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# Delayed Analysis of Hydrogen-Methane Breath Samples

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**Conflict of Interest**
The authors have no financial conflicts of interest.

## ABSTRACT

**Purpose:** Hydrogen-methane breath tests are used to diagnose carbohydrate malabsorption and small intestinal bacterial overgrowth. The COVID-19 pandemic has driven the modification of procedures as breath tests are potentially aerosol-generating procedures. We assessed the effect of delayed analysis of breath samples, facilitating the at-home performance of breath testing.
**Methods:** Children provided two breath samples at every step of the lactose breath test. The samples were brought back to the clinic, and one set of samples was analyzed immediately. The second set was stored at room temperature and analyzed 1-4 days later.
**Results:** Out of the 73 "double" lactose breath tests performed at home, 33 (45.8%) were positive. The second samples were analyzed 20 to 117 hours after the first samples (41.7±24.3 hours). There was no significant difference in the hydrogen concentration between the first and second sets (Z=0.49, $p$=0.62). This was not the case for methane, which had a significantly higher concentration in the second breath samples (Z=7.6).
**Conclusion:** Expired hydrogen levels remain stable in plastic syringes if preserved at room temperature for several days. On the other hand, the delayed analysis of methane appeared to be less reliable. Further research is needed to examine the impact of delayed analysis on methane and hydrogen concentrations.

**Keywords:** Breath tests; Hydrogen; Methane; Lactose intolerance; Child

## INTRODUCTION

Hydrogen-methane breath tests are widely used. Hydrogen ($H_2$) and methane ($CH_4$) measurements in exhaled breath are reliable and non-invasive methods for diagnosing carbohydrate malabsorption and small intestinal bacterial overgrowth, based on the knowledge that human cells are unable to produce these gases. Their production is the result of the bacterial fermentation of carbohydrates [1].

The COVID-19 pandemic has driven the modification of the methodology of many procedures to decrease the risk of transmission of infection. Breath tests require the presence of both the child and parent in the clinic for several hours. They are also potentially aerosol-generating procedures that increase the risk of viral transmission [2]. Consequently, it was decided that

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breath tests should not be performed in the hospital but at home. A "home-breath test" was proposed to the parents of all consecutive children above the age of 5 years who were referred for a lactose breath test. Overall, parents and children favored the idea of performing the breath tests at home, particularly during the weekend. This resulted in an overload of the samples to be analyzed after the weekend. To allow for better management of the time-consuming analysis of breath samples, we assessed the effect of a delayed analysis of breath samples for $H_2$ and $CH_4$ levels.

## MATERIALS AND METHODS

The parents came to the day clinic, received written instructions, a link to a demonstration video that was developed by the nurses for this purpose, and an at-home testing kit. The parents were instructed by the nurses on how to collect breath samples. Informed consent was obtained to perform a "double" breath test. The study was approved by the local ethical commitee.

All parents received 14 plastic syringes (Henke-Ject 50 mL Luer-lock, Tuttlingen, Germany) with a three-way stopcock and a mouthpiece. The substrate administered was lactose. After providing two baseline breath samples and drinking the lactose solution (2 g/kg with a maximum of 50 g), the children had to exhale into two syringes every 30 minutes for 180 minutes. This procedure yielded two sets of breath samples, one intended for an "early" and one for a "late" analysis. Parents were asked to write down any symptom occurring during or up to three hours after the test on a form developed by the nurses, indicating the number and timing of measurements. The syringes were returned to the clinic the same as the test or the following day. The analysis for one set of samples was performed immediately after the samples were returned. The second set of seven samples was stored at room temperature and analyzed after 1-4 days. All breath samples were analyzed using a QuinTron MicroLyzer (QuinTron Instrument Company, Milwaukee, WI, USA).

Breath tests were categorized as positive when either $H_2$ was increased by ≥20 ppm or $CH_4$ by ≥10 ppm above the initial baseline value [3,4]. Valid breath samples had a $CO_2$ correction factor of ≤2.5.

The at home "double" breath tests were performed from June to September 2020. During the first month, the delayed analysis was performed on all second sets of breath samples, regardless of the result of the early analysis. Afterwards, only samples corresponding to positive early analyses were preserved for a late analysis. The aim of this study was to assess potential hydrogen/methane loss in breath samples in the analysis that was performed several days after the sample collection.

## RESULTS

During the four-month study period, a total of 73 "double" hydrogen-methane breath tests were performed at home. The mean age of the children was 9.8 years (standard deviation [SD], ±3.2 years; median age, 9.4 years). Since parents recorded the appearance of symptoms, malabsorption and intolerance could easily be differentiated (although the clinical outcome was not the goal of this study) [5].


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During the first month, all sets of breath test samples (n=32) were analyzed a second time, independent of the results of the early analysis. All positive tests were again positive with the late analysis, and all negative tests were confirmed as negative as well. Since the study objective was loss of hydrogen or methane during preservation, and since the first 18 negative early analysis results remained negative, thereafter, only samples corresponding to positive early results were analyzed a second time.

Of the 73 tests performed at home, one test was not interpretable, as the child was uncooperative and many samples were invalid. Of the 72 remaining breath tests, 39 were negative, and the early results for 33 samples (45.8%) were positive; 32 due to a rise in hydrogen ≥20 ppm and 1 with a significant increase in both methane and hydrogen. One of these 33 tests could not be used for further analysis, as only the last sample of the early read-out showed an increase in $H_2$, and the last sample of the late read-out was a failure. The late analysis of the 32 positive tests were performed 20 to 117 hours after the first analysis, depending on the availability of the nurses (mean±SD: 41.7±24.3 hours).

The 32 positive breath tests yielded 448 (224 paired) breath samples, of which 3 samples had a $CO_2$ correction factor ≥2.5 and were discarded along with their corresponding samples. For the remaining 442 (221 paired) samples, there was a non-normal distribution of data. Mean $H_2$ baseline value for the first analysis was 23.7±23.2 ppm (**Table 1**); for the second analysis, this was 23.2±25.0 ppm. The increase in $H_2$ concentration in exhaled breath over the baseline was highly variable: 36-341 ppm in the first analysis and 24-361 ppm in the second analysis.

The mean $H_2$ concentration in the first samples was 86.4±92.0 ppm; for the second sample, this was 85.6±94.2 ppm. Using the Wilcoxon signed-rank test, no significant differences in $H_2$ concentration between the first and second breath samples were detected (Z=−0.49, $p$=0.62). There was a very high correlation between the $H_2$ concentrations of the first and second breath samples (rho=0.96) (**Fig. 1**).

The mean $CH_4$ baseline value was 18.5±5.8 ppm for the early analysis and 20.4±7.0 ppm for the late analysis. Mean $CH_4$ concentration of the first samples was 21.6±10.0 ppm; for the second, this was 23.3±10.3 ppm. The Wilcoxon signed-rank test indicated that $CH_4$ concentration in the second sample was significantly higher than in the first breath samples (Z=−7.6), but there was still a clear correlation between the $CH_4$ concentration of the first and second breath samples (rho=0.88) (**Fig. 2**).

**Table 1.** Characteristics of the double breath samples

| $H_2$ and $CH_4$ parameter (ppm) | Early read-out (n=221) | Late read-out (n=221) |
|---|---|---|
| $H_2$ baseline (SD) | 23.7 (23.2) | 23.2 (25.0) |
| $H_2$ mean (SD) | 86.4 (92.0) | 85.6 (94.2) |
| $H_2$ median (IQR) | 55 (111) | 52 (102) |
| $CH_4$ baseline (SD) | 18.5 (5.8) | 20.4 (7.0) |
| $CH_4$ mean (SD) | 21.6 (10.0) | 23.3 (10.3) |
| $CH_4$ median (IQR) | 20 (7) | 22 (9) |

$H_2$: hydrogen, $CH_4$: methane, SD: standard deviation, IQR: interquartile range.


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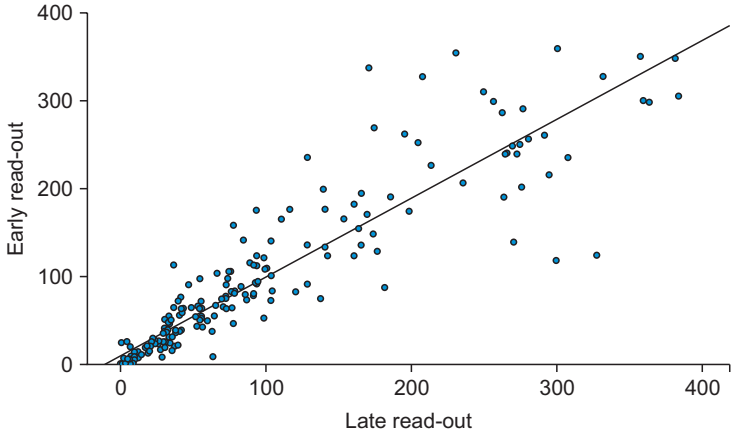


**Fig. 1.** Hydrogen concentration in early and late read-outs.

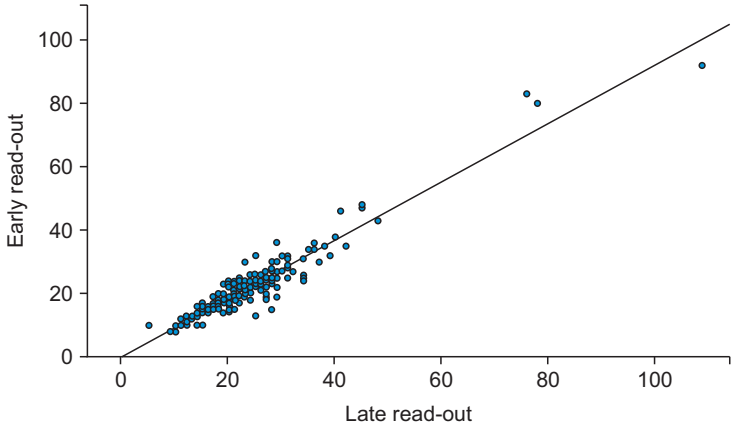


**Fig. 2.** Methane concentration in early and late read-outs.

## DISCUSSION

We demonstrated that storage of $H_2$ breath samples at room temperature can be performed in a reliable way without affecting the results for up to 5 days. However, further evaluation is required to determine if this is also applicable for $CH_4$ measurements.

Lactose malabsorption, referred to as "intolerance, is the most common type of carbohydrate malabsorption. Lactose is a disaccharide that is broken down by the lactase present on the top of the villi of the small intestinal brush border. When lactose is digested, nearly all mono- and disaccharides are absorbed in the small intestine. However, if lactose reaches the colon undigested, it is fermented by colonic bacteria, producing short chain fatty acids, carbon dioxide, hydrogen, and methane [6,7]. These gases are absorbed in the bloodstream and are exhaled via the lungs.

Measurement of exhaled gases is typically performed using clinic-based equipment, and stationary dedicated gas chromatographs are the gold standard [8]. A $CO_2$ correction factor was used to reduce errors caused by accidental contamination of breath samples by room air. $CO_2$ levels in alveolar air are stable at approximately 5%, whereas they are negligible in room


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air. $H_2$ and $CH_4$ levels in expired air can be adapted according to this constant alveolar $CO_2$ level, increasing the reliability and clinical utility of breath tests [9].

In 1975, it was shown that the concentration of $H_2$ remained stable for 21 days in Vacutainer tubes (Becton Dickinson, Rutherford, NJ, USA) and, in 1977, it was shown to remain stable for 47 days in foil bags [10,11]. The use of glass tubes has been questioned, since contamination of silicone-coated Vacutainer tubes with volatile reducing gases has been demonstrated [12]. Plastic syringes would appear to be a useful alternative when storage of large sample volumes is required. Perman et al. [13] found no loss of $H_2$ concentration in gas stored in plastic syringes over a 12 hours period. Rosado and Solomons [14] showed in 1983 that at sea level in a temperate environment, the loss of $H_2$ stored in plastic syringes fitted with three-way stopcocks is approximately 1 μL/L per day for each 25 μL/L of initial concentration. Applying these findings, the decline in diagnostic sensitivity over 48 hours would thus be minimal, and they concluded that plastic syringes can be used for the storage of breath test samples for several days before analysis [14]. Ellis et al. [15] found that at room temperature (~21°C), plastic syringes leak $H_2$ and $CO_2$ at rates of approximately 5% and 4% per day, respectively. At −20°C, the loss of $H_2$ and $CO_2$ was reduced to approximately 1% per day. As $H_2$ and $CO_2$ are lost at approximately the same rate during storage, correction of $H_2$ for $CO_2$ concentration reduces the error caused by this leakage [15]. The Rome Consensus Conference in 2009 recommended that the $H_2$ concentration in breath samples should be determined within 6 hours after sampling, because of the risk of leakage [3]. Breath samples for $H_2$ testing are stable for 6 hours at room temperature, and if analysis is delayed further, storage at −20°C has been recommended [15,16]. This statement was recently confirmed by the consensus published by the European Association for Gastroenterology, Endoscopy, and Nutrition; the European Society of Neurogastroenterology, and Motility; and the European Society for Pediatric Gastroenterology Hepatology and Nutrition consensus [5]. Our data indicate that $H_2$ levels remain stable for longer than 24 hours at room temperature in the material used.

As mentioned in the introduction, because of COVID-19, breath tests had to be performed at home and families preferred to do this over the weekend. This made analysis of the breath samples on the same day practically impossible as many tests were returned on Monday morning, leading to an excessive workload. It is unclear how delayed analysis would affect the results. We demonstrated that the correlation between the $H_2$ concentration of early and late analyses of breath samples was very high. Despite some differences in concentration between the early and late analyses, in all tests with a very high $H_2$ concentration, the correlation was almost perfect. The observed differences are presumably a reflection of the natural hydrogen variability in exhaled breath. This hypothesis is supported by the fact that the differences occurred in both directions, as shown in **Fig. 2**.

Regardless of whether the additional determination of $CH_4$ levels is clinically relevant or not, the overall detection rate of carbohydrate malabsorption is not significantly affected by additional measurements of $CH_4$ [5,17]. The possible increase in test accuracy related to this extra measurement must be weighed against the increased costs of equipment and more complex breath collection. Independent from measuring $CH_4$ to diagnose lactose malabsorption, $CH_4$ determination may be helpful in guiding constipation treatment [18]. According to our data, the methane concentration was slightly higher in the second sample. This was surprising, as we expected a loss of gas and not a gain. We tentatively hypothesized that this could be due to oral bacteria entering the syringe during exhalation. Higher $CH_4$ concentrations could theoretically lead to false-positive results. However, the result will


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then be indicative of malabsorption and not intolerance since symptoms will not develop in case of a false positive test result, thus, this will not have a clinical impact as no dietary restrictions are recommended in case of malabsorption. Otherwise, in this cohort, methane was of no added value in the diagnostic process.

Four molecules of $H_2$ can be converted into one $CH_4$ molecule, resulting in lower $H_2$ excretion [19]. For this reason, many studies have included $CH_4$ to improve the sensitivity of breath tests. However, the literature available so far is inconclusive, and the Rome Consensus does not recommend the measurement of breath $CH_4$ excretion to improve the diagnostic accuracy of the $H_2$ breath test [3]. The North American Consensus from 1981 states that the optimal criterion to define excessive $CH_4$ production is not clear and suggests a cut-off value for "methane positivity" of ≥10 ppm at any point of the test, and not a rise from baseline [4]. Since the 2003 study by Vernia et al. [20] on the effect of a predominant methanogenic microbiome on lactose breath tests, a rise of 10 ppm for $CH_4$ above baseline is generally considered positive for a lactose breath test.

Based on our results and considering the current non-uniform recommendations about $CH_4$, we would recommend that $CH_4$ concentrations not be considered when performing a delayed analysis of breath samples. A limitation is that the two breath samples the children delivered every 30 minutes were not from the same exhalation. It would have been better to use a double-loop system, but it would have made the test more difficult for parents and children. Another limitation is that after the run-in period including 32 patients, delayed analysis was only performed in those breath tests that were positive at the early analysis.

As stated before, breath tests are easy, non-invasive, and reliable diagnostic tools. However, they are time-consuming for the patient, family, and healthcare staff. Postal kits for at-home testing, such as Cerascreen® or Gut-Chek®, can be found online, but these are not reimbursed by health insurance, and quality control is uncertain. In 2019, 28,748 $H_2$ breath tests were performed in Belgium, including both adult and pediatric patients [21]. Being able to take the test at home during the weekend meant that these children do not miss half a day of school and parents or adult patients do not have to take a day off from work. However, it is necessary for parents to have sufficient language and literacy skills to understand the instructions. A trip to the hospital can be a stressful event for a child, and children will be more comfortable in their home environment, especially when they are symptomatic, with abdominal pain, flatulence, or diarrhea. In addition, a higher respiratory rate due to anxiety can lead to a reduction in exhaled $H_2$, thus altering test results [22]. Of the 73 at-home breath tests over the past months, only one parent reported that the child did not finish the lactose solution. Children refusing to drink a solution is a common observation in the clinic. This could mean that children are more relaxed at home or less influenced by other children taking the test. Feedback from our nursing staff showed that the at-home procedure does not save much time, pointing out that they sometimes spend over an hour explaining the whole procedure to parents. However, it did make their day more predictable and there were fewer people in the day clinic during the day, providing a more relaxing environment for other patients.

In conclusion, lactose breath testing can be performed at home in a reliable manner, and it is a cost-saving and child-friendly alternative to the classic in-hospital testing. The expired $H_2$ level remained stable in the plastic syringes even if they were preserved at room temperature for more than 24 hours. $CH_4$ appeared to be less reliable. The minimal difference in $H_2$ concentration between the early and late analysis can be considered a clinically meaningful


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result and can serve as a basis for increased use of at-home testing in the future. More research is needed to examine the impact of delayed analysis on $H_2$ and $CH_4$ concentrations.

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