a hypothesis on what to look for, a targeted approach should be devised. However, when this is not the case or when an unbiased approach is preferred, untargeted analysis might result in a better overview of the metabolic composition of a sample.

In chapter 2, recent developments in the targeted chromatography–mass spectrometry analysis of biologically relevant endogenous carboxylic acids are reviewed, addressing specific issues for small organic acids, fatty acids, eicosanoids, and bile acids. Sample preparation, derivatisation techniques, separation and MS detection of these different carboxylic acid classes are evaluated. Ultimately, based on structural features, the reader is guided to the most versatile, sensitive and facile analytical methods for the carboxylic acid class under evaluation.

In chapter 3, a thorough evaluation of different column chemistries in combination with different mobile phases for fast liquid chromatography–mass
spectrometry (LC–MS) urinary metabolic profiling is presented. Three porous HILIC (hydrophilic liquid interaction chromatography) materials were investigated, next to core-shell C18-, XB-C18- and PFP-RPLC (pentafluorophenyl-reversed phase liquid chromatography) material. The performance of the selected column chemistries was tested in a non-targeted manner with pooled urine samples and in a targeted manner with a set of 54 common urinary metabolites. In order to evaluate the differential behaviour of the tested columns in a targeted manner, we applied a peak scoring algorithm. This algorithm takes into account several quality criteria such as retention time, dead time, peak height, and peak shape. In general, HILIC columns provide more retention for polar metabolites. When an experimental setup was performed by comparing different column chemistries for LC–MS in combination by different solvent conditions on a broader range of small (urinary) metabolites, it can be concluded from chapter three, that HILIC proved to be advantageous for the analysis of the 54 compounds tested. Also when an untargeted approach was executed by the analysis of human urine samples, more molecular features were found by using HILIC. Diol and amine chemistries performed better in terms of molecular features found, especially when compared to RPLC. This can be explained by the fact that urine contains mostly highly polar components. Interaction to a solid phase mostly relying on Van-der-Waals interaction, as is the case with RPLC, is not sufficient to retain most of these analytes. The field of metabolomics will benefit from HILIC

When an analyte is not readily ionisable, as is the case for malondialdehyde (MDA), other techniques then MS should be considered. This has become a well-established biomarker for oxidative stress. The most commonly used way to determine urinary MDA levels is the thiobarbituric acid (TBA) assay, which suffers from several drawbacks. The novel derivatisation methodology described in chapter
4 is based on the mild labelling of MDA with 2-aminoacridone, which can be carried out in aqueous citrate buffer at 40 °C, yielding a highly fluorescent substance. No further sample preparation than mixing with the necessary chemicals is necessary. The formed MDA derivative can conveniently be separated from the label itself and matrix constituents by gradient LC in less than 5 minutes on a cyano-based reversed-phase material. The method was validated with respect to matrix effects, linearity, selectivity and sensitivity. Values as low as 1.8 nM for the LOD and 5.8 nM for the LOQ (limit of quantification) could be achieved. Standard addition quantitation was applied for the determination of MDA in human urine samples.

Also, as described in chapter five, derivatisation can improve the analysis of small carboxylic acids significantly. As not all MS instrumentation is able to sufficiently cope with the very low masses, nor negatively charged analytes, increasing the weight and transformation to a protonatable derivative is shown to be effective. With commercially available N-methyl-2-phenylethanamine (MPEA), in combination with the water soluble carbodiimide EDC, we successfully derivatised clinically relevant carboxylic acids in biological matrices. The SPE-LC-method aids to a fast and automated sample clean-up, reducing the introduction of salts and other contaminants into the MS ion source, thus prolonging the reliable use of the instrument. Without the need for special ultra-high-pressure pumps, the use of the core–shell column material allows short analysis times of less than 10 min, including separation and re-equilibration, and resulting in sharp peaks with widths down to 10 s at the base. Furthermore, the new approach resulted in LODs which were low enough to determine the analytes of interest in relevant matrices down to 12 nM, where other methodologies show LODs in the µM ranges.

Chapter 6 presents a comprehensive gas chromatography (GC)–MS based targeted analytical platform for the simultaneous quantitative analysis of fatty acids
and sterols. As it is important to look at multiple classes at once, a one-pot derivatisation approach was described in this chapter. A GC–MS method for fatty acids and sterols was developed by combining two consecutive silylation reactions by N,O-Bis(trimethylsilyl) trifluoroacetamide (BSTFA) and N-methyl-N-tert-butylidimethylsilyl trifluoroacetamide (MtBSTFA), enabling the analysis of both classes. After validation in human plasma, also isotopologue analysis for flux analysis has been achieved. The validated method features short run times, straightforward sample pre-treatment allowing the analysis of both free and bound lipids and high sensitivity showing lower limits of quantification in the low ng/mL range. The method shows great potential for the screening of fatty acid synthase and cholesterol biosynthesis inhibitors as well as the comprehensive study of both biochemical pathways, using $^{13}$C-flux analysis. The described derivatisation methods would all benefit from automation as manual preparation is still relatively laboursome and will become impractical for larger sample cohorts. Automation will speed up the process and minimize the induced errors.