Gaseous reference standard for breath analysis

Laura Gagni [1*], *Giorgio Bissolotti,*[1]

[1] SIAD S.p.A., Gases of life District, Osio Sopra, Italia

Abstract. An increasing amount of research is going into detecting volatile organic compounds (VOCs) in exhaled breath to detect diseases and personalize treatment for patients: recently, these analytes has risen as potential biomarkers. The present work aims to examine different multi-component gas mixtures suitable for breath gas analysis. In addition, also the stability of these mixtures was investigated. The number of components tested were 31, divided in two gas mixtures A and B. These components are aldehydes, acetate compounds, aromatics compounds: the lists are available in the article. Gas components of mixture A and B were prepared in different concentration from 1 µmol/mol to 10 nmol/mol. All the mixtures were analytically verified and the expected analytical relative uncertainty is 10-15% for 10 nmol/mol mixture. The stability check is also performed after 1 year and 2 years. The mixtures demonstrated good stability, and tests are still on going.

1 Introduction

Research into volatile organic compounds (VOCs) in exhaled breath has grown substantially in recent years, as these analytes have emerged as valuable potential biomarkers for disease detection and personalized treatment. Once collected and analysed, VOCs can provide a rapid, accurate, non-invasive, and painless diagnostic tool for a wide range of diseases and health conditions.

However, breath samples are inherently challenging to collect because they are gaseous and can contain high levels of noise due to VOCs present in inhaled ambient air. For analytical purposes, gas chromatography–mass spectrometry (GC-MS) is widely recognized as the gold standard, as it enables the detection of chemically diverse compounds within complex mixtures with high sensitivity and across a broad dynamic range. GC-MS also allows accurate quantification of VOCs and provides high confidence in biomarker identification [1].

To ensure reliable results, the use of standards for calibrating detection systems is essential. This guarantees consistency across devices and prevents analytical drift over time. In practice, liquid-phase standards are more commonly used because they offer several advantages, including low cost, ease of handling, and suitability for a broad range of VOCs. They also allow the application of internal standard methods [2,3]. Nevertheless, research has shown that calibrating gaseous samples with liquid-phase standards can introduce substantial variability and measurement bias [4]. For this reason, gas-phase standards are often preferred for VOC analysis, as they help eliminate or reduce matrix effects by ensuring that both the sample and the calibration standard are in the same phase. Furthermore, gas-phase standard mixtures can be formulated to include all VOCs of interest, including isotopically labelled analytes at very low concentrations. The present work focuses on the preparation and analysis of multi-component gas mixtures suitable for breath analysis, with concentrations ranging from 1 µmol/mol down to 10 nmol/mol. The stability of these mixtures was also investigated. In total, 31 compounds were examined, divided into two gas mixtures. These compounds can be combined with other commonly used VOCs whose stability is already well established, even at very low concentrations.

2 Material and methods

2.1 Preparation of static standards

A total of 31 target compounds were selected and divided into two gas mixtures, A and B, to ensure their stability and to avoid incompatibilities among certain components. The compositions of the two mixtures are reported in Table 1 and Table 2. The producer prepared the mixtures starting from pure substances with the highest commercially available purity. All chemicals were GC-grade analytical standards from Sigma Aldrich, with stated purities of 98% or higher. Nitrogen was used as the balance gas, with a purity of 99.9995% mol, supplied by SIAD S.p.A.

The mixtures were filled into 10-litre aluminium cylinders made of 6061 alloy, supplied by Luxfer, and fitted with brass valves. Prior to filling, both the cylinders and the valves underwent a dedicated conditioning

* Corresponding author: laura_gagni@siad.eu

procedure designed to remove residual moisture and other impurities. Particular attention was given to the presence of water, both in the nitrogen used as balance gas and throughout the filling procedure. Following a gravimetric method inspired to ISO 6142-1, the mixtures were prepared by adding the components sequentially into the cylinders and weighing each addition using calibrated laboratory balances (Sartorius CCE60K2, Sartorius MAS5203S-100DR, and Sartorius MSA524S-100-DU)

Table 1. list of components in mixture A and relative CAS-Number

Mixture A		
N	Component	CAS Number
1	Styrene	100-42-5
2	Toluene	108-88-3
3	Deuterated Toluene	2037-26-5
4	(R)-Limonene	5989-27-5
5	Isoamyl Acetate	123-92-2
6	Perfluoro-2-methyl-3-pentanone	756-13-8
7	Acrolein	107-02-8
8	Acrylonitrile	107-13-1
9	Benzene	71-43-2
10	Deuterated Benzene	1076-43-3
11	Butyl Acetate	123-86-4
12	Cyclohexene	110-83-8
13	1,1-Dichloroethylene	75-35-4
14	Diethyl Ether	60-29-7
15	Ethyl Acetate	141-78-6
16	Fluorobenzene	462-06-6
17	1-Heptene	592-76-7
18	1- Hexene	592-41-6
19	Isoprene	78-79-5
20	Methyl Acetate	79-20-9
21	1- Octene	111-66-0
22	Propyl Acetate	109-60-4

Table 2. list of components in mixture B and relative CAS-Number

Mixture B		
N	Component	CAS Number
1	Valeraldehyde	110-62-3
2	Hexanal	66-25-1
3	Benzaldehyde	100-52-7
4	Octanal	124-13-0
5	Heptanal	111-71-7
6	Decanal	112-31-2
7	Nonanal	124-19-6
8	Isobutyraldehyde	78-84-2
9	Propionaldehyde	123-38-6

2.2 Production scheme

Gas mixtures A0 and B0 were prepared in two cylinders at a concentration of 2 µmol/mol. Starting from these two primary mixtures, additional diluted gas mixtures containing the same components were produced with gravimetric method. All mixtures were prepared in the cylinders described in paragraph 2.1, and at different times, in order to ensure process reliability and to enable stability testing. Further details are provided in Table 3.

Table 3. scheme of production of mixtures started from mixture A.

Mix code	Conc. Mix A in nitrogen	Mix code	Conc. final mixture in nitrogen
A0	2 µmol/mol		
A0 + N2	=	A1, A4	1 µmol/mol
A0 + N2	=	A5	100 nmol/mol
A0 + N2	=	A2	50 nmol/mol
A0 + N2	=	A3	10 nmol/mol

Mix code	Conc. Mix B in nitrogen	Mix code	Conc. final mixture in nitrogen
B0	2 µmol/mol		
B0 + N2	=	B1, B4	1 µmol/mol
B0 + N2	=	B5	100 nmol/mol
B0 + N2	=	B2	50 nmol/mol
B0 + N2	=	B3	10 nmol/mol

As mentioned earlier, the preparation of these gas mixtures was carried out at two different times. Mixtures A4, A5, B4, and B5 were prepared in August 2023, while all the others were produced in April 2023.

2.3 Analysis of gas mixture

All mixtures were analytically verified, and all results were positive. The instrument was calibrated using standards certified by SIAD S.p.A. Each mixture was also subjected to cross-verification, both against the other mixtures and against their parent mixtures A0 and B0. A GC-FID system (Agilent Technologies) was used to quantify all components. Separation was achieved using a Vocol column (Supelco), and the oven temperature was programmed from 40 °C to 200 °C to ensure adequate resolution of the analytes. Special attention was given to the sampling procedure to guarantee that a representative amount of gas reached the GC, thereby preventing losses of high-molecular-weight compounds. All components exhibited good chromatographic resolution, with the exception of diethyl ether and isoprene, which co-eluted due to identical retention times.

2.4 Stability tests

The stability assessment was carried out using mixtures A4, A5, B4, and B5. These mixtures were analysed following the methodology described in paragraph 2.3. They were certified in August 2023 and subsequently analysed in April 2024 (corresponding to 8 months of stability) and again in January 2026

(29 months of stability). All mixtures were verified through cross-comparison, pairing A4 with A5 and B4 with B5.

3 Result and discussion

3.1 Preparation results

The gas mixtures produced at different concentration levels have a target concentration, a gravimetric concentration and an analysed concentration. For all the mixtures and components, the agreement was very good with differences below 15 % as shown in Fig. 1 and Fig. 2. The difference between analytical value (x_a) at the time of production and the gravimetric value (x_g) for each component i is represented by the evaluation of the parameter D_i:

$$D_{\mathrm{i}} = (x_g - x_a)/x_g\% \quad (1)$$

Fig. 3 shows the results for mixture A4. The values of D_i exhibit an acceptable and fairly symmetrical distribution around zero: twelve components have D_i values below zero, while nine components show values above zero. Fig. 4 presents the results for mixture B5. In this case, the distribution is more unbalanced: six components display analytical concentrations significantly lower than their gravimetric values. The remaining components — propionaldehyde, isobutyraldehyde, valeraldehyde, and caproaldehyde— show good agreement between gravimetric and analytical concentrations.

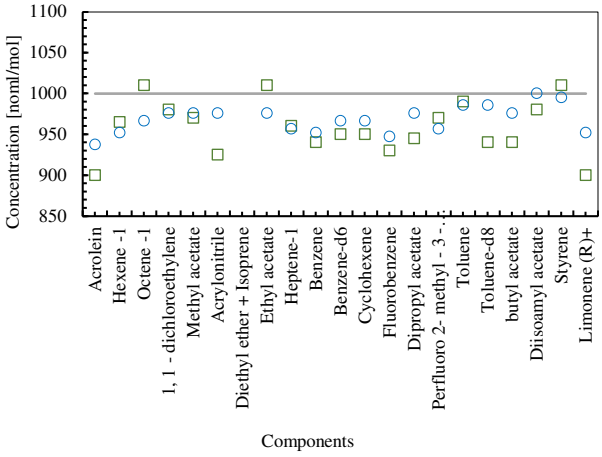

Fig. 1. For mixture A4 comparison of target concentration (line), gravimetric concentration (symbol □) and analytical concentration (symbol ○).

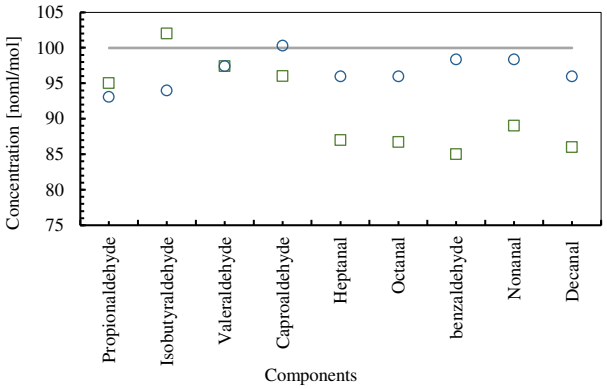

Fig. 2. For mixture B5 comparison of target concentration (line), gravimetric concentration (symbol □) and analytical concentration (symbol ○).

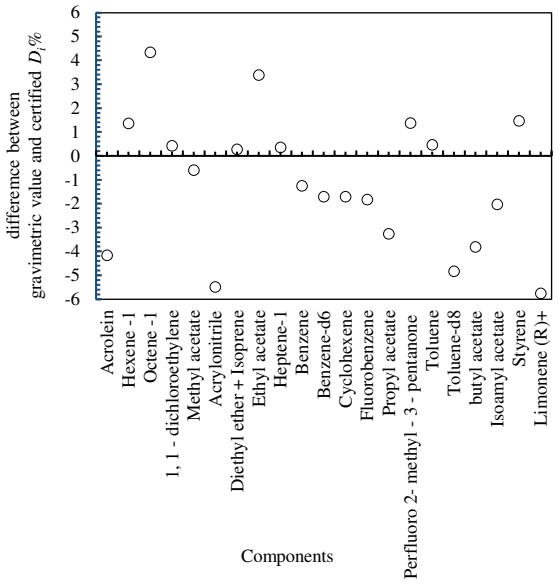

Fig. 3. For mixture A4 parameter D_i (symbol ○).

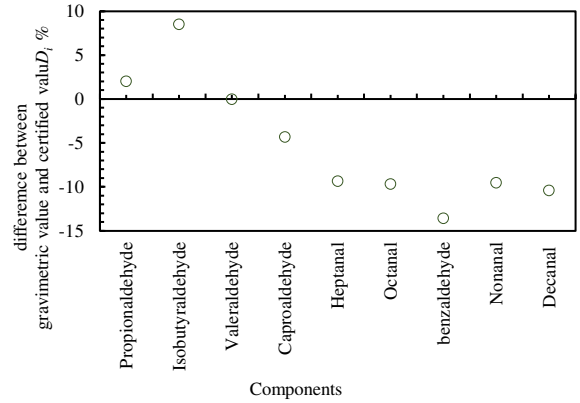

Fig. 4. For mixture B5 parameter D_i (symbol ○).

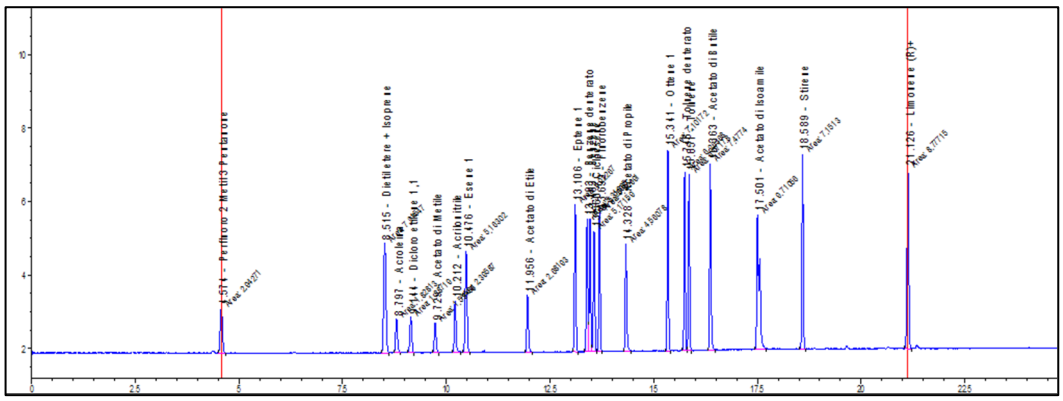

Fig. 5 GC- FID chromatogram for mixture A4.

All components showed good chromatographic resolution as shown in Fig. 5 for mixture A4 and Fig. 6 for mixture B5.

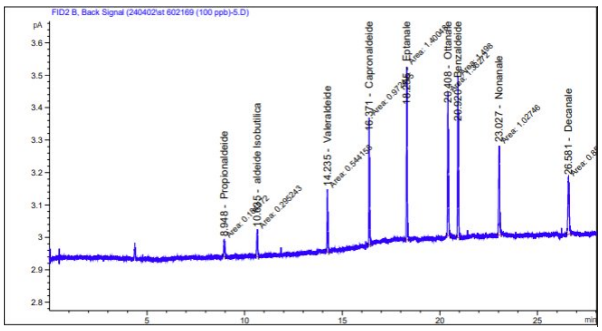

Fig. 6. GC- FID chromatogram for mixture B5.

3.2 Stability results

All the mixtures were verified with a cross control between them A4 with A5 and B4 with B5. This approach could be possible thanks to one of the most important properties of the FID : its extreme long linear range. The linearity has its limitations according to studies dealing with the linear behaviour of the detector response [5–7]. The mixture A4 was used to calibrate the instrument and analyse mixture A5. Fig 7. show the results.

All the components have a concentration of approximately 100 nmol/mol. The analysis performed in August 2023, April 2024 and January 2026 show excellent agreement for all components except ethyl acetate, propyl acetate and butyl acetate. For these components the consistency is observed only between the two later analyses (April 2024 and January 2026), while the certified value from August 2023 is lower than the values obtained during the stability assessments.

The results confirm the stability of great part of components in the mixtures which maintain their stability for more than 2 years at both levels of concentration. However, further investigation is required to better understand the behaviour of acetate compounds.

The mixture B5 was used to calibrate the instrument and analyse mixture B4. Fig 8. show the results.
The data indicate good stability for isobutylaldehyde and propionaldehyde while some critical issues emerge for high molecular weight aldehydes. In particular:

- for valeraldehyde, capraldehyde and benzaldehyde agreement is observed only

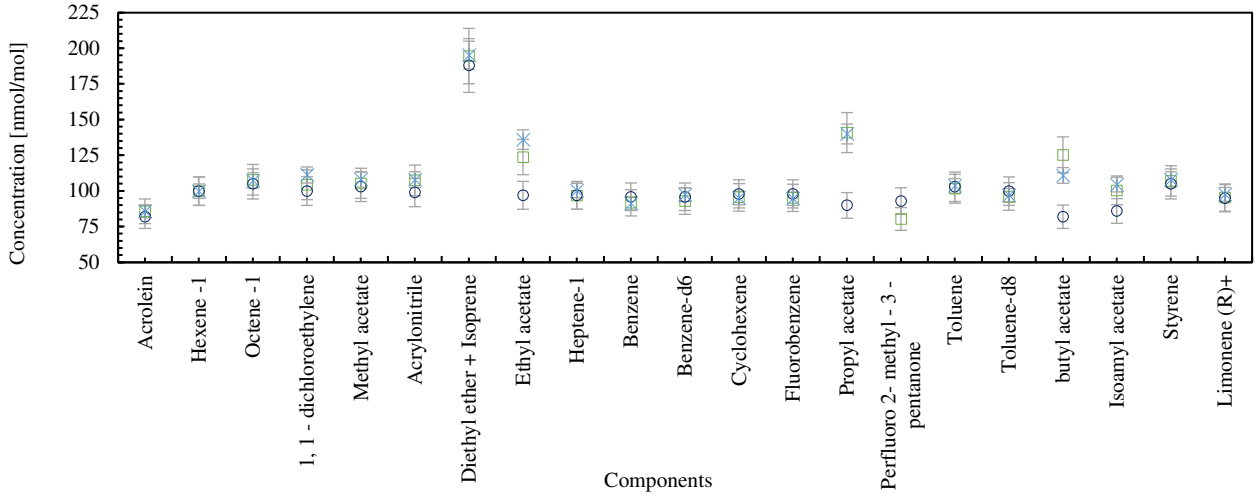

Fig. 7. Concentration of mixture A5 in nmol/mol: certified value (08/2023) (symbol ○); stability analysis value (04/2024) (symbol □); stability analysis value (01/2026) (symbol ×).

between the most two recent analysis (April 2024 and January 2026) whereas the certified value from August 2023 is higher than the stability measurements.

- Heptanal, octanal, nonanal and decanal exhibit a continuous decrease in concentration over time, indicating that these components are not stable.

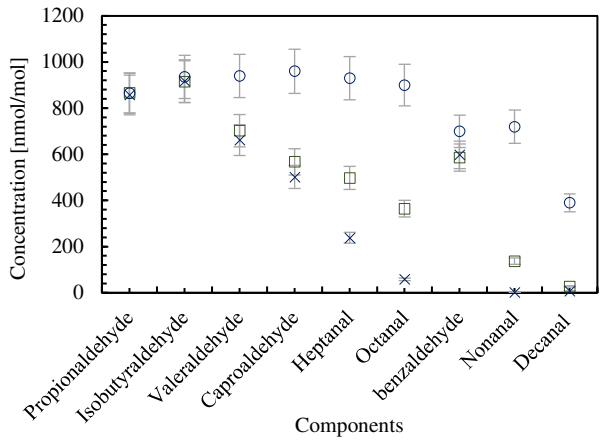

Fig. 8. Concentration of mixture B4 in nmol/mol: certified value (08/2023) (symbol ○); stability analysis value (04/2024) (symbol □); stability analysis value (01/2026) (symbol ×).

These findings are consistent with the results obtained during mixture preparation. The parameter D_i for the higher-molecular-weight aldehydes (heptanal, octanal, nonanal, and decanal) was approximately −10% or lower, suggesting that these compounds may undergo partial adsorption or degradation even shortly after preparation. This behaviour likely contributes to the observed instability during long-term storage. That indicates different possible scenario:

1. The theoretically weighed amount of product did not fully enter the cylinder and was partially lost during the filling operations.
2. The cylinder was correctly filled and the entire weighed amount was transferred, but the sampling procedure did not allow the full quantity to reach the GC-FID detector, resulting in an apparently lower concentration.
3. The components inside the cylinders were not stable, and their concentrations gradually decreased over time.

Further studies are required to better understand the behaviour of high-molecular-weight linear aldehydes, considering additional variables such as different concentration levels during stability testing..

4 Conclusion

The present study demonstrates the feasibility of producing stable calibration gas mixtures for breath analysis. The mixtures developed include aldehydes, acetate compounds, aromatic compounds, alkenes, and deuterated species—representative of the key VOC classes commonly investigated in breath research. Very low concentration levels can be achieved, with stability maintained for more than two years. Further work will focus on elucidating the behaviour of acetate compounds and higher-molecular-weight aldehydes, for which specific issues were observed.

Additionally, deeper interpretation may be obtained through supplementary analysis of the raw chromatographic data, taking advantage of the proportional relationship between FID signal intensity and the carbon content of hydrocarbons

References

1. White paper, Breath Biopsy® OMNI®: Advanced Global Breath VOC Analysis.
2. M.A. Iqbal, K.H. Kim, J.E. Szulejko, M.M. Rahman The selection of the standard phase (gas vs. liquid) and the related matrix effect on the direct injection gas chromatographic analysis of VOCs at sub–ppm levels. Atmos. Pollut. Res., 5 (3) (2014), pp. 563-571, 10.5094/APR.2014.065
3. C. Liaud, N.T. Nguyen, R. Nasreddine, S. Le Calvé. Experimental performances study of a transportable GC-PID and two thermo-desorption based methods coupled to FID and MS detection to assess BTEX exposure at sub-ppb level in air.
   Talanta, 127 (2014), pp. 33-42, 10.1016/j.talanta.2014.04.001 J.
4. Y.H. Kim, K.H. Kim: Effect of standard phase differences between gas and liquid and the resulting experimental bias in the analysis of gaseous volatile organic compounds. Anal. Chim. Acta, 714 (2012), pp. 98-103, 10.1016/j.aca.2011.11.042
5. McWilliam, I. G. "A study of the flame ionization detector", Journal of Chromatography A, 51, pp. 391–406, 1970. https://doi.org/10.1016/S0021-9673(01)96888-8
6. Slemr, J., Slemr, F., D'Souza, H., Partridge, R. "Study of the relative response factors of various gas chromatograph–flame ionisation detector systems for measurement of C2 –C9 hydrocarbons in air", Journal of Chromatography A, 1061(1), pp. 75–84, 2004. https://doi.org/10.1016/j.chroma.2004.10.037
7. Bromly, J. H., Roga, P. "Nonlinearities in Flame Ionization Detector Response", Journal of Chromatographic Science, 18(11), pp. 606–613, 1980 https://doi.org/10.1093/chromsci/18.11.60