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Stability of Volatile Organic Compounds in Exhaled Breath Samples: Comparative Evaluation of Storage Conditions Using HS-SPME-GC/MS

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Abstract

This study evaluated the stability of volatile organic compounds (VOCs) in exhaled breath samples under different storage conditions (storage at −20 °C vs. room temperature, 25 °C) and analysis times (0 h, 3 h, 6 h, and 12 h). Alveolar exhaled breath samples were collected from 30 volunteers in 500 mL Tedlar® bags, followed by analysis using headspace solid-phase microextraction (HS-SPME) coupled to gas chromatography–mass spectrometry (GC-MS). Seventy-three metabolites were putatively identified, of which 40 met the detection-frequency criterion (present in ≥70% of samples) under both conditions. After excluding two annotations considered physicochemically implausible for headspace analysis (glycine and cholesteryl benzoate), 38 common VOCs were retained. Freezing at −20 °C significantly influenced the variability of 30 of these 38 VOCs (FDR-adjusted $p < 0.05$). Specifically, freezing reduced the coefficient of variation (CV) of 20 of the 38 metabolites compared to samples kept at room temperature. Six metabolites, including isoprene, methoxyacetic acid, methylcyclohexane and 1-pentene, showed CV < 15% under both conditions. A quantitative drop of more than 90% of the total analytical signal was observed at room temperature by 12 h after sample collection, whereas freezing substantially attenuated, but did not fully eliminate, this signal loss. These findings highlight the influence of pre-analytical conditions on the integrity of volatile profiles, indicating that immediate cold storage is essential in breath biomarker studies. These results provide key criteria for the standardization of breathomics protocols.

Keywords: exhaled breath; breath biomarkers; volatilomics; stability of samples in the gas phase; clinical metabolomics

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1. Introduction

Volatile organic compounds (VOCs) constitute a diverse set of metabolites characterized by their low molecular weight, high vapor pressure, and low boiling point [1]. These compounds have attracted considerable interest in medicine because of their potential as diagnostic biomarkers. The importance of VOCs lies in their capacity to provide significant information about the body's metabolic processes, as well as serving as indicators of exposure to environmental toxins or pollutants [2]. For instance, breath acetone correlates with ketosis processes in diabetes, while volatile sulfur compounds such as methyl mercaptan are associated with liver dysfunction [3].

The origin of VOCs can be either endogenous, as a product of the body's normal or altered metabolism, or exogenous, deriving from exposure to environmental pollutants or external substances that are metabolically processed. In humans, approximately 2800 different VOCs have been identified, which are present in various biological matrices such as breath, saliva, blood, milk, semen, skin, feces, and urine [2]. The presence of these compounds in exhaled breath is the result of complex physiological dynamics. They include metabolic intermediates originating both systemically and locally in the respiratory tract and the oral cavity. Those of systemic origin are transported by the bloodstream to the pulmonary capillaries, where their transfer into alveolar air is governed by the concentration gradient and the blood:air partition coefficient of each compound. Because VOCs are part of the metabolome, they can be regarded as metabolites [4].

In this regard, a recent and promising metabolomic approach is 'volatilomics', defined as the study of volatile organic compounds produced in the human body and emitted through biological matrices such as breath, among others. Exhaled breath sampling particularly offers unique advantages: it is non-invasive, allows repeated sampling and reflects systemic metabolic changes in real time. Volatilomics has linked specific molecular signatures to oncological, respiratory, and neurodegenerative diseases. However, the low concentration of metabolites (often in the parts-per-billion range) and the high intra-individual variability pose critical analytical challenges for its clinical implementation [1,5–7].

Despite this potential, the high humidity of breath (>95% RH), environmental contaminants, and the chemical instability of the analytes require standardized protocols for sampling, storage, and analysis. Comparative studies reveal that within-subject breath VOC concentrations can vary by up to 40% over 6 h, which limits reproducibility and calls for sensitive preconcentration and detection techniques [3,8–12].

In this regard, the coupling of solid-phase microextraction with gas chromatography and mass spectrometry (SPME-GC/MS) helps to address these limitations. Selective preconcentration is achieved by using fibers with coatings such as polydimethylsiloxane/divinylbenzene (PDMS/DVB), which have been shown to adsorb low-polarity compounds with efficiencies exceeding 90% [13–15]. These mixed coatings exhibit a significantly higher extraction capacity compared to single-material fibers [16,17]. Furthermore, the minimization of interferences is achieved through headspace extraction (HS-SPME), which effectively reduces the carryover of non-volatile molecules and matrix components that could interfere with the analysis. Regarding chemical stability, recent research has demonstrated that the SPME technique preserves the integrity of volatile compounds such as terpenes and ketones during prolonged post-sampling periods. Specifically, validation studies have confirmed that SPME-GC/MS methods maintain their analytical performance and long-term stability, with tests demonstrating excellent results over extended periods [18–21].

However, even with robust analytical techniques, the clinical validity of these analyses critically depends on the temporal stability of the volatile profiles during the pre-analytical phase. Previous research has evaluated the persistence of VOCs in polymeric sampling containers. For example, earlier studies compared the stability of various breath constituents in Tedlar bags against other polymers (Kynar and Flexfilm), demonstrating the superiority of Tedlar in terms of lower background emission and greater stability of the chemical species, although they recommended that samples be analyzed preferably within the first few hours of collection to minimize losses [22,23].

In light of the above, the present study focuses on analyzing variations in the volatile profile of exhaled breath using SPME-GC/MS, with the aim of evaluating variability under different storage conditions (freezing at −20 °C vs. room temperature) and analysis times (0 h, 3 h, 6 h, and 12 h). This evaluation of volatile organic compounds in exhaled breath

is crucial to ensure reproducibility and reliability in the future development of diagnostic methods and the identification of potential biomarkers.

2. Materials and Methods

2.1. Characterization of Study Subjects and Collection of Breath Samples

A total of 30 adult volunteers aged between 18 and 73 years were recruited. The demographic characteristics and relevant clinical information of the study cohort are summarized in Table 1. All participants self-reported to be apparently healthy at the time of sampling, with no recent history of acute respiratory or metabolic diseases. All participants were thoroughly informed about the objectives and methodology of the study, providing written informed consent prior to inclusion. The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Institutional Ethics Committee, Universidad de Caldas, Facultad de Ciencias para la Salud (approval no. CBCS-031; date 27 July 2021). To reduce the presence of exogenous factors in the volatile profile, each volunteer was asked to strictly adhere to the following instructions for at least 8 h prior to sampling: refrain from consuming food, beverages, alcohol, or tobacco, and avoid the use of mouthwash or toothpaste. Breath collection was carried out at room temperature in a closed room, using 500 mL Tedlar® bags. Each participant was instructed to take a deep breath and discard the first portion of the exhaled air, in order to exclusively collect alveolar breath. Each volunteer provided a single sample, exhaling continuously until filling the total volume of one (1) collection bag, which was sealed immediately to prevent losses. As a quality control, a sampling blank was prepared by filling an identical bag with high-purity nitrogen gas (N2), which was stored and analyzed under the same experimental conditions as the biological samples. The 30 bags were placed in a frozen container for transport to the laboratory. Once there, the sample cohort was randomly divided into two equal groups: 15 samples were stored frozen at −20 °C, while the remaining 15 samples were stored at room temperature (25 °C). Each of these bags was sampled repeatedly by headspace extraction (HS-SPME) for GC-MS analysis at four evaluation times (0 h, 3 h, 6 h, and 12 h), counted from the moment each bag was sealed and not from the start of instrumental analysis.

Table 1. Clinical and demographic characteristics of the study cohort.

Characteristic	**Frozen Samples (n = 15)**	**Room Temperature, 25 °C Samples (n = 15)**
Age (years), *Mean* ± *SD*	36.0 ± 11.8	43.8 ± 11.3
Age range	18–60	27–73
Sex, *n (%)*		
- Female	9 (60.0%)	8 (53.4%)
- Male	6 (40.0%)	7 (46.6%)
Habits, *n (%)*		
- Smoking	3 (20.0%)	3 (20.0%)
- Alcohol	2 (13.3%)	4 (26.6%)
- Medication use	1 (6.6%)	2 (13.3%)

2.2. SPME Metabolite Extraction

The extraction of volatile organic compounds (VOCs) present in the breath samples was performed using the solid-phase microextraction (SPME) technique. Agilent Technologies

(5301 Stevens Creek Blvd Santa Clara, CA 95051 United States) SPME fibers with an 80 µm thick Divinylbenzene/Carbon WR/Polydimethylsiloxane (DVB/C-WR/PDMS) stationary phase (Agilent 5191-5874) were used. The fibers employed were previously conditioned according to the manufacturer's recommendations. The SPME fiber was inserted into the collection device and exposed directly to the headspace of the breath sample for an extraction time of 30 min, keeping the sample at room temperature. Since the efficiency of HS-SPME extraction depends on temperature for the proper thermodynamic partitioning of the analytes into the headspace, in the case of frozen samples, each bag was removed from the freezer and subjected to a thermal equilibration period at room temperature (25 °C) for 15 min prior to each extraction process (at 3 h, 6 h, and 12 h). This 15-min interval was defined a priori based on general recommendations for headspace thermal recovery; the internal bag temperature was not independently verified with a dedicated probe, so incomplete or variable thermal re-equilibration cannot be entirely excluded as an additional source of variability in the frozen samples, and this is acknowledged as a limitation of the present protocol. Immediately after the fiber was retracted, the bag was resealed and returned to the −20 °C and the fiber was inserted into the GC injection port. Desorption was carried out for 10 min at a temperature of 270 °C.

2.3. Untargeted Volatilomics by Gas Chromatography Coupled to Mass Spectrometry (GC-MS)

Following the thermal desorption of the extracted metabolites, volatilomic analysis was carried out using an untargeted approach via GC-MS. Chromatographic separation was performed on a Shimadzu GCMS-QP2010 Plus system. A DB-5MS capillary column (30 m × 0.25 mm × 0.25 µm) was used, employing high-purity helium (99.9999%) as the carrier gas at a constant flow rate of 1 mL/min. The electron ionization (EI) source was operated at 70 eV. The injection port was operated in splitless mode at 250 °C. The oven temperature was programmed as follows: initial temperature of 50 °C (held for 1 min), increased at a rate of 10 °C/min to 290 °C (held for 4 min), and then increased at a rate of 4 °C/min to 300 °C (held for 6 min). The transfer line temperature was set at 300 °C, and the ion source and quadrupole temperatures were maintained at 300 °C. Mass spectra were acquired in full-scan mode (m/z 50 to 500; scan speed of 1666 u/s). It should be noted that the DB-5MS stationary phase used in this study, being a low-polarity phase, provides comparatively limited chromatographic retention for the most volatile and polar VOCs (e.g., short-chain alcohols and ketones); this is acknowledged as an analytical limitation that may affect the resolution and retention-time-based identification confidence for this specific subset of compounds.

2.4. Data Acquisition and Pre-Processing

Data acquisition was performed using Shimadzu's GCMS Real Time Analysis software (GCMSsolutions v2.50). The raw data obtained from the GC-MS were processed using the GCMS Postrun Analysis software. Pre-processing included peak detection, deconvolution performed with AMDIS v2.73 software, retention time alignment, and peak area integration. Peak detection was carried out using the *centWave* algorithm, implemented in the *xcms* package, which identifies regions of interest (ROIs) based on peak width parameters (5–30 s), mass tolerance (±0.5 m/z), and intensity thresholds. For peak alignment across samples, a density-based grouping approach was employed, correcting retention time variations through the iterative grouping of peaks with maximum deviations of 5 s within each group (<0.5% as quality criterion). Impurities were filtered by combining mass thresholds (tolerance of ±0.5 m/z for predefined masses) and relative intensity (<5% of the global maximum peak). To minimize the contribution of background signals, all compounds detected in the Tedlar® bag blanks filled with high-purity N_2 and in the SPME fiber blanks

were evaluated; a feature was retained only when its mean peak area in the breath samples was at least three times its mean area in the corresponding blank, and features below this ratio were excluded from further analysis instead of being blank-subtracted. All processing was executed in RStudio v2025.05.0+496. Data transformation was performed using the *tidyverse (dplyr)* package, while the *ggplot2* package was used for results visualization and plotting. The stats package was used for univariate statistical tests (Wilcoxon, t-test), calculation of means and standard deviations, and *p*-value adjustment. Exploratory data analysis was conducted using the MetaboAnalystR package.

2.5. Metabolite Identification

Metabolites were identified by comparing the acquired mass spectra with the spectral databases of the NIST 14 library, considering as putative identifications those compounds showing a spectral similarity greater than 90% (individual similarity scores are reported in Table S1). In addition, retention indices (RI) were calculated for metabolites whose retention time fell within the elution range of an n-alkane mix (C8–C29) analyzed under the same chromatographic conditions as the samples. RI values were calculated with the linear-interpolation equation of Van den Dool and Kratz for temperature-programmed gas chromatography (Equation (1)):

$$RI = 100\left[n + \frac{t_R - t_n}{t_{n+1} - t_n}\right] \quad (1)$$

where t_R is the retention time of the analyte, t_n and t_{n+1} correspond to the retention times of the n alkanes eluting immediately before and after the compound, respectively, and n is the number of carbon atoms of the preceding alkane.

Experimental RI values were compared with reference values reported for 5% phenyl-methylpolysiloxane columns (DB-5/HP-5MS type) in the NIST Chemistry WebBook and the NIST 14 retention index library.

2.6. Data Processing and Statistical Analysis

To evaluate the overall sample loss, the percentage reduction of the Total Ion Chromatogram (TIC) Area was calculated using the untransformed data of the chromatographic peak areas over time, setting the 0 h time point as 100%. Subsequently, to evaluate the relative and individual stability of each metabolite, a frequency filter was applied to define the constitutive (common) compounds; only those metabolites present in at least 70% of the samples under both storage conditions were selected for downstream analysis. After this filtering, which reduced the initial list of 73 putative compounds to a subset of 40 metabolites (two of which, glycine and cholesteryl benzoate, were subsequently excluded as physicochemically implausible for headspace sampling of breath, leaving 38), the raw metabolomic data were subjected to the following preprocessing steps to ensure statistical integrity: normalization by sum, logarithmic transformation of intensities (log10 scale) to reduce bias from heteroscedasticity and approximate a normal distribution, and Pareto scaling to balance the contribution of high- and low-abundance metabolites.

The stability of each metabolite was quantified using the coefficient of variation (%CV), as shown in Equation (2).

$$\%CV = \left(\frac{Standar\ deviation}{Mean}\right) \times 100 \quad (2)$$

Given that each of the 30 volunteers provided a single sample bag ($n = 15$ for the frozen condition and $n = 15$ for room temperature), the mean and standard deviation (SD) were calculated between subjects for each evaluated time point. That is, for each metabolite, the mean and SD of the abundances obtained from the 15 individuals belonging to the same

storage condition at a specific time (0 h, 3 h, 6 h, 12 h) were calculated. Subsequently, the resulting CVs from each time point were averaged. Therefore, the reported CV encompasses the technical variability of the method and the chemical stability of the metabolite, as well as the biological variability inherent to the cohort evaluated under those conditions. Accordingly, the CV values reported throughout this study should be interpreted as an index of group-level analytical reproducibility under each storage condition, rather than as a pure, compound-specific measure of chemical degradation. Because several metabolites fell below the limit of detection at 6 h and 12 h, particularly at room temperature (Figure 1), ratios of the peak area at each time point to the 0 h area (At/A0) would be undefined or strongly biased for these compounds; compound-specific temporal behavior is therefore presented in Figure 1, which shows the individual observations of each subject at each time point, and overall signal retention is quantified from the TIC. The absence of a complete per-compound recovery analysis is acknowledged as a limitation of the present study.

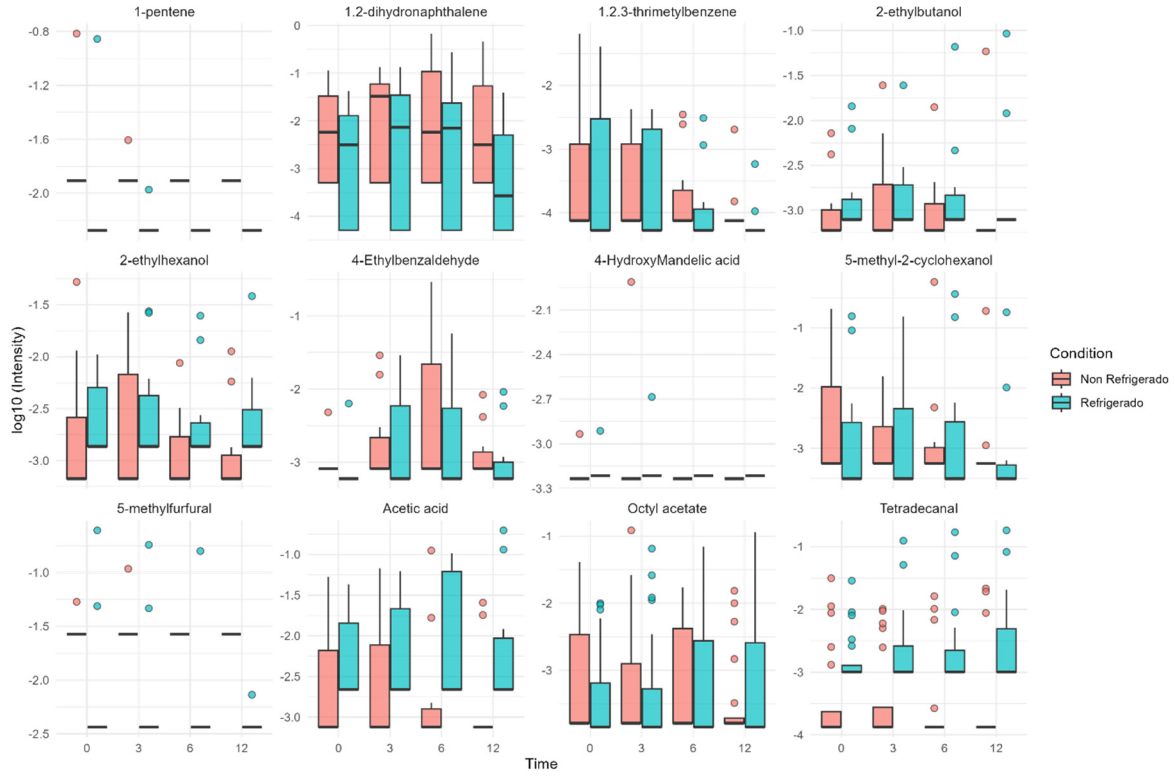


Figure 1. Temporal dynamics of the relative intensity (log10 transformation) of the 12 most representative metabolites, comparing frozen storage at −20 °C (blue) and at room temperature (pink) at 0 h, 3 h, 6 h, and 12 h. Boxes represent the interquartile range, and individual points indicate the observations per subject. Note: The absence of data points or boxes at later time points (particularly at 6 h and 12 h) is due to the metabolite abundance falling below the instrument's limit of detection (missing values due to non-detection), which is direct evidence of analyte degradation and loss in the headspace.

For each condition (frozen vs. room temperature) and the respective sample analysis times, the stability criteria were defined as follows: CV < 15%: High stability (robust metabolites for biomarkers). CV 15–30%: Moderate stability (requires validation). CV > 30%: High variability (potential exclusion or cautious analysis). Statistical comparison: The Wilcoxon test was used to determine significant differences in CV% between conditions. A t-test was performed to compare abundances between conditions at each time point (assuming data normality after transformation). The Benjamini–Hochberg adjustment (FDR < 0.05) was

applied to control for false positives in multiple simultaneous comparisons. All analyses were performed in RStudio v2025.05.0+496.

2.7. Quality Assurance and Quality Control (QA/QC)

Instrumental quality assurance during HS-SPME-GC-MS runs was implemented through a protocol based on standards and methodological blanks. For continuous monitoring of system stability, a mixed standard solution containing 1-chlorononane (not reported in breath), acetic acid and acetone was used, selected to cover a representative range of volatilities and retention times. Prior to metabolite extraction from breath samples, this standard mixture (0.05 mg L^{-1} in water) was sampled by HS-SPME from Tedlar® bags at 25 °C to monitor fiber extraction efficiency and mass spectrometer sensitivity.

To verify chromatographic system suitability, an n-alkane standard mixture was injected at the beginning of each sequence, with the aim of confirming analytical signal robustness, ensuring chromatographic resolution and assessing peak symmetry. To suppress carry-over and avoid cross-contamination, a conditioning protocol of the stationary phase was applied, interspersing injections of n-hexane as solvent approximately every five chromatographic runs to purge residual hydrocarbons from the capillary column; this instrumental maintenance step was unrelated to sample collection and was applied uniformly across both storage conditions. Environmental blanks (empty Tedlar® bags purged with N_2) were also processed under identical microextraction conditions. The compounds detected in these blanks and the criterion used to exclude background features are described in Section 2.4.

3. Results and Discussion

3.1. Relative Stability of Breath Samples and Changes in the Metabolic Profile According to Storage Conditions

A total of 73 metabolites were putatively identified in the exhaled breath samples collected from the healthy volunteers. In order to rigorously evaluate the stability of these volatile organic compounds (VOCs) against pre-analytical factors, the samples were subjected to two contrasting storage conditions in Tedlar® bags: frozen storage at −20 °C and storage at room temperature (25 °C). From the comparative analysis of the chromatograms, and after applying the frequency filtering criterion described in the methodology (presence in at least 70% of the samples under both conditions), a subset of 40 common metabolites was obtained; after excluding two physicochemically implausible annotations (Section 2.6), 38 metabolites were retained. These compounds exceeded the baseline analytical reproducibility threshold, ensuring that their subsequent longitudinal stability evaluation was not biased by missing values. The list, systematically grouped by chemical class along with their relative abundances and retention times, is presented in Table 2.

Table 2. Stability of volatile organic compounds in exhaled breath: coefficients of variation (CV%) and statistical significance between storage conditions.

RT	Putative Identification	*m/z*	Class	CV% 25 °C	CV% −20 °C	*p_adj*	RI
3.963	2-ethylbutanol	102	Alcohol	17.07	19.99	5.98×10^{-6}	-
2.9655	2-methyl-1-butanol	72	Alcohol	20.1	17.82	3.3×10^{-5}	-
12.108	5-methyl-2-(propan-2-yl)cyclohexan-1-ol (Menthol)	156	Alcohol	30.24	36.17	0.0002	1102
9.137	Tetrahydrogeraniol	158	Alcohol	15.38	19.11	2.85×10^{-5}	-

Table 2. *Cont.*

RT	Putative Identification	*m*/*z*	Class	CV% 25 °C	CV% −20 °C	*p_adj*	RI
8.675	4-Ethylbenzaldehyde	134	Aldehyde	25.01	20.65	9.42×10^{-6}	-
5.885	5-methylfurfural	110	Aldehyde	6.85	22.56	5.89×10^{-10}	-
8.407	Benzaldehyde	106	Aldehyde	42.27	27.37	0.0434	-
17.04	Tetradecanal	212	Aldehyde	20.87	22.38	1.24×10^{-13}	-
3.8	hexanal	100	Aldehyde	34.18	37.71	0.0169	-
3.256	Methylcyclohexane	98	Alkane	6.24	7.29	1.55×10^{-15}	-
6.3285	Octane	114	Alkane	29.27	20.91	4.94×10^{-5}	800
1.89	1-pentene	70	Alkene	9.49	10.22	1.55×10^{-15}	-
1.6015	Isoprene	68	Alkene	2.82	3.23	3.28×10^{-14}	-
4.407	Methylcyclohexene	96	Alkene	37.85	43.92	0.1857	-
4.4265	N,N-Dimethylacetamide	87	Amide	71.16	84.32	0.0747	-
13.4845	1,2-Dihydronaphthalene	130	Aromatic	50.52	46.01	0.0148	1175
7.408	1,2,3-Trimethylbenzene	120	Aromatic	20.57	21.94	0.0005	871
2.27	Benzene	78	Aromatic	60.63	52.22	0.0131	-
6.773	Cumene	120	Aromatic	21.54	22.9	7.34×10^{-5}	840
4.467	O-xylene	106	Aromatic	44.21	42.47	0.055	-
3.124	Toluene	92	Aromatic	28.39	26.86	0.0061	-
5.5395	4-Hydroxymandelic acid	152	Carboxylic acid	6.68	2.98	3.28×10^{-14}	-
1.9015	Acetic acid	60	Carboxylic acid	26.56	30.17	8.98×10^{-6}	-
4.419	Butyric acid	88	Carboxylic acid	55.56	49.25	0.0597	-
20.0	Hexadecanoic acid	256	Carboxylic acid	31.76	31.6	7.77×10^{-6}	1554
2.0765	Methoxy acetic acid	90	Carboxylic acid	1.87	1.85	1.42×10^{-15}	-
20.6405	Octadecanoic acid	284	Carboxylic acid	23.39	12.17	1.97×10^{-11}	1594
3.556	Propionic acid	74	Carboxylic acid	37.5	40.9	0.3952	-
1.5695	Acetone	58	Ketone	38.67	40.83	0.2432	-
1.9845	Ethylacetate	88	Ester	36.72	34.21	0.0147	-
23.848	Lauryl acetate	228	Ester	24.32	18.71	7.6×10^{-8}	1815
9.0987	Methyl salicylate	152	Ester	24.12	28.98	3.63×10^{-6}	-
9.1025	Octyl acetate	172	Ester	24.84	27.73	1.49×10^{-7}	-
10.249	butyl glyoxylate	130	Ester	21.91	10.38	1.26×10^{-9}	1009
2.13	methylpropionate	88	Ester	21.83	21.71	5.8×10^{-9}	-

Table 2. *Cont.*

RT	Putative Identification	*m*/*z*	Class	CV% 25 °C	CV% −20 °C	*p_adj*	RI
5.4675	Alpha pinene	136	Terpene	40.17	32.0	0.3947	-
6.9875	Limonene	136	Terpene	56.64	49.74	0.7033	851
6.779	Terpinene	136	Terpene	13.6	9.35	6.11×10^{-10}	840

CV: coefficient of variation; RT: retention time; *m*/*z*: mass-to-charge ratio; *p*_adj: *p*-value adjusted for multiple comparisons (FDR). CV% values represent the relative variability of each metabolite under storage conditions, refrigerated (−20 °C) and unrefrigerated at room temperature. The *m*/*z* value listed corresponds to the diagnostic/quantifier ion selected during chromatographic deconvolution and does not necessarily represent the molecular ion; for several compounds it reflects a characteristic fragment ion rather than the nominal molecular weight.

Before examining differential stability, it is useful to summarize the chemical-class composition of the 38 common metabolites listed in Table 2, given its physiological relevance. The identified compounds span alcohols, aldehydes, alkanes, alkenes, aromatic hydrocarbons, carboxylic acids, esters, a ketone, an amide, and a terpene family, reflecting the broad metabolic diversity of the human breath volatilome. Several of these classes have well-documented endogenous origins: isoprene (alkene) is considered a byproduct of the mevalonate/cholesterol biosynthetic pathway; acetone (ketone) reflects ketone-body metabolism and lipid catabolism; aldehydes such as hexanal and tetradecanal are commonly associated with lipid peroxidation and oxidative stress; carboxylic acids such as octadecanoic and hexadecanoic acid relate to fatty-acid β-oxidation and, in part, to microbiota-derived metabolism; and aromatic compounds such as toluene, benzene, and cumene are more frequently linked to exogenous exposure (e.g., environmental or dietary sources) than to endogenous metabolism. This chemical-class perspective provides a physiological framework for interpreting the differential storage stability described below, since compounds sharing a chemical class tend to share broadly similar physicochemical behavior (e.g., volatility, polarity, and reactivity), even though, as shown next, stability can still vary considerably within a class.

To complement the cross-sectional CV approach, degradation dynamics were also analyzed longitudinally within each sample by evaluating the trends of relative intensities over time (Figure 1), using the chromatographic analysis at 0 h as a baseline reference (100%) for the quantification of overall loss (TIC).

A first group of compounds exhibited an intrinsically high baseline stability. Metabolites such as isoprene and methoxyacetic acid showed very low variability (CV < 4%) in both frozen and room-temperature samples. However, the adjusted *p*-values demonstrated statistically significant differences ($p_adj < 0.05$) between both storage conditions. This indicates that, although their overall stability is good, the storage temperature still exerts a statistically measurable effect on their retention. The high stability of low-molecular-weight compounds such as isoprene has been previously documented in Tedlar® matrices, demonstrating a strong resistance to adsorption onto the polymer walls [24]. This low variability indicates that these metabolites are ideal candidates to form the human breath baseline. The identification of these stable compounds ensures that the alterations observed in future studies reflect true metabolic changes rather than mere sampling artifacts [22].

On the other hand, many metabolites from diverse chemical families—such as carboxylic acids, esters, and aldehydes—showed a significant reduction in their CV under freezing conditions compared to samples at room temperature ($p_adj < 0.05$). Lipid compounds and long-chain fatty acids such as octadecanoic acid, as well as butyl glyoxylate and lauryl acetate, showed a notable improvement in their analytical reproducibility when the bags were stored at −20 °C. This pattern suggests that freezing is an effective measure

to preserve the chemical integrity of most VOCs, mitigating losses due to diffusion through the Tedlar® film, atmospheric oxidation reactions, and volatility exacerbated by vapor pressure at room temperature. Previous research has indicated that compounds with higher molecular weights and greater reactivity undergo losses of up to 20–40% within the first few hours if temperature is not controlled [25].

The dependence of this stability on the chemical family becomes evident when grouping the results presented in Table 2. For instance, within the ester class, butyl glyoxylate and lauryl acetate significantly reduced their variability when frozen (from ~22–24% to ~10–19%; $p_adj < 0.05$), whereas the remaining esters showed similar (ethyl acetate, methyl propionate) or higher (octyl acetate, methyl salicylate) CVs under freezing. The carboxylic acid family also behaved heterogeneously: octadecanoic acid (from 23.39 to 12.17%), 4-hydroxymandelic acid (from 6.68 to 2.98%) and butyric acid (from 55.56 to 49.25%) showed lower CVs when frozen, whereas acetic acid (26.56 vs. 30.17%) and propionic acid (37.50 vs. 40.90%) showed slightly higher CVs under freezing, and hexadecanoic and methoxyacetic acids were practically unaffected. Aldehydes likewise showed marked intra-class heterogeneity. On the one hand, aromatic aldehydes such as benzaldehyde improved their stability under frozen storage, reducing their CV from 42.27 to 27.37%. On the other hand, more polar aldehydes and furan derivatives evidenced a significant loss of stability when frozen. Illustratively, the compound 5-methylfurfural tripled its CV under cold conditions (from 6.85 at room temperature to 22.56% at −20 °C). This divergent behavior within aldehydes highlights the need to consider the intrinsic physicochemical properties of each analyte, suggesting that, for more polar or water-soluble compounds, freezing at −20 °C may induce detrimental processes, such as the partitioning or dissolution of the metabolite into condensed water droplets on the bag walls due to the high intrinsic relative humidity of exhaled breath [22].

Furthermore, the presence of metabolites with extremely high variability under both evaluation conditions (CV > 50%), such as benzene and N,N-dimethylacetamide, suggests the influence of exogenous and endogenous factors beyond thermal control. This instability may be attributed to temporal physiological oscillations, environmental cross-exposure dynamics, or potential background emissions from the collection bag material itself, a phenomenon widely described in the use of polymers for environmental analysis [26].

Consistent with the above, the diversity of observed responses reinforces the premise that the optimal storage temperature in volatilomics is not universal but rather depends on the chemical family of the target analyte. These results provide fundamental guidelines for the standardization of pre-analytical protocols using HS-SPME extraction, demonstrating that the selection of robust biomarkers requires combining statistical rigor with strict thermodynamic control of gaseous samples.

3.2. Potential Mechanisms of Stability and Instability of Metabolites Present in Breath Samples

Figure 1 shows the temporal dynamics of the relative intensity (logarithmic) of the 12 metabolites with the highest presence in the analyzed samples, comparing the frozen (blue) and room-temperature (pink) conditions at different chromatographic analysis times (0, 3, 6, and 12 h) after sample collection. Each box summarizes the intensity distribution for that metabolite and condition at each time point.

Interquartile distributions (boxes) that maintain a constant median over time indicate that the metabolite is stable, while a reduction in the signal suggests possible degradation, adsorption, or volatilization. The progressive degradation of the gaseous matrix is visually evidenced by the absence of observations (missing values) at the later analysis times (6 h and 12 h), which reflects that the metabolite concentration fell below the instrument's limit of detection, occurring more frequently in room-temperature samples. For example, the

temporal dynamics of 1,2-dihydronaphthalene illustrate the complexity of this stability: rather than a continuous linear degradation, the intensity exhibits fluctuations throughout the 12-h period under both conditions. However, the overall variability is quantitatively more pronounced in the samples subjected to room temperature, as supported by the overall statistical analysis (room-temperature CV = 50.52% vs. frozen CV = 46.01%; $p_adj = 0.014$). Nevertheless, both CVs exceeded 30%, so this compound should be classified as highly variable under both conditions.

Acetic acid shows a trend toward lower values in the room-temperature condition, especially at intermediate time points. On the other hand, the plots of 5-methylfurfural and 4-hydroxymandelic acid exhibit low baseline intensities and narrow interquartile distributions, which is in numerical agreement with the low coefficients of variation reported in Table 3. However, consistent with statistical analysis, while 4-hydroxymandelic acid visually confirms greater stability under frozen storage (CV decreases from 6.68% to 2.98%), 5-methylfurfural evidences the opposite pattern, losing stability under cold conditions. For the majority of the metabolites, the general trend demonstrated that when samples were frozen, intensities remained higher and more stable throughout the 12-h evaluation period.

Table 3. Stability classification of selected exhaled-breath metabolites under the two storage conditions.

RT	Putative Identification	*m*/*z*	Class	CV% 25 °C	CV% −20 °C	*p_adj*	Short Interpretation
2.08	Methoxy acetic acid	90	carboxilic acid	1.87	1.85	1.40×10^{-15}	Highly stable in both conditions
1.6	Isoprene	68	alkene	2.82	3.23	3.30×10^{-14}	Highly stable in both conditions
5.54	4-HydroxyMandelic acid	152	carboxilic acid	6.68	2.98	3.30×10^{-14}	More stable when frozen
20.64	Octadecanoic acid	284	carboxilic acid	23.39	12.17	2.00×10^{-11}	More stable when frozen
10.249	Butyl glyoxylate	130	ester	21.91	10.38	1.30×10^{-9}	More stable when frozen
23.85	Lauryl acetate	228	ester	24.32	18.71	7.60×10^{-8}	More stable when frozen
5.885	5-methylfurfural	110	aldehyde	6.85	22.56	5.90×10^{-10}	More stable at room temperature
4.43	Butyric acid	88	carboxilic acid	55.56	49.25	0.06	High variability under both conditions
4.43	N,N-dimethylacetamide	87	amide	71.16	84.32	0.075	High variability under both conditions
2.27	Benzene	78	aromatic	60.63	52.22	0.013	High variability, slightly better when frozen

The intrinsic stability of metabolites is a phenomenon that has been previously documented in metabolomic studies, where it has been demonstrated that certain compounds exhibit greater resistance to degradation under various experimental conditions. This variability in stability is determined by three main factors: physicochemical properties, biological function, and environmental handling conditions. At the molecular level, polarity and susceptibility to processes such as oxidation or hydrolysis make primary metabolites and basic cellular compounds—such as amino acids and carbohydrates—intrinsically more resistant to degradation than unsaturated lipids, aldehydes, or secondary metabolites, which undergo greater fluctuations. Furthermore, external factors such as temperature, humidity, and exposure to oxygen drastically alter these metabolic profiles. This thermal vulnerability has been confirmed in various biological matrices, such as urine and dried blood, where refrigerated storage successfully preserves the vast majority of compounds, whereas exposure to room temperature induces rapid degradation and drops of up to 40% in their concentration [13,27,28].

3.3. Impact of Sample Storage on the Selection of Potential Biomarkers

Rigorous control of pre-analytical storage temperature is critical for preserving the chemical integrity of potential gaseous biomarkers. Unlike liquid matrices (such as plasma or blood), where degradation is primarily driven by enzymatic or bacterial activity, the loss of VOCs in breath samples stored in polymers like Tedlar is mediated by physicochemical processes such as diffusion through the material, adsorption onto the bag walls, and reactivity, among others. By reducing the temperature to −20 °C, the decrease in kinetic energy attenuates the volatility and permeability of the metabolites in the polymer, preventing their escape or degradation. Through untargeted metabolomics, it is possible to identify differential stability patterns. For example, the results of this study show that methoxyacetic acid could be considered a potential quality control marker due to its high stability across different temperatures. Cholesteryl benzoate, initially annotated in the dataset, was excluded from the stability analysis because its molecular weight (490 Da) and negligible vapor pressure make its presence in the headspace of a breath sample physicochemically implausible; its signal most likely reflects contamination or contact of the SPME fiber with the bag surface. In contrast, 5-methylfurfural was less stable when stored frozen [29,30].

Consistent with the above, Figure 2 (Volcano Plot) provides a comprehensive visual representation of the statistical significance and magnitude of the differences in metabolite abundance between both storage conditions. To construct this plot, the preprocessed relative intensities (with Log10 transformation and Pareto scaling) corresponding to all post-sampling time points were consolidated, globally comparing the group kept at room temperature ($n = 15$) against the group under frozen storage at −20 °C ($n = 15$). The magnitude of the change was evaluated by directly calculating the median difference, while statistical significance was determined using the nonparametric Wilcoxon rank-sum test (Mann–Whitney U test) for independent samples. To control the false positive rate derived from multiple comparisons, the *p*-values were adjusted using the Benjamini–Hochberg False Discovery Rate (FDR) method, setting the significance threshold at $p_adj < 0.05$. The analysis reveals three distinct categories of metabolites based on their response: Metabolites significantly more abundant in the room-temperature condition, such as 5-methylfurfural, methyl salicylate, and hexanal, are located in the upper left region of the plot, indicating that they present statistically significant negative median differences ($p < 0.05$). These compounds show greater stability or formation under room-temperature storage conditions, suggesting they may be sensitive to low temperatures or benefit from chemical processes occurring at room temperature.

On the other hand, metabolites significantly more abundant in the frozen condition, such as tetradecanal and benzaldehyde, are located in the upper right region, demonstrating positive median differences with high statistical significance. These metabolites evidence greater stability under frozen storage, which supports the importance of cold storage in preserving the integrity of certain volatile compounds in exhaled breath samples. Octadecanoic acid (frozen CV: 12.2% vs. room-temperature: 23.4%, $p < 0.001$) indicates that freezing preserves its abundance. This implies that these metabolites can be used to optimize storage protocols. Finally, there are the metabolites without significant differences. Most metabolites are distributed in the central region of the plot, close to the X-axis (median differences close to 0) and with low -log10 (*p*-value) values, indicating that they do not present significant changes between conditions. These compounds can be considered stable and independent of the storage condition, making them suitable candidates for further biomarkers.

Significantly altered metabolites can be grouped into: Lipids and fatty acids, such as butyl glyoxylate and lauryl acetate (median difference > 0.5, $p < 0.05$), which suggest alterations in membrane synthesis or energy storage [31,32]. Amino acids and their derivatives, such as 4-hydroxymandelic acid (frozen CV: 2.98% vs. room-temperature: 6.68%), could

indicate alterations in phenylalanine metabolism or oxidative stress [31,33]. Metabolites with low CV values and significant differences, such as isoprene, may correlate with stable physiological processes, such as the baseline production of volatile compounds [33,34]. Long-term metabolite stability suggests robust homeostatic mechanisms, whereas unstable metabolites reflect potential environmental influence [34,35].

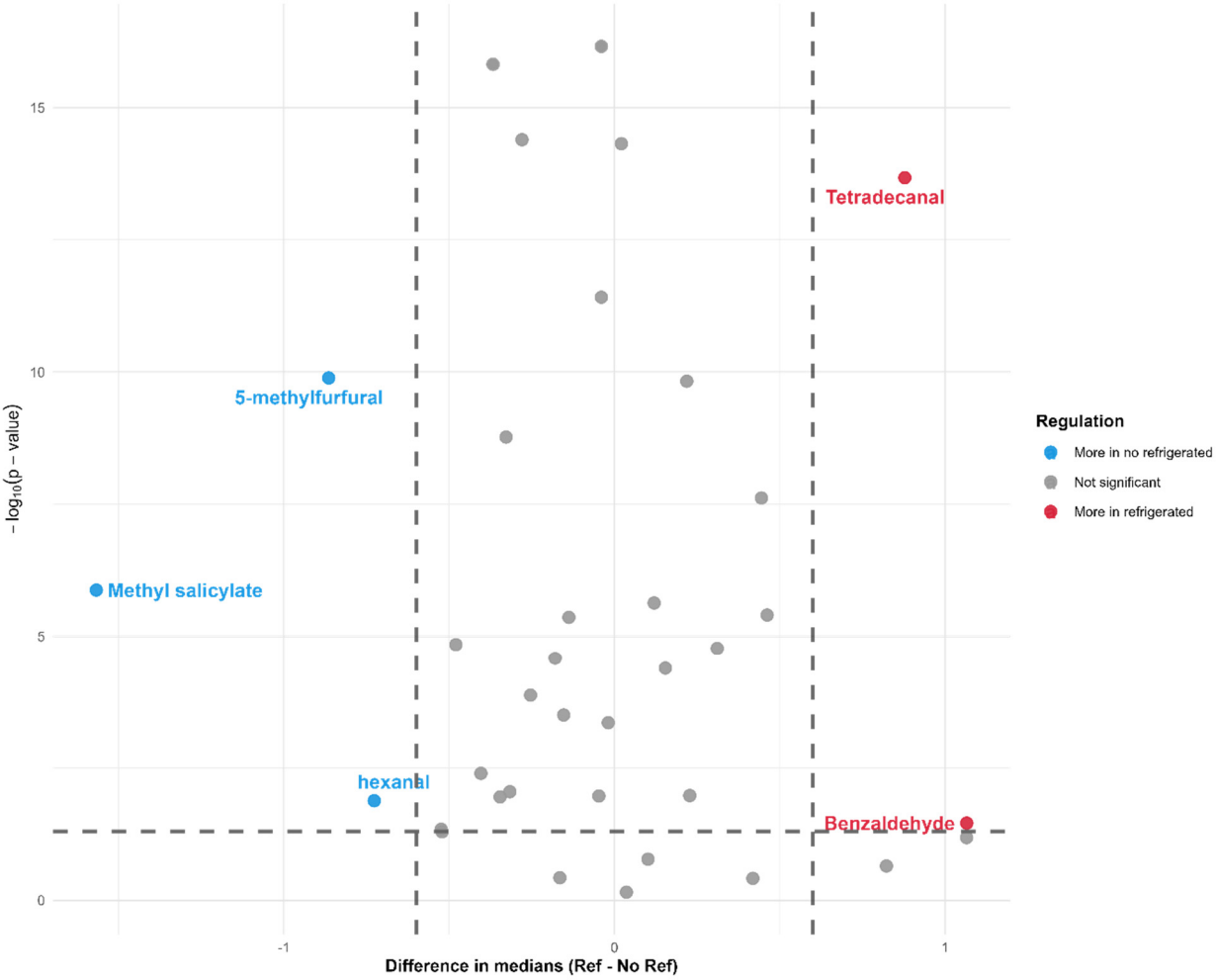


Figure 2. Volcano plot analysis of breath metabolites: comparison between frozen and room-temperature samples. Blue dots represent metabolites that are significantly more abundant in room-temperature samples, red dots indicate greater abundance in frozen samples, and gray dots correspond to metabolites with no significant changes.

3.4. Changes in the Chromatographic Profile of Samples Relative to Storage Conditions

Figure 3 displays chromatographic profiles of representative subjects, illustrating the predominant longitudinal trends in the cohort. It is important to highlight that the observable differences in peak magnitude at the initial time point (0 h) between panel A and panel B mainly reflect inter-individual biological variability of the volunteers. Figure 3A illustrates the comparatively greater stability of volatile metabolites under frozen storage: across the four evaluated time points (0 h, 3 h, 6 h, and 12 h), the main chromatographic peaks, at retention times of 8.1 and 12.0 min, remain largely overlapping, with intensities for the main peak staying in the range of 7.5×10^6. This temporal consistency indicates that freezing substantially preserves the chemical integrity of most breath VOCs; however, a modest decline in the intensity of some peaks was still observed even under frozen storage, consistent with the compound-specific (rather than uniform) degradation pattern

already described for individual metabolites in Table 2. Freezing at −20 °C should therefore be interpreted as markedly attenuating, rather than completely eliminating, oxidation, hydrolysis, and thermal degradation processes over the 12-h study period [35].

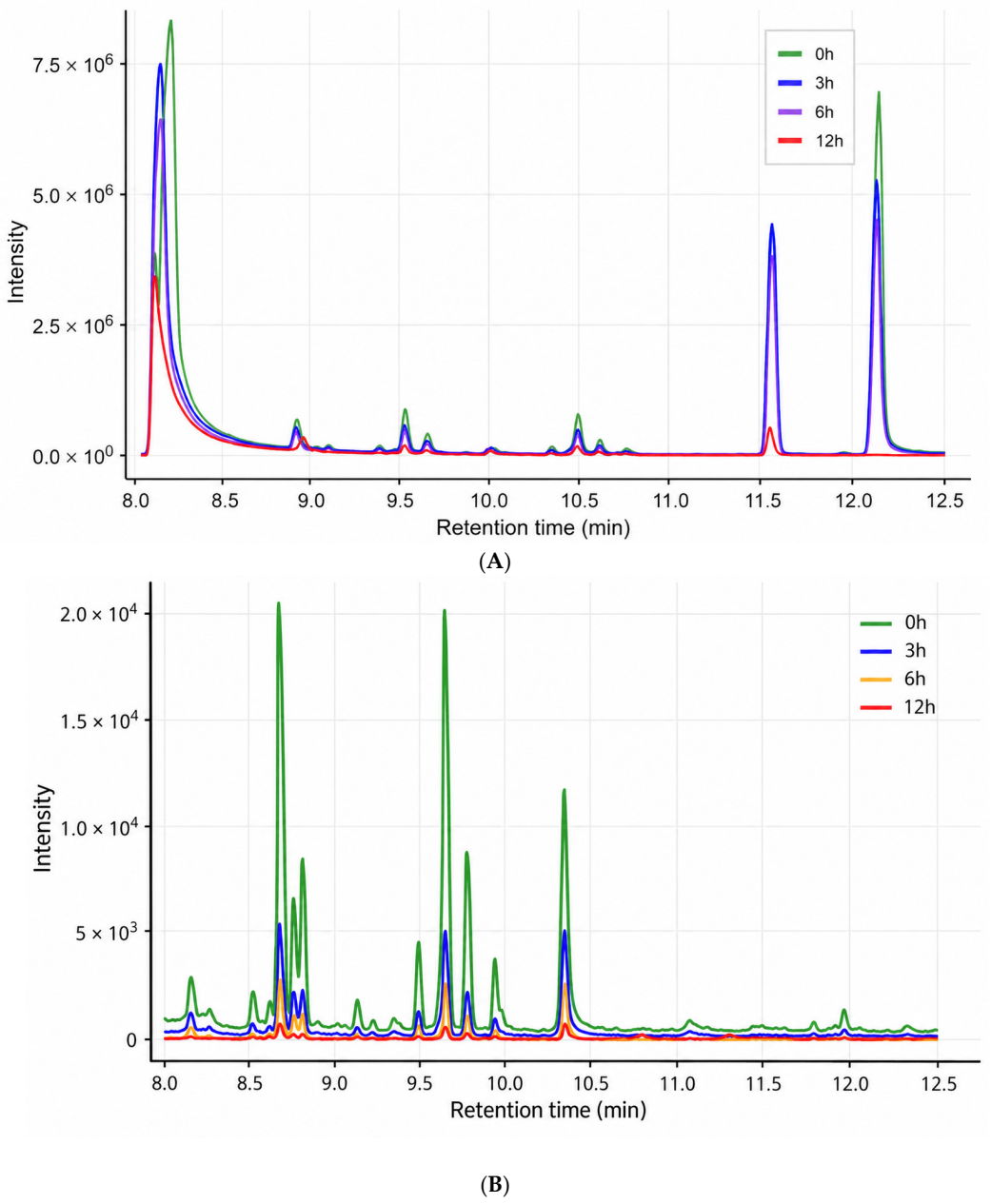


Figure 3. Chromatograms of exhaled breath samples analyzed using HS-SPME-GC-MS. (**A**) Exhaled breath sample cooled to −20 °C after sampling. (**B**) Exhaled breath sample stored at room temperature. Note: The chromatograms shown in panels (**A**,**B**) correspond to different individuals (given the unpaired study design); therefore, differences in absolute intensities at the initial time point (0 h) reflect normal inter-individual biological variability in exhaled breath. Degradation is evaluated by observing the relative decrease in signal within the same individual over time, rather than by comparing absolute peak heights between (**A**) and (**B**).

In contrast, Figure 3B illustrates the progressive degradation of volatile compounds at room temperature. At the initial time point (0 h, green line), a metabolite-rich profile is observed with multiple intense peaks between 8.3 and 10.2 min, reaching maximum intensities of 2.0×10^7. However, this metabolite concentration decreases over time. When

the analysis is performed at 3 h (blue line) post-sampling, intensities have dropped substantially, and by 12 h (red line), most of the peaks have disappeared almost completely, being reduced to baseline levels. Total Ion Chromatogram (TIC) analysis based on raw, unnormalized areas revealed that this visual loss represents a quantitative drop of more than 90% of the original overall analytical signal at room temperature. This massive loss of the total analyte mass in the headspace is consistent with previous studies reporting significant instability due to diffusion and reactivity of thermolabile VOCs [12,35].

The degradation observed under room-temperature conditions can be attributed to multiple simultaneous processes, such as the atmospheric oxidation of unsaturated compounds, accelerated volatilization due to increased vapor pressure, and potential interaction with condensed water vapor within the storage system [35,36]. The most susceptible compounds likely include aldehydes, short-chain ketones, and sulfur compounds, which are known for their low thermal stability [12,35].

The attenuation of degradation observed under freezing suggests that frozen storage for up to 12 h may be acceptable for the more stable analytes identified here, which would provide logistical flexibility for multicenter clinical studies; nevertheless, analysis as soon as possible after collection remains preferable, and compound-specific validation is advisable for metabolites identified as highly variable in Table 2, and data for these metabolites should be interpreted with caution [36–38].

3.5. Impact of Volatile Metabolite Instability on the Identification of Biomarkers for Clinical Diagnostic Use

The results of this research have demonstrated that VOCs present in breath samples are susceptible to post-sampling storage conditions. Although the analysis of these metabolites via HS-SPME-GC-MS requires minimal sample manipulation, the handling and storage of the sample prior to analysis represents a crucial step that directly impacts the results. The instability of VOCs represents a critical challenge for the reliable identification of potential biomarkers in breath metabolomics studies. Thermal degradation, oxidation, and the selective volatilization of certain metabolites can alter the chromatographic profiles of the samples, generating analytical artifacts that compromise the reproducibility of the results. Thermolabile compounds, such as volatile sulfur compounds (H_2S, CH_3SH) and aldehydes (hexanal, nonanal), show losses of 30–60% after 6 h at room temperature [22,39], while ketones such as acetone exhibit greater stability (<5% degradation at 4 °C) [38]. This variability in metabolite concentration and the formation of new VOCs depend on storage conditions, which introduces biases in cross-sectional studies, where apparent differences between groups could reflect pre-analytical instability rather than genuine physiological phenomena [39,40].

Sample collection and storage protocols are aspects that must be taken into account in the experimental design. Previous studies report that exhaled-breath VOCs stored under refrigerated conditions can retain the large majority of their original signal over periods of a week or more, whereas storage at room temperature leads to substantially faster losses within 48 h; however, reported retention percentages and time windows vary depending on the specific adsorbent or bag material, storage temperature, and compound class used in each study, so direct quantitative comparability with the present Tedlar®/−20 °C system should be interpreted with caution rather than as a strict equivalence. Furthermore, differential adsorption on materials such as Tenax TA® varies according to molecular polarity: non-polar hydrocarbons (e.g., isoprene) show recoveries of 95%, compared to 70% for polar alcohols (methanol, ethanol) under identical conditions. These technical factors can mask true biomarkers or generate false positives, particularly for low-concentration compounds (<1 ppb) [41,42].

To counteract these effects, it is advisable to implement storage temperature control, preferably freezing the samples at −20 °C immediately after collection. Chromatographic analysis should be performed within 6 h post-sampling. The use of stable standards is recommended for the correction of analytical losses. Once biomarker candidates for the studied pathology are identified, their degradation kinetics must be analyzed. This is necessary because metabolic instability is not uniform; while alkanes can maintain stable profiles for weeks, other compounds may degrade rapidly [22], whereas sulfur and oxygenated compounds require shorter analytical windows (<4 h) [38]. This demonstrates that biomarker validation in breathomics requires the integration of metabolomic stability studies. Consequently, standardized protocols that control preanalytical variables [39,41], will allow for the discrimination between technical fluctuations and biologically relevant signals for studies with subsequent robust diagnostic applications.

A limitation inherent to the longitudinal sampling design of this study should be noted. Due to the thermodynamic requirements of the HS-SPME technique, the frozen samples experienced brief heating cycles prior to each temporal analysis (3 h, 6 h, and 12 h) to ensure the release of the analytes into the headspace. Although these repeated thermal fluctuations could exacerbate the degradation of VOCs, the results showed that the samples subjected to freezing maintained superior stability compared to those kept continuously at room temperature. This suggests that storage at −20 °C remains beneficial even with repeated handling. In addition, because the same sample bag was used for all four time points, each preceding HS-SPME extraction itself removes a fraction of the remaining headspace analyte mass; this repeated-extraction effect constitutes an additional, non-isolated depletion source that is superimposed on genuine chemical degradation and cannot be fully disentangled from it using the current cross-sectional CV-based design.

4. Conclusions

This study on the stability of volatile organic compounds (VOCs) in exhaled breath samples highlights the critical importance of preanalytical control in clinical metabolomic research. The comparative analysis of the coefficient of variation (CV) confirms that storage and handling conditions constitute a determining factor to ensure the reproducibility of results and methodological robustness in precision medicine studies. Statistical analysis based on %CV shows that some constitutive baseline metabolites present high intrinsic stability (CV < 15%), regardless of storage conditions. This characteristic positions them as reliable references for comparisons between pathological states.

Furthermore, the stratification of VOCs according to their stability allows for the identification and exclusion of potential false positives derived from degradation processes, as well as the prioritization of compounds with lower variability as biomarkers of clinical interest. The identification of stable metabolites and the understanding of their variability patterns provide substantial evidence for the development of non-invasive diagnostic strategies. The implementation of these approaches can favor the early detection of pathologies through exhaled breath analysis, optimizing diagnostic sensitivity and specificity within a personalized medicine framework.

To strengthen analytical consistency, it is recommended to establish differentiated protocols that consider the selection of appropriate adsorbents and the application of cryopreservation during transport, especially in the case of mercaptans and aldehydes. Likewise, it is suggested to limit the interval between collection and analysis of furans to a maximum of 12 h. The systematic adoption of these measures will favor the standardization of sampling and analysis procedures, promoting the effective transfer of metabolomic research to the clinical setting.

The findings of this study indicate that immediate freezing at −20 °C significantly influences the cross-sectional profile of volatile compounds (30 of 38 metabolites showed FDR-adjusted $p < 0.05$). Specifically, the data suggest that cold maintenance attenuates the analytical signal loss suffered by thermolabile compounds at room temperature at 12 h, promoting lower statistical variability (CV reduction in 20 metabolites). However, since these CV calculations include inter-individual biological variability, the preanalytical response is heterogeneous and strongly dependent on the chemical family, with a subgroup of compounds (18 metabolites) presenting increased variability under frozen storage. These observations provide fundamental criteria for methodological standardization in clinical volatilomics.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/molecules31224178/s1, Table S1: Volatile organic compounds putatively identified in exhaled breath by HS-SPME-GC-MS.

Author Contributions: E.V.S.: Conceptualization, Data curation, Formal Analysis, Investigation, Methodology, Writing—original draft. G.T.O.: Investigation, Writing—review and editing, Supervision. J.A.J.G.: Project Administration, Supervision, Writing—review and editing. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: The raw GC-MS data and the processed data matrices supporting the findings of this study are available from the corresponding author upon reasonable request.

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