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Patterns of volatile organic compounds in excrements of preterm neonates

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Abstract

Background: As neonates are susceptible for many diseases, establishing non-invasive diagnostic methods is desirable. We hypothesized that volatile organic compounds (VOCs) could be successfully measured in diaper samples.

Methods: We performed a feasibility study to investigate whether ambient air-independent headspace measurements of the VOC profiles of diapers from premature infants can be conducted using ion mobility spectrometer coupled with multi-capillary columns (B & S Analytik GmbH).

Results: We analysed 39 diapers filled with stool ($n = 10$) or urine ($n = 20$) respectively, using empty diapers as a control ($n = 9$). A total of 158 different VOCs were identified, and we classified the content of the diapers (urine or stool) according to their VOC profiles with a significance level of $p < 0.05$.

Conclusions: We have developed a novel method to study headspace VOC profiles of biosamples using ion mobility spectrometry coupled with multi-capillary columns. Using this method, we have characterized the VOC profiles of stool and urine of preterm neonates. Future studies are warranted to characterize specific VOC profiles in infections and other diseases of the preterm neonate, thus establishing quick and noninvasive diagnostics in the routine care of the highly vulnerable preterm and term neonates.

KEYWORDS

IMS, ion mobility spectrometry, neonatology, noninvasive diagnostics, VOC, volatile organic compounds

Abbreviations: AIS, Amnion infection syndrome; BPD, Bronchopulmonary dysplasia; C, Celsius; CAS, Chemical abstract service; CI, Confidential interval; CrP, C-reactive protein; DT, Decision tree; IL-6/-8, Interleukin-6/Interleukin-8; IMS, Ion mobility spectrometry; IVH, Intraventricular haemorrhage; L, Left; MCC, Multi-capillary column; MCC/IMS, Ion mobility spectrometer coupled to multi-capillary column; NEC, Necrotizing enterocolitis; NICU, Neonatal intensive care unit; P, Peak; PFA, Perfluoroalkoxy alkane; RDS, Respiratory distress syndrome; R, Right; RIP, Reactant ion peak; ROP, Retinopathy of prematurity; SARS-CoV-2, Severe acute respiratory syndrome coronavirus; VOCs, Volatile organic compounds.

1 | INTRODUCTION

Every tenth child worldwide is born prematurely. A state of immaturity of all organ systems including the immune system is associated with prematurity demanding intensive medical care treatment. Diagnosis of infections is difficult in preterm infants as they are often linked to nonspecific symptoms. Diagnostic procedures are not merely painful for premature infants, but other major problems include the time to diagnosis and treatment, costs and sensitivity of existing diagnostic tests. In daily clinical practice, markers of systemic inflammation and immune responses like C-reactive protein, procalcitonin, interleukin-6 and interleukin-8, provide limited sensitivity, especially at the onset of an infection. Time-consuming laboratory tests can delay adequate treatment for hours. In consequence, empiric antibiotic treatment is often started due to clinically suspected sepsis before the laboratory results are known. A fast and nearly instant diagnostic tool could reduce unnecessary exposure to antibiotics in suspected, but unconfirmed sepsis. Blood cultures often yield results 24–48 h or even later and are only positive in approximately 0.5%. Rapid diagnosis and prompt initiation of therapy significantly improves outcome.[1]

Analysis of volatile organic compounds (VOCs) is an innovative approach for noninvasive diagnostics. VOCs are produced in the body during physiological and pathophysiological processes and are emitted via skin, exhaled air, urine, stool and other body secretions. Many diseases are linked to alterations in specific VOC profiles. Neonates are at high risk for inflammatory disease and sepsis. Necrotizing enterocolitis (NEC) is an acute inflammatory disease of the intestine which primarily affects preterm infants and is a leading cause of morbidity and mortality in the neonatal intensive care unit. Diseases like NEC have been shown to be detectable by VOC detectors.[2,3] Electronic nose devices are based on pattern recognition, measurements can be conducted quickly and at the bedside.

Yet those devices do not allow qualitative VOC analysis, therefore identification of substances as potential biomarkers is not possible. With our established method, ion mobility spectrometry is coupled to multi-capillary columns (MCC/IMS) and allows the measurement of VOC profiles and the specification of individual VOCs using a reference data set. It is also possible to assign individual substances to most of the detected VOCs. VOCs were detectable by various other methods including GC/MS, SIFT-MS, sensors and IMS.[4] The possibility to obtain time series every 15 minutes is an advantage of MCC/IMS, especially in comparison to different mass spectrometric methods. Thus, serial measurements with MCC/IMS allow insight into the dynamics of VOC release. In general, the moisture content of exhaled breath samples is a major problem for most analytical methods except SIFT-MS and MCC/IMS. On the other hand, bedside and on-site applications of MCC/IMS were reported since 2017 as medical products based on MCC/IMS technology were introduced into the market.

MCC/IMS analyses provide results within a few minutes and can be used for instant bedside diagnostics. Previous studies revealed the high potential of VOC analysis and its application for noninvasive diagnostics.

For continuous monitoring of propofol in human breath, the Exhaled Drug Monitor (*Edmon*) was launched in 2017 as classified medical product within the European Union by B. Braun Melsungen AG, Center of Breath Research. In addition, the MCC/IMS technology is undergoing trials in the detection of infectious diseases. Proof-of-concept studies showed the great potential of MCC/IMS in the detection of respiratory viruses like severe acute respiratory syndrome coronavirus 2 and influenza virus.[5–7] Also, bacterial strains were found to emit specific VOCs.[8,9] Differences in the VOCs profile in rats'' exhalome when suffering from sepsis versus when suffering from haemorrhagic shock were found using MCC/IMS.[10]

We now want to establish MCC/IMS for noninvasive diagnostics in neonatology. We could previously demonstrate that VOC profiles can be detected in the incubator atmosphere of neonates, and we were able to differentiate between empty and full incubators using the MCC/IMS in a proof-of-principle study.[11] Moreover, we found a potential biomarker for amnion infection syndrome (AIS) using VOC analysis via MCC/IMS.[12] In this study, we aimed to proof if we can generate VOC profiles originating from stool and urine and whether these specimens can be used for diagnostics. Since stool and urine are obtained noninvasively as a waste product, they seem to be suitable for easy diagnostics in preterm infants but also in term infants.

The aim was to establish the method of headspace measurements independent from ambient air for measuring and distinguishing faecal and urine samples and to create specific VOC profiles using MCC/IMS as novel noninvasive, cost-effective and precise method.

2 | METHODS

2.1 | Patients

This study was performed at the Department of Paediatrics, Saarland University Medical Center, Homburg (Germany) after approval by the Ethical committee Saarland

(reference 276/17). Written and informed consent was obtained upfront from all parents. All acquired data were recorded and processed in a pseudonymized form. Inclusion criteria were birth weight <2.000 g or gestational age <32 weeks and treatment on the neonatal care units (NICU) at the Saarland University Hospital, Homburg (Germany). 10 preterm infants were included and clinically characterized (Table 1 and Table S1).

TABLE 1 Sample classification

a: Urine

Infant ID	Sex	Gestational age [weeks]	Birth weight [g]	Sampling day	Diaper size
22	F	28 + 4	980	D7	0
22	F	28 + 4	980	D8	0
22	F	28 + 4	980	D9	1
26	M	29 + 6	950	D9	0
26	M	29 + 6	950	D10	0
28	M	32 + 2	1360	D6	0
28	M	32 + 2	1360	D8	0
28	M	32 + 2	1360	D10	1
29	F	32 + 2	1605	D4	0
29	F	32 + 2	1605	D6	0
29	F	32 + 2	1605	D7	0
29	F	32 + 2	1605	D8	0
30	M	32 + 2	1570	D3	0
30	M	32 + 2	1570	D5	1
31	F	32 + 2	1405	D7	0
32	M	33 + 2	1580	D1	0
32	M	33 + 2	1580	D2	0
32	M	33 + 2	1580	D4	0
32	M	33 + 2	1580	D7	0
32	M	33 + 2	1580	D8	0

b: Stool

Infant ID	Sex	Gestational age [weeks]	Birth weight [g]	Sampling day	Diaper size	Amount	Consistency	Colour
21	M	28 + 4	990	D7	0	4	B	II
21	M	28 + 4	990	D8	0	4	B	II
21	M	28 + 4	990	D10	0	4	B	II
22	F	28 + 4	980	D1	0	3	C	II
22	F	28 + 4	980	D2	0	4	C	II/IV
24	F	28 + 6	1310	D3	0	3	A+C	III/IV
24	F	28 + 6	1310	D9	0	4	B	II
25	F	30 + 5	1610	D4	0	1	A	I
25	F	30 + 5	1610	D9	0	4	B	II
32	M	33 + 2	1580	D3	0	4	B	II

Note: We used diapers (Pampers, Procter & Gamble Service GmbH, Schwalbach/ Taunus, Germany) from a total of 10 preterm infants. For each sample, infants' ID, sex, gestational age, birth weight and diaper size are indicated (F – female, M – male, D – day). (A) Parameters of the samples containing urine. (B) Samples with stool are classified additionally regarding a stool chart[13] referring to the amount, consistency and colour of the stool samples (Amount - 1: smear, 2: up to 25%, 3: 25%–50%, 4: more than 50%; Consistency – A: watery, B: soft, C: formed, D: hard; Colour – I: yellow, II: orange, III: green, IV: brown, V: meconium, VI: white).

2.2 | Sample collection and processing

The diapers were collected daily as part of the morning care routine without generating additional stress for the patients. We used diapers (Pampers, Procter & Gamble Service GmbH, Schwalbach/Taunus, Germany) of 10 preterm infants and classified the diaper contents regarding stool consistency, colour and quantity using a paediatric stool scale[13] (Table 1). Fresh diaper pieces served as control. Sample processing was performed according to a standard operating procedure after preliminary measurements. The surface layer of the diaper impregnated with urine or stool was cut into four pieces of a size of 1×1.5 cm. The pieces were immediately stored at −80°C. The samples were thawed at room temperature 30 minutes before the measurement. The samples were tested individually. For further information about sample inclusion, see Figure S1.

2.3 | Data assignment/Headspace measurement

To perform headspace measurements of VOCs derived from the faecal and urine samples, we used an MCC/IMS BreathDiscovery (B & S Analytik GmbH). It was placed on a metal cart with a laptop computer and connected to a synthetic air supply (20.5 ± 0.5% O_2 in N_2, Alphagaz™ 1 Air, AIR LIQUIDE Deutschland GmbH). The pre-separation was performed using an OV-5 (5% - diphenyl, 95% - dimethylpolysiloxane) MCC (Multichrom Ltd.). The device and sampling parameters are given in Table S2. The methods for VOC analysis were published earlier.[11] Concerning the IMS, the standard operation conditions are used as recommended by the supplier of the instrument. IMS was regularly calibrated using standardized reference mixture ("R06" calibration liquid, B & S Analytik GmbH, Table S3). A laboratory bottle (100 ml, Schott Duran®, DURAN Group GmbH) heated to 37°C served as a sample container. A closed system was established: A large laboratory bottle (1000 ml, gas reservoir, Schott Duran®, DURAN Group GmbH) was connected to the small bottle (100 ml, Schott Duran®, DURAN Group GmbH) via a perfluoroalkoxy alkane tube that was led through the caps. Both bottles were filled with synthetic air as carrier gas. Another tube connected the cap of the small laboratory bottle and the sampling input of MCC/IMS device (Figure 1). Data were acquired using VOCan v3.7 (B & S Analytik GmbH). The measurement process is illustrated in Figure 2, and the precise sequence of our analysis is included in Table S4.

2.4 | Statistical analysis

We evaluated the data acquired by MCC/IMS using the software VisualNow 3.7 (B & S Analytik GmbH). All peaks were characterized by their specific combination of retention time and drift time (corresponding $1/K_0$-value). The databank layer (BS-MCC/IMS-analyses database) was used for peak referencing and determination of retention times and $1/K_0$-values. Here, pure analytes were measured 10 times each and comparison with parallel measurements using GC/MS standard procedures was performed.[14,15] Peak intensity (in volts) was considered as an indirect measure of compound concentration. A specific threshold was calculated for each peak and comparison. Box and Whisker plots and a rank sum test using Mann–Whitney U-test and Bonferroni post hoc analysis correction were performed. The n-value was set at $n = 10$ for diapers filled with stool, $n = 20$ for diapers filled with urine and $n = 9$ for empty diapers. The α-level was defined to be 0.05, and the p-value (one-tailed) was determined to be <0.05. Significant peaks ($p < 0.05$, 95% confidential interval [CI]) were used for further evaluation with decision trees (DT).

3 | RESULTS

We analysed a total of 39 diapers filled with stool ($n = 10$) or urine ($n = 20$) and empty diapers ($n = 9$), respectively. We identified 158 signals (peaks) from each comparison such as between empty diapers and diapers with urine, between empty diapers and diapers with stool and between stool and urine, respectively (Figure 3D, Table S5). We found 17 peaks exclusively assigned to empty diapers in each differentiation. After Bonferroni post hoc analysis correction, peak P98 showed the highest sensitivity, specificity, positive and negative predictive value with 99.9% each in a significance level with $p < 0.05$ (>95% CI) for empty diapers (Figure 4a, Table 2, Table S6a,c). Comparing empty diapers to diapers with urine, 48 relevant peaks could be assigned to urine, amongst peak P20 reaching the highest sensitivity, specificity, positive and negative predictive value of 99.9% each for urine (significance level with $p < 0.01$ (>99% CI after Bonferroni post hoc analysis correction; Figure 4b, Table 2, Table S6a).

Regarding the comparison between empty diapers and diapers with stool, 85 peaks were assigned to stool (Table S6b). From those peaks, peaks P13 and P83 showed the highest sensitivity, specificity and positive and negative predictive value (99.9% each) at a significance level with $p < 0.05$ (>95% CI) after Bonferroni post hoc analysis correction (Figure 4c, Table 2, Table S6b).

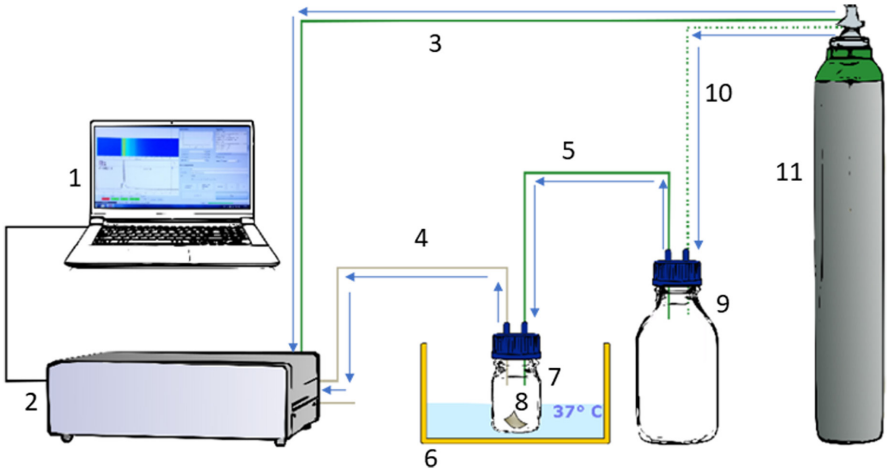

FIGURE 1 Experimental design. We used an MCC/IMS BreathDiscovery (B & S Analytik GmbH) to perform headspace measurements of VOCs derived from the faecal and urine samples. (1) Laptop; (2) MCC/IMS BreathDiscovery (B & S Analytik GmbH); (3) Connecting tube between MCC/IMS and synthetic air supply gas bottle; (4) Connecting tube between MCC/IMS and sample container; (5) Connecting tube between gas reservoir and sample container; (6) Water bath; (7) Sample container: small laboratory bottle (100 ml); (8) Diaper sample; (9) Gas reservoir: large laboratory bottle (1000 ml); (10) Connecting tube between synthetic air supply gas bottle and gas reservoir; (11) Synthetic air supply gas bottle; PFA tubes (green) as drift gas supply; PFA tube (grey) as sample input. Data acquisition was directed and recorded using VOCan v3.7 (B & S Analytik GmbH).

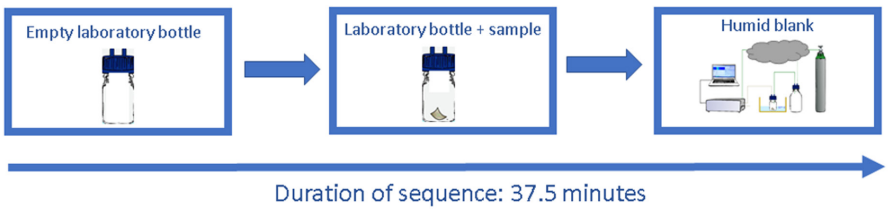

FIGURE 2 Scheme of the measurement procedure. First, we analyse an empty laboratory bottle (Figure 1 (7)) to establish a reference and a control point that shows potential contaminations in the MCC/IMS and the tubes. Then, we place our sample inside the laboratory bottle for a second analysis. Following reference measurement and measurement of the sample, a rinsing process, so-called humid blank, takes place to warrant equal conditions for every sample

For the distinction between stool and urine in diapers, 55 relevant peaks with a significance level of at least $p < 0.05$ (>95% CI) were found (Table S6c). These peaks differ in their signal intensity; 53 of these peaks were found in diapers with stool and two peaks in diapers with urine. Considering those peaks, peak P99 showed the best sensitivity, specificity and positive and negative predictive value of 90%, 80%, 69.2% and 94.1%, respectively, in a significance level with $p < 0.001$ (>99.9% CI) for stool (Figure 3a–c). Overall, a total of 20 peaks exclusively assigned to diapers with stool were found in every comparison. The chemical identification of our compounds is shown in Table S7.

The DT reached a sensitivity of 70% / 95% / 99.9% and a positive predictive value of 87.5%/86.4%/99.9% regarding stool, urine and empty diapers, respectively. Referring to the DT, a total of three peaks enabled the differentiation of stool, urine and empty diapers regarding their signal intensities. The peak named P103 allows to differentiate between empty diapers (with a signal intensity ≤0.008 V) and diapers with stool and urine, respectively (with a signal intensity >0.008 V). Regarding signal intensities >0.102 V, stool is identified via peak P160. The peak P1 enables further differentiation regarding a signal intensity ≤0.102 V: a signal intensity ≤0.005 V stands for urine and a signal intensity >0.005 V for stool (Figure 4D).

In an additional experiment, it was shown that VOC profiles of twins differ from each other (Figure S2).

4 | DISCUSSION

Intensive care treatment and diagnostics are often affiliated with invasive procedures. Establishing a noninvasive, painless method by means of VOCs would be best for vulnerable patients like preterm infants. In the present study, we showed that faecal and urine samples in a diaper can be distinguished based on their VOC profiles

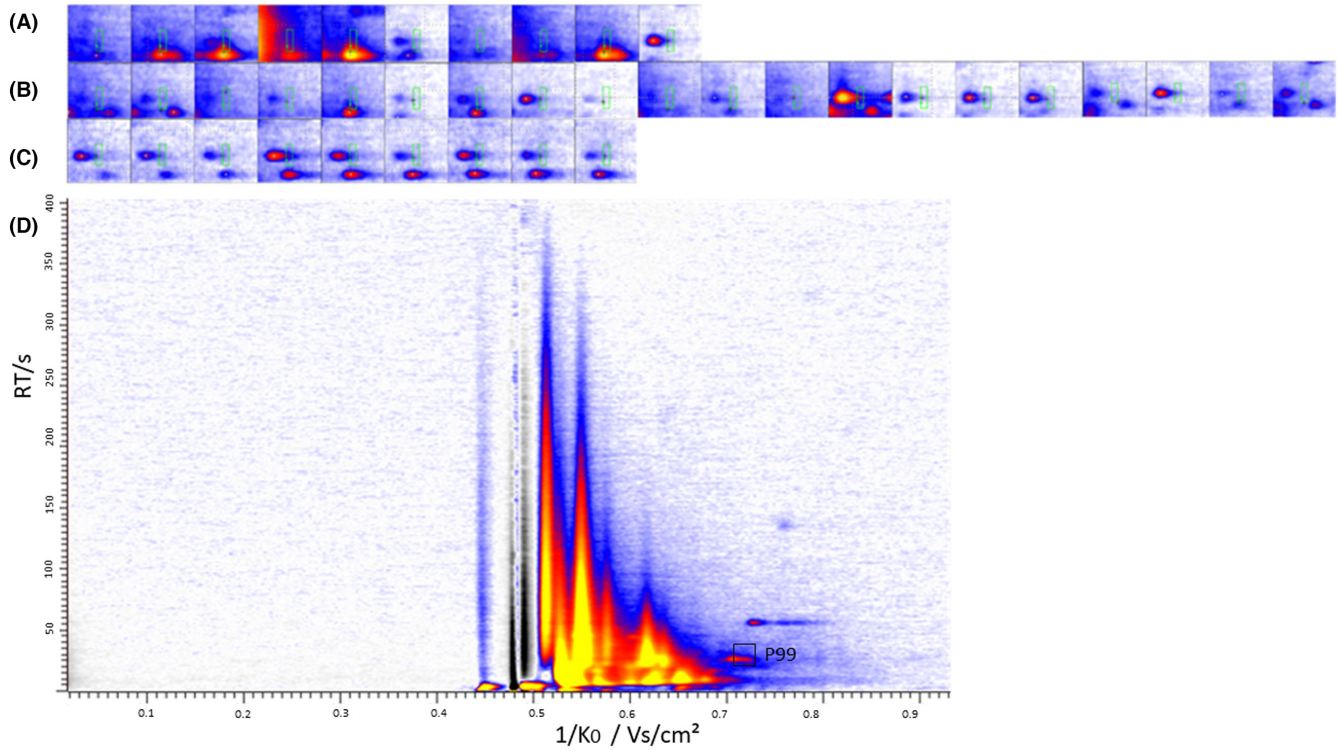

FIGURE 3 Intensity distribution of peak P99 and representative chromatogram. (A)-(C) Shown are the cut-outs of MCC/IMS-chromatograms of peak P99 for every single measurement. In (A) we showed diapers with stool, in (B) diapers with urine and in (C) empty diapers as a control. (D) Heatmap: The y-axis of the heat map represents the retention time RT, and x-axis represents the inverse ion mobility $1/K_0$, a transformation of the drift time. The colours display the signal intensities with increasing values from white over blue and red to yellow. White = no signal, blue = low signal, red = medium signal, yellow = high signal.

using MCC/IMS which implies a proof of principle as a first step in establishing a new diagnostic tool. Besides establishing a low-contact intensive care treatment, VOC analysis via MCC/IMS might allow rapid and precise diagnostics in preterm infants. Current studies using MCC/IMS conducted breath analysis, headspace measurement of biosamples and the measurement of incubators atmosphere.[11,16,17] Chemical substances such as 2-hexanone and butanal detected via MCC/IMS were found to be connected to endotoxic shock or sepsis in rats.[10] Besides, it was shown that bacterial strains of *Escherichia coli* emitted specific VOCs. Even malignant tumours, as lung cancer, seem to be detectable by MCC/IMS.[16]

Other methods such as electronic nose device showed the potential of VOC analysis in stool samples of patients suffering from diseases like inflammatory bowel disease (IBD) and NEC.[2,3] De Meij et al. showed that it was possible to distinguish infants with NEC from healthy controls using an electronic nose device.[3] Stool samples of healthy children's stool samples differed significantly from those suffering from IBD.[2] Regarding breath analysis, patients suffering from diseases like rheumatoid and psoriatic arthritis could be discriminated from healthy control groups using electronic nose devices.[18] Some studies were able to differentiate various bacteria species.[19,20] Also, microbial strains from bronchoalveolar lavage deriving from ventilated patients were discriminated from other using electronic nose technique.[21]

Many studies also conducted analysis on urine samples. Pathogens of urinary tract infections could be differed from each other using ChemPro 100i (Environics Inc.). Urine is best suited to detect metabolic disorders such as diabetes: using electronic nose device patients suffering from diabetes could be distinguished from healthy controls. The detection of colorectal cancer seems to be possible by VOC analysis out of urine samples.[22] Preterm infants are immature in terms of their metabolism which can lead to metabolic imbalances like hyper- and hypoglycaemia. Unfortunately, these irregularities result in frequent blood samplings causing distress and pain.[23,24] Using noninvasive diagnostics like MCC/IMS might facilitate quick and painless detection of glucose levels in urine of premature infants.

We established a method to proceed VOC analysis of faecal and urine samples using MCC/IMS.

Further studies with an increased number of subjects should be conducted to examine the potential of ion mobility spectrometry for diagnostic purposes. The method seems to be suitable for VOC analysis of biosamples as it is able to detect substances with extremely low

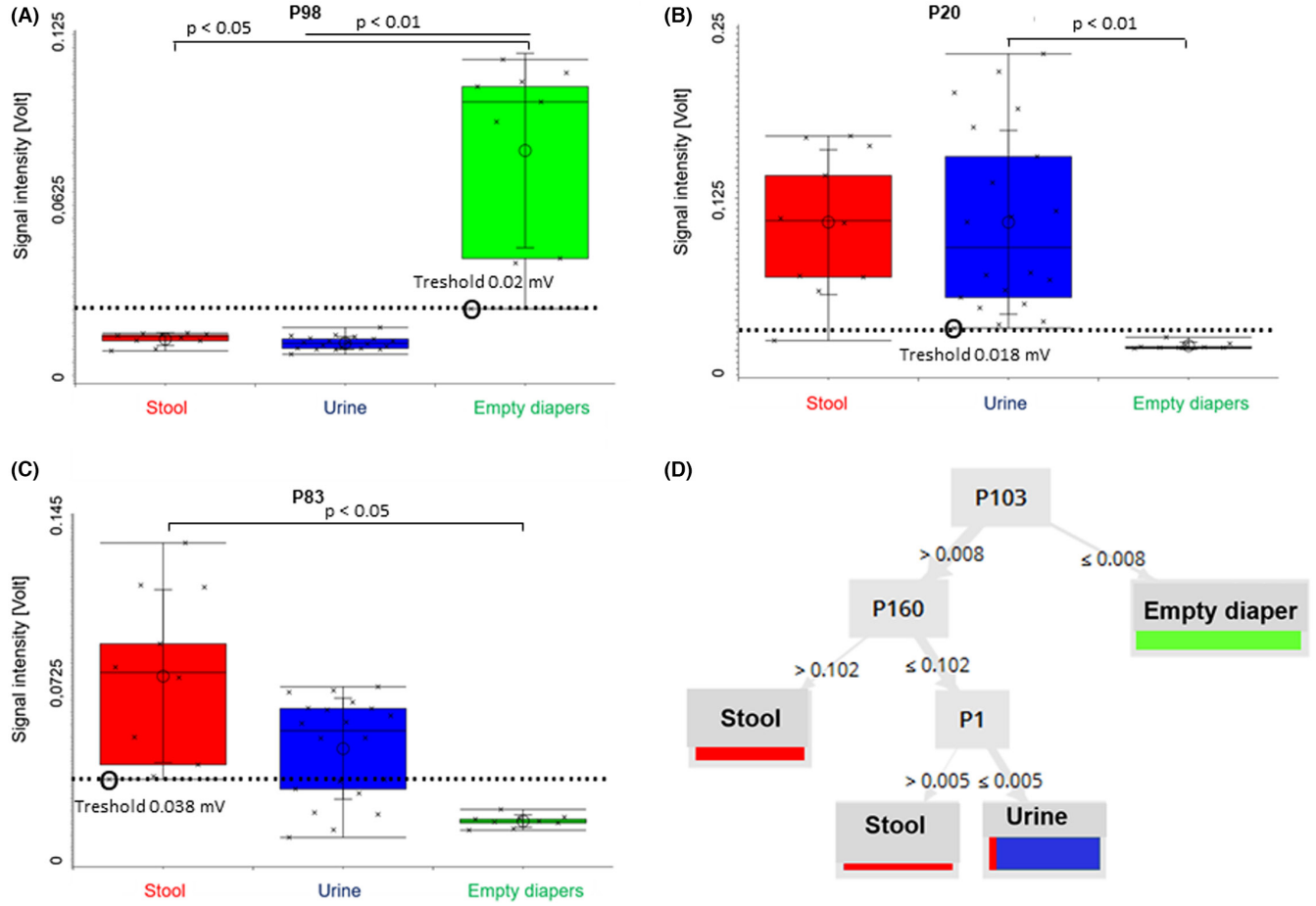

FIGURE 4 Box and Whisker plot of relevant peaks of the comparison of empty diapers ($n = 9$) and diapers with urine ($n = 20$) or stool ($n = 10$), respectively. Measurements are marked with crosses. (A) Peak P98 exclusively assigned to empty diapers showed the highest sensitivity, specificity, positive and negative predictive value with 99.9% each (significance level with $p < 0.05$ (>95% CI)). (B) After Bonferroni post hoc analysis, correction peak P20 reached the best sensitivity, specificity, positive and negative predictive value (99.9% each, $p < 0.01$ [>99% CI]) regarding urine in the differentiation between empty diapers and diapers with urine. (C) Comparing empty diapers and diapers with stool, peak P83 was assigned to a sensitivity, specificity, positive and negative predictive value of 99.9% each in a significance level with $p < 0.05$ (>95% CI) after Bonferroni post hoc analysis correction. (D) A decision tree based on three compounds is shown. Samples are grouped according to the means of the peak intensity of each compound, at which point, the maximum number of samples is classified correctly. Relative numbers of classified all samples stool are blue, urine are green, and empty diapers are red. Discrimination of the three groups is possible. The decision tree reached a sensitivity of 70%/95%/99.9% and a positive predictive value of 87.5%/86.36%/99.9% regarding stool, urine, and empty diapers, respectively.

concentrations (pg/L).[25] As electronic nose devices are only able to detect patterns of VOCs profiles, MCC/IMS enables to precisely identify substances as well. Our measurements can be conducted quickly within a few minutes at the bedside. Moreover, MCC/IMS is suitable for bedside diagnostics as it can be transported on a cart. It is possible to run the mobile MCC/IMS using a small gas bottle containing synthetic air.[11]

Headspace measurements enable VOC analysis in a closed system and independently from ambient air. Other methods such as VOC analysis from exhaled breath are subject to many influences like oral hygiene, food habits, age and gender. Dead space air potentially causes a dilution of the VOC concentration as well as differences in sample conditions leading to measurement bias. In addition, exhaled breath analysis is difficult to accomplish in neonates as it requires the patient's compliance and the use of a mouthpiece which conflicts with the principle of minimal handling. Compared to other methods of VOC analysis such as electronic nose devices which are based on pattern recognition, IMS enables a qualitative analysis of VOCs. The main strength is the ability to assign substances to the spectra. Single substances therefore can be identified as originating from disinfectants or as potential bacterial metabolites.[8,26]

In our study, we identified seven signals that could be assigned to substances using a database (B. Braun Melsungen-Database/ BS-MCC/IMS-analyses database)

TABLE 2 Statistical analyses for the model set and validation set for peaks P98, P20 and P83

	P98		P20		P83	
Best direction	**Empty diaper > Stool**	**Empty diaper > Urine**	**Urine > Empty diaper**	**Urine > Stool**	**Stool > Empty diaper**	**Stool > Urine**
Best threshold	0.02	0.02	0.018	0.018	0.038	0.079
Classified right	19	29	29	21	19	26
Classified wrong	0	0	0	9	0	4
True positive	10	20	20	1	10	6
False positive	0	0	0	0	0	0
True negative	9	9	9	20	9	20
False negative	0	0	0	9	0	4
Sensitivity [%]	99.9	99.9	99.9	10	99.9	60
Specificity [%]	99.9	99.9	99.9	99.9	99.9	99.9
Positive predictive value [%]	99.9	99.9	99.9	99.9	99.9	99.9
Negative predictive value [%]	99.9	99.9	99.9	69	99.9	83.3
Accuracy [%]	99.9	99.9	99.9	70	99.9	86.7
Significance level (Mann–Whitney U-test)	<0.001	<0.001	<0.001	–	<0.001	<0.05
Significance level (Bonferroni correction)	<0.05	<0.01	<0.01	–	<0.05	–

(Table S7). Peak P9 represents 2-pentylfuran and was present in both urine and stool and showed exemplary a high sensitivity/specificity/positive predictive value/negative predictive value (95%/99.9%/99.9%/99.9%) in a significance level with $p < 0.01$ after Bonferroni *post hoc* analysis correction for urine (Table S6) and a sensitivity/ specificity/ positive and negative predictive value of 99.9% each in a significance level with $p < 0.05$ after Bonferroni *post hoc* analysis correction for stool (Table S6) in the respective comparisons to empty diapers. An association between 2-pentylfuran (P9) and Aspergillus was described.[26] Peak P52 was identified as 5-methyl-2,1-methylethylcyclohexanol. Junger et al. showed that benzonitrile (P32) is associated with bacterial strains and might be an *Escherichia coli*, *Enterobacter cloacae*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Serratia marcescens*, *Staphylococcus epidermidis* and *Streptococcus pneumoniae*.[8] Nevertheless, 5-methyl-2,1-methylethylcyclohexanol (P32) assigned to stool in differentiation between empty diapers and stool only showed a sensitivity/specificity/positive and negative predictive value of 70%/99.9%/99.9%/75% (in a significance level with $p < 0.05$; Table S6). Peak P91 represents 2-hexanone and is a metabolite of the bacterium *Escherichia coli* that is associated with endotoxin shock and septic shock, respectively, in animal models.[9] 2-hexanone was exclusively found in stool reaching a sensitivity/specificity/positive and negative predictive value of 90%/88.9%/90%/88.9% (significance level with $p < 0.01$; Table S6) comparing empty diapers and stool and a sensitivity/ specificity/ positive and negative predictive value of 20%/99.9%/99.9%/71.4% (significance level with $p < 0.05$; Table S6) comparing stool and urine, respectively. Those peaks should be re-evaluated in further studies with a higher sample count.

One limitation of our study is that we conducted headspace measurement of diaper samples. Additional measurements are required to determine the VOC profiles for stool and urine in the infant's environment or the incubator's atmosphere respectively.[11] Patient size was small in the present study, and our results have to be validated on a higher number of patients. Nevertheless, the cohort was homogenous and a lot of attention was paid on sample quality with regard to storing conditions and processing. One strength was the consideration of a school classification focusing on amount, colour and consistency of stool.

We processed and stored all samples adhering to a standard operating procedure. Differences in VOC profiles of breastfed versus formula-fed preterm infants were found.[27] In our cohort, all preterm infants were formula-fed which implies a homogeneity in this aspect (Table S2). To emphasize the accuracy of our method, we showed that diamniotic twins differ significantly

from each other regarding their VOCs profile. In this analysis, a total of 59 significant ($p < 0.05$ (>95% CI)) peaks, including 8 significant peaks after *Bonferroni* post hoc analysis correction were detected via VOC analysis using MCC/IMS. Peak P104 showed the best values for sensitivity, specificity, positive and negative predictive value with 99.9% each in a significance level with $p < 0.05$ (>95% CI) (Figure S2). Reasons for the differences might be attributed to internal factors like changes in the gut microbiome or different sex, but also in external factors like contact to various nursing staff.

Dominianni et al. demonstrated that faecal samples should be stored at −80°C to ensure stability.[28] One study conducted by electronic nose device showed a better signal intensity for samples stored at −80°C.[29] Our diapers containing urine and stool had been stored at −80°C and were thawed prior to the measurements; therefore, VOC profiles of native samples might differ from those in our study. Furthermore, after all the impact of the gut microbiome on VOC profiles remains unknown and has to be considered in future studies. Artefacts like the overload of signal intensity must be considered as well. One reason for too intense signals might be ammonia detected in urine. Therefore, our sampling system and the MCC/IMS itself were constantly purged with synthetic air and were rinsed using a special humid blank. Nevertheless, the storage time of stool and urine in the incubator upfront processing might cause an unknown bias and has to be investigated more in detail.

Currently, there is only limited evidence about VOCs changes over time. Our analysis showed a tendency towards changing VOCs profiles over time. We hypothesize that there might be differences in food quantity or quality or in medication, as well as in the respective microbiome, another aspect could be changes in the stool consistency, amount and colour. Nevertheless, we observed a persistence of the relevant peaks of our study. Hence, further studies should address the consistency of VOCs profiles.

Overall, a variety of environmental conditions and the sampling processing seem to influence VOC analysis using MCC/IMS. Therefore, we regularly calibrated the IMS using a standardized reference mixture (R06-calibration liquid, B & S Analytik GmbH, Table S3), as recommended by the supplier, and refer to the stability of the MCC/IMS shown in the literature.[30] We admit that MCC/IMS enables the identification of most VOCs. Using additional methods like gas chromatography coupled to mass spectrometry (GC/MS) enables the identification of the analytes and should be realized to perform quantification of the analytes down to the ng/L- and pg/L-range in future.[14,15] However, our study reveals a high potential for VOC analysis using MCC/IMS. It could contribute to reduce neonatal stress on the NICU through the establishment of a noninvasive diagnostic tool. IMS might become part in the setup of a future low-contact incubator in future. We aim to determine volatile biomarkers and/or patterns of volatile biomarkers to use MCC/IMS as a novel, noninvasive and instant diagnostic tool. Our distant objective is to gradually replace conventional, invasive diagnostics by noninvasive methods.

AUTHOR CONTRIBUTIONS

MB: conception and design, sample collection and processing, measurement performing, collection and assembly of data, data analysis and interpretation, manuscript writing, final approval of manuscript. ET: coordination of sample collection, attainment of parents' written consent, manuscript writing, final approval of manuscript. NNT: attainment of parents' written consent, manuscript writing, final approval of manuscript. EK, RS, SM: manuscript writing, final approval of manuscript. JIB: data analysis and interpretation, manuscript writing, final approval of manuscript. MZ: conception and design, data analysis and interpretation, financial support, manuscript writing, final approval of manuscript. SGF: conception and design, data analysis and interpretation, manuscript writing, final approval of manuscript. All authors have read and agreed to the published version of the manuscript.

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CONFLICT OF INTEREST

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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