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Preservation of exhaled breath samples for analysis by off-line SESI-HRMS: proof-of-concept study

To cite this article: Rosa A Sola-Martínez *et al* 2024 *J. Breath Res.* **18** 011002

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NOTE

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OPEN ACCESS

RECEIVED

28 August 2023

REVISED

17 November 2023

ACCEPTED FOR PUBLICATION

29 November 2023

PUBLISHED

13 December 2023

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Keywords: breath analysis, off-line breath analysis, SESI-HRMS, breath storage, gas sampling bag

Supplementary material for this article is available [online](#)

Abstract

Secondary electrospray ionization-high resolution mass spectrometry (SESI-HRMS) is an established technique in the field of breath analysis characterized by its short analysis time, as well as high levels of sensitivity and selectivity. Traditionally, SESI-HRMS has been used for real-time breath analysis, which requires subjects to be at the location of the analytical platform. Therefore, it limits the possibilities for an introduction of this methodology in day-to-day clinical practice. However, recent methodological developments have shown feasibility on the remote sampling of exhaled breath in Nalophan[®] bags prior to measurement using SESI-HRMS. To further explore the range of applications of this method, we conducted a proof-of-concept study to assess the impact of the storage time of exhaled breath in Nalophan[®] bags at different temperatures (room temperature and dry ice) on the relative intensities of the compounds. In addition, we performed a detailed study of the storage effect of 27 aldehydes related to oxidative stress. After 2 h of storage, the mean of intensity of all *m/z* signals relative to the samples analyzed without prior storage remained above 80% at both room temperature and dry ice. For the 27 aldehydes, the mean relative intensity losses were lower than 20% at 24 h of storage, remaining practically stable since the first hour of storage following sample collection. Furthermore, the mean relative intensity of most aldehydes in samples stored at room temperature was higher than those stored in dry ice, which could be related to water vapor condensation issues. These findings indicate that the exhaled breath samples could be preserved for hours with a low percentage of mean relative intensity loss, thereby allowing more flexibility in the logistics of off-line SESI-HRMS studies.

1. Introduction

In recent years, the development of personalized medicine has been one of the priorities for the medical and scientific community. Thus, most of the emphasis has focused on the search for diagnostic and therapeutic monitoring methods adapted to the needs of each patient. In this context, it has been suggested that human exhaled breath could be a potential source of biomarkers [1–3]. Indeed, it is possible to distinguish a wide variety of volatile organic compounds (VOCs) in exhaled breath, whose concentration can fluctuate based on the health state and

metabolism [4]. In addition, the implementation of breath analysis in clinical practice is interesting due to the widespread availability of human exhaled breath and the non-invasive procedure for breath collection [5]. Therefore, several studies have tried to identify biomarkers in exhaled breath for pathologies, such as asthma [6–8], lung cancer [9], chronic obstructive pulmonary disease [10], diabetes mellitus [11, 12], cystic fibrosis [13, 14], infectious diseases [15–17] and cardiorespiratory conditions [18].

Sampling and measurement of exhaled breath can be conducted in two different ways: on-line breath analysis, where exhaled breath is analyzed directly

(i.e. in real-time), and off-line breath analysis, where a breath container (e.g. gas sampling bag, thermal desorption tubes, etc) is used, allowing collection and storage of exhaled breath prior to analysis [4, 19, 20]. On the one hand, the gold standard technique for off-line breath analysis is gas chromatography coupled to mass spectrometry (GC-MS), which allows a previous preconcentration step of the exhaled breath samples [21]. On the other hand, flow ion selection tube—mass spectrometry (SIFT-MS) [22] and proton transfer reaction—mass spectrometry (PTR-MS) [23] are widely used methodologies in on-line breath analysis, since a direct analysis of the exhaled breath can be carried out without any pretreatment. However, the lack of previous steps, such as sample preconcentration or separation by gas chromatography, may limit the sensitivity and selectivity of analytical results. Another technique with intrinsic characteristics for on-line breath analysis is secondary electrospray ionization-mass spectrometry (SESI-MS). This is an atmospheric pressure ionization alternative [24, 25], which can be coupled to multiple types of mass analyzers, including high-resolution mass spectrometry (HRMS) [19, 26]. This fact, together with the progress in standardization and metabolic coverage, makes it one of the most promising approaches to achieve the introduction of breath analysis into clinical practice [5, 19, 26–28].

Real-time on-line breath analysis by mass spectrometry offers several benefits such as shorter analysis times, faster results and less sample handling, which reduces the possibility of alterations to the exhaled breath samples during collection and storage [19, 29]. However, it also has drawbacks such as the need for the subject to be at the location of the analytical platform. Unfortunately, many patients cannot be transported or are unable to perform active and prolonged exhalations (e.g. intensive care patients, neonates, infants, etc). Therefore, especially for non-cooperative passive patients, it is a container required to collect the exhaled breath samples that subsequently can be transported to the analytical platform [30]. In this regard, PTR-MS and SIFT-MS techniques have been successfully employed in several studies to analyze exhaled air previously stored in gas sampling bags of different type [5, 31–33]. Along the same lines, we have recently presented the first protocol for off-line breath analysis using SESI-HRMS being successfully tested in both adults and neonates [30]. Moreover, the conventional real-time method was compared with the new off-line method (breath samples were collected in Nalophan® bags and quickly analyzed within 10 min of collection) by computation of the Lin's Concordance Correlation Coefficient (Lin's CCC) [34], and 1249 *m/z* signals with Lin's CCC > 0.6 were identified [30].

Once the applicability of the off-line method has been checked [30], the present paper describes a pilot study which evaluated the conservation of the exhaled

breath samples in Nalophan® bags over time under different conditions (room temperature and dry ice), in order to optimize the management and handling of the sample storage as well as its effect on the reproducibility and repeatability of breath analysis by SESI-HRMS. Room temperature and dry ice storage conditions were selected as being compatible for easy and economical transport of exhaled breath samples collected at different locations (e.g. hospitals of other cities) to the analytical platform. Special focus has been placed on investigating the impact of storage on 27 aldehydes belonging to three different chemical families (2-alkyl, 4-hydroxy-2-alkyl and 4-hydroxy-2,6-alkyl) related to lipid peroxidation. These aldehydes have been robustly identified in exhaled breath condensate and exhaled breath by SESI-HRMS [35].

2. Material and methods

2.1. Study participants and breath sampling

For the present study, four healthy, non-smoking volunteers (two men and two women; range 26–38 years) were recruited. During October 2021 to December 2021, the four subjects completed several visits to the University Children's Hospital Basel (UKBB, Switzerland) for exhaled breath sample collection. All samples were collected and analyzed in the same room. The exhaled breath collection was carried out by means of the off-line device described by Decrue *et al* [30], which consists of a Nalophan® bag of approximately 2 l volume and 700 cm² surface (Nalophan® NA, 20 ± 5 μm thick, Kalle) coupled at one end to a mouthpiece (Hudson RCI®) and at the other end to a tube (Rotilabo® PTFE, 6 mm, 8 mm, length 90 mm, Carl Roth®) connected to a valve (VHK2-08F-08F, SMC Switzerland). The Nalophan® bags were prepared the day before the visit of the subjects. Subjects fasted for at least 1 h before sample collection. Subjects exhaled into the Nalophan® bags with a deep exhalation, so mixed breath sample (alveolar and dead space) were collected. During each visit, besides a bag at time zero, a couple of bags were collected for each storage time (one stored at room temperature 20 °C–25 °C and other on dry ice). Dry ice was at –80 °C at the beginning of storage. Even Nalophan® bags were collected per participant: one was analyzed immediately after filling by SESI-HRMS and was identified as the reference sample (zero-time bag), two were analyzed 10 min after breath sampling (one stored at room temperature and the other on dry ice), two were analyzed 60 min after breath sampling (one stored at room temperature and the other on dry ice), and two were analyzed 120 min after breath sampling (one stored at room temperature and the other on dry ice) (figure 1). The intensity of metabolites could decrease in consecutive exhalations [27]. Therefore, to avoid this bias, the times (0 min, 10 min, 60 min and 120 min) and temperature conditions (room temperature and dry ice) were randomized

among the 7 bags collected per visit. Moreover, two additional Nalophan® bags were collected in 14 of the visits and analyzed 24 h after breath sampling (one was stored at room temperature and the other on dry ice).

2.2. Ethical statement

The study protocol was approved by the local Ethics Committees (Northwest and Central Switzerland Ethics Committee 2018-01324) in accordance with the guidelines of the Declaration of Helsinki. Written informed consent from participants was obtained at the time of recruitment.

2.3. Breath analysis

Exhaled breath samples were analyzed using a SESI ion source (SuperSESI, FIT, Spain) coupled to a high resolution mass spectrometer (Q-Exactive Plus, Thermo Fisher Scientific, Germany) following the protocol described above [27, 30, 36]. Once the storage period had been completed, the exhaled breath contained in the Nalophan® bags was infused into the ion source. All bags were measured at room temperature. Bags stored on dry ice were brought to room temperature for a few minutes before analysis to temper. The ion source was equipped with a mass flow controller that ensured a constant flow of exhaled breath into the ion source, set at 0.3 l min^{-1} . Data acquisition was performed in full scan mode (positive polarity and m/z range 70–1000) with a resolution of 140 000 (at m/z 200) using Q-Exactive Tune software. For electrospray generation, a $20 \mu\text{m}$ inner diameter TaperTip silica capillary emitter (New Objective, USA) and 0.1% formic acid in water were used. Prior to exhaled breath measurements, a gaseous standard of α -Terpinene was analyzed daily to test the system suitability of the breath analysis platform. The signal intensity of protonated α -Terpinene ($m/z = 137$) had to be higher than 10^9 a.u. for a quality control to pass [28].

2.4. Data analysis

Data preprocessing was conducted with MATLAB (version 2020b, MathWorks Inc., USA), and data postprocessing with R (version 4.0.5). The protocol followed for data preprocessing after off-line breath analysis using SESI-HRMS is described in detail elsewhere [30]. The area under the curve of each of the m/z signals or mass spectral features was determined and normalized to the overall exhalation time window. After data preprocessing, a data matrix with the m/z signal intensities detected in exhaled breath samples was obtained. The data matrix was 5th-root transformed to approach normality. Afterwards, the relative intensities of each m/z signal were calculated for the different conditions (10 min-room temperature, 10 min-dry ice, 60 min-room temperature, 60 min-dry ice, 120 min-room temperature and 120 min-dry ice) expressed as a

percentage with respect to the bag at zero time. Thus, the mean relative intensity (%) was defined as the average of the normalized intensities expressed as a percentage of the m/z signals at each condition based on the bag at time zero. Furthermore, the list of m/z signals that showed a Lin's CCC > 0.6 in the comparison between the off-line breath analysis and real-time on-line breath analysis strategies using SESI-HRMS performed by Decrue *et al* [30], and the list of m/z signals selected by the DOPAEx (Determination of Optimal Procedures for Analysis of Expired Breath by Secondary Electrospray Ionization-Mass Spectrometry) project [28] were considered in data analysis for filtering the data matrix obtained in the present study. Mass spectral features of the three sets were matched with a range of ± 2 ppm. Although the m/z lists obtained by Decrue *et al* [30] and DOPAEx project [28] were used for m/z signal selection in the present study, the data analysis was only performed on the data from breath samples collected in the present study.

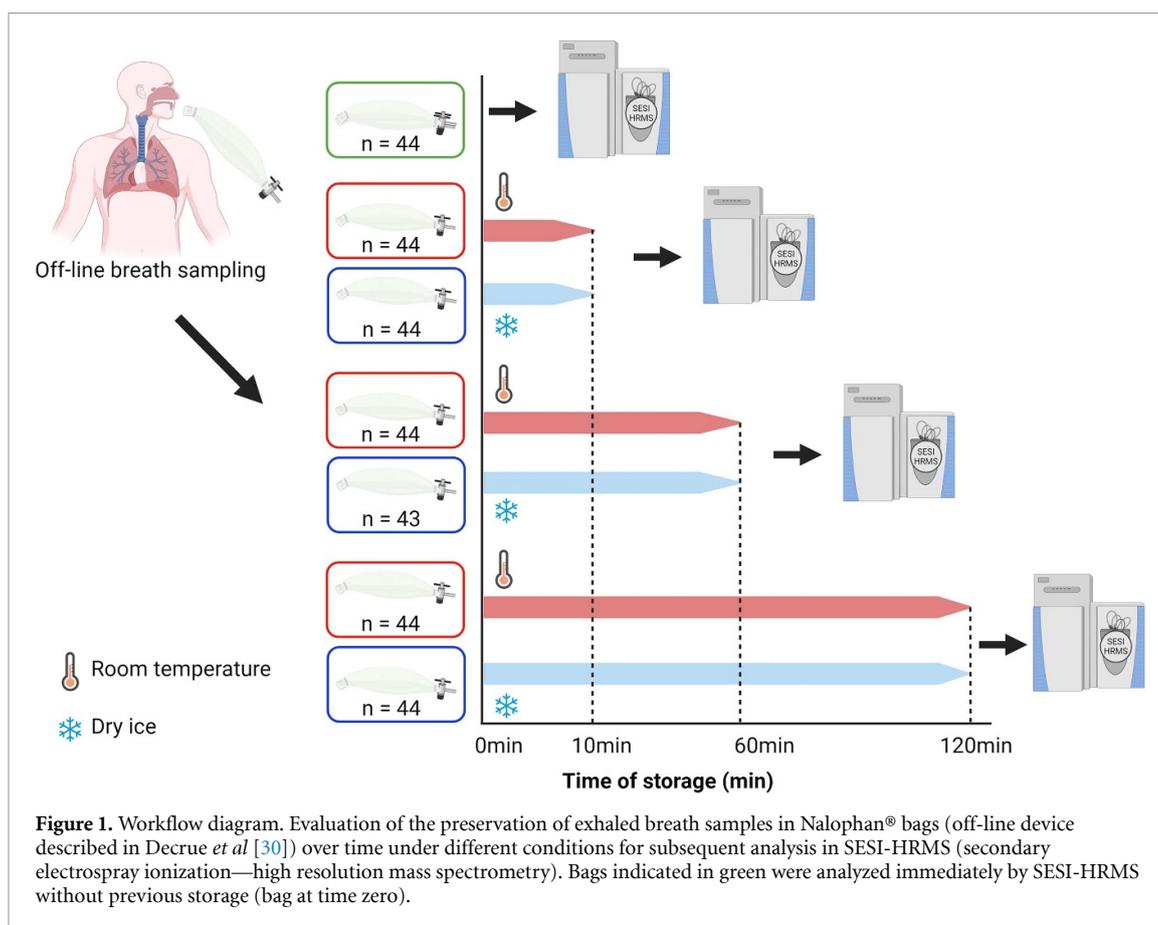
In addition, the impact of 24 h storage on the relative intensities in exhaled breath samples of those aldehydes with Lin's CCC > 0.6 in the offline/online comparison performed by Decrue *et al*. The data distribution was evaluated by Lilliefors tests. Differences in relative intensities between samples stored at different time periods (10 min, 60 min, 120 min and 24 h) were assessed using a Kruskal–Wallis test followed by multiple testing correction via false discovery rate (FDR) estimation. Then, differences in relative intensities between samples stored for the same period under different conditions (room temperature and dry ice) were assessed by a Wilcoxon signed-rank test (dependent samples analysis). The significance threshold for all analysis was p -value < 0.05.

Finally, the ALOGPS 2.1 tool (www.vcclab.org/lab/alogps/) [37, 38] was used to predict the water solubility expressed as logS of 27 aldehydes detected in exhaled breath (C8–C16 2-alkenals, C8–C16 4-hydroxy-2-alkenals and C8–C16 4-hydroxy-2,6-alkadienals), based on the SMILES (Simplified Molecular-Input Line-Entry System) nomenclatures of these compounds. Depending on the predicted logS value, aldehydes were classified as soluble if they showed a logS greater than -2 , as slightly soluble if they showed a logS between -2 and -4 , and as insoluble if they had a logS less than -4 [39].

3. Results

3.1. Effect of time and temperature on exhaled breath conservation

The subjects included in this study performed a total of 44 visits and figure 1 shows the workflow diagram of the present study. After storage, water condensation was observed on some bags, especially on bags stored on dry ice. One of the breath samples stored on dry ice for 1 h had to be excluded due to an acquisition



error (i.e. negative mode mistakenly chosen). After data preprocessing, a data matrix with the intensity values of 2681 m/z signals detected per sample was generated for the 307 exhaled breath samples collected.

The sample reference at time zero was used to determine the loss of intensity of the m/z signals over time, and the mean of all relative intensities for each time point was calculated. As illustrated in figure 2(A), the mean relative intensities remained above 80% for the samples stored in the bags for 2 h, both at room temperature and on dry ice. Subsequently, the data matrix obtained in the present study was filtered based on the estimation of the agreement of measurements with off-line and on-line methods carried out by Decrue *et al* [30] (figure 2(B)). Thus, 870 m/z signals with Lin's CCC > 0.6 were detected in this previous study. In this sense, it was observed that the intensities of these m/z signals at 2 h were reduced on average by less than 25% with respect to the bag at zero time. On the other hand, 383 m/z signals were filtered when the list elaborated by the DOPAEx project was used (figure 2(C)). It was observed that the average intensities of these features at 2 h were very similar to those measured at zero time, with the average relative intensity being higher than 95% in samples stored at room temperature and higher than 85% in the samples preserved in dry ice. Finally, out of these 383 m/z signals, 111

were extracted based on having a Lin's CCC > 0.6 in the comparison between off-line and on-line methods (figure 2(D)). Then, in this subset, it was observed that the average decrease of the relative intensities at 2 h with respect to the bag at zero time was practically null in the samples stored at room temperature, and less than 10% in the samples stored in dry ice.

3.2. Aldehydes in exhaled breath: effect of storage conditions

After checking the appropriate preservation of the exhaled breath samples in the bags, we focused on the effect of storage on aldehydes due to their clinical relevance as oxidative stress markers and their previous robust identification in exhaled breath by SESI-HRMS [35].

Figure S1 shows the changes of mean relative intensity for the m/z signals of 27 aldehydes belonging to the 2-alkenyl, 4-hydroxy-2-alkenyl and 4-hydroxy-2,6-alkadienyl families, as a function of storage time. Figure 3(B) shows the effect of the carbon chain length of the aldehydes on the intensity levels of the m/z signals under different conditions of storage. In addition, figure 3(A) shows the Lin's CCC values obtained by Decrue *et al* [30] in the comparison between the off-line and on-line approaches for the 27 aldehydes. In the study of Decrue *et al* [30], in the offline approach, exhaled breath samples were collected in sampling gas bags and immediately analyzed

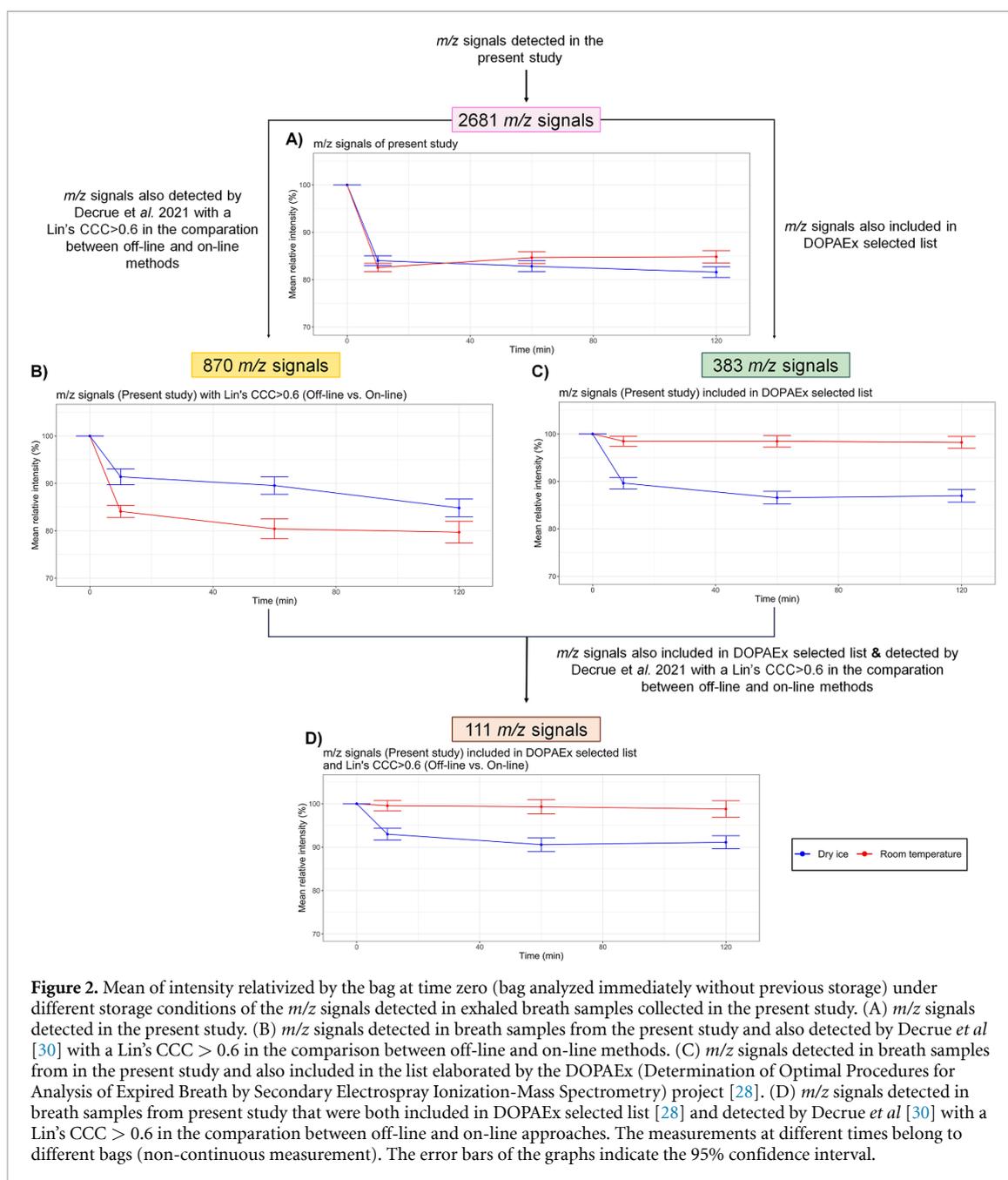


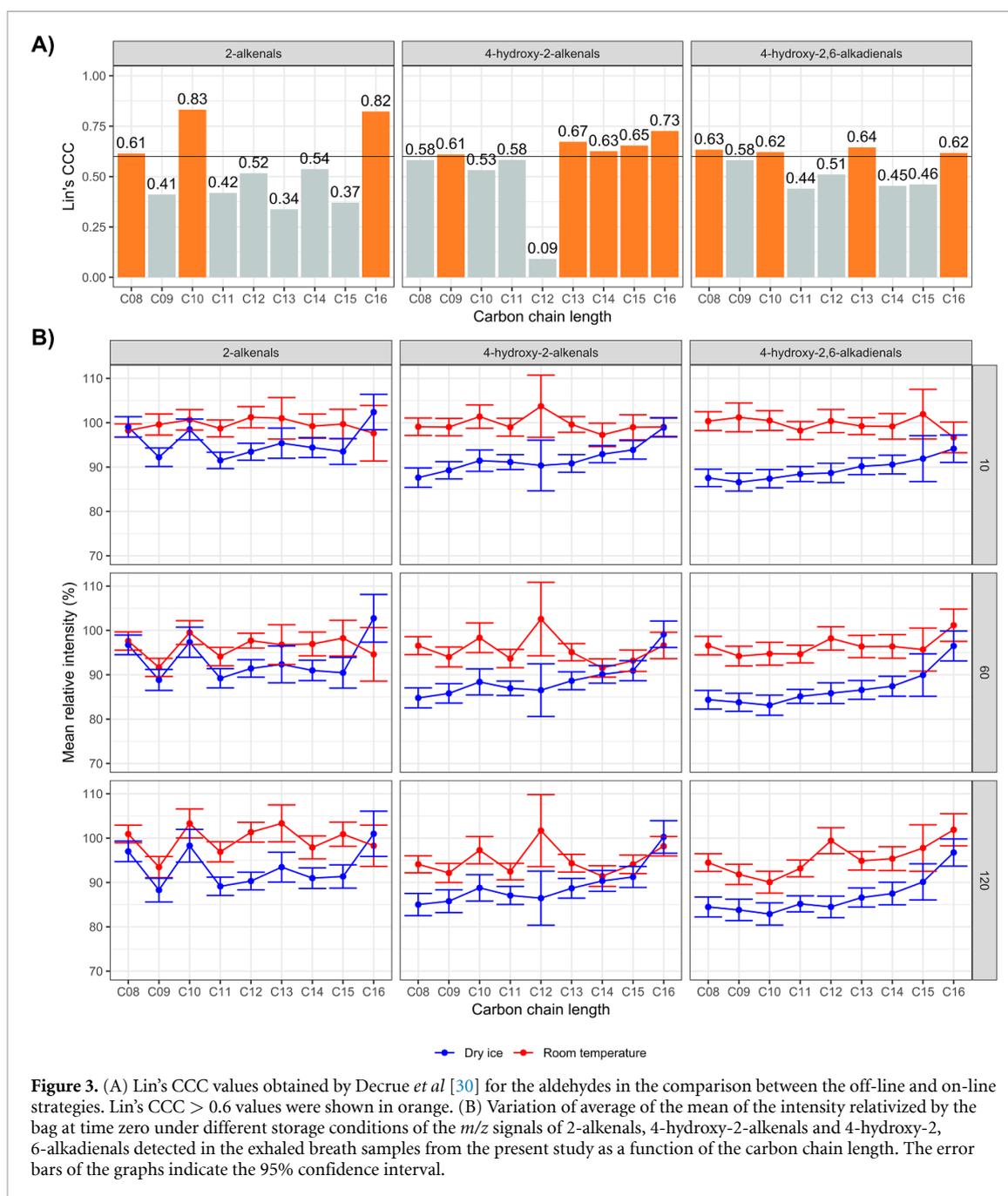
Figure 2. Mean of intensity relativized by the bag at time zero (bag analyzed immediately without previous storage) under different storage conditions of the m/z signals detected in exhaled breath samples collected in the present study. (A) m/z signals detected in the present study. (B) m/z signals detected in breath samples from the present study and also detected by Decrue *et al* [30] with a Lin's CCC > 0.6 in the comparison between off-line and on-line methods. (C) m/z signals detected in breath samples from in the present study and also included in the list elaborated by the DOPAEx (Determination of Optimal Procedures for Analysis of Expired Breath by Secondary Electrospray Ionization-Mass Spectrometry) project [28]. (D) m/z signals detected in breath samples from present study that were both included in DOPAEx selected list [28] and detected by Decrue *et al* [30] with a Lin's CCC > 0.6 in the comparison between off-line and on-line approaches. The measurements at different times belong to different bags (non-continuous measurement). The error bars of the graphs indicate the 95% confidence interval.

by SESI-HRMS, so these samples are preserved as the zero bag in the present study. The estimation of the mean relative intensities for all aldehydes under every condition was very accurate, with narrow 95% confidence intervals, except in the case of 4-hydroxy-2-dodecenal, where a larger data dispersion and a lower Lin's CCC value than the rest of the aldehydes were observed. Furthermore, although a few of the mean relative intensity values are above 100% in figures 3 and S1, for all aldehydes 100% is within the 95% confidence interval.

As visible in figures 3 and S1, the mean relative intensity of 27 aldehydes remained above 80% after 2 h of storage under both conditions. For most aldehydes, the most pronounced decrease in the mean relative intensity occurred between 10 and 60 min

of storage whilst remaining almost stable between 60 and 120 min. In general, the average decrease in relative intensities was greater for the shorter carbon chain length aldehydes of the 4-hydroxy-2-alkenyl and 4-hydroxy-2,6-alkyldienal families.

As for the effect of temperature, all aldehydes maintained almost equal intensity levels after being stored for 10 min at room temperature and in the bags at zero time (mean relative intensity around 100%). Furthermore, except for 2-hexadecenal and 4-hydroxy-2-hexadecenal, the exhaled breath samples were better preserved in the bags stored at room temperature, as the mean relative intensities of the compounds were higher than those detected in the bags stored on dry ice. The decrease in mean relative signal intensity, not exceeding 15% in any case, was more



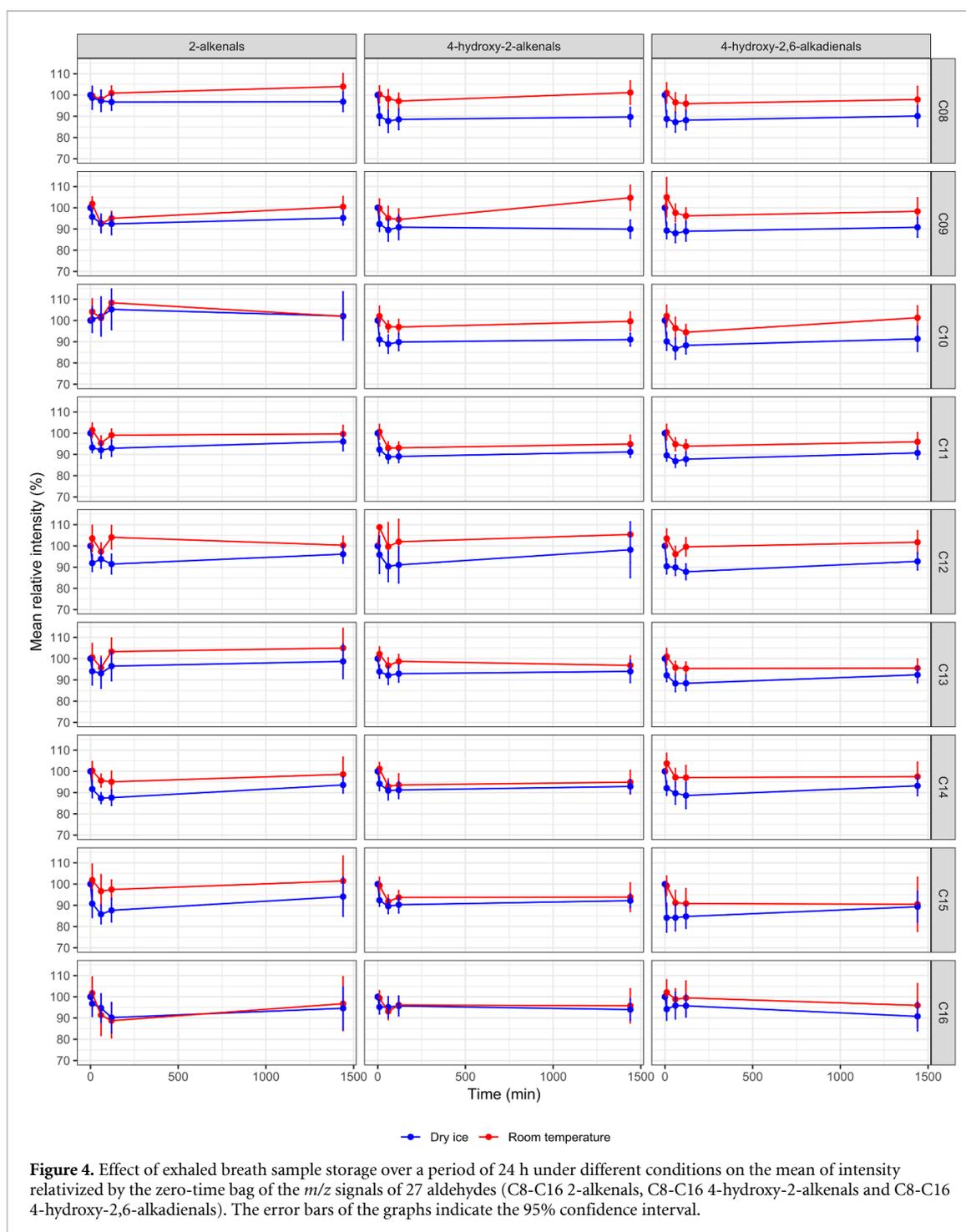
pronounced for the short chain aldehydes of the two families with a hydroxyl group, and these differences diminished with increasing carbon chain length, as shown in figures 3(B) and S1.

Then, the effect of storage of exhaled breath in bags for 24 h was assessed. For this purpose, in 14 of the 44 visits, subjects also filled two additional Nalophan® bags with exhaled breath which were stored for 24 h, keeping one of the bags at room temperature and the other on dry ice. The samples were then analyzed by SESI-HRMS. Figure 4 shows the evolution of the mean of the intensity relativized by the bag at zero time of the m/z signals of the 27 aldehydes studied over the 24 h of storage.

For all aldehydes, the mean relative intensity remained above 80% at 24 h of storage, with very

little changes observed after 2 h of storage. Again, the mean relative intensities were found to be higher for samples stored at room temperature than those stored on dry ice, although the difference was generally no more than 15%.

Figure 5 shows the mean relative intensities of the m/z signals of the 12 aldehydes that showed Lin's CCC value > 0.6 when comparing the off-line and on-line methods performed by Decrue *et al* [30] (figure 3(A)). As can be seen, the mean relative intensity was conserved above 90% at 24 h of storage. For storage at room temperature (figure 5(A)), significant differences in the mean relative intensity of selected aldehydes were observed between samples stored for only 10 min (mean relative intensity ~100%) and the rest of the samples stored for a longer period, with the

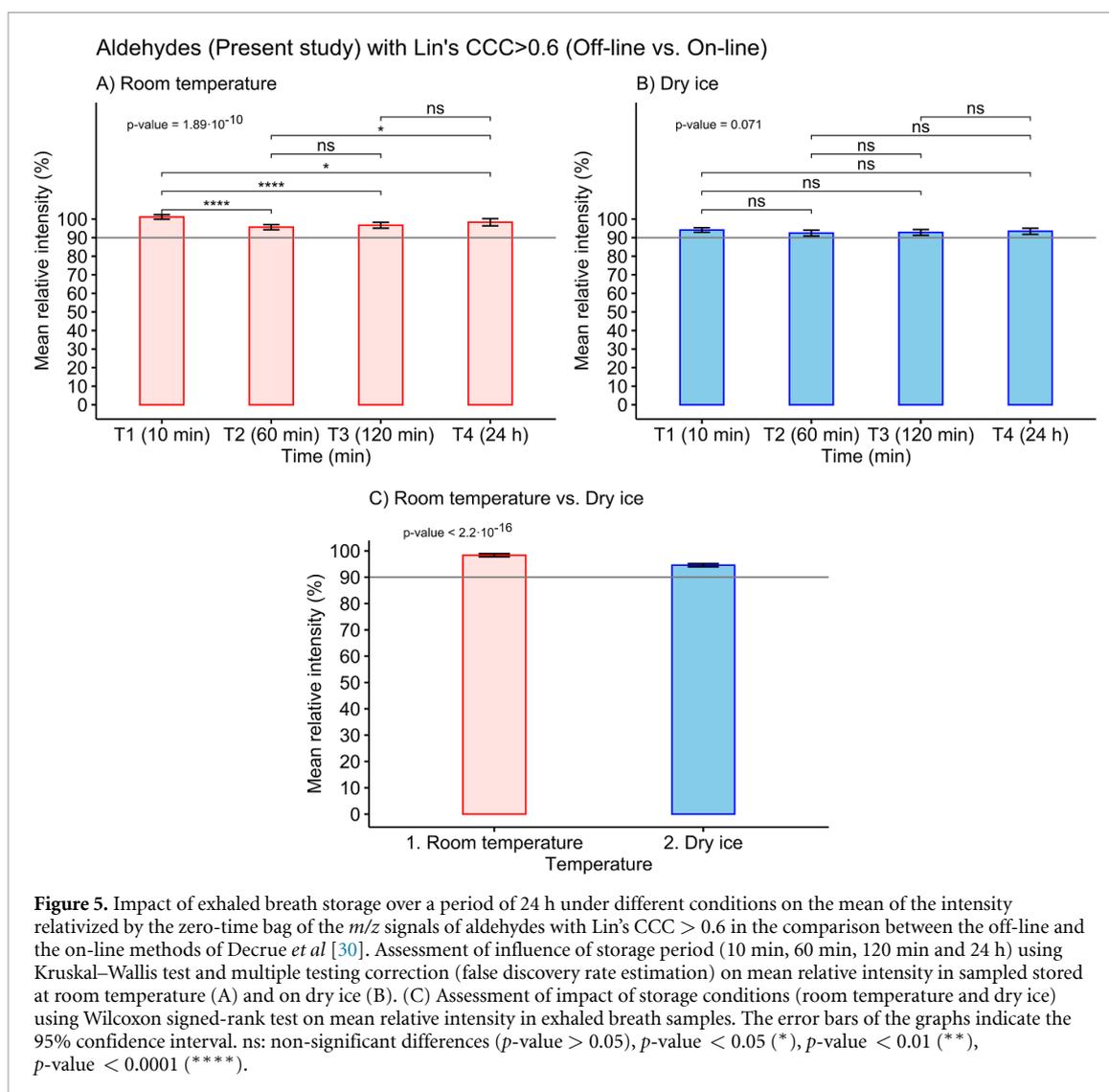


biggest drop in intensity being less than 10% for the other periods (60 min, 120 min and 24 h). Moreover, no significant differences were observed between the samples stored for 60 min and 120 min, nor between those stored for 120 min and 24 h. As for storage on dry ice (figure 5(B)), no significant differences in the mean relative intensity of the selected aldehydes were observed between the samples stored at different time points. Nevertheless, for the samples stored on dry ice, the mean relative intensity of the selected aldehydes was below 95% for all storage periods. In this regard, as can be seen in figure 5(C), comparing

samples stored at room temperature and on dry ice, the mean relative intensity of the selected aldehydes was significantly higher in the samples stored at room temperature.

3.3. Water solubility of aldehydes

To assess the relationship between the chemical structure of the aldehydes and the storage time, their water solubility was determined. Then, predicted water solubility of the 27 aldehydes expressed as $\log S$ was determined using the ALOGPS 2.1 tool [37, 38]. According to the predicted values,



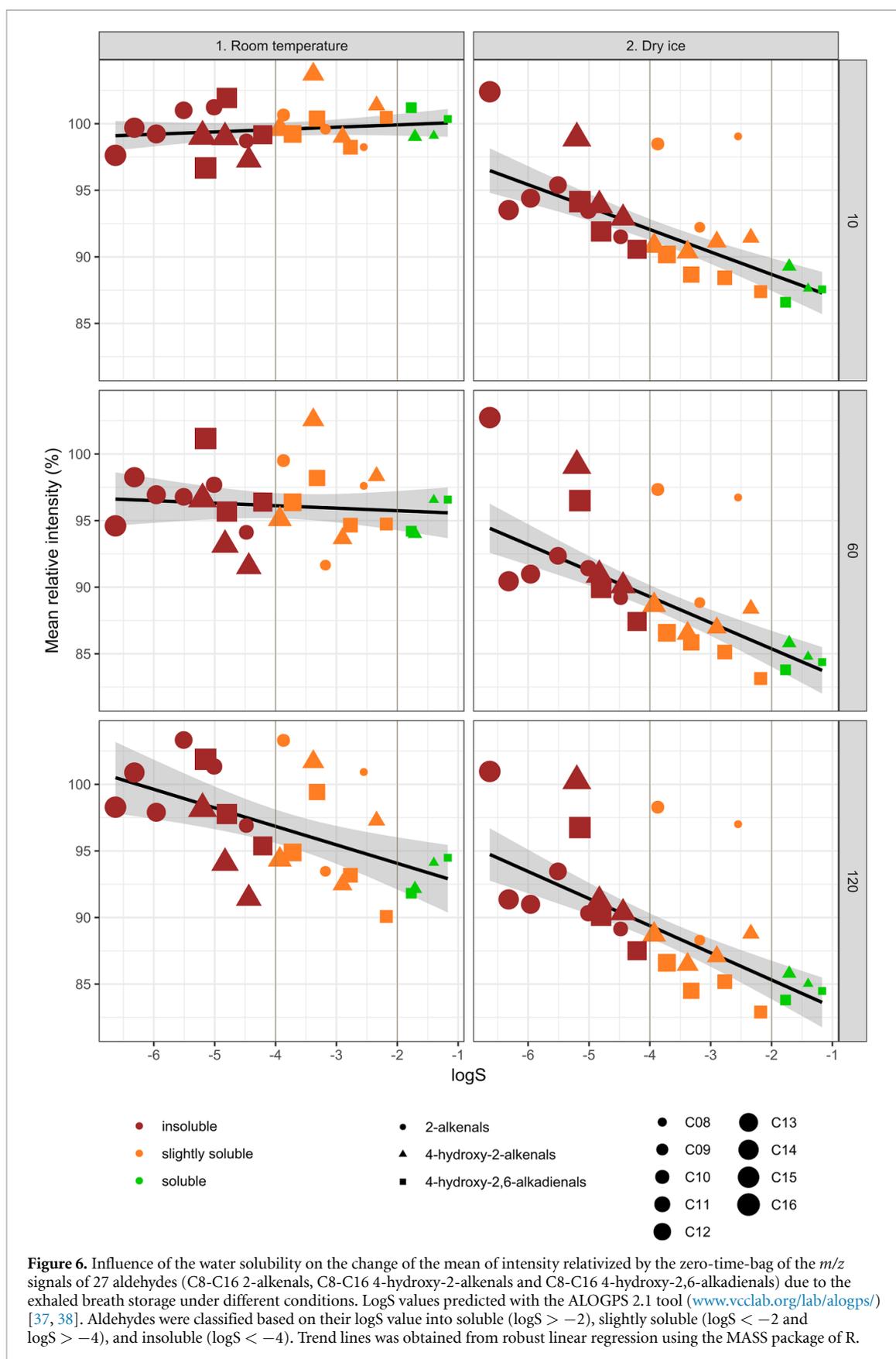
4-hydroxy-2-alkenals and 4-hydroxy-2,6-alkadienals presented a higher water solubility than 2-alkenals (figure S2). The aldehydes of the two families with a hydroxyl group showed very similar $\log S$ values. The slopes were similar for all the three aldehyde families and the water solubility decreased proportionally with the carbon chain length. On the other hand, most of the 2-alkenals were insoluble in water ($\log S$ values below -4), and only the three shortest chain aldehydes of this family were slightly soluble in water ($\log S$ values between -2 and -4) (figure S3).

Figure 6 shows the relationship between the water solubility of the 27 aldehydes and the variation of the mean relative intensity after the exhaled breath samples storage under different conditions. As can be seen, the mean relative intensities and $\log S$ values showed a linear relationship. The water-soluble compounds ($\log S$ values greater than -2) suffered a greater mean loss of relative intensity than those that were classified as insoluble or slightly insoluble. Furthermore, this difference was more evident in dry ice than at room temperature and increased when the storage time before breath analysis was longer. In

this regard, in general, compounds considered insoluble in water ($\log S < -4$), such as 2-hexadecenal (figures S2 and S3), presented higher mean relative intensity values than water-soluble or slightly water-soluble compounds after storage on dry ice for 2 h.

4. Discussion

SESI-MS is an established technique that offers numerous advantages for the field of breath analysis such as high sensitivity, detection of a wide range of compounds, among others [19, 28, 40]. In this regard, SESI-MS has traditionally been used for on-line breath analysis [19, 31]. However, an off-line device recently developed and tested by Decrue *et al* [30] has also enabled the application of SESI-HRMS in off-line breath analysis. Thus, it was demonstrated that more than a thousand m/z signals can be detected with a moderate-to-high degree of agreement to the measurements performed by the conventional method of direct real-time breath analysis (Lin's CCC > 0.6 when comparing the off-line and on-line methods) [30]. Therefore, it would not be necessary for subjects



to come to the laboratory where SESI-HRMS platform is located, which is very important especially for passive non-cooperative patients [30]. Furthermore, a previous study as part of the DOPAEx project has

confirmed the reproducibility and the metabolic coverage of SESI-HRMS for human breath analysis. In this regard, a reproducible list of m/z signals has been established by multicenter trials in different countries

around the world [28]. So, after this multicenter validation [28] and the establishment of a protocol for offline breath collection and the comparison of online and offline methods [30], the present study evaluated the impact of storage times (10, 60 and 120 min) of exhaled breath samples preserved in two conditions (room temperature and dry ice) prior to SESI-HRMS analysis (figure 1). The establishment of protocol for the correct storage of exhaled breath samples is essential so as to benefit from the inherent advantages of the off-line breath analysis strategy, as well as the possibility to easily transport samples and to centralize breath analysis in a single laboratory [20, 21, 32, 41]. Thus, all samples could be analyzed using the same analytical platform, reducing batch effects [28]. On the other hand, the most established online analytical systems such as SIFT-MS and PTR-MS allow the quantification of metabolites [42–45]. Nevertheless, despite the advances of the last few years [46], absolute quantitative analysis of a wide range of compounds in exhaled breath by SESI-HRMS remains one of the weaknesses of this technique [19]. In this sense, in the present study, the relative intensities of the m/z signals detected in exhaled breath were considered.

As can be seen in figure 2(A), for all the m/z signals detected in this study, there was only a minimal loss of intensity with respect to the bag at time zero, with mean relative intensity values above 80% after storage for 2 h. This reduction in the mean relative intensity during storage could have been caused either by adsorption of the compounds on the surface of Nalophan®, by diffusion of the VOCs through the bag walls and/or by condensation [47]. Nonetheless, Nalophan® bags show lower adsorption of VOCs than other gas sampling bags [46]. Furthermore, the 870 m/z signals detected with high degree of agreement between both breath analysis approaches (Lin's CCC > 0.6 in the comparison of off-line and on-line methods by Decrue *et al* [30]) (figure 2(B)), also retained similar mean intensity levels to those of the zero-time bag, since the decrease in mean relative intensity was less than 25% at 2 h of storage. Notably, the intensity losses, over storage, were even lower when only considering the 111 m/z signals included in the list elaborated in the recent standardization study (DOPAEx project) [28] and with Lin's CCC > 0.6 in the comparison between the off-line and on-line methods (figure 2(D)). In fact, for this subset of m/z signals, the mean relative intensity after 2 h of storage was around 90% for samples stored on dry ice and around 100% for samples stored at room temperature. Consequently, the results obtained by Decrue *et al* [30] stating the applicability of an off-line method for breath analysis by SESI-HRMS within a few minutes of sampling, can be extrapolated after 2 h of storage without an important reduction in the intensity of the m/z signals.

Furthermore, in this study, the effect of storage up to 24 h of exhaled breath samples on the mass spectral features of 27 aldehydes from three chemical families (2-alkenals, 4-hydroxy-2-alkenals and 4-hydroxy-2,6-alkadienals) was analyzed in detail. Aldehydes, in particular 4-hydroxy-2-nonenal (4-HNE), have been widely reported as potential biomarkers of oxidative stress related to numerous pathologies [48]. For this reason, recent studies have focused on the analysis of aldehydes in exhaled breath by different mass spectrometric techniques [49, 50]. As shown in figures 3(B), 4 and S1, the mean relative intensity remained above 80% for all aldehydes after 2 h of storage. A few of the mean relative intensity values at different storage time points exceed 100%. Values above 100% could be related to the emission of pollutants from the gas sampling bags [51]. However, 100% is within the 95% confidence interval of the mean relative intensity for all aldehydes. Furthermore, in the particular case of 4-hydroxy-2-docenal, a high dispersion of the data was observed in line with the low value of Lin's CCC obtained in the comparison with the on-line method by Decrue *et al* (Lin's CCC = 0.09). This phenomenon could be explained by the existence of different isomers of this compound with different degrees of affinity for the Nalophan® surface of the bags [30]. In general, the decrease in the mean relative intensities of the aldehydes occurred mainly during the first hour of storage and remained constant even 24 h after breath sampling (figure 4). In fact, no significant differences were observed in the mean relative intensity of the 12 selected aldehydes (Lin's CCC > 0.6 at comparison of the off-line and on-line methods by Decrue *et al* [30]) between exhaled breath samples stored for 2 h and 24 h (figures 5(A) and (B)). Moreover, a storage time of 10 min at room temperature (a period equivalent to the transport of samples within the same facility or between contiguous buildings) resulted in a variation of the mean relative intensity close to zero for all aldehydes (mean relative intensity around 100%).

In addition, for most aldehydes, the mean relative intensities remained higher for samples stored at room temperature than for those stored on dry ice, regardless of the storage period (figures 4 and S1). This behavior was also observed for the mass spectral features included in the list elaborated by the DOPAEx project [28] (figure 2(C)) as well as for the m/z signals of the 12 aldehydes with a Lin's CCC > 0.6 in the comparison between the off-line and on-line method (figure 5(C)). In fact, the mean relative intensity of these aldehydes in samples stored at room temperature was significantly higher than those stored in dry ice (p -value < 2.2×10^{-16}). In this sense, we reasoned that the differences observed between the two conditions, room temperature and dry ice, could be related to the condensation of the water vapor present in the exhaled breath samples, produced by the temperature fluctuation [52]. Water

vapor accounts for a high percentage of exhaled breath and the humidity of the samples can have a substantial influence on the preservation process [32, 53]. Indeed, previous studies that monitored humidity in samples stored in gas sampling bags for subsequent analysis by PTR-MS found that humidity decreased over time [32, 33]. Beauchamp *et al* [32] reported high humidity losses in Tedlar® and Nalophan® bags. The humidity dropped to the humidity level of the room air during the first hours of bag storage, so the losses could be mainly caused by diffusion through the bag walls [32]. Thus, the decrease of humidity during the beginning of breath sample storage could explain the faster decrease in mean relative intensity of most aldehydes in the first 60 min of sample preservation in the present study. In this regard, in off-line breath analysis by GC-MS, where samples have to be preconcentrated, there are several strategies to minimize the effect of humidity, such as the use of thermal desorption tubes which contain sorbent materials that are not very sensitive to the presence of high water (e.g. Tenax TA) [31, 54, 55]. However, in analytical systems with inherent characteristics for on-line breath analysis without a sample preconcentration step [1], the selection of a high storage temperature is important to reduce problems related to water vapor condensation [32]. Nevertheless, high temperature storage may induce an increased emission of contaminants from the gas sampling bags, such as 2-methyl-1,3-dioxolane and 1,2-ethanediol in the case of Nalophan® bags [56]. On the other hand, it has been suggested that heating exhaled breath samples to body temperature (37 °C) could keep water vapor in a gaseous state and avoid condensation issues [47, 51], but it could also lead to an increase of artifacts. Therefore, the simplicity of the storage protocol must be considered versus the benefits and disadvantages of heating the samples. For this reason, in this study, preservation of exhaled breath samples at room temperature was selected. Furthermore, prolonged storage of exhaled breath samples at high temperatures could increase oxidation reactions on some metabolites such as aldehydes [57]. However, in the present study, no significant differences were observed in mean relative intensity of the 12 selected aldehydes between samples stored for 2 h and 24 h both at room temperature and dry ice (figures 5(A) and (B)). On the other hand, conservation of the samples on dry ice produced a condensation effect of water vapor due to the temperature difference, but the impact of dry ice storage on the mean relative intensity was not equal for the 27 aldehydes (figures 3(B) and S1). Indeed, the largest decrease in mean relative intensity after storage on dry ice was found for the short-chain aldehydes of the two families with a hydroxyl group. This could be caused by the different water solubility of aldehydes (figures S2 and S3 and 6), being water-soluble compounds more sensitive to the effects of water vapor condensation

[47, 52]. As can be seen in figure 6, there is a linear association between the mean relative intensities of the aldehydes and their logS values. Furthermore, when data from samples stored at room temperature were compared with those stored on dry ice, this study shows that the slope of change of the relative mean intensity of aldehydes was more pronounced on dry ice, where water vapor condensation is more likely to occur.

5. Study limitations

The present study has some limitations to be noted. On the one hand, it was not possible to perform an absolute quantitative analysis of the intensity values of the m/z signals detected in exhaled breath samples. In addition, some parameters were not monitored during the collection and storage process of the exhaled breath samples, such as humidity in the samples, evolution of bag temperature over storage time, diffusion rates of the compounds and inspiratory flow rates. On the other hand, even though many exhaled breath samples were analyzed (more than 300 samples), they only came from four subjects. Furthermore, although in the present study the impact of storage under different conditions on a large list of m/z signals (2681 m/z signals) has been assessed, only the effect of water solubility on 27 aldehydes has been studied. Thus, in future studies, it could be interesting to further evaluate a broader range of compounds and recruit a large cohort of subjects.

6. Conclusion

The present study represents a step further in the analysis of human exhaled breath by SESI-HRMS. In fact, the findings of this pilot study show that the off-line sample collection methodology previously developed by our group [30] is useful for exhaled breath samples storage for at least 2 h with reduced losses of mean relative intensity, which depends on the compounds and the storage temperature. Thus, for all m/z signals detected in breath samples of the present study, the relative mean intensity losses were lower than 20%. However, for the m/z signals that are included in a selected list supported by multicenter validation [28] and had a Lin's CCC > 0.6 in the comparison between the off-line and on-line method in a previous study [30], the loss of the mean relative intensity was substantially lower (mean relative intensity after 2 h of storage was about 90% for samples stored on dry ice and about 100% for samples stored at room temperature). Furthermore, it is advisable to keep the sampling bags at room temperature to avoid water condensation which would affect the preservation of the exhaled breath samples, especially to the most water-soluble compounds. The possibility to store and transport the exhaled breath samples without alterations offers greater logistical flexibility in the

application of the technique, allowing, for example, to recruit patients from different hospitals.

Data availability statement

All data that support the findings of this study are included within the article (and any supplementary files).

Acknowledgments

Dr Fabienne Decrue is gratefully acknowledged for providing training to R A S-M on the sample bag manufacturing. We thank Mélina Richard for coordinating the study, as well all healthy volunteers participating in this study. This work is part of the Zurich Exhalomics project under the umbrella of the University of Medicine Zurich/Hochschulmedizin Zürich. This study was funded by the Fondation Botnar, Switzerland (Professorship to Pablo Sinues), and the Swiss National Science Foundation (Grant No. 320030_173168). Rosa A Sola-Martínez is recipient of a Grant ‘Margarita Salas’ from University of Murcia (133/MSJD/22), financed by the Ministry of Universities, by the European Union—NextGenerationEU and the Recovery, Transformation and Resilience Plan.

Conflict of interest

The authors declare the following competing financial interest(s): P S is co-founder of Deep Breath Intelligence AG (Switzerland), which develops breath-based diagnostic tools. K D S is a consultant for Deep Breath Intelligence AG (Switzerland). The University Children’s Hospital Basel is a shareholder of Deep Breath Intelligence AG (Switzerland).

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