**Development and Application of a HILIC UHPLC-MS Method for Polar Fecal Metabolome Profiling**

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**Abstract**

The fecal metabolome is a complex mixture of endogenous, microbial metabolites, and food derived compounds. Hydrophilic interaction liquid chromatography (HILIC) enables the analysis of polar compounds, which is a valuable alternative to reversed-phase liquid chromatography in the field of metabolomics due to its ability to retain a greater portion of the polar metabolome. The objective of the study was to find the optimal chromatographic solution to perform non-targeted metabolomics of feces by means of HILIC ultra-high-pressure liquid chromatography mass spectrometry (UHPLC-Q-TOF-MS). The performance was systematically investigated analyzing a pooled fecal sample, and mixtures of 150 metabolites from different families, including for example amino acids, amines, indole derivatives, fatty acids and carbohydrates. Three different stationary phases (zwitterionic, amide and unbonded silica) were operated at three pH values (4.6, 6.8 and 9.0), and three salt gradient conditions (5–5, 5–10 and 5–25 mM ammonium acetate). Amide and zwitterionic stationary phases performed similarly at low pH, with highest number of detected standards, which increased by increasing the salt gradient. The amide column showed slightly better performance in terms of separation of isomers and peak widths and remarkably good performance at basic pH, with highest number of metabolite features in the non-targeted analysis. The zwitterionic column operated best in terms of number of detected standards, retention time distribution of standards and metabolite feature across whole chromatographic run. Thus, the zwitterionic column was proven to suit for non-targeted analysis of fecal samples, resulting in good coverage of especially amino acids and carbohydrates.

**Keywords:** hydrophilic interaction liquid chromatography; HILIC; UHPLC-MS; metabolomics; non-targeted; feces

**Highlights**

* Systematic evaluation of eighteen HILIC conditions for standards and fecal sample
* Three HILIC stationary phases, pHs and salt gradients were studied
* Amide column performed best at low and high pH and zwitterionic at low pH
* Zwitterionic phase showed good retention time repartition and coverage of standards
* Best results for non-targeted analysis of stool with amide and zwitterionic column

1. **Introduction**

Metabolome profiling studies are often performed using reversed-phase (RP) chromatography because of its robust and reproducible separation characteristics and the coverage of a wide range of metabolites. However, most biological matrices contain plenty polar metabolites, which cannot be retained on RP stationary phases. Thus, hydrophilic interaction liquid chromatography (HILIC) became more and more popular in the field of metabolomics for separation of polar compounds. The term HILIC was first introduced by Andrew J. Alpert to describe the combination of hydrophilic stationary phases and organic mobile phases [1]. HILIC offers a wide variety of different retention mechanisms and column selectivities. Stationary phases can be classified in neutral ligand (e.g. amide), charged (e.g. unbonded silica) and zwitterionic phases (e.g. sulfobetaine) based on the characteristics of the functional group. Besides the different column chemistries, the separation properties in HILIC are also dependent on the pH value and buffer salt concentration [2]. The most commonly used buffers are ammonium formate or acetate. HILIC has often been used for analyzing hydrophilic compounds in urine and plasma to increase the coverage of the metabolome [3-6]. Yet, HILIC represents a challenge in metabolomics because it is less reproducible and requires longer equilibration time than RP chromatography [7, 8]. Only a few studies used HILIC for the analysis of fecal samples so far [9-14], and mostly in combination with RP measurements. Feces represents a challenging matrix influenced by many factors such as diet, gut microbiota, and the host metabolism. The optimal fecal sample preparation for a targeted HILIC-MS/MS method was already investigated [10]. However, there is currently no study comparing different HILIC columns and conditions for non-targeted metabolome profiling in feces.

In the present work we aimed to find the optimal chromatographic settings for non-targeted metabolomics of fecal samples using HILIC-MS. Therefore, three different HILIC columns (zwitterionic, amide and unbonded silica) were compared at different pH values (depending on the column specifications) and three salt gradient conditions. Parameters were evaluated in terms of number of detected features, separation of isomers, precision and repartition of the metabolic features along the chromatographic runs using standard mixtures and fecal samples.

# Materials and Methods

## Reagents and materials

A set of 150 analytical grade standards were purchased from different vendors as summarized in Table S1. Four/nine standard mixtures were prepared to a final concentration of 0.0122/0.0139 mg/mL in 75% acetonitrile (LiChrosolv®, hypergrade for LC-MS, Merck KGaA, Darmstadt, Germany). MilliQH2O was derived from Milli-Q Integral Water Purifcation System (Billerica, MA, United States of America). Methanol (LiChrosolv®, hypergrade for LC-MS), ammonium acetate (NH4Ac) and ammonium hydroxide (≥ 25% in water) were obtained from Merck KGaA, Darmstadt, Germany. Glacial acetic acid was purchased from Biosolve, Valkenswaard, Netherlands.

## Fecal sample preparation

Metabolite extraction from infant stool samples was prepared as described in Bazanella et al. [15]. A pooled methanol stool extract (n=468) was evaporated at 40°C (Savant, SPD121P, SpeedVac Concentrator, ThermoFisher Scientific, Waltham. Massachusetts, United States of America) and reconstituted with 75% acetonitrile to perform HILIC coupled to mass spectrometry (MS) analysis.

## LC-MS conditions

The pooled fecal sample and a set of 150 metabolites (Table S1) were analyzed on a time of flight mass spectrometer (maXis, Bruker Daltonics, Bremen, Germany), coupled to an UHPLC system (Acquity, Waters, Eschborn, Germany). Internal calibration of the mass spectrometer (MS) was done by injecting ESI-L Low Concentration Tuning Mix (Agilent, Santa Clara, CA, United States of America). External Calibration of the MS was ensured by injecting ESI-L Low Concentration Tuning Mix (1:4 diluted in 75% acetonitrile) in the first 0.3 min of each LC-MS run, introduced by a switching valve. Mass spectra were acquired in positive and negative ionization mode (+/-ESI). ESI parameters were as follows: nitrogen flow rate of 10 L/min, dry heater of 200°C, nebulizer pressure of 2.0 bar and capillary voltage of 4500 V. Data was acquired in line and profile mode with an acquisition rate of 5 Hertz, within a mass range of 50-1500 Da.

## Chromatographic conditions

Three different HILIC columns were compared: a charge modulated hydroxyethyl amide (zwitterionic) HILIC column (iHILIC®-Fusion UHPLC column, SS, 100x2.1 mm, 1.8 µm, 100 Å, (HILICON AB, Umea, Sweden)), an ethylene Brigded Hybrid (BEH) amide (ACQUITY UPLC BEH Amide column, 100x2.1 mm, 1.7 µm, 130 Å, (Waters, Eschborn, Germany)) and a solid-core particle (unbonded silica) CORTECS HILIC column (CORTECS UPLC HILIC column, 100x2.1 mm, 1.6 µm, 90 Å (Waters, Eschborn, Germany).

A stock solution of 0.5 M NH4Ac was either adjusted to pH 4.6 (acidic), 6.8 (neutral) or 9.0 (basic) with glacial acetic acid or 25% ammonium hydroxide, respectively.

Mobile phases for HILIC separation consisted of 5 mM NH4Ac in 95% acetonitrile (A) (A; acidic, neutral or basic) and 5, 10 or 25 mM NH4Ac in 5% acetonitrile (B; acidic, neutral or basic). Elution of metabolites was performed with a flow rate of 0.5 mL/min, using a 0.1 – 99.9% phase B gradient over 7.5 min. After a pre-run time of 5 min at 0.1% B, 0.1% B was kept for 2 min with increasing to 99.9% B within 7.5 min. 99.9% B was kept constant for 2.5 min with fast decrease to 0.1% B within 0.1 min. The column oven temperature was set to 40°C and 1 μL sample was injected by partial loop (10 μL). Weak and strong wash consisted of 95% acetonitrile and 10% acetonitrile, respectively.

## Data processing and analysis

Raw LC-MS data were processed with Genedata Refiner MS software (Genedata GmbH, Munich, Germany), including chemical noise subtraction, intensity cutoff filter, calibration, chromatographic peak picking, deisotoping, blank subtraction and metabolite library search with The Human Metabolome Database (HMDB) for MS1 level (±0.005 Da) for targeted and non-targeted analysis of standards and stool samples [16]. Metabolite subclass classification was done with ClassyFire Batch [17] by the Fiehn Lab (<http://cfb.fiehnlab.ucdavis.edu/>) and the subclass name was taken for metabolite classification for standards and annotated metabolites. Standard classification was done by translating compound name into InChIKeys with the chemical translation service (<http://cts.fiehnlab.ucdavis.edu/>) [18].

# Results and discussion

We evaluated the behavior of three HILIC columns under three different pHs (acidic, neutral or basic) and salt gradient conditions (5–5, 5–10 or 5–25 mM NH4Ac) in acetic acid/ ammonium hydroxide buffered conditions. All UHPLC columns (iHILIC Fusion, BEH Amide and Cortecs HILIC) were assessed under acidic conditions, two (iHILIC Fusion, BEH Amide) were performed under neutral and one (BEH Amide) under basic conditions, depending on the vendor specifications for each column (pH range iHILIC Fusion: 2–8, BEH Amide: 2–12, Cortecs HILIC: 1–5). In total, we analyzed 150 different metabolite standards, classified into amino acids, fatty acids, tricarboxylic acids, indole derivatives, carbohydrates, amines, flavones and others and a pooled fecal sample with in total 18 different chromatographic settings in positive and negative electrospray ionization mode. The majority of HILIC application based studies in targeted and non-targeted metabolomics are performed with particle sizes above 2 µm [8]. Few studies performed analyses using sub-2 µm HILIC columns with different numbers of tested compounds [3, 4, 6, 19, 20].

## Evaluation of the HILIC conditions using standards

The number of detected metabolites ranged between 62.7% and 77.3%, with highest percentages given under acidic (average: 71.9%) or basic (average: 73.8%) pH, but having limited column chemistry (BEH Amide) for comparison at pH 9 (Fig. 1A). Under neutral pH, only 66.3% of all measured standards were detected. Under acidic conditions, the increase of NH4Ac salt resulted in a higher percentage of detected compounds for all column chemistries with the highest values for iHILIC Fusion (77.3%) and BEH Amide (76.7%) under 5–25 mM NH4Ac conditions (Fig. 1A). We could also observe that iHILIC Fusion and BEH Amide had higher signal to noise ratios compared to Cortecs HILIC, despite same injected concentrations on all columns (exemplarily shown for indole-3-lactic acid in Fig. S1). However, iHILIC Fusion showed broader peak widths. Twenty metabolites couldn’t be detected in all 18 conditions, possibly being under limit of detection for the used concentration (~13 ppm).

## Analysis of resolution of isomers and selectivity of metabolite classes

In our set, we had 15 isomeric pairs (36 substances with 2, 3 or 4 isomers within the pairs) for which we analyzed chromatographic separation. Here, we considered the following, 2 pairs (both detected and separated or no separation), 3 pairs (3 or 2 detected, 3, 2 or 1 resolved or no separation), 4 pairs (4, 3 or 2 detected with 1, 2, 3 or 4 resolved or no separation), summarized in Table S2. Almost all settings reached over 50% of separated isomers, except for Cortecs HILIC pH 4.6, 5–5 mM NH4Ac (Fig. 1B). Highest percentage of isomer separation (≥ 75%) was reached for BEH Amide (acidic, 5–10 mM, and neutral, 5-5/10/25 mM). Under acidic conditions the increase of the salt gradient led to an increase of separation of isomers for iHILIC Fusion, whereas BEH Amide and Cortecs HILIC didn’t show such a behavior (Fig. 1B). At pH 6.8, less isomers were separated with iHILIC Fusion and BEH Amide. BEH Amide performed well at basic pH, reaching ~74% for 5–5 and 5–25 mM and a small decrease at 5–10 mM condition. For example, for iHILIC Fusion (acidic, 5–25 mM), we could detect 32 substances and resolve 8 pairs (Table S2), whereas for BEH Amide with highest % of separated isomers (neutral at all gradients), we could only detect 20 (5–5 mM) or 24 (5–10 mM and 5–25 mM) substances and resolve 4 or 5 pairs, respectively. Some representative examples, analyzed at acidic, 5-25 mM NH4Ac condition for each column, are shown in Fig. S2. For example, under acidic conditions, BEH Amide was able to resolve the 4 L-leucine isomers (L-isoleucine : L-leucine : L-norleucine : L-tert-leucine) into two peaks consisting of (L-leucine + L-norleucine and L-isoleucine + L-tert-leucine, Figure S2) at 5-25 mM NH4Ac.

Overall, under basic and acidic pH, we observed higher coverage, regarding the subclass comparison (Fig. 2). Amino acids, peptides and analogues (AAs) were mostly preferred under acidic conditions using iHILIC Fusion and BEH Amide with an 87.9% coverage for iHILIC Fusion at 5–25 mM condition, which is similar to Sampsonidis et al. [19]. They showed high coverage of amines and amino acids for amide and zwitterionic columns at pH 4. Flavones and amines are 100% covered by Cortecs HILIC using any salt gradient, whereas a full coverage of amines is also shown for BEH Amide (acidic; Fig. 2). The class of flavones was also fully detected by the BEH Amide and iHILIC Fusion at 5–25 mM NH4Ac concentration. We observed higher counts of the carbohydrates and carbohydrate conjugates (Carb) class for iHILIC Fusion, with 72.3% under 5–25 mM condition and BEH Amide (basic; all salt gradients). Indolyl carboxylic acids and derivatives (ICAs) were preferred under acidic conditions with 100% for iHILIC Fusion for 5–10 mM and 5–25 mM NH4Ac. Moreover, neutral pH resulted in full coverage of ICAs, using iHILIC Fusion at all salt gradients (Fig. 2). Tricarboxylic acids and derivatives (TCAs) and fatty acids and conjugates (FAs) were poorly recovered at each pH, but FAs had higher counts (8/12, 66.7%) for BEH Amide at neutral and basic pH for all salt gradients. Altogether, iHILIC Fusion at acidic pH or BEH Amide at basic pH had the best abilities to cover high percentage of metabolite classes.

## Retention time distribution and precision of analyzed standards

Preferably, retention time distribution should be uniformly given across the whole retention time axis with as few as possible compounds detected in the void volume. For example, Cortecs HILIC showed poor retention for the tested compounds, resulting in a high density between 0.0 and 2.5 min under acidic conditions for all salt gradients (Fig. 3A). BEH Amide and iHILIC Fusion had higher densities between 2.5 and 10 min at low pH throughout all salt gradients. The small peak density at ~8 min at 5–5 mM NH4Ac, for iHIILIC Fusion moved to earlier retention times by increasing the salt gradient. Neutral pH resulted in lower retention times for BEH Amide for all salt gradients whereas no big differences were observed for this column at basic pH (Fig. 3A). Precision was assessed by calculating the coefficient of variation (CV) in % of the peak areas of triplicate injections for all detected metabolites. Most of the detected peaks showed CVs below 20% (Fig.3B). Highest density of CV% ≤ 20% was observed for acidic conditions at highest concentration of NH4Ac for iHILIC Fusion, followed by BEH Amide.

## Non-targeted evaluation of the HILIC conditions using fecal sample

We compared the same chromatographic conditions for a pooled feces sample. The objective was to maximize the number of detected metabolite features with good reproducibility and repartition of these features along the chromatographic run. We calculated the total number of metabolite features as a sum of isotope clustered features and singletons, for which no matching isotopes were found by the used software. Among all tested conditions, BEH Amide operated at basic pH provided the highest number of metabolite features (pos: 4026 at 5–25 mM NH4Ac, neg: 1045 at 5–10 mM NH4Ac) (Fig. 4A, S3). At low pH, most features were detected with iHILIC Fusion in positive mode (3958, 3402 and 2827 for 5–5, 5–10 and 5–25 mM NH4Ac, respectively) and with Cortecs HILIC in negative mode (949, 867 and 634 for 5–5, 5–10 and 5–25 mM NH4Ac, respectively). Interestingly, the number of detected metabolite features decreased by increasing the NH4Ac concentration at pH 4.8, whereas the opposite was true for pH 9 (pos mode). Overall, the number of detected metabolite features in feces was much higher in positive mode, compared to negative mode, independently of the chromatographic settings. The opposite was reported by Marcobal et al., however, they analyzed mouse feces and used a column which is strictly speaking classified as aqueous normal-phased, thus, no adequate comparison is possible [12]. For annotation with HMDB only isotope clustered features were used. Here, the maximal number of HMDB annotated features was reached with iHILIC Fusion at acidic pH and 5–5 mM NH4Ac in positive mode with 493 annotations (Cortecs HILIC acidic pH and 5–5 mM NH4Ac with 150 annotations in negative mode, Fig. S3) and the least annotations with iHILIC Fusion 5–25 mM NH4Ac at neutral pH in positive and negative mode.

In order to gain an overview of the metabolite classes detected in feces under the 18 different conditions, the HMDB annotated features (RT > 0.8 min) were classified with ClassyFire Batch and the ten most abundant subclasses were selected for representation (Fig. 4B). Overall, the highest number of metabolites were classified under acidic, the fewest under neutral conditions. The most abundant metabolite class in negative mode was “bile acids, alcohols and derivatives”. Here, the Cortecs HILIC was the most prominent one (5–10 and 5–25 mM NH4Ac). Also the compound classes fatty acids, benzoic acids, carbonyl compounds and carbohydrates and conjugates/ derivatives reached the highest numbers with Cortecs HILIC for all salt gradients at acidic pH. In positive mode, the most abundant metabolite class was “amino acids, peptides and analogues”, followed by glycerophosphocholines and glycerophosphoethanolamines. Especially the iHILIC Fusion column detected the highest numbers in those compound classes, followed by Cortecs HILIC, while numbers were slightly decreasing with increasing NH4Ac concentrations. In contrast, BEH Amide detected more of the mentioned classes under basic conditions. Moreover, with iHILIC Fusion more carbohydrates and carbohydrate conjugates, as well as triterpenoids were found compared to the other columns.

From the 150 evaluated standards approximately 50 were identified in feces under acidic and basic pH. At neutral pH less than 30 were found with BEH Amide and roughly 40 with iHILIC Fusion. A detailed list of detected standards in feces in consideration of possible isomer separation can be found in Table S3. Virgiliou et al. detected more metabolites under neutral conditions using BEH Amide compared to acidic pH, however, their findings aren’t referred to fecal samples [6].

## Retention time repartition and precision of metabolite features of fecal sample

The best repartition along the chromatographic run was observed for iHILIC Fusion under acidic conditions with lowest density between 0–2.5 min and highest density between 2.5–7 min (Fig. 5A). Additionally, only with iHILIC Fusion features with retention times above 8 min were detected. At neutral pH, both, iHILIC Fusion and BEH Amide, showed worse repartition with higher densities for early retaining metabolites (0–2.5 min). The precision of the triplicates gave overall good results for all tested conditions (Fig. 5B). Under acidic and neutral pH the CV% values improved with increasing salt gradient. The best results with the highest density under 20% were achieved for BEH Amide 5–25 mM NH4Ac at pH 4.6. Also iHILIC Fusion reached good CV% values, especially at acidic pH and 5–25 mM NH4Ac. In comparison, Cortecs HILIC showed the worst density distribution.

# Conclusion

In the present study we compared three different HILIC chemistries operated under acidic, neutral and basic pH and increasing salt gradients. The performance was evaluated for a set of 150 endogenous compounds and infant stool samples, in terms of number of detected features or metabolite classes, separation of isomeric pairs, distribution along the chromatographic run and precision. The major goal was to find a HILIC method which covers the broadest space of polar metabolites in feces. The unbonded silica Cortecs HILIC column was not convincing in regards of retention time distribution, precision and number of recovered standards. Overall, BEH Amide and the zwitterionic iHILIC Fusion performed similarly satisfying under the studied acidic conditions, whereas neutral pH achieved the worst results. For basic pH no comparison between columns was possible, due to limitations in pH tolerances, though the results of the non-targeted analysis were very promising and would require more detailed examination. The increase of salt gradient concentration improved the number of detected standards. The opposite behavior was observed in terms of number of metabolite features in the non-targeted analysis of feces. However, high salt gradients reduce the risk of carry-over for late eluting compounds, especially for zwitterionic columns. BEH Amide showed sharp peaks, slightly better separation of some isomers and increased coverage of amines and fatty acids. The iHILIC Fusion column demonstrated throughout the highest number of detected standards and metabolite features in the non-targeted analysis at acidic pH. Furthermore, it showed the best repartition of the metabolic features along the chromatographic run and a good coverage of metabolite classes like amino acids and carbohydrates in standards and non-targeted analysis of feces. In summary, we think that using the zwitterionic iHILIC Fusion column under acidic conditions offers the best prerequisites to study the polar fecal metabolome in our future non-targeted metabolomics studies.

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**Figure Captions**

**Fig. 1. Detection of analyzed standards and separation of isomeric substances under different HILIC conditions**. Comparison of analyzed standards under acidic, neutral and basic pHs for iHILIC Fusion, BEH Amide and Cortecs HILIC at 5–5 mM, 5–10 mM and 5–25 mM NH4Ac. (A) Percentage of detected metabolites in each condition, taken into account number of detected against injected metabolites (n=150). (B) Percentage of separated isomeric pairs for each condition, taking into account number of detected isomeric metabolites and number of separated isomers.

**Fig. 2. Selectivity of metabolite subclasses for the different HILIC conditions.** Standards were translated by the Chemical Translation Service and classified by ClassyFire using compound name and InChIKey information; compounds were classified into eight different categories, including amino acids, peptides and analogues (AAs), Flavones (Flav), Amines (Am), Carbohydrates and carbohydrate conjugates (Carb), Indolyl carboxylic acids and derivatives (ICAs), Tricarboxylic acids and derivatives (TCAs), Fatty acids and conjugates (FAs) and Others (Oth; with less than 4 compounds per subclass).

**Fig. 3. Retention time and precision distribution of detected standards.** (A) Density distribution of retention times across the chromatographic run for each HILIC condition and (B) Precision of triplicate injections of analyzed standards, expressed in percentage of coefficient of variation (CV%).

**Fig. 4. Non-targeted analysis of stool sample performed at 18 different HILIC conditions.** (A) Number of detected metabolite features, categorized into singletons (mass and retention time), isotope clustered features (compounds with recognized isotope pattern and retention time) with or without (“unknowns”) HMDB annotation (±0.005 Da, MS1), analyzed in positive ESI mode. (B) Metabolite features were annotated with HMDB and categorized by ClassyFire into different subclasses using InChIKey information. Top ten subclasses are illustrated for negative or positive ESI mode across all eighteen different HILIC runs.

**Fig. 5. Retention time repartition and precision of metabolite features detected in stool sample.** (A) Retention time distribution in min of metabolite features across the 18 HILIC chromatographic runs. (B) Precision of metabolite features from triplicate injections, expressed in CV%.