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In Vivo Detection of Nitrous Oxide in Blood and Saliva Following Recreational Use

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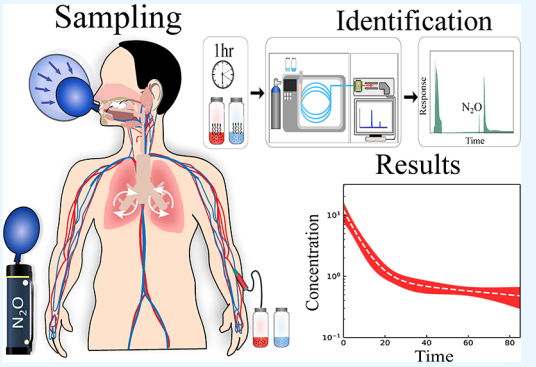
ABSTRACT: Nitrous oxide (N_2O) is commonly used as a recreational drug, and the Dutch police have reported numerous traffic incidents related to its misuse. At the same time, the *in vivo* pharmacokinetics of N_2O remain underexplored. In light of these facts, this study aimed to detect and quantify N_2O in blood and saliva after inhalation exposure of a recreational dose. Consequently, a pilot *in vivo* study was conducted with six participants inhaling N_2O using a party balloon, comparable to urban habits. A headspace gas chromatography-mass spectrometry method was used to detect N_2O levels in blood and saliva. N_2O was detectable in both blood and saliva for over 60 min post-exposure. Concentrations in blood followed a two-phase exponential decay with a crossover time of 16.7 ± 4.7 min between the two phases. Calculated blood concentrations were found at 0.4−20 mL of N_2O/L blood. Salivary concentrations did not exhibit an exponential decay pattern and were only clearly distinguishable from baseline levels during the first 15 min, as low N_2O concentrations were also detectable in saliva of participants who had not inhaled N_2O gas. This points toward the presence of endogenous sources of N_2O giving rise to detectable concentrations in the oral cavity. This study underscores the possibility of using blood for detecting recent N_2O inhalation and highlights the need to consider background levels in saliva when interpreting results. Further research is necessary to elucidate the mechanisms underlying baseline concentrations of N_2O in the saliva matrix.



INTRODUCTION

The recreational use of nitrous oxide (N_2O) has increased in Europe since 2000, starting with small-scale whipping cream canisters.[1] The introduction of large N_2O tanks in recent years has contributed significantly to its increase in misuse.[2] Known as "laughing gas", N_2O is popular for its rapid euphoric and dissociative effects, usually achieved by inhaling 100% gas from a party balloon filled from whipping cream canisters or N_2O tanks.[1] Excessive misuse poses serious health risks, including neurotoxicity, thromboembolic events, and asphyxiation.[3] Additionally, disorientation and loss of motor control resulting from N_2O inhalation pose substantial dangers when driving or operating machinery.[4] In line with this, there has been an increase in (inter)national media and police reports concerning impaired driving, where the Dutch National Police have reported more than 900 traffic accidents involving serious injury or fatal outcomes.[5,6]

The increase in N_2O related problems has compelled the Netherlands, Denmark, and Belgium to implement bans on recreational use of nitrous oxide use. Also, in the United Kingdom (U.K.), the possession of N_2O for recreational use was banned in 2023.[7] Recreational N_2O usage is legal in most countries, where N_2O is readily available via online retailers, catering outlets, festivals, and clubs at low prices, most likely adding to N_2O's popularity.[8] In 2019, N_2O was identified as the 10th most prevalent drug of abuse by the global drug survey, with the Netherlands and the U.K. being the most prominent countries in terms of recreational use.[9] Consequently, the need for appropriate screening tools or roadside tests for Driving Under Influence of Nitrous Oxide (DUINO) is growing. However, knowledge of the pharmacokinetics of N_2O and the mechanisms underlying behavioral impairments remains incomplete, and the exact pathways through which nitrous oxide exerts behavioral effects have yet to be fully elucidated.

In this respect, decades ago, Moyes and co-workers reported residual impairment in the postacute phase that lasted for up to 30 min.[10] Moyes and co-workers found that the driving skills of dental students, who were exposed to medical N_2O (50 or

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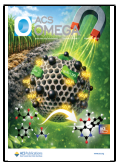

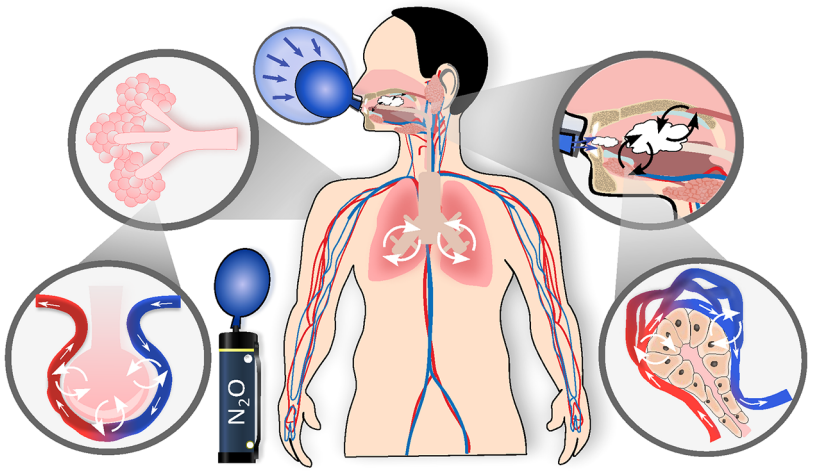

Figure 1. In this study six participants inhaled N_2O through a balloon, the N_2O entered and rapidly equilibrated with blood through the alveoli of the lungs. Similarly, N_2O equilibrated with saliva and the salivary glands either through direct contact or via blood. Saliva and blood samples from the participants were collected, and N_2O concentrations were quantitatively determined using HS-GC-MS.

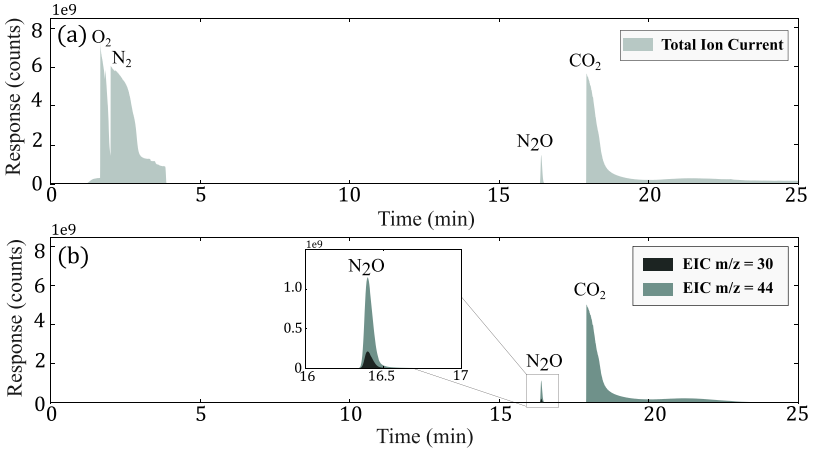

Figure 2. (a) A chromatogram showing the TIC of a spiked sample with a calculated sample concentration of 1 mL/L N_2O. (b) A chromatogram showing the EIC of m/z = 30 and 44 of the same sample. The retention times of O_2, N_2, N_2O, and CO_2 were 1.8, 2.4, 16.4, and 18.1 min, respectively.

70% N_2O in O_2), worsened after exposure.[10] Specifically, errors significantly increased following exposure to N_2O, particularly during the middle of the test period, i.e., 10−15 min postexposure.[10] In their study, blood N_2O concentrations were not determined. In turn, more recently, blood samples from traffic cases were retrospectively studied by Lindholm and co-workers using headspace gas chromatography coupled with mass spectrometry (HS-GC-MS).[11] N_2O was detected in 52/62 samples, with calculated sample concentrations ranging from 0.1 to 48 mL of N_2O/L blood. Yet, their detected concentrations could not be linked to time and magnitude of post-exposure.[11]

Demonstration of recent N_2O in a roadside drug test necessitates confirmation of the molecule's presence in a suspect-derived biofluid such as blood, saliva, urine, or exhaled breath.[12] Biofluids including blood and saliva have been shown to be suitable, and generally accepted matrices for the screening and determination of drug use.[13−15] Unlike other common drugs such as cocaine, (meth)amphetamine, heroin, or cannabis, N_2O is absorbed and distributed in the body as the result of pressure gradients and equilibrium of gas tension in the alveoli, blood, saliva, and surrounding tissues.[16] Owing to the low solubility of N_2O in blood, equilibrium is reached quickly, leading to rapid pharmacodynamic effects.[17] In the current pilot study, which included six participants, we investigated the relationship between the alveolar N_2O concentration and the N_2O concentrations measured in saliva and blood following exposure to a recreational dose using HS-GC-MS (Figure 1).

The most commonly utilized analytical technique for measuring N_2O is GC-MS in an operational forensic setting, which involves manual collection, transport, and storage of samples.[18,19] For example, studies of exposure of operating theater staff to ambient N_2O successfully analyzed N_2O using GC-MS on urine, blood, and exhaled breath samples.[20,21] In 2014 Giuliani et al. described a method for the quantification of N_2O in biological samples collected from a lethal intoxication due to recreational inhalation of N_2O using HS-GC-MS.[22]

In our previous work, we investigated the longevity of N_2O in exhaled breath of 24 participants in a clinical trial for screening purposes in forensic and law-enforcement applications.[6] In that study, exhaled breath samples were collected from participants and analyzed using infrared spectroscopy to

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quantify the concentration of gaseous N_2O. The results indicated that the exhaled breath samples contained N_2O concentrations of approximately 100 ppm, even 100 min after administration. As part of the same research program, the current pilot study focused on examining the persistence of N_2O in the blood and saliva of six participants. This current pilot study primarily aimed to evaluate the feasibility of detecting N_2O in blood and saliva postrecreational use and to explore the potential applications of HS-GC-MS in forensic and roadside testing scenarios.

RESULTS

In Vivo N_2O Concentration

In Figure 2a, a chromatogram of a N_2O (g) sample in water with a calculated N_2O (g) sample concentration of 1 mL/L is shown. The neutrals of O_2, N_2, N_2O, and CO_2 were effectively separated over the column without necessitating any special sample preparation. Figure 2b shows the extracted ion chromatograms of m/z = 30 and 44. The raw chromatograms at m/z 30 and 44, together with the corresponding mass spectra of a representative blood sample, are provided in the Supporting Information (Section: GC-MS Data).

A total of 72 saliva samples and 60 blood samples were collected from 6 (5 male and 1 female) participants of a meanage of 26 ± 4 years, an average weight of 77 ± 15 kg, and an average height of 176 ± 4 cm. The samples were weighed, producing a mean sample mass of 1.4 ± 0.8 g for saliva samples and 3.0 ± 0.6 g for blood samples. Of the six participants, three reported occasional smoking, one reported daily smoking, and two reported never smoking. No other relevant medical history was indicated by the participants.

The resulting calculated sample concentrations of the saliva samples are presented in Table 1. Figure 3a shows the calculated sample N_2O blood concentrations of the combined single and double exposures, including the 95% confidence interval. The blood N_2O concentration decay pathway can be characterized by two compartments. The concentration followed an exponential decline from a calculated sample concentration of 20 mL/L in Figure 3a, region I, followed by a slower decay down to a calculated sample concentration of 0.4 mL/L in Figure 3a, region II. No noticeable differences between the single dose in Figure 3b and the double dose in Figure 3c could be observed.

Table 1. Calculated N_2O Concentrations in Saliva and Blood Samples of Participants, Grouped by Time Range[a]

time range (min)	time $t_{subjects}$ ± SD (min)	single + double $C^0_{N_2O}$ ± SD (mL/L)	placebo $C^0_{N_2O}$ ± SD (mL/L)
		Saliva	
0–15	2.0 ± 0.0	13.4 ± 3.6	0.7 ± 0.4
16–30	18.7 ± 1.2	2.9 ± 2.2	1.8 ± 1.4
31–45	35.3 ± 0.9	2.1 ± 2.1	1.5 ± 1.5
46–60	55.7 ± 1.6	1.7 ± 1.7	0.8 ± 0.6
>60	74.2 ± 5.0	0.9 ± 0.6	
		Blood	
0–15	1.6 ± 0.5	13.6 ± 4.7	0.022 ± 0.003
16–30	18.9 ± 1.6	2.4 ± 2.1	0.022 ± 0.005
31–45	36.7 ± 3.5	1.6 ± 1.9	0.026 ± 0.004
46–60	64.3 ± 9.3	1.2 ± 1.3	0.021 ± 0.003
>60	73.2 ± 4.2	1.1 ± 1.1	
	response lab air (counts)	response blood matrix (counts)	$C^0_{N_2O}$ in blood matrix (mL/L)
mean (n = 3)	6186 ± 490	5916 ± 853	0.023 ± 0.002

[a]Single- and double-dose conditions are combined. For t > 60 min, incidentally a sample was available, as data collection was primarily focused on the first hour postexposure. The bottom section reports GC-MS baseline responses in laboratory air and in the blood matrix, including the corresponding calculated concentration for the blood matrix.

To describe the concentration of N_2O gas in blood we employed a series two-compartment model of the respiratory system.[23−25] For describing N_2O in blood, the model was extended including the gas exchange between the alveoli and the pulmonary veins. The exchange mechanisms between the salivary glands, the veins, and the upper respiratory tract is not included in the model.

The two-compartment series model of the respiratory system assumes an uneven distribution of N_2O gas in the lungs. The series gas redistribution model is made of two compartments, V_1 and V_2, connected in series and coupled to the ambient air. In Figure 1, a schematic representation is shown. The distal volume V_1 represents the lumping of homogeneous alveoli, and V_2 represents the volume present in the trachea up to the upper respiratory tract.[25] The distal volume is connected to the proximal volume by a gas-exchange rate λ_1. The proximal volume is coupled to the ambient air; this is controlled by the exhalation gas-exchange rate λ_2. Furthermore, we model the pulmonary gas exchange at the alveoli using the Ostwald blood-gas partition coefficient κ.[26] The N_2O in the alveoli is in equilibrium with the pulmonary capillary, i.e., the blood−air barrier between the alveolar air and the pulmonary capillary.

The full derivation of this model is shown in the Supporting Information and results in two separate regions that can be defined by their half-life: the first region (I), dominated by a (fast) exhalation process, and the second region (II), described by gas exchange between the alveoli and the upper respiratory system.[27] The time-dependent decline of N_2O concentration in the blood phase following exposure was described by a biexponential decay model (eq 1).

In this model, the first term ($C^0_{bl}\, e^{-\tilde{\lambda}_1 t}$) represents the rapid washout phase dominated by alveolar exchange and elimination from highly perfused tissues, while the second term ($C^1_{bl}\, e^{-\tilde{\lambda}_2 t}$) accounts for the slower release of N_2O from poorly perfused compartments back into the circulation. For the present data, the fitted parameters were C^0_{bl} = 11.0 ± 2.0 mL/L, C^1_{bl} = 0.89 ± 0.34 mL/L, $\tilde{\lambda}_1$ = 0.159 ± 0.032 min^{-1} (fast phase), and $\tilde{\lambda}_2$ = 0.0072 ± 0.0063 min^{-1} (slow phase).

$$C^0_{N_2O}(t) = C^0_{bl}\, e^{-\tilde{\lambda}_1 t} + C^1_{bl}\, e^{-\tilde{\lambda}_2 t} \quad (1)$$

The crossover time between the two phases, τ_{12}, is given by eq 2, corresponding to the point at which both terms in eq 1 contribute equally to $C_{bl}(t)$. For the present data, the τ_{12} was found to be 16.7 ± 4.7 min.

$$\tau_{12} = \frac{\log(C^1_{bl}) - \log(C^0_{bl})}{\tilde{\lambda}_1 - \tilde{\lambda}_2} \quad (2)$$

Figure 3a displays the decay of the calculated sample N_2O concentration in blood over time in the samples collected from participants who received either a single or double dose of N_2O. The concentration decay of the single dose is shown in Figure 3b and the concentration decay of the double dose is

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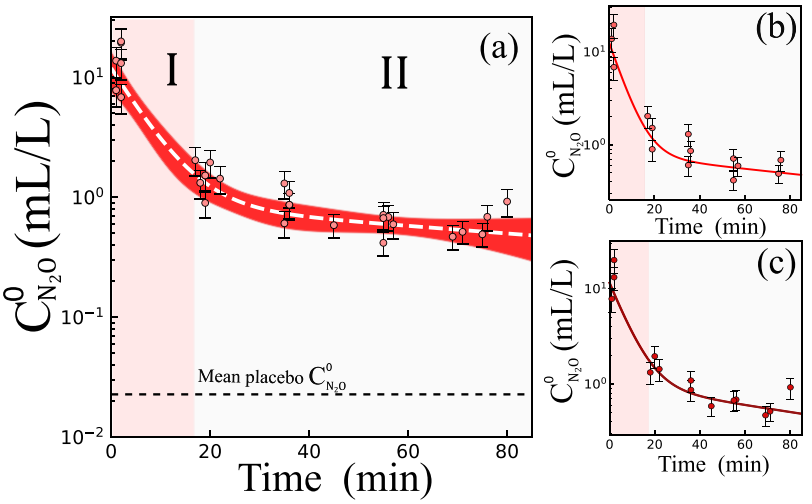

Figure 3. (a) Calculated sample N_2O concentration $C^0_{N_2O}$ (mL/L) in whole blood of the participants (n = 6) monitored in time after inhalation of N_2O at t = 0. The calculated sample N_2O concentration follows a two-phase exponential decay over time (white-dashed). (b) The fast decay process is depicted by region I (pink). (c) The slower decay process is depicted by region II (gray). The red area is the 2σ confidence interval, and the black dashed line is the mean concentration of the placebo group.

shown in Figure 3c. The calculated sample concentrations of all samples ranged between 0.4 and 20 mL/L.

DISCUSSION AND CONCLUSION

The goal of this study was to evaluate the feasibility of detecting N_2O in blood and saliva following a representative recreational dose and to explore potential forensic and law-enforcement applications by analyzing saliva and blood samples from six participants. In our previous work, we have demonstrated that N_2O can be detected in exhaled breath beyond 60 min after administration, suggesting that breath analysis may be a viable method for detecting recent N_2O use.[6]

At each sampling interval, both blood and saliva samples were collected. In total, 72 saliva samples and 60 blood samples were obtained. The difference in the number of samples between matrices was due to practical and participant-related constraints. For blood, several participants were unable to provide all intended samples, owing to difficulties with venous access. For saliva, in a few instances, participants experienced a dry mouth, which prevented the collection of sufficient material for analysis. In addition, very few samples were collected beyond the 1 h time point, as the operating theater was available for a maximum duration of 1 h.

To date, to the best of our knowledge, no published studies have systematically explored the detection of N_2O in blood or saliva following a representative recreational dose. Previous work by Lindholm and co-workers demonstrated that N_2O could be detected in blood samples obtained from individuals suspected of DUINO, with concentrations ranging from 0.1 to 48 mL/L using HS-GC-MS. However, due to the retrospective nature of their study, the timing of N_2O exposure relative to sampling remained unknown, limiting interpretation in a forensic setting.[11]

The present study demonstrates the feasibility of detecting N_2O in blood following recent recreational use by employing an HS-GC-MS method within 1 h after inhalation. Nevertheless, confirmation in larger cohorts and in real-world samples will be necessary before these findings can be reliably translated to roadside testing.

The calculated concentrations that we have found in our population of participants (0.4−20 mL/L) were within the same order of magnitude as those reported by Lindholm et al.[11] Although we measured blood concentrations within 1 h after exposure, when higher levels would be expected, we nevertheless observed lower concentrations. This discrepancy may be attributable to several factors, including differences in calibration procedures, interindividual variability, sample temperature, or methodological sensitivity, as well as our relatively small sample size. Sample analysis was conducted at lab temperature (18−22 °C); however, the actual sample temperature during GC-MS analysis was neither measured nor used for any correction. Because ambient air already contained analyte concentrations above the method limit of detection (LOD), all blanks reflected the true background rather than a zero-analyte condition. Consequently, very low-level sample results close to this background should be interpreted with caution, and a formal statistical LOD based on replicate analyte-free blanks could not be established under these conditions.

Since these aspects were not specifically examined in the present study, the exact cause remains uncertain. Further research addressing calibration approaches, interindividual differences, and methodological sensitivity in larger cohorts will be necessary to clarify these deviations.

The results of our study suggest that N_2O in blood might follow a similar decay profile as observed for exhaled breath.[6] This apparent biexponential decay likely reflects an initial rapid elimination phase followed by a slower release phase; however, this interpretation is based on general pharmacokinetic principles rather than being directly evidenced by our experimental data. The limited number of blood samples in this study, combined with unknown interindividual differences in breathing patterns, resulted in high uncertainties when modeling the slow decay process.

Detection of N_2O in saliva was also investigated in the present study, as saliva is a commonly used matrix for recent drug detection due to the noninvasive nature of the sampling procedure.[13] Based on the conditions applied in this study, saliva appeared to be an unsuitable matrix for N_2O detection

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following recent recreational use using the HS-GC-MS method. Even though sample collection is less invasive for saliva compared to blood, the interpretation of N_2O concentrations in saliva in our study was more complex due to the presence of relevant background levels. Surprisingly, these relevant background calculated sample concentrations in the range of 1.7−2.1 mL/L N_2O were found in the saliva of participants before exposure to N_2O and in the saliva of participants undergoing the placebo condition (Table 1).

This background concentration is possibly the result of microbial activity in the oral cavity.[28] Previous studies found that N_2O can be produced endogenously in the oral cavity through microbial activity in dental plaque, where salivary nitrate (NO_3^-) is metabolized via denitrification, resulting in the formation of nitric oxide (NO), N_2O, and dinitrogen (N_2).[29,30] This background concentration of N_2O complicates the forensic distinction between users with low concentrations and nonusers with elevated background levels.

The suitability of saliva as a forensic biofluid for N_2O detection requires extensive baseline studies across diverse populations to refine interpretation of these results in forensic settings. Consequently, although saliva collection is non-invasive, testing for N_2O in this matrix may be less suitable for forensic or law-enforcement applications compared to blood testing using the methodology employed in our study. The suitability of alternative saliva collection methods was not assessed in this study; however, such methods may help to reduce background concentrations of endogenous N_2O, which could interfere with the measurements. The study of Habobe et al. showed that distinct saliva collection methods can show significantly different results on biomarker concentrations.[31]

Future research on N_2O should focus on obtaining a more detailed understanding of the presence of the compound in blood, through measurements at shorter intervals immediately following use and extending beyond the 1 h period applied in this study. The small sample size (n = 6) for two dose conditions and a placebo control in this pilot study limits the generalizability of the findings. Future studies should aim to include a larger and more diverse participant pool to improve statistical robustness and applicability. We therefore recommend increasing the number of participants and collecting prestudy information on participants' diet and oral health, particularly for saliva analysis. Finally, a dose−effect relation of N_2O should be established in order to relate driving impairment to blood or exhaled breath concentrations.

METHODS

Study Design

The current investigation was part of a single-blind, randomized placebo-controlled, crossover clinical trial that was conducted in an operating room of the Leiden University Medical Center (LUMC) from September 2021 to November 2023 by Jiménez et al.[6] Medical-ethical approval was obtained from the medical ethics committee of the academic hospital Maastricht, and Maastricht University (Registration date: 28-01-2022, Trial Number: EudraCT 2021-003242-20), and the study protocol and procedures were executed and supervised by researchers from Maastricht University in accordance with the Declaration of Helsinki (1964) and its most recent amendments (2013). The blood and saliva samples were acquired from six participants for N_2O analysis in this study at time intervals of 10−15 min for 1 h. In this study, participants were exposed to three ad libitum dosing conditions, i.e., placebo (A), single (B), and double (C) as shown in Table 2. All participants in this study underwent all three dosing conditions of the study on three separate test days.

Table 2. Description of the Three Experimental Dosing Conditions

condition	type of experiment	description
A	placebo	participant receives two doses of compressed air (4 L)
B	single dose of N_2O	participant receives one dose of compressed air (4 L), followed by one dose of N_2O (4 L/8 g)
C	double dose of N_2O	participant receives two doses of N_2O (4 L/8 g per dose)

N_2O Analysis

A HS-GC-MS method consisting of preparation, equilibration, and identification steps was developed to separate and quantify N_2O. Calibration standards were prepared from a 100% (v/v) cylinder of medical grade N_2O (ATC: N01AX13, Niontix, 268 Linde Gas Benelux, Schiedam, the Netherlands). Background levels were assessed using measurements of empty vials containing laboratory air, blood, and saliva samples collected from participants prior to exposure. Further details are provided in the Supporting Information (Section: GC-MS Data).

Calibration samples were prepared using either water or whole blood retrieved from the Dutch bloodbank Sanquin (Amsterdam, the Netherlands). Sample stability was assessed by conducting triplicate analyses on identically prepared samples, measured sequentially at approximately 25 min intervals. Additional methodological details on sample stability are available in the Supporting Information (Section: GC-MS data). A balloon was filled with the calibration gas (N_2O, 100% (v/v)), connected with the vial (50 mL) containing either water or blood (10 mL), and equilibrated for 1 h. After equilibration the N_2O (g) concentration in water or blood stock solutions were demonstrated to be at the maximum concentration, i.e., 0.64 mL/mL (20 °C) and 0.47 mL/mL (37 °C).[32,33]

The water or blood stock solutions were used to prepare a calibration series by diluting the stock solution with fresh blood or water to the desired concentration. Next 1 mL was transferred to a HS vial (10 mL) and equilibrated for at least 1 h before analysis with HS-GC-MS. The blood and saliva samples collected from the participants were directly transferred to a HS vial (10 mL), transported to the lab, and allowed to equilibrate for at least 1 h before analysis.

All samples were analyzed using an automated Agilent Technologies 7890B HS-GC-MS instrument (Agilent Technologies, Palo Alto, CA). The headspace (HS) was formed at room temperature (RT, 18−22 °C) on the autosampler tray. Using a Gerstel multipurpose sampler, 1 mL of HS was sampled with a heated (90 °C) gastight injection needle. This was injected splitless at 200 μL/s onto an Agilent Technologies 7890B GC (Agilent, CA) equipped with a heated injector (275 °C). After a splitless time of 90 s, the split vent was opened at 50 mL/min. The injected sample was transferred to an Agilent Technologies J&W, CP-molsieve 5 Å 30 m × 0.32 mm × 10 μm analytical column (Agilent). The end of the column was connected via a 0.5 to 0.5 micro union (Trajan SGE siltite, Ringwood, Australia) with a 5 m × 0.32 mm × 430 μm VSD retention gap column (Trajan SGE, Ringwood, Australia). Column flow was 1.5 mL of He/min. The oven program was 40 °C (1 min), with heating at 10 °C/min to 210 °C, followed by heating at 30 °C/min to 350 °C and maintained for 5 min. The temperature of the transfer line to the MS was 275 °C.

Sampling

In vivo exposure to N_2O was carried out at the LUMC, where sample collection and preliminary sample processing were also performed. Samples were transferred to airtight HS vials in the operating theater of the LUMC directly upon collection, as described below, in 10 mL HS vials with a screw cap with septum (BGB Analytik Benelux B.V.,

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Harderwijk, the Netherlands). After collection, the vials were labeled and stored at room temperature in a Styrofoam box that was sealed tight and transported for approximately 45 min to the TNO laboratory via a courier service (Biologistic Services, Schiphol-Rijk, the Netherlands) for N_2O analysis the same day.

To correct for variations in sampled mass of saliva or blood, the N_2O concentration in the samples were corrected using the phase ratio equation $\beta = \frac{V_g}{V_s}$, where V_g is the volume of the HS and V_s is the volume of the sample.[34] The resulting calculated sample concentration $C^0_{N_2O}$ is defined by the headspace partition eq 3:

$$C^0_{N_2O} = C^g_{N_2O}\left(\kappa_i + \frac{V_T - V_s}{V_s}\right) \quad (3)$$

where $C^g_{N_2O}$ is the measured gas-phase concentration, which is proportional to the GC-MS detector response. β is the phase ratio, V_T is the total vial volume of 10 mL, and κ_i denotes the partition coefficient; for blood/N_2O and water/N_2O, $\kappa_{blood} = \kappa_{H_2O} = 0.47$.[26]

Blood Sample Collection. Participants' blood samples were collected through a peripheral intravenous cannula inserted into a vein on the dorsum of the nondominant hand before the administration of experimental gas (N_2O or compressed air). To prevent blood clots in the cannula, the cannula was filled with a saline solution 0.9% (m/V) after placement. When a blood sample was collected, this solution was first removed by withdrawing approximately 1 mL of fluid using a syringe through the cannula. Subsequently, a new syringe was used to draw approximately 2 mL of blood in an anticoagulated (ethylenediaminetetraacetic acid, EDTA) blood collection tube. This blood was then transferred to a 10 mL HS vial closed with a screw cap upon injection of the sample into the vial and weighed.

Saliva Sample Collection. To stimulate saliva production, the participant was instructed to chew on a piece of 3 × 3 cm paraffin wax foil (Parafilm, Merck, Darmstad, Germany), hereinafter referred to as Parafilm, for 2 min without swallowing the saliva, in a similar fashion as Dlugash et al. and Al Habobe et al.[31,35] After the 2 min period, the participant expectorated the generated saliva into a polypropylene container. The collected saliva was immediately transferred to a preweighted 10 mL HS vial, capped, and then reweighed to determine its volume assuming a density of 1.0 g/mL.[31]

■ ASSOCIATED CONTENT

Data Availability Statement

The data supporting the findings of this study are available from ACTA and the Dutch National Police. Due to licensing restrictions, the data used in this study are not publicly accessible. For data requests, please contact the corresponding author. Access to the data will be granted upon reasonable request, pending approval from both ACTA and the Dutch National Police.

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.5c09572.

Calibration curves of water and whole blood; inclusion criteria and medical questionnaire (PDF)

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Author Contributions

D.D.J. and F.V. designed the study and conceived the experiment(s). D.D.J., P.D., and T.G. helped develop and performed the HS-GC-MS analysis of all samples. D.D.J., J.B., and M.S. analyzed the results. D.D.J. and J.B. made the figures. All authors reviewed the manuscript.

Notes

The authors declare no competing financial interest.

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