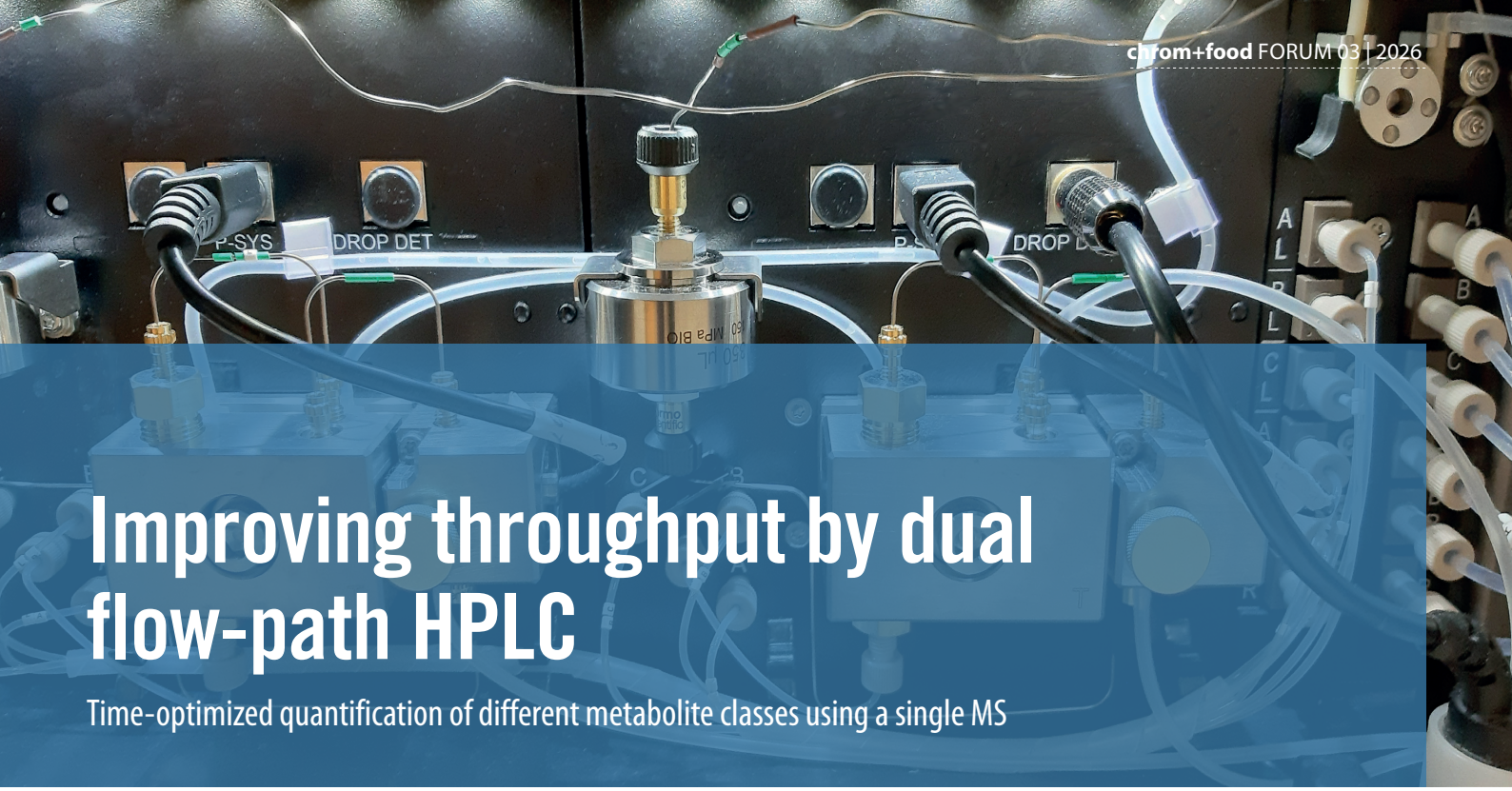

Improving throughput by dual flow-path HPLC

Time-optimized quantification of different metabolite classes using a single MS

Dr. Christian Faist | Edda Tacke | Prof. Dr. Heiko Hayen

Biomedical studies often involve the analysis of a wide range of analytes. Their varying physicochemical properties frequently hinder analysis by a single method, making it necessary to employ different chromatographic approaches, with the disadvantage of increased analysis time. Therefore, we developed a time-optimized LC-MS approach using only one mass spectrometer, but utilization of two orthogonal chromatographic techniques (reversed phase-HPLC and HILIC) in parallel.

The field of metabolomics involves the analysis of a wide variety of compounds. Within metabolomics, the gut microbiome has emerged as a major focus of interest due to its association with numerous processes within the human body, e.g. host-microbe interactions on the immune system [1]. Short-chain fatty acids (SCFAs) constitute a major group of gut microbiome-derived metabolites. Another metabolite that is known to be associated with cardiovascular diseases is trimethylamine *N*-oxide (TMAO) [2, 3]. It is also synthesized in the gut through microbial processes, primarily by converting dietary components such as the amines carnitine, betaine and choline to trimethylamine (TMA), which can then be further metabolized into TMAO in the liver [4].

Since these two metabolite classes differ strongly in their physicochemical properties, they usually require two distinct analytical methods. Therefore, we developed a dual-HPLC-MS method based on a HPLC system comprising two separate flow-paths using a dual split-sampler and dual gradient pump to allow time-saving analysis of SCFAs and amines (e.g., TMAO and carnitine) in human blood samples.[5] This approach involved a two-step sample preparation strategy comprising SCFA derivatization and amine processing from the same sample, which is depicted in Figure 1.

The method allows recovery of SCFAs and amines (such as trimethylamine *N*-oxide and carnitine) from a single human serum sample. SCFAs were derivatized to their corresponding 3-nitrophenylhydrazones for improved

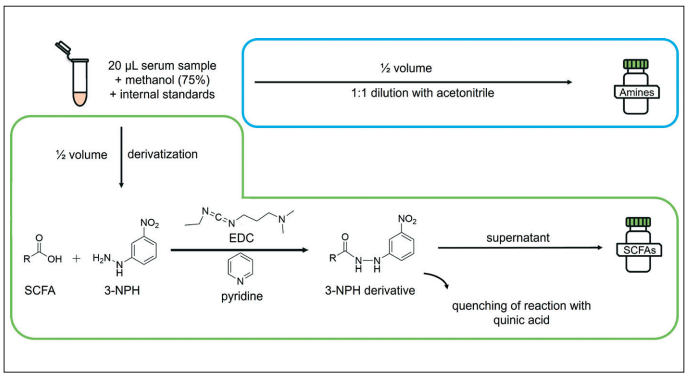

Fig.1: Illustration of the sample preparation of the amines and derivatization of SCFAs

RP-HPLC separation and electrospray ionization efficiency, whereas amines could be separated and detected in their native forms on a Diol-HILIC phase.

Quantification was performed using an external calibration series and additional internal standards. The stable isotopically labelled internal standards $^{13}C_2$-acetate, d2-propionate and d7-butyrate were used for quantification of their unlabeled counterparts, whereas the latter was also used as surrogate internal standard for iso-butyrate in the RP-LC approach. For the highly polar amines in the HILIC setup, d9-TMAO and d3-carnitine were used as internal standards.

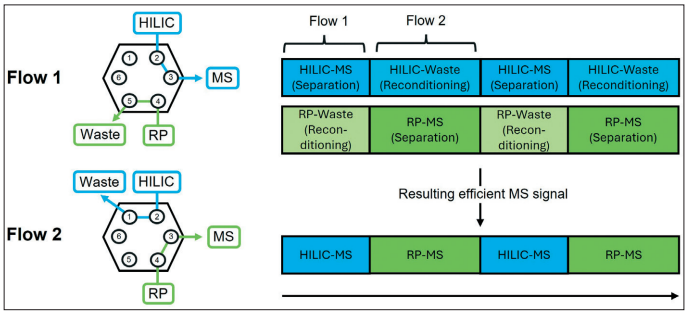

Fig. 2: Schematic overview of the valve setup and the alternating operation of the HILIC and RP-HPLC

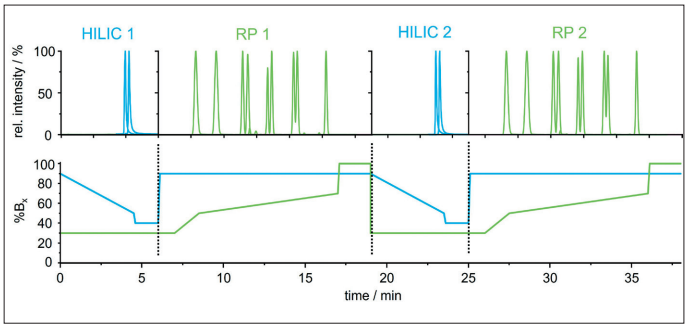

Fig. 3: Interleaving of the HILIC und RP separations. The 6-way valve was switched at each dotted line according to the valve configuration depicted in Figure 2.

Chromatographic conditions and dual-HPLC-MS setup

For all chromatographic separations, the Thermo Scientific Vanquish Flex Duo system was used. It includes a dual split autosampler, a dual gradient pump, a column oven and two 6-port valves. Amine separation was performed using an Accura Triart Diol-HILIC bioinert column (50 × 2.1 mm, 1.9 µm; YMC). Solvents used for the separation were A_{amine}: 10 mM aqueous ammonium formate (pH 4, adjusted with formic acid) and B_{amine}: acetonitrile. The total time of the HILIC analysis was 13 min with a flow rate of 0.4 mL/min.

Separation of the derivatized SCFAs was achieved by RP chromatography using a Kinetex Core-Shell C18 (150 × 2.1 mm, 2.6 µm; Phenomenex) column equipped with a SecurityGuard ULTRA precolumn (C18, 4.0 × 2.1 mm). The binary eluent system A_{RP}: water + 0.01% formic acid and B_{RP}: methanol + 0.01% formic acid was selected. Total RP analysis required 16 min at a flow rate of 0.4 mL/min.

Both chromatographic setups using two columns, two pumps and two eluents were combined, sharing the dual split autosampler, column oven and detector. The combination of both methods was achieved using a six-port valve as illustrated in Figure 2.

In valve position I, the flow is directed through the HILIC column into the MS, while the eluent for the RP separation is guided to the waste. This is the case in the first 6 minutes of the measurements. In the following seven minutes, valve position II is used where the eluent is directed through the RP column to the MS. During this time, the flow was directed into the waste for the first 1.5 min via a second six-port valve to remove excess derivatization chemicals. The valve positions are switched continuously as the measurements proceed.

For detection, a hybrid quadrupole-orbitrap-MS (Q Exactive Plus, Thermo Fisher Scientific) equipped with electrospray ionization source was utilized. For RP analysis, the negative ionization mode was applied, whereas detection of the HILIC-based analysis was carried out in positive electrospray ionization mode.

In order to analyze the two different metabolite classes, non-polar derivatized SCFAs and polar amines, two complementary separation techniques are required due to their different physicochemical properties. Assembly of the different HPLC set-ups and rinsing of the columns require a significant amount of time. Furthermore, HPLC methods, especially in HILIC, usually have long re-equilibration times during which the MS is practically inoperative, waiting for the next measurement. To overcome this unproductive usage of detector time, the HILIC und RP chromatography were interleaved as illustrated in Figure 3.

Both methods were connected via a six-port valve and switched as demonstrated in Figure 2. After the separation step in the HILIC gradient (6 min), as the rinsing step begins, the system switches to the RP method whose eluent then enters the MS. Since the HPLC-system consists of a dual split sampler, the second injector can proceed to inject the next sample, while the first injector is used for the ongoing HILIC measurement. During the RP runtime, the HILIC method can be re-equilibrated without the MS being idle. Once the separation step of the RP method is complete (19 min), the system returns to the next HILIC measurement and the RP column can be re-equilibrated. As a result, both measurements can be performed together in 19 min, whereas the individual methods would take a combined total of 29 min. This leads to a considerable time saving of 37%, which is particularly beneficial for large sample batches.

Conclusion

A dual HPLC-MS method was developed enabling quantification of two different classes of metabolites in a time-optimized approach. Due to the different physicochemical properties of SCFAs and the amines, a two-part sample preparation procedure was established for this purpose, including the derivatisation of SCFAs. The separation of SCFAs was optimized to obtain baseline separation of the iso-butyric acid/butyric acid isomer pair, which is particularly important in the biomedical context. Using six-port valves and a dual split sampler, RP and HILIC chromatography were combined in such a way that the MS is always actively involved in data acquisition through efficient interleaving of the rinsing and separation steps of both methods. This considerably reduced the time required for RP and HILIC measurement from 29 min for the combination of both individual methods to 19 min in the dual-HPLC-MS setup, corresponding to a total time reduction of 37%. The method has been successfully validated and demonstrates high chromatographic robustness and reproducibility for metabolite quantification in human serum samples. As the MS is acquiring data throughout the whole separation step of the chromatographic runs, this method is not limited to target analysis, but can also be extended to non-target approaches. Additionally, any two chromatographic methods can be interconnected, as long as their rinsing and reconditioning steps are compatible.

Acknowledgements

The authors acknowledge YMC Europe GmbH for providing the Accura Triart Diol-HILIC column. The authors would also like to thank Prof. Dr. Alexander Oberhuber, Dr. Thorsten Eierhoff and Claudia Kemming (Clinic for Vascular and Endovascular Surgery, University Hospital Münster, Münster, Germany) for providing the human serum samples and for the assistance with sample preparation.

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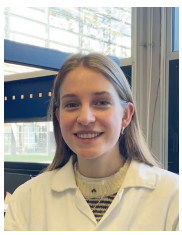

Authors | Contact

Prof. Dr. Heiko Hayen | Dr. Christian Faist | Edda Tacke
University of Münster
Institute of Inorganic and Analytical Chemistry
Department of Analytical Chemistry
Corrensstr. 48 | 48149 Münster | Germany
https://www.uni-muenster.de/Chemie.ac/hayen/index.html