Simultaneous measurement of inhaled air and exhaled breath by double multicapillary column ion-mobility spectrometry, a new method for breath analysis: results of a feasibility study

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Shareable abstract (@ERSpublications)
Simultaneous analysis of inhaled air and exhaled breath by a newly invented double MCC-IMS device shows that exhaled breath contains confounding exogeneous analytes and only a smaller number of truly endogenous VOCs, which can be used for further analysis https://bit.ly/3HGVzV5

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Abstract

The high sensitivity of the methods applied in breath analysis entails a high risk of detecting analytes that do not derive from endogenous production. Consequentially, it appears useful to have knowledge about the composition of inhaled air and to include alveolar gradients into interpretation.

The current study aimed to standardise sampling procedures in breath analysis, especially with multicapillary column ion-mobility spectrometry (MCC-IMS), by applying a simultaneous registration of inhaled air and exhaled breath.

A "double MCC-IMS" device, which for the first time allows simultaneous analysis of inhaled air and exhaled breath, was developed and tested in 18 healthy individuals. For this, two BreathDiscovery instruments were coupled with each other.

Measurements of inhaled air and exhaled breath in 18 healthy individuals (mean age 46±10.9 years; nine men, nine women) identified 35 different volatile organic compounds (VOCs) for further analysis. Not all of these had positive alveolar gradients and could be regarded as endogenous VOCs: 16 VOCs had a positive alveolar gradient in mean; 19 VOCs a negative one. 12 VOCs were positive in >12 of the healthy subjects.

For the first time in our understanding, a method is described that enables simultaneous measurement of inhaled air and exhaled breath. This facilitates the calculation of alveolar gradients and selection of endogenous VOCs for exhaled breath analysis. Only a part of VOCs in exhaled breath are truly endogenous VOCs. The observation of different and varying polarities of the alveolar gradients needs further analysis.

Introduction

The high sensitivity of methods applied in exhaled breath analysis entails a high risk of detecting analytes that do not derive from endogenous production. They need to be regarded as exogenous, and thus confounding, volatile organic compounds (VOCs): pollutants. Therefore, exhaled breath analysis needs standardisation and validation for its clinical usefulness, as described in European Respiratory Society (ERS) recommendations [1].

For interpretation of relevant physiological and pathological VOCs, as well as circadian and day-to-day variations, Wallace *et al*. [2] postulated in 1996 that a greater number of collectives needs to be studied.

The relevance of variations could be shown in time series of exhaled breath by calculating alveolar gradients [3] and by times series of room air [4, 5]. The inspiration of confounding, site-specific

exogenous analytes may result in a transfer to the examination room and not only in their detection in exhaled breath, but also in an expiration of new analytes [4, 5]. This implicates a misinterpretation of such analytes as endogenous ones. Notably, a comparison of individuals or patient groups with different diseases as well as studies at different sites bears this risk of false classification by exogenous and site-specific, but not disease-specific, analytes [5, 6]. Furthermore, VOCs in room air, as well as in exhaled breath, may not only exhibit circadian fluctuations, but also variations of peak intensities and alveolar gradients within longer periods of time [7].

As a consequence, for exhaled breath analysis it appears useful to have knowledge about the composition of inhaled air, mainly room air. Additionally, alveolar gradients should be included in interpretation.

First examinations of alveolar gradients by Phillips *et al*. [8] showed that 50% of VOCs in exhaled breath have a negative alveolar gradient. Further studies by Phillips [9] could detect increasing numbers of VOCs with varying proportions of negative and positive alveolar gradients. Only 27 VOCs out of >3000 VOCs were consistently observed among the 50 healthy subjects, confirming wide interindividual variations in healthy individuals.

Accordingly, regarding analytes only in exhaled breath may lead to different results compared to a consideration of analytes and their peak intensities in inhaled air and exhaled breath with calculation of alveolar gradients [5]. This is associated with a dramatic reduction of the number of discriminating VOCs [10].

Following the ERS/American Thoracic Society recommendations [1] for breath analysis, which outlines a framework regarding local conditions and standardisation of sampling procedures, we believe the best course would be to standardise sampling procedures in breath analysis using multicapillary column ion-mobility spectrometry (MCC-IMS). This would be done by applying a simultaneous registration of inhaled air and exhaled breath. For this we developed a “double MCC-IMS” device, which for the first time allows simultaneous analysis of inhaled air and exhaled breath, and we tested it in a pilot study in healthy individuals.

Methods

MCC-IMS

The study was performed using two BioScouts, consisting of a BreathDiscovery instrument and a spirometer (SpiroScout). Therefore, the measurements of exhaled breath and inhaled air were made by ion mobility spectrometry (IMS), coupled to a multicapillary column (MCC) (BioScout; B&S Analytik, Dortmund, Germany). The major parameters of the MCC-IMS and of peak analysis are described elsewhere [7, 11–21]. In the spectrometer, either a 550 MBq (BreathDiscovery 01) or a 95 MBq (BreathDiscovery 31) nickel-63 β-radiation source were applied for the ionisation of the drift gas. The difference in the activity has no effect to the results, because in all cases sufficient ionisation was realised.

The REDMON (B. Braun Melsungen, Dortmund, Germany) purifies the room air to provide it as operating gas for the BreathDiscovery. Room air is conducted through activated carbon and a molecular sieve to dry and filter the air.

The IMS is connected to a polar multicapillary column (type OV-5; Multichrom, Novosibirsk, Russia), which was used as a pre-separation unit. The analytes of a 10-mL sample of inhaled air and, respectively, exhaled breath, were sent through its 1000 parallel capillaries, each with an inner diameter of 40 mm and a film thickness of 200 nm. The total diameter of the pre-separation column was 3 mm. The relevant MCC and IMS parameters are listed in table 1.

Connecting a BreathDiscovery to a SpiroScout (Ganshorn Medizin Electronic, Niederlauer, Germany) allows a flow-triggered sample of exhaled breath. Sampling starts when a minimum volume is exhaled, which can be adjusted (standard setting 500 $mL \cdot min^{-1}$).

Breath sampling with double MCC-IMS (double-SpiroScout)

For simultaneous measurement of inhaled air and exhaled breath, two BreathDiscovery instruments were used and coupled with each other. Each BreathDiscovery was provided with one REDMON. Because technically it was not possible to connect two BreathDiscovery machines with one SpiroScout or two SpiroScouts with one BreathDiscovery, two independent systems were connected mechanically (figure 1). T-pieces were custom-made. Therefore, the lack of marked standard mechanical possibilities was overcome, by connecting and fitting both ends with transparent adhesive tape. Normally the SpiroScout starts breath sampling through expiratory flow signals. By rotating the second SpiroScout by 180°, it was

TABLE 1 Parameters and adjustments for the BioScouts

Sample	100 mL·min^{-1}
MCC	150 mL·min^{-1}
Drift	100 mL·min^{-1}
Pump	0
Temperature MCC	40°C, isothermal
Polarity	Positive (+)
Humidity	Off
Airflow valve	Open
Sample valve	Sampling
Spectra count	1500
Average RT	5 s
Average DT	5 s
Measuring programme	Pump
Pump flow	300 mL·min^{-1}
Sampling control	Volume-controlled

MCC: multicapillary column; RT: retention time; DT: drift time.

not necessary to reverse the flow signal. As a result, the respective SpiroScout displays detected flow in different directions, which enabled sampling of inhaled breath. When inhaling and exhaling through both fitted SpiroScouts, exhaled breath was recognised in the proximal and inhaled air in the distal. The sampling procedure started in the moment when both SpiroScouts were active, by the operator informing the test subject to start breathing through the sampling system. A nose clamp was used to avoid breathing through the nose. The samples of inhaled air and exhaled breath were carried to the respective BreathDiscovery instruments, where further separation of the VOCs and visualisation of their resulting peaks was processed.

Comparison of volume flows of inhaled air and exhaled breath

First, it was tested whether both SpiroScouts detected the flows correctly and comparably, and whether they were supplied with sufficient amounts of sample volume; the flow–volume curves of inspiration and expiration for each SpiroScout mirrored each other (figure 2). The red line indicates the integral volume of the flow curves. The yellowish colour within the flow curve and the flow integral marks the time space

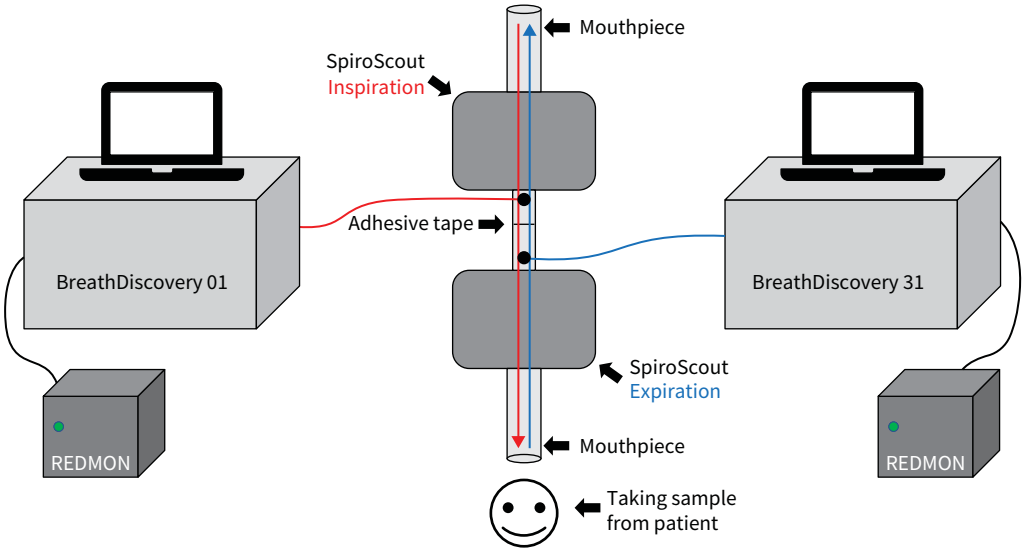

FIGURE 1 Schematic experimental setup consisting of two BreathDiscovery, two REDMON and two SpiroScout instruments. The rear SpiroScout was turned around 180° and connected to the proximal one. Each SpiroScout was linked to one BreathDiscovery, that is connected to a REDMON which provided the operating gas. The red line indicates the path of the inhaled air moving towards the patient through the SpiroScouts and reaching the BreathDiscovery 01. The blue line shows the path of exhaled breath moving away from the patient and reaching the corresponding BreathDiscovery.

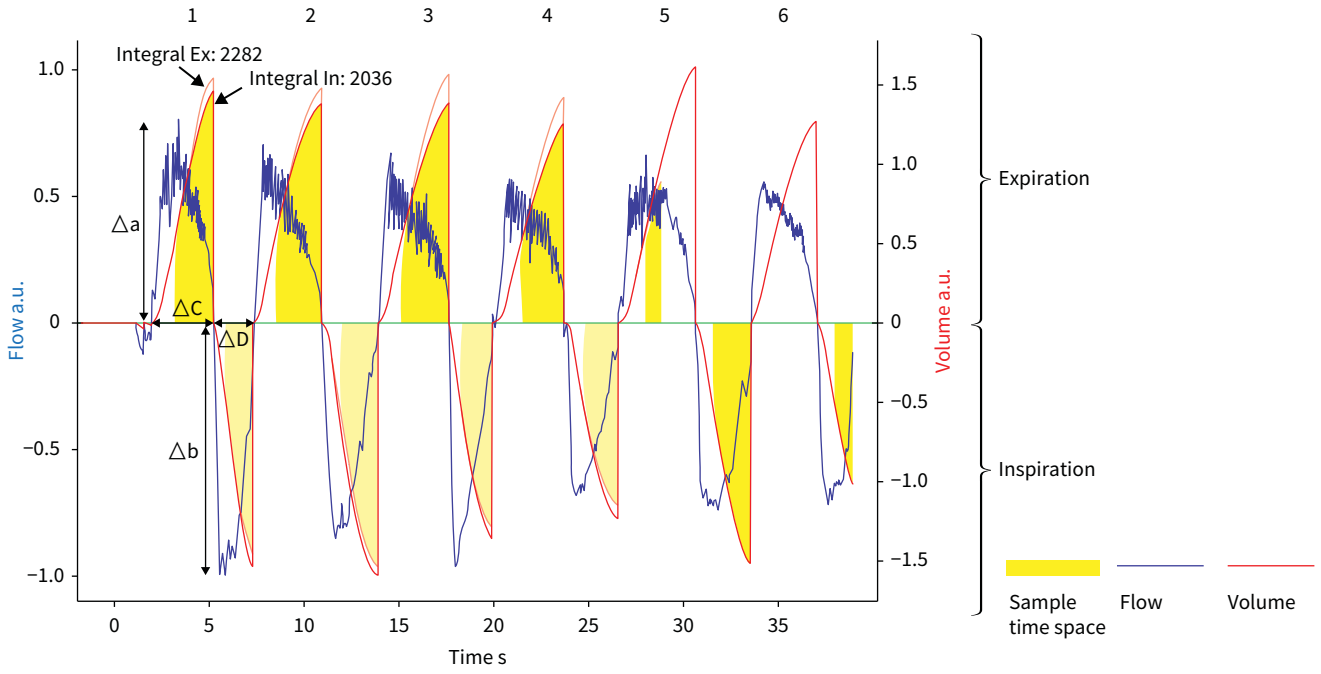

FIGURE 2 Comparison of volume and flows during inspiration and expiration by mirroring them on top of each other. The left y-axis shows the flow, while the right y-axis indicates the volume (flow rate over time). The time in seconds is shown on the x-axis. The flow is proportional to $L \cdot s^{-1}$ and the volume to litres. Although the instruments are not calibrated, they are equal. Δa and Δb indicate the rise of the flow at expiration and inspiration, respectively. The duration of either expiration or inspiration is indicated by ΔC and ΔD. Each SpiroScout should recognise both directions of flow, even if only one is collected and analysed. The area of the integrals Ex (expiration) and In (inspiration) show that although their flows are mirrored and should be the same, they differ slightly from each other. a.u.: arbitrary units.

within which the volume of exhaled breath and inhaled air are above the limit (a volume of 500 mL in figure 2) chosen for starting the sampling procedure. Summing up all yellow areas, respectively time spaces, within the flow curves results in a sampling time of 10 s and represents a complete sample that is analysed. The duration of 10 s is chosen to guarantee a complete air exchange within the sample loop. Additionally, the setup could be checked for possible leaks due to the new connection between the two BreathDiscovery machines.

Numbers from one to six in figure 2 indicate the number of breaths during the sampling procedure. To reach the sampling time of 10 s, four breaths were needed for inspiration, and six for expiration. The difference between inspiration and expiration is because the duration of expiration is longer than that of inspiration (ΔC>ΔD). A comparison of the flow curves also shows different peak flows, with an expiratory increase to 0.75 arbitrary units (a.u.) (Δa), and an inspiratory increase to 1 a.u. (Δb), resulting in a higher volume per time and a shorter duration of inspiratory sampling. Because the parameters for sampling refer to the sampling duration, but not to the volume, this results in more inspirations needed to reach the total time of 10 s.

Analysis of inhaled air and exhaled breath

After the BreathDiscovery instruments had finished the analysis of the samples taken from inhaled air and exhaled breath, chromatograms were generated for visual interpretation as well as files that could be opened within the software VisualNow (B&S Analytik). The measurement files visualise three dimensions: peak positions by drift and retention time and peak intensities.

The feasibility of the method was first proved in a single healthy person by analysing 12 selected VOCs. Afterwards, 18 healthy persons had simultaneous analysis of inhaled air and exhaled breath by the presented double MCC-IMS method.

Results

Comparison of single heatmaps of inhaled air and exhaled breath

The heat maps (figure 3) represent the spectra of drift time and retention time and the peak heights of a single individuum. The peak heights correlate with the intensity of the VOCs, which is underlined by the

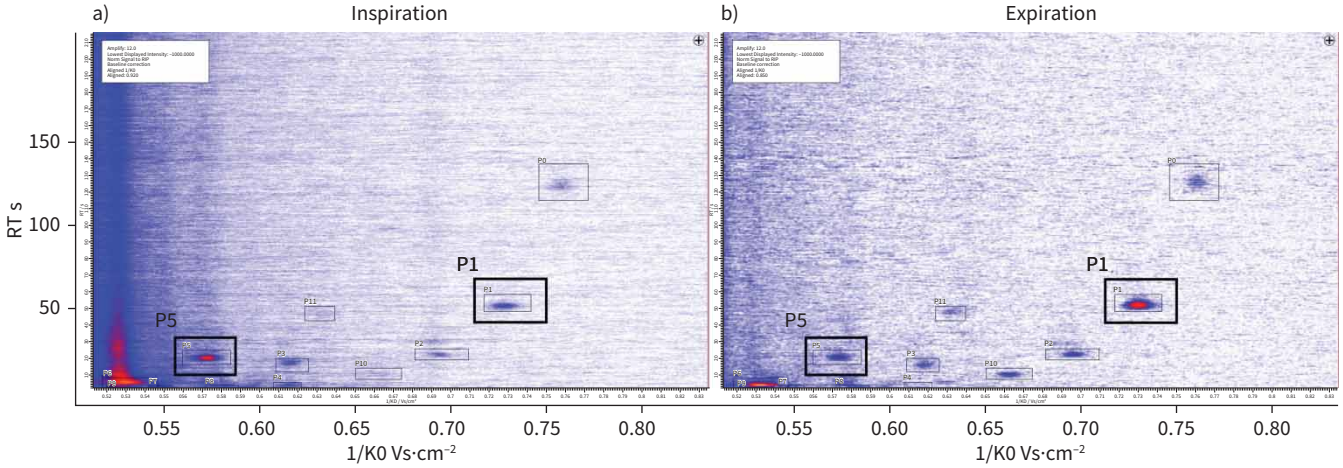

FIGURE 3 Three-dimensional spectrum of a) inhaled air and b) exhaled breath. The y-axes indicate the retention time (RT) in seconds; the x-axes the drift time in 1/K0 Vs·cm^{-2}. The third axis, the peak intensity, increases from white to yellow colour. At expiration, peak P1 alone can be visually recognised as more intense compared to the inspiration. However, peak P5 can be seen more intensely during inspiration (cf. black frames). For detailed peak analysis intensities have to be measured and compared.

colour range, with white being the lowest and yellow the highest intensity. In figure 3a, the analytes of inspiration are shown; figure 3b shows the analytes of expiration. For a better comparison of product ions in inhaled and exhaled air, the RIP was cut out.

The product ions of inhaled air and exhaled breath differ in their peak intensities (figure 3). Peak 5 in inspiration is visually recognisable as more intense (cf. black frames). In comparison, P1 in expiration is more intense (cf. black frames). However, it is not always the case that the peak differences can be distinguished visually so clearly. Therefore, the indication and evaluation of the numeric peak intensity values is needed.

Comparison of inhaled air and exhaled breath in 18 healthy persons

18 healthy persons (mean age 46±10.9 years; nine men, nine women) had measurements of inhaled air and exhaled breath for comparison. 35 different peaks could be identified and were put together to create a set (figure 4). The horizontal rows show the 35 different peaks found in every person. The 18 upper rows correspond to the patients' inspiration, the lower 18 rows to their expiration. Peak intensities reach from white being the lowest to yellow being the highest intensity. Remarkable differences of peak intensities of exhaled breath and inhaled air are already detectable visually, *e.g.* at peak P1. This peak is more intense peaks on inhaled air compared to exhaled breath (c.f. black frame).

Differentiation of endogenous VOCs

The visual comparison shows a significant difference of single peak intensities between inspiration and expiration ($p<0.001$). For more precise evaluation, alveolar gradients from peak intensities of exhaled and inhaled VOCs were calculated.

The calculation showed that not all 35 peaks in exhaled air had positive alveolar gradients and can be regarded as endogenous VOCs.

For example, in case of P1 in the second column of figure 4, the measurements of inhaled air in nearly all patients show a dark blue to reddish spot, whereas, in contrast, in exhaled breath only light blue spots are present. Regarding the measured intensity values, table 2 shows the mean positive alveolar gradients of 16 peaks. 12 of these peaks were positive in >12 of the healthy subjects; five peaks in >15 of them.

Table 3 shows the mean negative alveolar gradients of the remaining 19 peaks. 16 of these peaks were negative in ⩾15 of the healthy subjects.

Discussion

For the first time to our knowledge, we describe a method using a double MCC-IMS that enables simultaneous measurement of inhaled air and exhaled breath, thus facilitating the calculation of alveolar gradients and selecting endogenous VOCs for exhaled breath analysis.

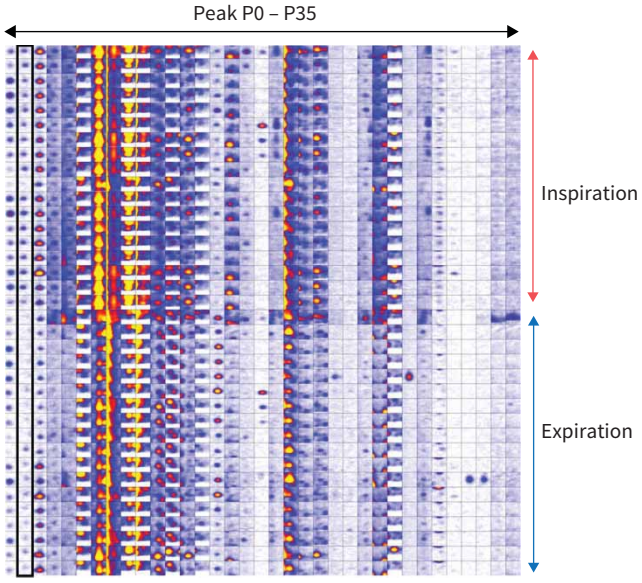

FIGURE 4 Peak images of all peaks found from all 18 healthy test subjects. The images are created from the peak windows from the three-dimensional spectrum of each measurement. Peak intensities reach from white being the lowest intensity to yellow being the highest. The top 18 rows result from inhaled air (rows at the height of the red arrow); the lower 18 show exhaled breath (rows at the height of the blue arrow). Each column shows one out of 35 peaks, ascending from left to right and starting at peak P0. The black frame circles peak P1. Here the difference between the more intense peaks on inhaled air compared to exhaled breath can be made visually.

By applying the presented method, it can be shown that only a part of VOCs in exhaled breath are truly endogenous VOCs. More than half of the VOCs chosen for analysis had negative alveolar gradients and only 16 peaks had positive alveolar gradients in a mean. However, only five peaks had positive alveolar gradients in most subjects, and only two peaks in all subjects.

This finding has an important impact on the interpretation of VOCs. This concerns their metabolism as well as their relevance for disease classification. As Phillips and co-workers [8, 9, 22] have shown,

TABLE 2 Positive alveolar gradients of the 16 peaks

	Alveolar gradient
P5	0.0045±0.0150
P7	0.0242±0.0448
P9	0.0100±0.0288
P10	0.0223±0.0285
P11	0.0204±0.0261
P13	0.0030±0.0025
P14	0.0293±0.0266
P20	0.0930±0.1208
P23	0.0005±0.0009
P24	0.0013±0.0087
P26	0.0011±0.0104
P27	0.0025±0.0103
P28	0.0009±0.0057
P32	0.0008±0.0023
P35	0.0011±0.0049
P36	0.0011±0.0046

Data are presented as mean±SD.

TABLE 3 Negative alveolar gradients of 19 peaks

	Alveolar gradient
P0	−0.0031±0.0031
P1	−0.0076±0.0040
P2	−0.0243±0.0273
P3	−0.0032±0.0033
P4	−0.0048±0.0078
P6	−0.2429±0.0927
P8	−0.0781±0.0685
P12	−0.0306±0.0352
P19	−0.0076±0.0038
P21	−0.0007±0.0022
P22	−0.0005±0.0067
P25	−0.0042±0.0053
P29	−0.0083±0.0058
P30	−0.0015±0.0036
P31	−0.0284±0.0326
P33	−0.0026±0.0022
P34	−0.0016±0.0030
P37	−0.0009±0.0036
P38	−0.0002±0.0042

Data are presented as mean±SD.

considering only exhaled VOCs does not lead to the conclusion that all VOCs in exhaled breath represent truly endogenous ones. The simultaneous measurement of inhaled air and exhaled breath by double MCC-IMS certifies this. Even most of the detectable VOCs in exhaled breath have lower intensities than in inhaled air and cannot be regarded as endogenous ones. This confirms the data of PIZZINI *et al.* [10]: the number of discriminant VOCs, which can be used for exhaled breath analysis, is much lower than the number of VOCs detectable in exhaled breath when calculating alveolar gradients. However, in contrast to our simultaneous analysis, PIZZINI *et al.* [10] used a separated analysis of room air and exhaled breath.

The observed negative alveolar gradients, which result from higher peak intensities in inspiration than in expiration, may have different explanations. VOCs may either be attributable to original room air [4], other external factors such as clothes and perfume or to VOCs which are transferred by the patient from former locations to the examination room [5]. Contamination caused by a disinfectant at a location different to the examination room were shown [5] to result in significantly higher intensities of VOCs not only in exhaled breath, but also in the examination room, compared to corresponding baseline measurements. Such constellations often occur in hospitals where disinfectants are routinely used. This strengthens the necessity of evaluating either if inhaled air is possibly contaminated by such confounding analytes or if newly detected VOCs are a result of an endogenous metabolisation or possibly an induction of an inflammatory process by such irritant analytes.

Furthermore, as was seen in indoor time series [3, 4, 23], indoor VOCs are dependent on room airing and may exhibit different behaviours. In this case, the concentration decreases, increases or changes cyclically over time

GAIDA *et al.* [6], even after having excluded confounding cleaning agents, still found locational differences and features that influenced exhaled breath analysis in COPD patients. Thus, by comparing persons or patient groups with different diseases as well as studies at different sites, this could implicate a risk of misclassification, if VOCs are exogenous and caused by the location, but are not disease specific. This confirms the relevance of obtaining additional information about local analytes and their influence on room air, especially inhaled air, and on exhaled breath.

These particularities not only have implications on the interpretation of exhaled breath, but also highlight the necessity to overcome these confounding influences by a method-specific standardisation of the sampling procedure. For this, the constant ventilation of the examination room with fresh air, and especially the inspiration of synthetic air, were regarded as appropriate to overcome such exogenous influences [5, 24]. However, much more than a constant ventilation of the examination room with fresh air,

and beyond a wanted reduction of confounding exogenous analytes, inspiration of synthetic air may also reduce relevant endogenous analytes [4, 5]. This, as well as reported variations of peak intensities in synthetic air [23], can influence the height and polarity of the alveolar gradient [4, 5].

A further approach is the calculation of alveolar gradients by analysing room air, as was done mainly by Phillips and co-workers [8, 9, 22, 25, 26]. They found that 50 VOCs with the highest alveolar gradients mostly comprised benzene derivatives, acetone, methylated derivatives of alkanes, and isoprene [27]. Until now, only a few further studies regarded alveolar gradients [3, 10, 28, 29]. They also calculated them by measuring exhaled breath and room air separately. However, when room air was used as a reference, it was assumed that inhaled air not only contains the same VOCs, but at the same concentration as room air. But as we showed in another study using double MCC-IMS (unpublished data), this does not provide reliable information about inhaled air. The composition of room air and inhaled air may not be identical; but if so, the VOCs detected in both may have different peak intensities, thus potentially even leading to different alveolar gradients. Therefore, simultaneous measurements of inhaled air are preferable.

The interpretation of negative alveolar gradients is challenging. Negative gradients do not necessarily mean that the related VOCs are not valuable for further interpretation. The degree of their reduced intensity, either because of metabolisation or absorption, might provide additional and valuable information. In case of pentane in normal subjects, Phillips *et al*. [22] made a subdivision into "passive equilibrators", who did not appear to excrete pentane in the breath and represent the majority; "metabolizers", who actively catabolised inhaled pentane; and "manufacturers", who excreted more pentane than they inhaled. Furthermore, the gradient was found to be higher in cystic fibrosis patients, especially in those with exacerbations, than in healthy controls, with an inverse proportionality to forced expiratory volume in 1 s [30]. Pollutants in inhaled air, *i.e.* exogenous compounds, are partially retained in the exhaled breath and were found to follow a close compound-specific linear relationship between the exhaled and inhaled air concentrations [31]. However, there are no further conclusive data. In the future, simultaneous measurement of inhaled air and exhaled breath may offer further insights, especially concerning the metabolisation and resorption of VOCs, a process which may also be disease specific.

When calculating alveolar gradients, a further question arises concerning the minimal significant intensity, which allows VOCs to be regarded as relevant ones for further interpretation of exhaled breath analysis. Therefore, VOC-specific cut-off values need to be defined, which exclude analytes, if their alveolar gradients fall below them. Some authors [28, 29] only included VOCs if they had a concentration in exhaled breath that was $\geqslant$15% higher than in room air, like a threshold. This seems arbitrary, because the significant difference and the polarity of the alveolar gradient may exhibit variations over time [3, 5, 32], even when the sampling procedure is standardised. Besides that, the aforementioned studies only used room air instead of truly inhaled air for calculation of alveolar gradients.

Unfortunately, there is no consensus on whether alveolar gradients [25, 26, 28, 29, 33, 34] or absolute concentrations at peak intensities of analytes [35–37] should be used in exhaled breath analysis. With reference to such complex interactions between expiration and uptake of VOCs, some authors [36, 37] even doubt that a simple subtraction of peak intensities is appropriate.

However, standardised simultaneous measurement of alveolar gradients by a method like double MCC-IMS may open a new field and give further answers to these questions.

There are some limitations to our study. We used two BreathDiscovery instruments from different production series. This may have an influence on the peak intensities. Further studies using test substances are necessary to ascertain that measurements with both BreathDiscovery series result in identical peak intensities.

Moreover, the integral volumes of both devices at the first breath differed by ~10%, with the inspiratory volume being less. This is probably due to a greater dead space between the mouthpiece and the distal BreathDiscovery, which measures inspiration, compared to the proximal BreathDiscovery and the mouthpiece. Because the volume flow process of both devices matches, variations of ~10% might be neglected. Otherwise, we cannot exclude that neither differences up to 10% nor a possible rebreathing of exhaled breath out of the dead space influences the alveolar gradient. This might explain why the alveolar gradients of the peaks varied between some subjects. A daily variation of alveolar gradients has been described by Bunkowski *et al*. [3] in time series when calculating alveolar gradients by measuring room air. In order to exclude the position of the BreathDiscovery, which was chosen for inspiratory and expiratory measurements, as an influential factor, additional measurements with the BreathDiscovery

instruments switched may give further insights. Connecting both SpiroScouts using a Y-piece with an inspiration and expiration valve is a further option to reduce dead space and to prevent a possible influence of a rebreathing effect on the alveolar gradient.

Furthermore, the connection of both SpiroScouts might be a potential source of leakage.

The calculation of alveolar gradients is not yet automatised. However, this can be overcome in the next steps by developing special computerised programmes.

We only tested healthy individuals. However, data about exhaled breath analysis alone and additionally calculated alveolar gradients in a comparison of healthy individuals and COPD patients are in preparation. So, whether alveolar gradients provide a higher sensitivity and specificity than an analysis of exhaled VOCs alone can be determined.

In conclusion, we present for the first time a feasibility study about simultaneous measurement of inhaled air and exhaled breath by using a double MCC-IMS device. The initial data show that an onsite calculation of alveolar gradients by this method may allow a more precise selection of truly endogenous VOCs for exhaled breath analysis.

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This study is registered at www.clinicaltrials.gov with identifier number NCT00632307 and the German Registry of Clinical Studies with identifier number DRKS00000026. The authors confirm that the data supporting the findings of this study are available within the article. Additional data supporting the findings of this study are available from the corresponding author (M. Westhoff) on request.

Ethics statement: The study was approved by the ethics committee of the University of Münster. Consent to participate was provided through written consent.

Author contributions: All authors contributed to the development of double MCC-IMS equally. M. Westhoff and M. Friedrich contributed to data acquisition. All authors contributed to search and review of literature, drafting the manuscript, and revising the final draft. All authors have read and approved this manuscript.

Conflict of interest: M. Westhoff has nothing to disclose. M. Friedrich is member of the staff of Braun Melsungen AG, Branch Dortmund, Center of Competence Breath Analysis, Dortmund. J.I. Baumbach is member of the staff of Braun Melsungen AG, Branch Dortmund, Center of Competence Breath Analysis, Dortmund.

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