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OPTIMIZED SAMPLING CONDITIONS FOR FECAL VOLATILE ORGANIC COMPOUNDS ANALYSIS BY MEANS OF FIELD ASYMMETRIC ION MOBILITY SPECTROMETRY.

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Conflicts of interest

The department of Gastroenterology and Hepatology of the VU University Medical Center has no conflicts of interest to disclose

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TGJ de Meij and NKH de Boer were the guarantors of this article.

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JA Covington and AN Wicaksono analyzed the results.

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Abstract (259 words)

Background Fecal volatile organic compounds (VOCs) are increasingly considered as potential non-invasive, diagnostic biomarkers for various gastrointestinal diseases. Knowledge of influence of sampling conditions on VOC outcomes is limited. We aimed to evaluate effects of sampling conditions on fecal VOC profiles and to assess under which conditions an optimal diagnostic accuracy in the discrimination between pediatric inflammatory bowel disease (IBD) and controls could be obtained.

Methods Fecal samples from *de novo* treatment-naïve pediatric IBD patients and healthy controls (HC) were used to assess effects of sampling conditions compared to the standard operating procedure (reference standard), defined as 500mg of sample mass, diluted with 10mL tap water, using field asymmetric ion mobility spectrometry (FAIMS).

Results A total of 17 IBD (15CD and 2 UC) and 25 HC were included. IBD and HC could be discriminated with high accuracy (accuracy=0.93, AUC=0.99, $p<0.0001$). Smaller fecal sample mass resulted in a decreased diagnostic accuracy (300mg accuracy=0.77; AUC=0.69, p=0.02; 100mg accuracy=0.70, AUC=0.74, p=0.003). A loss of diagnostic accuracy was seen towards increased numbers of thaw-freeze cycles (one cycle: accuracy=0.61, AUC=0.80, p=0.0004, two cycles: accuracy=0.64, AUC=0.56, p=0.753, three cycles: accuracy=0.57, AUC=0.50, p=0.5101) and when samples were kept at room temperature for 180 minutes prior to analysis (accuracy=0.60, AUC=0.51, p=0.46). Diagnostic accuracy of VOC profiles was not significantly influenced by storage duration differences of 20 months.

Conclusion Application of 500mg sample mass analyzed after one thaw-freeze cycle, showed best discriminative accuracy for differentiation of IBD and HC. VOC profiles and diagnostic accuracy were significantly affected by sampling conditions, underlining the need for implementation of standardized protocols in fecal VOC analysis.

Introduction

Analysis of volatile organic compounds (VOC) is a relatively new technique within the field of metabolomics. VOCs are carbon-based chemicals originating from both physiological and pathophysiological processes in the human body. Fecal VOCs are considered to reflect microbiota composition, function and interaction with the host [1, 2]. They are increasingly considered to have potential as biomarker in the diagnostic work-up and monitoring of various gastrointestinal diseases, e.g. inflammatory bowel disease (IBD), colorectal cancer and even sepsis [3-11]. Various studies have demonstrated the diagnostic potential of VOCs in both pediatric and adult IBD populations, by analyzing VOCs deriving from urine, exhaled breath and feces [6, 12-14]. The majority of studies on fecal VOCs have been performed using Gas Chromatography/Mass Spectrometry (GC/MS), allowing for identification of individual VOCs on molecular level. This technique is expensive, time-consuming and requires specialized personnel and is therefore not suitable for utilization in a clinical setting [15]. Pattern recognition based techniques, like electronic noses (eNose) and field asymmetric ion mobility spectrometry (FAIMS), are examples of instruments that are lower in expense and faster, allowing for their application as a non-invasive biomarker in clinical practice. However, traditional eNoses contain sensors that are notorious for batch-to-batch variation, fouling and ageing effects and sensors drift [14, 16]. Novel measurement of VOCs using physical techniques, coupled with pattern recognition, like FAIMS, have a higher sensitivity and minimal drift. It achieves separation by measuring the differences in mobility of ionized molecules in high-electric fields.

Data on the potential influence of sampling and storage methods on fecal VOC profiles are scarce. We aimed to evaluate effects of environmental factors and sampling conditions on fecal VOC profiles, using FAIMS. In addition, we aimed to assess under which conditions an

optimal diagnostic accuracy could be obtained in the differentiation between pediatric IBD and controls. This may lead to the development of rationale-based standardization protocols on fecal VOC analysis, paving the way towards reliable comparisons between different study outcomes, and implementation of VOC-based diagnostics in clinical practice.

Methods

Study design

This case-control study was performed at the outpatient clinic of the pediatric gastroenterology departments in two tertiary referral hospitals, the VU University medical center (VUmc) and the Emma Children's Hospital, Academic Medical Center (AMC), both located in Amsterdam, The Netherlands.

Study participants

Inflammatory bowel disease

IBD subjects were selected from an existing cohort of *de novo* treatment-naïve pediatric patients, consisting of 125 subjects (78CD, 47UC), aged 4 to 17 years, recruited between October 2013 and July 2017 at the VU University Medical Centre (VUmc) and Academic Medical Centre (AMC). Diagnosis of IBD was based on endoscopic, histologic and radiologic findings, according to the revised Porto-criteria[17]. Localization and behavior of IBD were classified during endoscopy, based on the Paris Classification[18]. Physician Global Assessment (PGA) combined with levels of C-reactive protein (CRP) and fecal calprotectin (FCP) were used as an index of the clinical disease activity [19, 20]. All IBD patients were asked to collect a fecal sample prior to endoscopy and bowel preparation [14]. Inclusion criteria also included sufficient fecal material for VOC analysis (3.4 grams per subject). Exclusion criteria were use of antibiotics, probiotics or immunosuppressive therapy in the three months prior to inclusion, a concomitant diagnosis of a gastrointestinal disease or immunocompromised disease (i.e. HIV, leukemia) and abdominal surgery (except for appendectomy). In addition, children with proven infectious colitis (parasites in stools, or positive stool culture for S*almonella spp., Shigella spp.,Yersinia spp., Campylobacter spp.,* or toxigenic *Clostridium spp.)* were excluded.

Healthy controls

Healthy controls (HC) were children aged 4 to 17 years selected from elementary and high schools in North-Holland, the Netherlands between June 2016 and December 2016. All participants were asked to collect a fecal sample, and complete a questionnaire on abdominal symptoms, bowel habits, including consistency of stool using the Bristol stool chart, medication use and medical history[21]. Exclusion criteria for HC were similar to IBD, with the addition of diagnosis of IBD and/or a functional gastrointestinal disorder according to the Rome IV criteria based on the questionnaires.

Matching procedure

From the original cohort of 125 IBD patients, 106 were not eligible for this study due to insufficient quantities of the fecal samples. A total of 17 IBD patients (15CD, 2UC) could be matched on age at sample collection and gender with 25 participants in the HC group.

Ethical considerations

This study was approved by the Medical Ethical Review Committee (METc) of the VU University Medical Centre (VUmc) under file number 2016.393, and by the local medical ethical committee of the Emma Children's Hospital (AMC). Written informed consent was obtained from all parents, and from the children in case of age over 12 years.

Sample collection IBD and controls

All study participants collected fresh fecal samples in a stool container (Stuhlgefäß 10ml, Frickenhausen, Germany). Patients with IBD collected their fecal sample prior to endoscopy and bowel lavage. Participants were instructed to store the fecal samples in the freezer at home directly after collection. The samples were transported to the hospital in cooled

condition, using cooling elements or ice cubes. Directly upon arrival in the hospital the samples were stored in the freezer (-24 °C) until analysis.

Sample preparation IBD and controls

The influence of fecal sample mass, number of thaw-freeze cycles, duration of storage in room temperature, were assessed by comparing VOC-profiles derived from subsamples taken from the original fecal sample of each HC and IBD subject. The subsamples were weighted on a calibrated scale (Mettler Toledo, AT 261 Delta Range, Ohio, United States), labelled and re-stored in a -24°C freezer until further handling. We compared the variables of interest with our standard operating procedure (reference standard), defined as a mixture of 500mg of feces, diluted with 10mL tap water and kept in room temperature for 10 minutes prior to analysis. These reference standard settings were chosen since they were used in several previous studies on fecal VOC profiling in a range of gastroenterology diseases and have provided us with positive results [14, 22].

Variables of interest

Effect of fecal sample mass on diagnostic accuracy of fecal VOC profiles was assessed by comparing subsamples weighing 100mg and 300mg with the reference standard mass of 500mg.

The influence of the number of thaw-freeze cycles on the diagnostic accuracy was analyzed by comparing the reference standard to subsamples, which underwent one, two and three additional thaw-freeze cycles. For every additional cycle, the sample was kept at room temperature for 10 minutes and subsequently kept on dry ice until the sample was frozen.

In order to assess the effect of duration of storage at room temperature on the diagnostic accuracy, VOC from samples kept at room temperature (18 degrees) for 180 minutes were compared to the reference standard. Variables of interest are presented in Table 1.

As described above, the effect of every variable on the diagnostic accuracy of fecal VOCs were assessed by comparing IBD subjects with HC. In addition, we assessed the influence of the variables on the VOC pattern. By combining the HC and IBD subjects, we were able to compare the variables to the reference standard.

FAIMS analysis

For this study a commercially available FAIMS instrument (Lonestar®, Owlstone, Cambridge, UK) was used. Prior to the analyses, the FAIMS instrument was checked for contamination using air and water blanks. The fecal samples were thawed to room temperature for ten minutes prior to VOC analysis, and manually homogenized after diluting the fecal sample with 10mL tap water by using a micropipette of 5000µl. The Lonestar® was setup as used in previous studies[14, 22, 23]. To transport the sample headspace into the Lonestar®, compressed air (0.1MPa) was used as the carrier gas. This air meets the European Pharmacopoeia criteria for medical air and its composition, pressure, temperature and water density are checked for continuality regularly. When entering the Lonestar, this carrier gas is filtered by a Carbon filter (Restek, Bellefote, VS). The flow rate was set on 2.0L/min, the temperature for the sample holder was set at 35°C, for the lid at 70°C and 100°C for the filter region. After every sample run, the Lonestar® was refreshed using 5mL of tap water. Furthermore, the dispersion field was set between 0% and 100% (in the ratio of the high electric field to low electric field) and passed through 51 equal settings. The compensation voltage was set between +6V and -6V in 512 steps for each dispersion field. All samples were

analyzed randomly. Each fecal sample was analyzed three times subsequently, resulting in three matrices, taking 540s to perform. In order to preclude environmental effects, the first matrix was excluded from analyses since this measurement includes the heaspace gas generated from both the sample and the environment (e.g. air in tubes). For the statistical analysis, only the second matrix was used for optimal diagnostic potential. The third measurement was made as a back-up file, but was not used in this study. The raw data output was analyzed at the School of Engineering, University of Warwick, United Kingdom[15].

Statistical analysis

The demographic data of each group (IBD patients and healthy controls) were compared using the Man-Whitney-U test for non-parametric continues data, and the Fisher's exact test for dichotomous data using SPSS Statistics (version 22, IBM, NY, USA). As previously reported, the FAIMS produces high dimensional data in terms of the number of features and covariates measured per sample. Therefore, a data compression was performed before feature identification and classification. Each FAIMS data (sample) consists of 52224 data points in a 2D matrix. Data compression was undertaken by applying a 2D discrete wavelet transformation. For the variables of interest in which the accuracy to discriminate between IBD and HC was assessed, feature selection and classifier training were performed to 90% of data (training set) and class predictions were produced from 10% of the data set (test set), in a 10-fold cross validation. The Wilcoxon-rank-sum test was used to calculate p-values in the training sets to identify which features best for disease prediction. From this, 4 statistically important features were used. Four classification algorithms were applied, Sparse Logistic Regression, Random Forrest, Gaussian Process and Support Vector Machine. A receiver operator characteristic (ROC) curve was created to predict the area under the curve (AUC),

p-values, sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and diagnostic accuracy. For the influence of sampling method on VOC composition, in which IBD and HC samples were combined and measurements of the same subjects samples were repeated, data were analyzed using SPSS statistics 22 (IMB). The raw sensor data was recombined with feature selection using the Wilcoxon rank sum tests. Paired t-tests were performed to assess the potential of the features to discriminate between sample handling methods. Scatterplots for the discrimination between samples were created for each variable of interest. Axes depict the recombination of the raw sensor data by means of features. Individual VOC profiles are illustrated as marked points. The intersection of the lines deriving from the invidual VOC profiles demonstrates the mean VOC profile of this specific variable of interest.

Post hoc analyses

Our main target of this study was to assess the optimum sampling method to discriminate between IBD and healthy controls based on VOC analyses by means of FAIMS. We found there is a gap of knowledge on the effects of sample storage duration on VOC integrity. Therefore, the effect of duration of storage in the freezer was analyzed by repeating measurements from a previous study, conducted by van Gaal et al. in which fecal VOC profiles of 36 *de novo* IBD patients were compared to 24 HC[14]. Based on the availability of fecal samples from this study, 10 IBD (all CD) subjects and 10 HC could be included for reassessment of VOC profiles. Storage time differed 20 months between the measurements, with a median storage time in the freezer of 43 months for the first and 63 months for the second measurements. Baseline characteristics and disease specifics of these study subjects are described in Table 3. For both these analyses, the reference standard was used.

Diagnostic accuracy to detect IBD as well as the difference in VOC profile were assessed using the statistical analyses described above.

Results

Baseline characteristics

Seventeen *de novo,* treatment-naïve pediatric IBD patients (15CD, 2UC) were selected from the original cohort and were matched to 25 HC. Patient characteristics are shown in table 2. There were no significant differences in age, sex and sample age between IBD and HC. The range of the sample age was, however, larger in the IBD group compared to HC.

For the assessment of the influence of sample age on diagnostic accuracy, fecal samples of 10 IBD patients (CD only) and 10 HC were selected from the previous study and re-measured[14]. Patients characteristics for this variable are described in Table 3.

Fecal VOC profiles per variable of interest

The results of the VOC analysis displayed per variable of interest are shown in Table 4. For each analysis, the outcome of the Sparse Logistic Regression is noted. A complete overview of the data generated by the four different classification models is given in supplemental Table 1a-1d.

Standard operating procedure

By application of the reference standardsettings, IBD and HC could be differentiated with high accuracy (Accuracy, AUC (95% CI), Sensitivity, Specificity, PPV, NPV, P values; 0.93, 0.99 (0.96 – 1), 0.94, 0.96, 0.94, 0.96, 1.178e-10)(*Table 4, Supp table 1a-1d, Figure 1)*. A typical FAIMS pattern (flame) of both the IBD samples and control samples is depicted in *Figure 2.*

Sample mass

IBD could be differentiated from HC using a lower sample mass, but diagnostic accuracy decreased compared to reference standard for both 300 mg per sample (Accuracy, AUC (95% CI), Sensitivity, Specificity, PPV, NPV, P values; 0.77, 0.69 (0.52 – 0.86), 0.88, 0.44, 0.52,

0.85, 0.02101) and 100 mg per samples (Accuracy, AUC (95% CI), Sensitivity, Specificity, PPV, NPV, P values; 0.70, 0.74 (0.59 – 0.90), 0.76, 0.72, 0.65, 0.82, 0.00364)(*Table 4, Supp table 1a-1d, Figure 1)*.

Thaw-freeze cycles

After adding one extra thaw-freeze cycle to reference standard, a decrease in diagnostic accuracy was observed (Accuracy, AUC (95%CI), sens, spec, PPV, NPV, P values; 0.61, 0.80(0.65-0.94), 0.76, 0.80, 0.72, 0.83) (*Table 4, Supp table 1-4, Figure 1)*. After addition of a second and third thaw-freeze cycle, differences in VOC profiles between IBD and HC dissolved (Accuracy, AUC (95%CI), sens, spec, PPV, NPV, P values; 0.64, 0.56 (0.38 – 0.74), 0.76, 0.48, 0.50, 0.75, 0.7534 and 0.57, 0.50 (0.32 – 0.69), 0.47, 0.72, 0.53, 0.67, 0.5101 respectively)*(Table 3, Supp table 1a-1d)*.

Duration of storage at room temperature

After keeping the samples at room temperature for 180 minutes prior to VOC analysis, differences in VOC outcome between IBD and HC dissolved (Accuracy, AUC (95%CI), sens, spec, PPV, NPV, P values; 0.60, 0.51 (0.32 – 0.70), 0.59, 0.68, 0.56, 0.71, 0.4596)(*Table 4, Supp table 1a-1d)*.

Influence of sampling method on overall VOC composition

In order to assess the influence of sampling conditions on the detected VOC patterns, HC and IBD subjects were combined to form one single study group. The comparisons between the four features are shown in Table 5. Differences in VOC pattern between sampling methods are depicted in Figure 3. Both fecal samples weighing 300mg and 100mg demonstrated a significantly different VOC profile compared to the standard reference mass of 500mg (Feature 1,2 and 4: p-value <0.001 for and feature 3: p-value=0.027 for 300mg,

feature 1-4: p-value<0.0001 for 100mg). All of the variables in thaw-freeze cycles differed to a similar extent from the reference standard (feature 1-4: p-value <0.0001 for all variables). A similar difference as with the previous variables was seen when comparing the VOC profiles of the reference standard to the VOC profiles of samples kept at room temperature for 180 minutes prior to VOC analysis (feature 1-4: p-value<0.0001).

Post hoc analyses:

Duration of storage in freezer

The diagnostic accuracy to discriminate IBD from controls was not influenced by differences in duration of storage time prior to VOC analysis (43 versus 63 months) (Accuracy, AUC (95%CI), sens, spec, PPV, NPV, P values; 0.75, 0.75 (0.53 – 0.97), 0.70, 0.80, 0.78, 0.73, 0.0262 versus 0.75, 0.73 (0.49 – 0.97), 0.80, 0.70, 0.73, 0.78, 0.0376) *(Table 4, Supp table 1a-1d, Figure 1)*. The VOC composition of the two variables showed a significant difference in three features (feature 1, 2 and 3 with p-values of <0.0001, <0.0001 and 0.021, respectively)(*Table 5, Figure 3*).

Discussion

In the present study, VOC profiles and diagnostic accuracy were influenced significantly by altering sampling conditions. Application of 500mg fecal sample mass diluted with 10mL, thawed for 10 minutes prior to analysis after a single thaw-freeze cycle, showed the best discriminative accuracy for differentiation of pediatric IBD and HC.

To our knowledge, this is the first published study to assess under which sampling conditions an optimal accuracy can be obtained in the differentiation between pediatric IBD and healthy state, by analyzing the fecal volatile metabolome using FAIMS. Studies assessing optimization of sampling methods for fecal metabolome analyses have mainly focused on gas chromatography – mass spectrometry (GC-MS), nuclear magnetic resonance spectroscopy (NMR-spectroscopy) and liquid chromatography – mass spectrometry (LC-MS), which are targeted and untargeted methods for identification of specific metabolites. These studies may hypothetically provide guidance to standardization for the pattern-based FAIMS technique. The results of this study will be discussed and compared to the available literature in the following sections. Regarding sample mass, similar results to eNose, GC-MS, NMR-spectroscopy and LC-MS studies on the fecal metabolome, in both humans and rats, were found in our study, showing a difference between the use of 500mg from that of lower masses [16, 24, 25]. Deda and colleagues have shown that the sample weight to volume ratio has a major effect on the number and signal intensity of features detected in fecal samples with GC-MS. This also applied for the spectral signal intensity when using NMR-spectroscopy, and for the peak area intensity when using LC-MS[24]. The increased accuracy to differentiate between IBD and HC when using a larger fecal mass, as observed in our study, may be explained by this increase of richness in number and intensity of VOCs.

Observed differences in VOC profiles between fecal samples enduring one versus multiple thaw-freeze cycles, are in line with previous research on VOC patterns using different eNose devices [16, 26]. It could be hypothesized that these effects are caused by changes in microbiota composition or function, although in a previous study no differences were found in microbiota composition between analyses of fresh samples versus samples frozen at minus 80 degrees and subsequently thawed prior to analysis [27]. A recent study suggested a release of microbial intracellular contents following thaw-freeze cycles, possibly explaining the effects of thaw-freeze cycles on VOC outcome[28]. In our study, it was shown that the diagnostic accuracy decreased with the addition of one extra thaw-freeze cycle, and that IBD could not be differentiated from HC after addition of multiple thaw-freeze cycles. Consequently, future studies on fecal VOC should limit the number of thaw-freeze cycles prior to analysis to a maximum of two.

Consistent with the results from a previous study on fecal VOCs using an eNose device, we measured significant differences between fecal VOC profiles measured directly after thawing (as used in the reference standard) and after 180 minutes stored at room temperature with an accuracy of 0.84 [16]. Furthermore, it was demonstrated that the diagnostic accuracy decreased when samples were kept at room temperature for 180 minutes (AUC= 0.53). These results are in line with a previous study on the impact of storage conditions on crude fecal samples measured by NMR-spectroscopy, showed that metabolic variation was influenced by storage at room temperature and 4 °C[28]. The metabolic profiles of fecal samples did not change after keeping the samples at room temperature for 1 hour. However, samples stored for a longer time prior to the analyses gradually shifted. The overall changes that were seen included decreased levels of fumarate, succinate, glutamate and increased levels of methanol, phenylalanine and alanine and short chain fatty acids like

acetate, butyrate, propionate and valerate. To a lesser extent, the same shifts were seen in samples kept at 4 °C, which indicates that the lower temperature slows down the impact on sample integrity, resulting in less alterations in the metabolic profile. In another study comparing VOC profiles of fecal samples kept at 1°C for 14 hours prior to GCMS analysis, there were no significant changes in VOC profiles before and after 14 hours [25]. Since the differences between IBD and HC in this study were analyzed by means of pattern-recognition, specific metabolic alterations cannot be elucidated in this study. However, it could be hypothesized that the unstable VOC composition when keeping the samples at room temperature, is caused by ongoing fermentation by the fecal microbiota. Since fermentative processes have shown to be reduced at lower temperature, this could explain why VOC integrity remained stable when samples were kept at 1 and 4 degrees in previous studies[28]. Another explanation is the emission of volatiles in the sample, and contamination with background volatiles. Fermentation, emission and contamination could be avoided by measuring the sample directly after collection. However, clinical implementation of VOC analyses would then become a logistic challenge.

Literature on the influence of storage time of fecal VOCs is scarce. In a study assessing VOC profiles of urine using a similar FAIMS method to the current study, a nine-month shelf-life for urine samples was suggested after it was shown that chemical information was lost over time, regarding both diversity and concentration of gas emission[29]. In addition, in a previous study assessing the effect of sample age on serum VOCs measured by GCMS a significant difference in metabolite composition was already seen after storage of three weeks in the freezer [30]. In the current study, fecal VOC profiles for IBD, seem less influenced by storage time compared to the previous studies on urine and serum, keeping a similar (high) diagnostic accuracy after storage of 43 months and 63 months. Interestingly,

the samples chosen for this comparison, were used in a larger study by van Gaal et al. where an area under the curve of 0.76 was found after a mean storage time of 23 months for the IBD group (25CD, 21UC) and 39 months for the HC group[14]. The increase in the AUC of this sub analysis, although analyzed at the same moment, can be explained by the fact that the remaining samples only consisted of CD patients and HC. In the previous study, the AUC for the differentiation between CD and HC was 0.90. There is, however, an important consideration to this post hoc analyses. The diagnostic accuracy was only assessed after a median storage duration of 43 and 63 months. Since there are no previous measurements, it could be possible that massive changes in VOC composition have influenced diagnostic accuracy in the initial months after collection. We cannot exclude this influence based on this study.

The main strength of this study is that we used an IBD group and an HC group to assess not only differences in VOC patterns between sampling methods, but also to assess the influence on the diagnostic accuracy for disease detection. In addition, we used the same subjects' samples for each of the analysis, accounting for various confounding factors of influence on fecal VOCS (e.g. smoking habits, medication use, diet). During each experiment, the remaining variables of interest were kept the same, ensuring optimal comparison based on the variable of interest. Our study also has several limitations. Most importantly, for the influence of storage time on diagnostic accuracy for IBD, we have made use of raw data of a previous study and have re-assessed samples with sufficient sample mass. For this analyses we were only able to include CD patients, and no UC. In addition, sample age differed between groups, which could have influenced diagnostic accuracy by the influence of metabolic degradation on VOC profiles at both measurements. Second, we have made use of unfiltered and unsterilized tap water for sample dilution, and compressed medical air as

carrier gas. This protocol was chosen since it has been found a reliable sampling method for the differentiation between various diseases and healthy controls based on fecal VOCs[31-34]. To avoid VOC profile contamination, we have run air and water blanks which were checked on contamination peaks, and met the cleanliness criteria. In addition, we have analyzed the samples in a random order, and have excluded the first matrix of every sample analyses to avoid air contamination. However, we cannot fully guarantee exclusion of VOC contamination by differences in tap water composition between measurements. Third, we have not explored the difference between the diagnostic accuracy when using fresh versus frozen samples. As previously described, this seems of important influence on urine and serum VOCs. However, since a diagnostic accuracy of 0.99 was found in this study, we believe that freezing our samples has not significantly influenced our study outcomes. Last, it is possible that optimized fecal sampling conditions are disease specific, and fecal VOC biomarkers to diagnose IBD might have different sensitivity to variations in sampling method compared to fecal VOC biomarkers for other gastrointestinal diseases. We did, however, find significant differences in VOC profiles between sampling methods, emphasizing the importance of the use of one standardized sampling method. Furthermore, it is important to point out that we made use of pattern-recognition in this study, which complicates the assessment of the influence of specific metabolites. We have chosen to validate specifically the FAIMS method since this device is an easy-to-use tool which could be suitable for clinical implementation [35].

This study highlights the need for one standardized methodology, in both research setting and when using VOCs analysis as a (future) clinical tool. Based on this and previous study results, we would like to suggest to use a standardized protocol with preferably fecal sample masses of 500mg, no more than one thaw session prior to VOC analysis, and analyzation of

samples directly after thawing or, if impossible, keeping the samples frozen until further analyses. Future studies should assess the difference in diagnostic accuracy between fresh samples and frozen samples, and the influence of storage duration using multiple measurement moments after sample collection.

In conclusion, this study showed a high discriminative accuracy to differentiate between IBD and HC when using the standard operating procedure. It was shown that the use of less than 500mg, multiple thaw-freeze cycles, storage at room temperature and storage in freezer all influence the diagnostic accuracy. We therefore suggest to use one standardized protocol when performing fecal VOC analysis. In addition, further studies should focus on finding IBD specific VOCs to allow for targeted pattern-recognition.

Table 1. Variables of interest

Variables of interest	Faecal sample mass (mg)	Thaw-freeze cycles (N)	Time out of freezer (min)
Reference standard	500	0	10
Mass variable 1	300	0	10
Mass variable 2	100	0	10
Thaw-freeze variable 1	500	1	10
Thaw-freeze variable 2	500	2	10
Thaw-freeze variable 3	500	3	10
180 minutes out of freezer	500	0	180
Storage time 1	500	0	10
Storage time 2	500	0	10

Table 2. Baseline characteristics

	Inflammatory bowel disease			
	Crohn's disease (n=15)	Ulcerative colitis (n=2)	Healthy controls (n=25)	*p-value*
Sex, male (n, [%])	10[66.7]	0[0]	14[56]	0.858
Age, yr (median[IQR])	13.0[11-15]	[10-16]*	12.0[4.0]	0.614
Sample age, mos (median [IQR])	11.0 [2-16]	[11-26]*	11.0[1.0]	0.376
Physician's global assessment				
Quiescent	1	0	NA	
Mild	9	2	NA	
Moderate	5	0	NA	
Severe	0	0	NA	
Fecal calprotectin (µg/g) (median[IQR])	1936[1006-2390]	[1800-2734]*	NA	
CRP (mg/l) (median[IQR])	24.3[2.5-42]	2.5**	NA	
[1]*Crohn's disease localization*				
Ileal (L1)	1	NA	NA	
Colonic (L2)	5	NA	NA	
Ileocolonic (L3)	6	NA	NA	
Proximal disease (L4)	1	NA	NA	
[1]*Crohn's disease behavior*				
B1 (NSNP)	14	NA	NA	
B1p (NSNP+p)	0	NA	NA	
B2 (S)	1	NA	NA	
B2p (S + p)	0	NA	NA	
B3 (P)	0	NA	NA	
B3p (P + p)	1	NA	NA	
[1]*Ulcerative colitis localization*				
Proctitis (E1)	NA	1	NA	
Left-sided (E2)	NA	1	NA	
Extensive (E3)	NA	0	NA	

Table 2. All values were obtained at study inclusion. Localization was obtained by ileocolonoscopy and esophagogastroduodenoscopy before treatment initiation, and magnetic resonance enteroclysis. Abbreviations: IQR, interquartile range; NA, not applicable; NSNP, non-stricturing non-penetrating; S, stricturing; P, penetrating; p, peri-anal disease. [1]Based on Paris classification for inflammatory bowel disease (24) *min-max values ** one missing value

Table 3. Demographics sample analysis of the influence of duration time on VOC profiles

	Crohn's disease (N=10)	Healthy controls (N=10)	p-value
Sex, male (n, [%])	5[50]	2[20]	0.350
Age, yr (median [IQR])	14.1[3.38]	7.8[3.72]	0.007
Sample age first measurement, mos (median[IQR])	23.4 [21-31]	52.2 [51-52.4]*	0.000
Sample age second measurement, mos (median[IQR])	43.2[41-51]	71 [70-72]*	0.000
Physician's global assessment			
Quiescent	0	NA	
Mild	0	NA	
Moderate	3	NA	
Severe	7	NA	
Fecal calprotectin (µg/g) (median[IQR])	1067[1218]	NA	
CRP (mg/l) (median[IQR])	29[29]	NA	
[1]*Crohn's disease localization*			
Ileal (L1)	0	NA	
Colonic (L2)	3	NA	
Ileocolonic (L3)	7	NA	
Proximal disease (L4)	5	NA	
[1]*Crohn's disease behavior*			
B1 (NSNP)	8	NA	
B1p (NSNP+p)	0	NA	
B2 (S)	0	NA	
B2p (S + p)	0	NA	
B3 (P)	1	NA	
B3p (P + p)	1	NA	

Table 3. All values were obtained at study inclusion. Localization was obtained by ileocolonoscopy and esophagogastroduodenoscopy before treatment initiation, and magnetic resonance enteroclysis. Abbreviations: IQR, interquartile range; NA, not applicable; NSNP, non-stricturing non-penetrating; S, stricturing; P, penetrating; p, peri-anal disease. [1]Based on Paris classification for inflammatory bowel disease (24) *one value missing.

Table 4. Performance characteristics for the differentiation between IBD and Healthy for all of the variables of interest by faecal VOC analysis.

Analysis	p-value	Accuracy	AUC (± 95% CI)	Cut-off	Sensitivity (± 95% CI)	Specificity (± 95% CI)	PPV	NPV
Reference standard (17IBD, 25HC)	1.178e-10	0.93	0.99 (0.96 - 1)	0.0014	0.94 (0.71 - 1)	0.96 (0.8 - 1)	0.94	0.96
Mass variable 1 (17IBD, 25HC)	0.02101	0.77	0.69 (0.52 - 0.86)	0.47	0.88 (0.64 - 0.99)	0.44 (0.24 - 0.65)	0.52	0.85
Mass variable 2 (17IBD, 25HC)	0.003642	0.70	0.74 (0.59 - 0.9)	0.44	0.76 (0.5 - 0.93)	0.72 (0.51 - 0.88)	0.65	0.82
Thaw-freeze variable 1 (17IBD, 25HC)	0.0004713	0.61	0.8 (0.65 - 0.94)	0.49	0.76 (0.5 - 0.93)	0.8 (0.59 - 0.93)	0.72	0.83
Thaw-freeze variable 2 (17IBD, 25HC)	0.7534	0.64	0.56 (0.38 - 0.74)	0.66	0.76 (0.5 - 0.93)	0.48 (0.28 - 0.69)	0.5	0.75
Thaw-freeze variable 3 (17IBD, 25HC)	0.5101	0.57	0.5 (0.32 - 0.69)	0.063	0.47 (0.23 - 0.72)	0.72 (0.51 - 0.88)	0.53	0.67
180 minutes out of freezer (17IBD, 25HC)	0.4596	0.60	0.51 (0.32 - 0.7)	0.13	0.59 (0.33 - 0.82)	0.68 (0.46 - 0.85)	0.56	0.71
Storage duration, first measurement (10CD vs 10 HC)	0.0262	0.75	0.75 (0.53 - 0.97)	0.47	0.7 (0.35 - 0.93)	0.8 (0.44 - 0.97)	0.78	0.73
Storage duration, second measurement (10CD vs 10 HC)	0.0376	0.75	0.73 (0.49 - 0.97)	0.58	0.8 (0.44 - 0.97)	0.7 (0.35 - 0.93)	0.73	0.78

Table 4. For each analysis, the best Sparse Logistic Regression outcome is shown. Sensitivities, specificities, p-values and AUCs are reported for the respective optimum cut-points.. Abbreviations: AUC, area under the curve; PPV: positive predictive value; NPV: negative predictive value. *Reference standard is defined as 500mg sample, diluted in 10mL water, thawed 10 minutes to room temperature.

Table 5. Paired feature analyses per variable of interest with corresponding p-values

Variables of interest	Feature 1 (p-value)	Feature 2 (p-value)	Feature 3 (p-value)	Feature 4 (p-value)
Sample mass (mg)				
500 vs 300	*<0.0001*	*<0.0001*	*0.027*	*<0.0001*
500 vs 100	*<0.0001*	*<0.0001*	*<0.0001*	*<0.0001*
Number of freeze-thaw cycles				
Measured directly vs one cycle	*<0.0001*	*<0.0001*	*<0.0001*	*<0.0001*
Measured directly vs two cycle	*<0.0001*	*<0.0001*	*<0.0001*	*<0.0001*
Measured directly vs three cycle	*<0.0001*	*<0.0001*	*<0.0001*	*<0.0001*
Kept at room temperature				
180 Minutes	*<0.0001*	*<0.0001*	*<0.0001*	*<0.0001*
Storage time				
First vs second measurement	*<0.0001*	*<0.0001*	*0.021*	0.825

Table 5. P-value < 0.05 is considered significant.

1

Figure 1. Receiver operating characteristics for each variable of interest for the differentiation between inflammatory bowel disease and healthy state

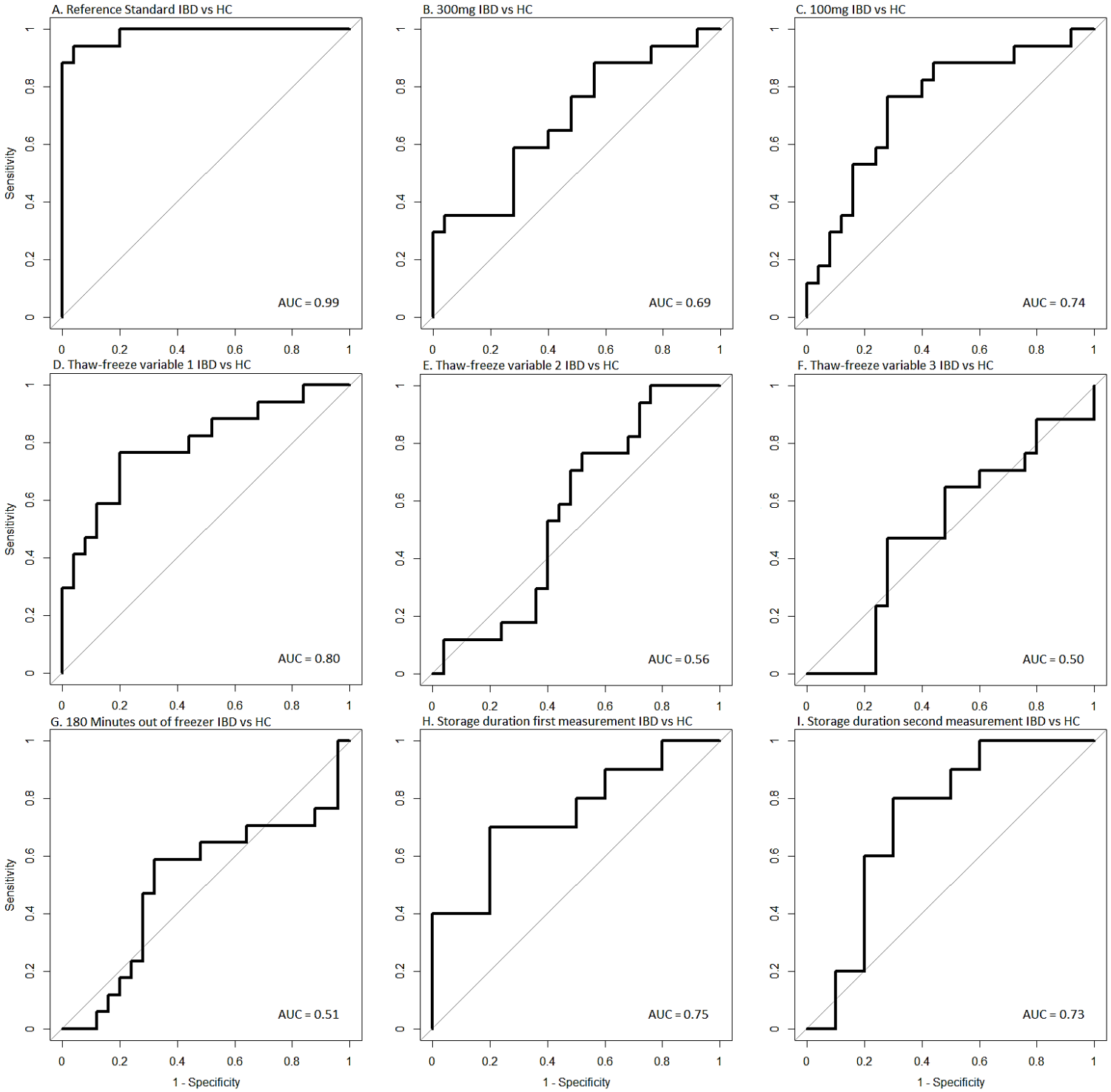

Figure 1. All receiver operating characteristic curves are obtained by Sparse Logistic Regression analyses. Abbreviations: AUC, area under the curve; IBD: Inflammatory bowel disease; HC: Healthy controls.

Figure 2. Typical FAIMS pattern of patients with inflammatory bowel disease and healthy controls

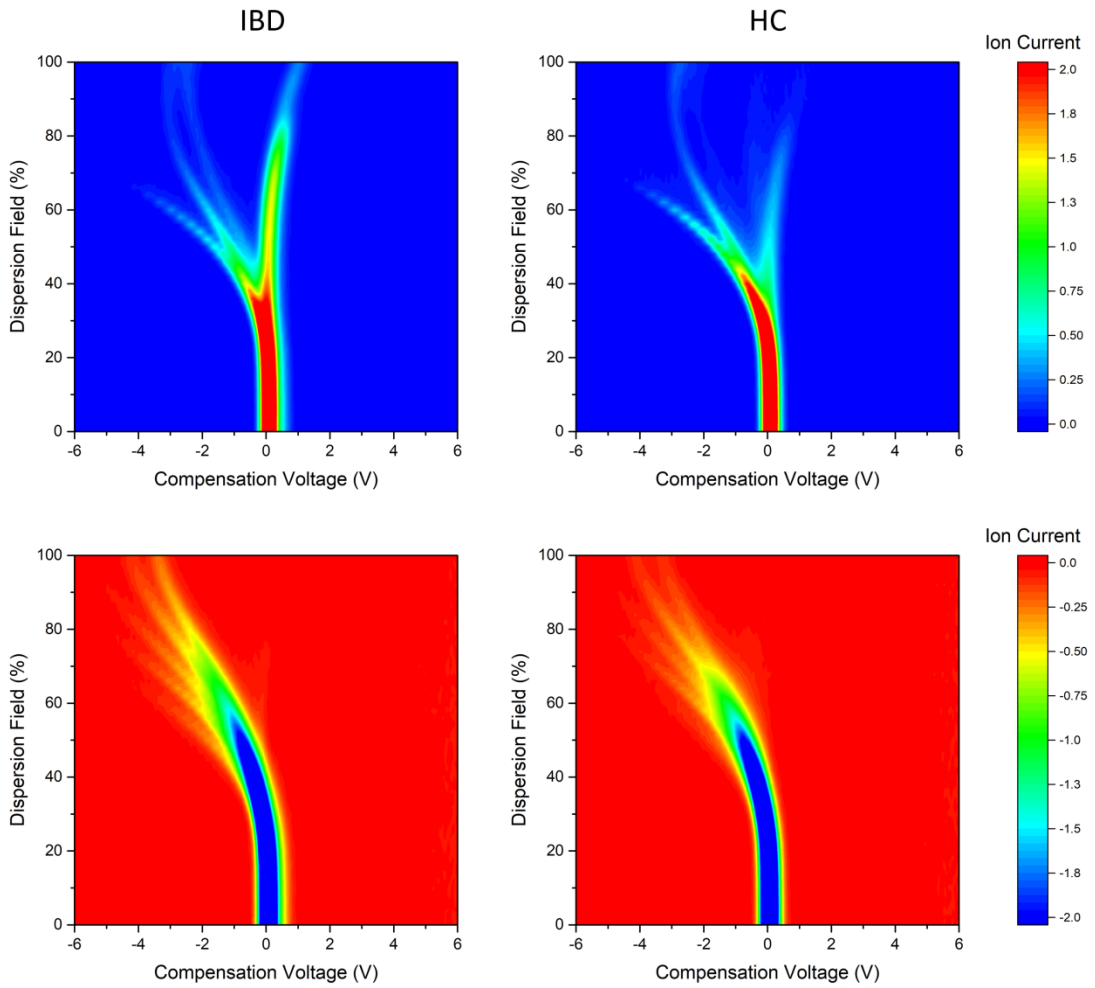

Figure 2. Depicted with a blue background are the positive ion currents. Depicted with a red background are the negative ion currents.

Figure 3. Scatterplot for the differentiation between sampling methods measured by field asymmetric ion mobility spectrometry

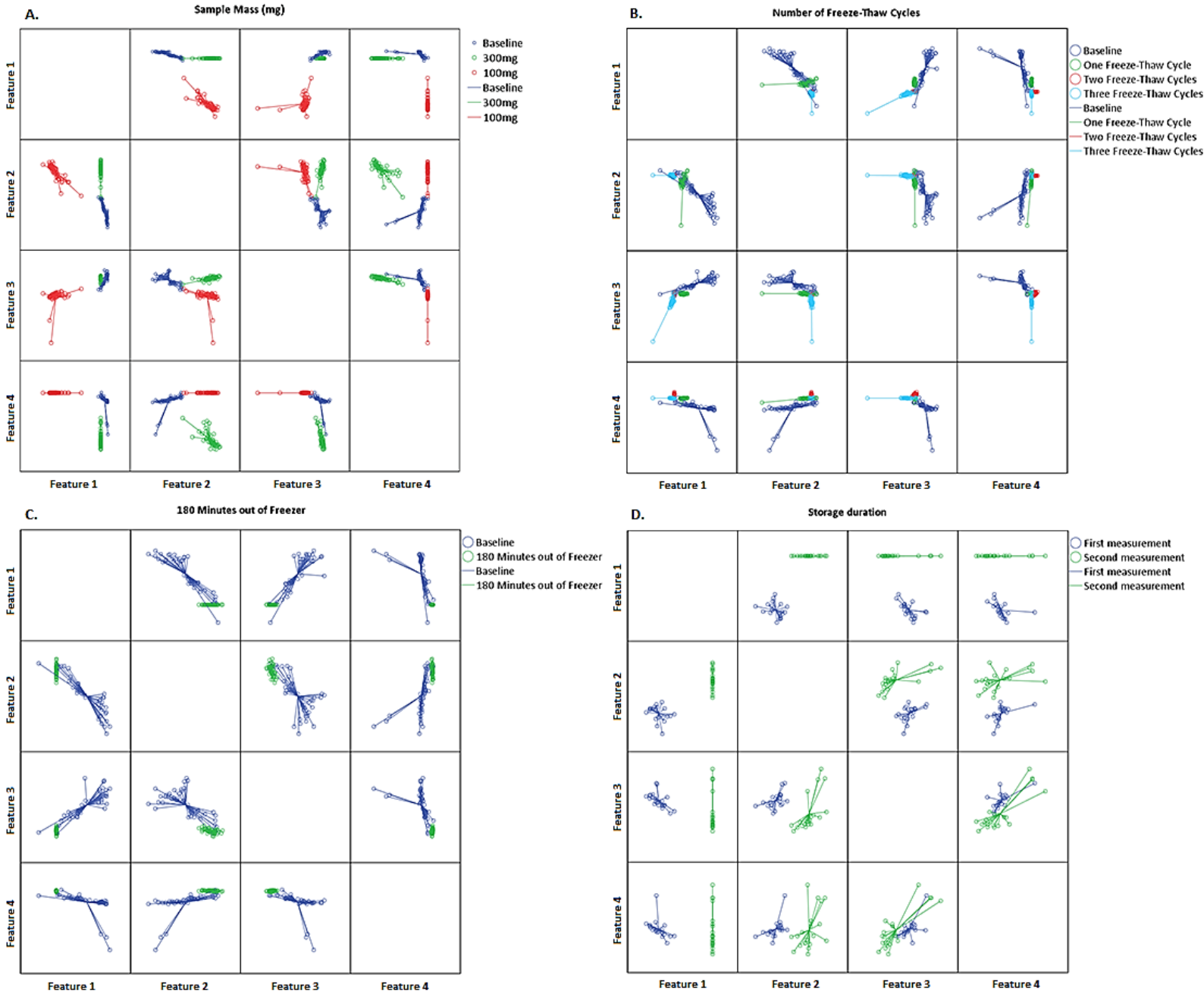

Figure 3. Scatterplot for the differentiation between sampling methods measured by field asymmetric ion mobility spectrometry, including (a) sample mass; (b) number of freeze-thaw cycles; (c) 180 minutes out of freezer; (d) storage duration. Axes depicted are recombinations of the raw sensor data by means of feature selection using Wilcoxon rank sum analyses, creating four features per measurement. The marked points are the individual VOC signals. The intersection of the lines deriving from the individual signals are the mean VOC profile of that specific variable.

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