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New strategy for carbon monoxide poisoning diagnosis: Carboxyhemoglobin (COHb) vs Total Blood Carbon Monoxide (TBCO)

Abstract

Diagnosis of carbon monoxide (CO) poisonings has always been a challenging task due to the susceptibility to alterations of the optical state and degradation of blood samples during sampling, transport and storage, which highly affects the analysis with spectrophotometric methods. Methodological improvements are then required urgently because of increased reports of cases with discrepancies between results of the measured biomarker carboxyhemoglobin (COHb) and reported symptoms. Total blood CO (TBCO) measured chromatographically was thus proposed in a previous study as alternative biomarker to COHb. This approach was investigated in this study by comparing the two biomarkers and assessing the effects of various storage parameters (temperature, preservative, time, tube headspace (HS) volume, initial saturation level, freeze- and thaw- and reopening-cycles) over a period of one month. Results show that while for TBCO, concentrations are relatively stable over the observation period regardless of parameters such as temperature, time and HS volume, for COHb, concentrations are altered significantly during storage. Therefore, the use of TBCO as alternative biomarker for CO poisonings has been proposed, since it provides more valid results and is more stable even under non-optimal storage conditions. Additionally, it can be used to predict COHb in cases where sample degradation hinders optical measurement. Furthermore, a correction formula for COHb and TBCO is provided to be used in laboratories or circumstances where optimal storage or analysis is not possible, to obtain more accurate results.

Keywords: carbon monoxide poisoning; carboxyhemoglobin; storage conditions; blood analysis; TBCO; GC-MS; COHb

1. Introduction

Carbon monoxide (CO) is colloquially named *'silent killer'* because despite it being a tasteless, odorless and colorless gas, its toxicity and occurrence in the atmosphere are important [1]. Hundreds of people in many western countries and thousands in less developed countries are killed because of CO poisonings each year [2–6]. However, due to the non-specific symptomatology and low medical awareness, CO poisonings are often misdiagnosed, leading to a potentially higher number of deaths that should be attributed to CO [7,8]. Recent studies have shown an increasing number of long-term neurological sequelae attributed to CO exposure *a posteriori*, raising questions and doubts about the accuracy of our current knowledge on CO, its pathophysiological effects and methods for quantification [9–12].

Procedures for sample collection and storage are one of the main aspects in the practice of clinical and forensic analyses of biological specimens. Given the medical and legal implications of these types of analyses, it is crucial that standardized protocols are in place to allow for correct and accurate interpretation of the results obtained, which help to provide adequate diagnoses and treatment strategies in clinical cases and hold up in court in forensic cases [13–15]. The fundamentals of these protocols are driven by the biochemical, physical and toxicological processes that occur when a substance of interest in a biological matrix is extracted, transported and stored prior to analysis. Storage after analysis is also of interest, since in many cases, especially forensic, reanalysis of a sample after several days or weeks is a necessary step in the investigation [14].

In cases of CO poisonings, currently the most common biomarker for diagnosis is carboxyhaemoglobin (COHb), due to the known high affinity of CO for the oxygen-carrying protein in erythrocytes, which displaces oxygen and, thus, reduces the oxygen transport to tissues, leading to hypoxia [1,16]. Methods for COHb quantification have been developed in blood [17–19] as main matrix, since it is readily available in both clinical and forensic cases. Biochemical alterations are known to occur in blood samples over time due to catabolism of proteins, which can be exacerbated by exposure to elevated temperatures, light, air or other compounds that might have contaminated the sample [20]. Common practice is to store blood samples possibly frozen or at least refrigerated, with addition of an anticoagulant and in a place not exposed to light [13]. Samples are usually obtained by laboratories in standard volume tubes, therefore the volume of the HS is determined based on the volume of the sample, not the selected sampling tube. Furthermore, in a laboratory routine a blood sample is often employed for multiple analyses, including drug screenings and alcohol testing [21,22].

Since CO is a gaseous compound, samples obtained from individuals suspected of CO poisoning have the potential of additionally being very susceptible to exposure to air and frequent reopening. Even though the bond between CO and Hb is very strong, it is also a reversible reaction, which, over long periods, can lead to dissociation, releasing CO into the headspace (HS) of the sampling tube [10,23]. Frequent reopening can hence lead to analyte loss. Another consequence of COHb dissociation includes the potential influence of the ratio of sample volume to HS in the sampling tube on the amount of CO dissociating into the HS. The formation of an equilibrium between CO in blood and HS was proposed by the study group of Kunsman *et al.* [24], who observed loss of COHb in samples with a higher volume of air in the sampling tube. They also showed a decrease in COHb levels over time for samples that had a higher initial saturation level [24]. Other storage parameters that were investigated in previous studies include different temperatures and preservatives, which often were contradictory: some studies showed no or little change with storage over long periods of time and at elevated temperatures [25–27], while others showed decreased COHb levels for different preservatives [24,28].

These differences can be explained by the fact that the majority of these studies were mainly performed using spectrophotometric methods for analysis, which are known to be susceptible to optical changes in the blood quality [29].

To be able to avoid erroneous results derived from poor sample quality due to inadequate sample collection and storage conditions, gas chromatographic (GC) methods can be alternatively employed. GC methods lead to results that are independent of optical changes to the specimen and enable the measurement of the total amount of CO in blood (TBCO) and in the HS of the sampling tube, as an alternative to COHb [19,30,31]. In addition, the measurement of TBCO is in conformity with the pathophysiological mechanisms of a CO poisoning, which recent developments have shown to be related not only to COHb, but also free CO [32–34].

Therefore, with this study, we aim to evaluate the effects of storage parameters such as temperature, preservative, HS volume, reopening cycles, freeze- and thaw-cycles and the level of initial COHb saturation over a storage period of one month on the quantification of both COHb and TBCO, in order to determine the most appropriate practices for sample collection and storage in CO poisoning cases with delayed analyses or storage in non-optimal conditions. Furthermore, we compare the spectrophotometric technique of CO-oximetry to a gas chromatography-mass spectrometry-based one, introducing the concept of TBCO

measurements as a necessary addition to COHb measurements, which are more sensitive to the quality of the matrix and storage conditions.

2. Materials and Methods

2.1 Chemicals and reagents

Formic acid (reagent grade, purity ≥95%) was purchased from Sigma-Aldrich (St Louis, USA) and CO gas (99%) was from Multigas (Domdidier, Switzerland). To prevent degradation all formic acid solutions were prepared on a daily basis. The internal standard formic acid (^{13}C, 99%) was ordered from Cambridge Isotope Laboratories (Cambridge, UK). Sulfuric acid (≥97.5%) was purchased from Fluka (Buchs, Switzerland). For the *in vitro* study of storage parameters, bovine blood was obtained from a local butcher and collected in 1L polypropylene bottles, which were previously fixed with the four investigated preservatives to obtain concentrations equivalent to the respective concentrations in the blood collection tubes (Monovettes). Ethylenediaminetetraaceticacid (EDTA) salt dehydrate was purchased from Sigma-Aldrich (St Louis, USA), sodium fluoride (NaF) was provided by Fluka (Buchs, Switzerland), lithium heparin (LiH) was from Fresenius Medical Care (Bad Homburg, Germany) and sodium citrate (NaCit) was obtained from Merck (Darmstadt, Germany).

2.2 Materials

The AVOXimeter 4000 Whole Blood CO-Oximeter and cuvettes were obtained from International Technidyne Corporation - ITC (Edison, USA). S-Monovettes of following types: 2.6mL K3E (EDTA, 1.6 mg/mL), 3mL 9NC (NaCit, 0.106 mol/L), 2.7mL FE (NaF, 1 mg/mL, + EDTA, 1.2 mg/mL), 2.6mL LH (LiH, 16 IU/mL), were obtained from Sarstedt (Nürnbrecht, Germany). Precision sampling gas syringes equipped with a press button valve and with capacities of 500µL (for dilution) and 2mL (for injection) were purchased from VICI (Baton Rouge, LA, USA). Aluminum caps were from Milian (Vernier, Switzerland). All extractions were carried out in 20mL headspace vials from Agilent Technologies (Santa Clara, CA, USA).

2.3 Instruments and GC-MS conditions

AVOXimeter 4000 Whole Blood CO-Oximeter from ITC was used for all COHb analyses. Manufacturer guidelines were followed to obtain COHb concentrations.

Agilent 6890 N GC (Palo Alto, USA) equipped with a HP Molecular Sieve 5 Å PLOT capillary column (30 m x 0.32 mm x 30µm) purchased from Restek (Bellefonte, USA) was used for gas chromatographic analysis. The temperature program used was as follows: 50°C, held for 4 minutes; injector temperature was set at 180°C, the injector used in splitless mode, and the MS interface at 230°C. Helium was employed as carrier gas, at a flow rate of 40 mL/min.

An Agilent 5973 mass spectrometer (Palo Alto, USA) was used for detection, operating in electron ionization (EI) mode at 70eV. Selected Ion Monitoring (SIM) mode was used to acquire the signal for CO at m/z 28 and ^{13}CO at m/z 29.

2.4 Sample preparation

2.4.1 CO-fortification of blood

Blank bovine blood, which was obtained freshly from a local butcher for each study period, was added with the respective preservative immediately after collection and subsequently fortified with pure CO gas through bubbling for a certain amount of time. COHb saturation levels were checked before bubbling and after bubbling with a 10-minute-interval until the desired initial saturation level was reached. To ensure homogenization, the blood-containing bottles containers were agitated for 20 minutes, after which the final COHb concentration was determined by CO-Oximetry.

2.4.2 Calibration standards

An aliquot of fresh bovine blood, which was previously controlled by CO-oximetry and found at 0% COHb, is used as matrix for GC calibration. Calibration standard working solutions of formic acid (43 µmol/mL) and working solutions of the internal standard isotopically labelled formic acid (84 µmol/mL) were prepared daily *de novo* in order to prevent degradation. Calibration points were set in a working range between 0-208 nmol/mL HS, with points at 6.5, 13, 26, 52, 104, 156 and 208 nmol/mL HS (equivalent to 1.3, 2.6, 5.2, 10.4, 20.8, 31.2 and 41.6 µmol/mL in blood). Matrix effects were evaluated by preparing a blank sample with the matrix without any reagent. 10 µL of the working internal standard solution were added to each calibration sample before extraction, leading to a final concentration of 42 nmol of ^{13}CO/mL HS. All standards and samples were stored at +4°C when not in use.

2.4.3 Extraction procedure

CO in blood

100µL aliquots of blood were introduced in a 20mL HS-vial, followed by 10µL of the internal standard solution. For calibration points, the respective aliquots of formic acid solution were added. Aluminum caps of 11mm (i.d.) were first filled with 100µL sulfuric acid, which is used as both releasing agent for CO and reagent with formic acid/isotopically labelled formic acid to generate the CO/^{13}CO needed for calibration/quantification, and then carefully introduced into the HS-vial. The vial was immediately hermetically sealed with PTFE/silicone septum caps of 20 mm (i.d.). In order to ensure complete mixing of the liquids contained in the vial, the samples were vigorously shaken and vortexed. Extraction was finalized by heating the vials at 100°C for 60 minutes.

CO in HS

To determine the CO in the HS of the sampling tubes, the same procedure as for the CO in blood samples was used. To be noted is that while for CO in blood analyses, CO needed to be released with sulfuric acid in the HS vial, to analyze CO in HS, 250µL of HS were directly sampled from the sampling tube with a 500µL airtight gas syringe and injected into a HS-vial containing the internal standard that had previously been generated.

2.5 Analysis procedure

2.5.1 CO-oximeter

Approximately 50-100µL of blood were sampled from the sampling tube and placed into an Avoximeter 4000 Whole Blood CO-oximeter cuvette, which was then introduced in the Avoximeter 4000 Whole Blood CO-oximeter for analysis.

2.5.2 CO in blood determination via HS sampling and GC-MS analysis

1mL HS was sampled from the 20mL HS-vial containing the extract and injected in the GC-MS for analysis, which was performed following a previously validated method [19].

To ensure that no contamination from CO contained in the air affected the measurements, a 1mL aliquot of air in the analysis-room was additionally analyzed prior to sample analyses.

2.6 *In vitro* storage study

The *in vitro* study to evaluate several storage parameters was carried out over a period of one month, with samples analyzed on days 0, 1, 2, 4, 7, 14, 21 and 28.

Blood specimens were generated on day 0 to investigate following parameters at various levels:

- Temperature: room temperature (RT), refrigeration at +4°C, freezing at -20°C
- Preservative: EDTA, NaF, LiH, NaCit
- HS volume: <25%, 25-50%, >50% of the total tube volume
- Saturation levels: 10-20%, 30-40%, 50-70%
- Reopening cycles
- Freeze- and thaw-cycles

One set of samples used to investigate the reopening cycles were reanalyzed on each day of analysis, while another set of samples used to investigate all other parameters were analyzed once on day of analysis. To assess the freeze- and thaw-cycles, the samples for investigating the reopening cycles stored in the freezer were used. A total of 2376 blood samples were analyzed, which were distributed for each parameter and day of analysis as follows: 108

samples for each saturation level per day, of which 27 per preservative, 36 per temperature and 36 per HS volume.

2.7 Back calculation of COHb from CO

Various research groups have previously proposed formulae to back calculate COHb from CO measured through GC approaches [35–38]. We compare the CO concentrations measured with the AGS-GC-MS method and back calculated to COHb through the formula proposed by Cardeal *et al.* [37] with the COHb measured by CO-oximetry to establish statistical significance.

2.8 Statistical analyses

Since saturation level is expected to have the most significant effect on the data and to simplify the data analysis, the dataset was split into the three categories (high, medium and low saturation level) and used for modeling. Data was then checked for normal distribution and transformed accordingly (**Table 1**). Due to the upper detection limit of the employed CO-oximeter of 75%, a large portion of the samples analyzed with high saturation level was found at 75%, despite potentially being higher. This is not an issue from a pathophysiological point of view, since the value is clearly in the toxic range of COHb concentrations. However, from a statistical perspective, this generated a severely left-skewed distribution, which could not be corrected for through transformations. Therefore, censored regression was considered for statistical analyses of the data. “censReg” is a package in the statistical software R, which can be useful when faced with censored data. The way the software deals with the values in case of a right-sided censoring is that it estimates the values above the censored limit based on maximum likelihood with the data available [39]. This might, however, problematic, since the software returns estimated values that can exceed 100, which is the physiological limit for COHb saturation. To test whether the “censReg” function returnes values that on average were above 100, we replaced the values at 75% COHb with 80%, 90% and 100% and compared the regression results with the results from a regression excluding the values at 75% COHb as well as the results of a censored regression. Results are shown in **Table S1** in the Supplementary Material. Based on the results, the values estimated when using a censored regression are closer

to 100% COHb, which is highly unlikely. COHb concentrations above 75% are unlikely and not distinguishable from a pathophysiological perspective. Therefore, taking also into account that the number of samples with 75% COHb concentrations are only 10.14% of the total samples analyzed (241), we decided to not perform censored regression.

Non-parametric tests were used for assessment of single storage parameters in high saturation levels, but no assessment was possible with multiple storage parameters. Missing values in cases of instrument malfunctioning or due to advanced stage of sample degradation were completely excluded for statistical analyses. Kruskal-Wallis test for high saturation COHb levels, multiway analysis of variance (ANOVA) for the other saturation and response variables (response variables here are our measured variables, thus COHb and TBCO) as well as multiple linear regression (MLR) and comparisons via Student t test were used to assess effect of the investigated parameters and generate correction and prediction models. All statistical analyses were performed with R (version 3.3.1, 2016-06-21).

3. Results

3.1 Correlation between COHb and TBCO

Figure 1 shows the plot of results obtained for COHb vs results of the same samples for TBCO. A linear regression was applied to the data and the obtained linear regression line is depicted in red. A correlation factor (R^2) of 0.68 with a p-value well below the significance limit of 0.05 (p-value <2.22e-16) represents a moderate positive correlation between the two measures.

To determine whether the formula proposed by the study group of Cardeal *et al.* [37] is applicable to our method, we have used it to backcalculate the values obtained from the AGS-GC-MS measurements and compared the measured COHb with calculated COHb from TBCO values. A paired Student t test was performed to statistically compare the two groups. With a p-value of <0.05 (p-value <2.2e-16), it was determined that the groups are significantly different.

3.2 Influence of storage parameters

3.2.1 Relevance of CO in HS

The amount of CO released into the HS of the sampling tube was on average 0.0001 (±0.0002) µmol/mL for high COHb saturation, $4 \cdot e^{-0.5}$ (±0.0001) µmol/mL for medium saturation and $4.78 \cdot e^{-0.5}$ (±0.0001) µmol/mL for low saturation levels, with a maximum of 0.003 µmol/mL. To determine whether transferred amount was significant, the results for CO in blood were added to the results of CO in HS and compared to the results of CO in blood with a paired Student t test. With a p-value of <2.2e-16, the two groups were found to be significantly different. Average relative differences in values were found to be between ±0.01% (histogram of relative differences distribution in **Figure 2**).

3.2.2 Reopening and freeze- and thaw-cycles

3.2.2.1 Reopening

To determine the influence of reopening the sampling tubes on the results obtained from the measurements of COHb and TBCO, the unpaired Student t test was used to compare the samples that were reopened during the study period to samples that were not reopened. Results for both COHb and TBCO gave p-values >0.05, thus indicating that there is no statistically significant difference in results for samples that were reopened for reanalysis.

3.2.2.2 Freeze- and thaw-cycles

The effect of freezing and thawing a sample multiple times on the obtained measurement results for COHb and TBCO was determined by comparing results obtained from samples, which underwent multiple freeze- and thaw-cycles, to samples, which underwent only one freeze- and thaw cycle. The unpaired Student t tests for both COHb and TBCO lead to p-values >0.05, resulting in no statistically significant difference between the comparison groups. To further test whether the first freezing cycle had a major effect on the concentrations, we compared the results of samples that underwent one freeze- and thaw-cycle with the samples that did not. The Student t test for both COHb and TBCO gave p-values >0.05, thus affirming no statistical difference.

3.2.3 Multiway-ANOVA

To determine which and if any of the investigated parameters has a significant effect on the measures COHb and TBCO, an ANOVA was first carried out for each parameter and saturation level in relation to COHb and TBCO, respectively, with exception of high saturation COHb levels. Due to the inability of the data to reach normal distribution despite transformation attempts, the non–parametric Kruskal-Wallis test was employed to assess the different storage parameters one by one. Subsequently, an additive model selection process was performed, which consisted in the generation of several models through addition of one parameter in each new model, which were, in order, temperature, preservative, time (day of analysis) and HS volume. We were not able to investigate interactions between the parameters, since the number of outputs remaining after cleaning of data was not sufficient to the number required to obtain enough study power. ANOVA was then used to determine the significance of the parameters in the models (significance was obtained with a p-value < 0.05). Results are summarized in **Table 2**.

3.3 Correction model

To be able to generate a correction model applicable to COHb or TBCO measurements based on this dataset, first, the behavior of the response variables over time for each saturation level needed to be identified. Therefore, time plots for COHb and TBCO were produced (**Figures 3a-f**), with a black line going through the means of the COHb/TBCO concentrations for each day of analysis. The graphs show a general weak linear trend for all saturation levels and response variable. For high and medium COHb levels (**Figures 3a and 3b**), a weakly decreasing trend can be observed, whereas for low COHb saturation (**Figure 3c**) there is a slight decrease in the initial phase, followed by a plateauing towards second half of the month. For high TBCO levels (**Figure 3d**) there is a sudden drop after the first day, followed by a stabilization and weak decrease along the monitoring period. A similar drop can be seen for medium TBCO concentrations (**Figure 3e**) on day 7, which is again followed by a stabilization and generally a weak decrease towards the end of the storage period. TBCO in low concentrations (**Figure 3f**) shows a slightly increasing tendency. Generally, increasing

variation can be observed the higher the saturation level and the higher the number of storage days from t0.

Due to the general linear behavior, MLR analysis was selected and used for each response variable and each saturation level to determine the coefficient estimate for each parameter and their significance, based on following equation:

$$c = c_M - x_t\beta_t - \beta_P - \beta_T - \beta_V \qquad (1)$$

with c: corrected concentration of analyte of interest (here COHb in % or TBCO in µmol/mL), c_M: measured concentration of analyte of interest; x_t: number of days since sampling of specimen, β_t: coefficient estimate for time, β_P: coefficient estimate for selected preservative, β_T: coefficient estimate for selected storage temperature, β_V: coefficient estimate for selected HS volume.

Reference level for each parameter was selected based on common guidelines for sample collection and storage in toxicological analyses (if specified), with EDTA as reference for preservative, freezing as reference temperature, low HS volume (<25%) and day 0 as reference for time. Results of the MLR are summarized in **Table 3**. To be noted here that all results for TBCO are based on normalized data and, thus, coefficients need to be transformed back to be able to obtain the actual TBCO concentrations (e.g., for high saturation TBCO, log transformation was applied, therefore the back transformation involves application of the exponential function to the coefficient estimates).

3.3.1 Saturation level

For COHb, all parameters show statistical significance except HS volume 25-50% for high saturation levels, while all parameters are significant except storage in the fridge (+4°C) for medium saturation levels and storage in the fridge, preservatives NaF, LiH and NaCit and time are significant for low saturation levels.
For TBCO, across all saturation levels, all preservatives are significant as well as storage at room temperature (+20°C), while no statistical significance was found for the other investigated parameters.

3.4 Prediction Model

To be able to predict the COHb concentrations based on a measured TBCO value and the given storage conditions, the dataset was split into a modeling set and a testing set. The testing set was obtained by extracting the data of one repetition for each analysis. The modeling set was then employed to generate a prediction model based on the linear function of

$$c_{COHb} = c_{TBCO} - x_t\beta_t - \beta_P - \beta_T - \beta_V \qquad (2)$$

with c_{COHb}: concentration of COHb in [%], c_{TBCO}: concentration of TBCO in µmol/mL, x_t: number of days since sampling of specimen, β_t: coefficient estimate for time, β_P: coefficient estimate for selected preservative, β_T: coefficient estimate for selected storage temperature, β_V: coefficient estimate for selected HS volume.

Coefficients and standard errors of the model are found in **Table 4**.This model was then used to predict the COHb concentrations based on the TBCO values and storage parameters form the training set.

To evaluate efficiency of the prediction model, predicted values were compared with measured values with a Student t test, which resulted in a p-value above 0.05, thus indicating that the measured and predicted values are not statistically different.

Prediction efficiency was further confirmed by a linear regression of predicted and measured COHb concentrations, which resulted in a good correlation ($R^2 = 0.87$) and is shown in **Figure 4**.

4. Discussion

4.1 Correlation between COHb and TBCO

Before assessing each storage parameter and their potential impact on the measurement results, we first needed to determine the direction and magnitude of correlation between the employed methods, namely CO-oximetry and GC-MS. Previous studies have determined a strong positive correlation between COHb determined via CO-oximetry and CO measured by GC, with R^2 found generally above 0.9 for detection via flame ionization detector (FID) or residual gas analyser (RGA) [36,37,40,41] and 0.85 for detection via MS [31,38,42,43]. Additionally, Cardeal, Vreman and others have proposed formulae to back calculate COHb from the CO

measured through these GC methods, which are based on the correlation they obtained by comparing the two measurement methods [36–38,44].

Results from this study, however, do not confirm the results of these research groups. A weaker correlation between COHb measured by CO-oximetry and CO measured by GC-MS (R^2 = 0.68, see **Figure 1**) was determined. Furthermore, the statistically significant difference found between the measured values and the ones back calculated through the applied formula from Cardeal *et al.* (see section *3.2.1*)does not confirm results previously obtained by the research group [43]. Therefore, the formula seems to be unsuitable. One possible explanation for this discrepancy in results can be found in the different analytical approaches used by each research group. While Vreman uses GC-RGA for detection [35,36], Cardeal uses GC-FID [37] and Hao [38] and Varlet [43] GC-MS. The advantages and disadvantages of each detection method have been discussed thoroughly in the past [17,45,46]. Generally, it is determined that GC-FID is the most sensitive method for CO analysis, but time-consuming and impractical due to the additional need of a methanizer, which makes the instrument limited to only a specific analysis, while GC-MS is the most versatile, accurate, rapid and reproducible method for CO determination in blood [17,45]. In addition to a different detection method, the research groups also use different calibration and sample preparation approaches. Various acids and oxidizing agents have been employed as 'liberating agents' to release CO for analysis via GC, which can result in different recoveries and efficiencies in CO release, hence altering the final CO concentrations obtained [19,31]. Furthermore, the calibration solutions were prepared differently. All previous studies have performed a *flushing* step of the calibrators prepared from CO-bubbled blood, with the aim of removing the 'excess' CO and, thus, recover only the CO bound to Hb. However, this does not comply with the pathophysiology of CO poisonings: both bound and free CO are responsible for the toxicity mechanisms of CO in the human body [1,7,16,34]. Consequently, removing and not analyzing free CO can underestimate the true CO burden, potentially resulting in fatal misdiagnoses. Therefore, in this study we do not determine only the bound CO fraction, but the TBCO, which includes both free and bound CO. The amount of free CO was already found to be significant and may be one of the reasons for the discrepancy between our results and those from previous researchers regarding both the correlation of COHb and (TB)CO and the back calculation of COHb through formulae [30].

4.2 Influence of storage parameters

4.2.1 CO in HS

The bond between CO and Hb is very strong, due to the high affinity of CO for the hemoprotein, which leads to COHb as being considered the sole biomarker of CO exposure. It is often reported as constituting the major form CO acquires when crossing the lung-blood barrier, making up more than 90% of inspired CO [47]. However, recent studies have also acknowledged the incongruence between symptoms and measured COHb and the possibility of a higher percentage of CO not bound to Hb than previously assumed [7,8,16,30,34,48]. This can partially be explained by the reversible reaction between CO and Hb: despite the high affinity, there is still a part of CO that can go back to its unbound form, even though it most likely constitutes only a small fraction. This equilibrium can however be shifted towards free CO by an increased HS volume: since CO is a gas, it behaves according to the ideal gas law, and according to Le Chatelier's principle and the entropy laws, an increase in volume drives the gas molecules to shift and distribute towards the additional space, where the gas concentration is lower. An increased HS volume can, thus, increase the concentrations of CO in the HS significantly.

Based on the results of the measurements of CO in the HS of the blood tubes after statistical analysis, CO in HS is determined to be significant. However, statistical significance does not always reflect a significance from a biochemical point of view, and, thus, needs to be put into the right context. As represented in the histogram in **Figure 2**, relative differences are generally below 0.01% COHb, which from a pathophysiological perspective do not have an impact on the severity of the poisoning. Therefore, we conclude that there is not a significant amount of CO that is released into the HS of the blood tube during storage.

4.2.2 Reopening and freeze- and thaw-cycles

Exposure to air through repeated reopenings of the samples was reported to decrease COHb values, which is mainly due to a loss of CO through an increase in the available volume.This can cause a shift of the equilibrium of free CO driven by entropy [49]. Similarly, blood samples stored below freezing temperature that had to undergo multiple freezing and thawing cycles due to repeated measurements required showed reduced COHb values, even though at a lesser extent [28,38]. In this study, however, results showed that both reopening and freeze- and thaw-cycles did not have a significant impact on the measurement values for neither COHb nor

TBCO. Previous research into alterations to COHb values due to storage of blood samples showed mild reductions when observed for periods varying between 45 days and 2 years [24,28,38]. Considering that in the current study the observation period was of 28 days, this could explain the lack of significant alterations observed, reopening and freeze- and thaw-cycles may affect the COHb and TBCO values only at a later storage period. Furthermore, in the study performed by Chace *et al.* [28], samples were allowed free air exchange during the whole period of storage, whereas the samples in this study were reopened only on the days of analysis. Kunsman *et al*. stored the samples for a period of 2 years and reopened the tubes only for the second analysis, thus only accounting for one reopening and one freezing- and thawing-cycle [24]. Therefore, no substantial loss of CO could have occurred due to exposure to air or the freezing- and thawing-cycle, which is in accordance with the observed results. Hao *et al.* describe a substantial loss of COHb during storage over 45 days when measured with UV-spectrophotometry, while COHb back calculated from CO measured by HS-GC-MS is shown to be stable over the course of the storage period [38]. A similar behavior is confirmed in this study.

4.2.3 Storage parameters: temperature, preservative, time and HS volume

Various storage conditions have previously been investigated by multiple research groups, with results usually showing either increased or decreased COHb concentrations based on the storage temperature chosen, the preservative used to prevent blood clotting or exposure to different amounts of air over the course of prolonged storage time. However, there is also the possibility that these parameters affect the alteration of measurement differently based on whether the CO level in blood is high or low. While a parameter might have significant effects when low CO levels are present in the blood specimen, the variation in higher saturation levels might not be as significant, thus making that parameter to be considered for certain types of poisoning cases. Therefore, the different storage parameters were evaluated for each saturation level separately, which was not investigated previously by other research groups.

The Multiway-ANOVA (**Table 2, model 7**) shows that all investigated parameters significantly affect the COHb concentrations for all saturation levels (except HS volume for low COHb concentrations), which is in congruence with previous studies reporting CO changes due to these parameters [24,25,28,38]. This behavior is, however, different for TBCO, where HS volume does not influence the measurement results in any saturation level and time only

affects these at low concentrations. Hence, TBCO seems to be less affected by storage conditions compared to COHb, especially at medium to high ranges, which are of particular relevance for forensic cases.

This is further confirmed by the MLR analysis results (**Table 3**), where a higher number of parameters are found to be significant for COHb than TBCO. A closer look at the variables shows that all preservatives are influential for both COHb and TBCO measurements (the higher the magnitude of the coefficient estimates (in either positive or negative direction), the more significant their effect).

For TBCO the effects of the different preservatives are of similar magnitude and direction in each saturation level, except for NaF in high saturations, which shows a decrease in TBCO (-0.24), as opposed to the other two preservatives showing an increase (0.27 for LiH, 0.18 for NaCit). Additionally, TBCO in low saturation levels shows a decreasing trend (-0.20 for NaF, -0.10 for LiH), with only NaCit resulting increased levels (0.21).

For COHb, a clear trend can be observed with NaF, where a decrease is observed in all saturation levels and the magnitude decreasing from higher to lower saturation (-16.35, -13.92, -7.01). Since NaF is a weak anticoagulant, its effect might diminish during prolonged storage, therefore increasing the chance of blood clotting, which reduces the blood quality. Therefore, it is not surprising that a decrease in COHb is observed and that the effect is more evident with higher COHb concentrations. However, for LiH and NaCit, no consistency can be observed between saturation levels and the effect of the preservative. With LiH as preservative, the highest effect shows at medium COHb levels, with an average decrease of 13.49, whereas for the other saturation levels, increased COHb concentrations are observed (2.39 in high COHb levels, 5.30 in low COHb levels). Heparin is a widely used anticoagulant, especially in clinical toxicology and biochemistry, despite its high cost and short lasting action. It belongs to the family of glycosaminoglycans. The anticoagulant feature of this complex chemical structure are the sulfated pentasaccharide units, which have a high binding affinity for antithrombin III, a plasma protein that inhibits blood clotting [50,51]. Considering that Hb has a relatively high binding affinity for oxygen and that the CO-Hb bond is reversible, it is possible that at certain concentrations the sulfated pentasaccharide units of heparin interact with Hb, thus altering the measured COHb concentrations. Furthermore, LiH is employed as a liquid solution, rather than a salt as all other preservatives. This increases the potential for blood dilution, therefore leading to decreased COHb measurement results [52]. These explanations for the observed behavior

are all hypothetical, no specific study was conducted in the past assessing the effect of storage with heparin for blood samples used in CO poisoning determinations.

When using NaCit as preservative, the highest decrease in COHb is shown, similarly to LiH, in medium COHb ranges (-17.63), while a less significant decrease is reported in high saturations (-5.96) and an increase in low saturations (3.00). NaCit is, similarly to NaF, a weak anticoagulant, used primarily in blood transfusions and generally clinical blood samples, mainly due to its low cost and reversibility of the anticoagulant mechanism (chelation of calcium ions) [53,54]. Therefore, it is possible that at certain COHb concentrations, driven by a concentration gradient or chemical interactions, either the chelation of calcium or the bond between Hb and CO is reversed (which is a coordinated bond and not a covalent bond), leading to decreases in COHb. In addition, it has been previously reported in several studies that citrate alters the measurements of other compounds, such as gamma-hydroxybutyrate (GHB), leading to false positives. While the mechanism has not yet been elucidated, it was recommended that citrate as preservative should not be used for forensic drug analyses [55–57]. Therefore, we hypothesize that a similar reaction might take place for COHb measurements, even though further investigation is needed to confirm this.

Regarding HS volume and temperature, these are shown to be more influential at higher saturation levels for COHb concentrations, which is in accordance with results reported by Hao *et al.* [38], who also showed a more marked change in COHb concentrations with increased COHb saturation level. Storage at room temperature, as opposed to storage in the fridge or freezer, shows more prominent increases of high COHb concentrations. This is in accordance with biochemically- and bacterially-induced blood degradation, which is increased with higher temperatures. Results reported by Kunsman *et al.* [24] showing reduced COHb levels with increased exposure to air is also confirmed by the MLR results, with a negative and more significant coefficient estimate (β) reported for COHb levels (**Table 3**). However, this behavior is not shown with TBCO, for which HS volume, time and storage in the fridge or freezerdo not play an influential role. Only the choice of the preservative and storage at RT has a significant impact on TBCO measurements.

This supports our hypothesis that TBCO is more stable and less prone to significant changes due to temperature, time and air exposure, as opposed to COHb. COHb measurement by spectrophotometry is affected by changes to the optical blood quality, which are mainly due to erythrocyte catabolism occurring with time and also temperature changes, making the

measurement more challenging and in some cases even impossible (the instrument returns an error message). Furthermore, measurement by CO-oximetry is also affected by the amount of Hb present in the blood sample, with a range of 5-25 g/L limiting the measurements, which is especially relevant for forensic cases, where with high PMI, the blood quality is often altered, potentially leading Hb levels to exceed the instrument's limits [29]. This also leads to some instances where the measurement is not feasible. The majority of these factors are, however, not relevant for TBCO measurements. Optical blood changes, erythrocyte catabolism, shifts of CO from bound to free, redistribution or increases of Hb in the blood compartments – neither of these factors influence measurement of CO via GC-MS. The pre-analytical reaction that takes place does not differentiate free or bound CO, all CO is transferred to the gas phase and then analyzed with a GC-column specific for small molecules, thus reducing the potential interference of compounds present in the matrix. However, TBCO measurements are not 'immune' to PM generation of CO, which is more likely to occur when samples are stored at higher temperatures. This explains why TBCO is shown to be influenced by storage at room temperature. Nevertheless,TBCO measurement may constitute a more reliable method for quantification of CO in non-optimal sampling and storage circumstances.

4.3 Correction model

In this study, several storage conditions have been investigated over a prolonged period, with parameters influencing the measurement results differently based on the chosen conditions and saturation level. Therefore, the selection of appropriate storage conditions is essential in guaranteeing accurate and reliable results, which can determine whether a case is attributed to CO poisoning as cause of death, contributing factor or unrelated to death, with significant legal consequences. However, optimal conditions cannot always be guaranteed. Based on the laboratory equipment, resources, location and collaboration with local law enforcement and emergency departments, conditions of sampling and storage may vary. To be able to obtain consistent and accurate results across laboratories, we have used our data and MLR analysis to generate a correction model for both COHb and TBCO with parameters temperature, time, preservative and HS volume as input variables.

Equation (1) can be adapted to the case at hand: depending on whether COHb or TBCO is being measured, the coefficient estimates for the selected storage conditions (if they vary from the reference conditions, otherwise the variable is equal to 0) that are significant for the relevant

saturation level are back-transformed (if necessary), input into equation (1) and the corrected concentration is obtained.

As an example, if there is a blood sample that was stored with NaCit and >50% HS volume in the freezer for 28 days and obtained a COHb concentration of 35%, the corrected concentration would be:

$$c_{COHb} = 35.00\% - 28 * (-0.08) - (-17.37) - 0 - (-1.24) = 55.85\%$$

For a sample stored with the same conditions and with a measured TBCO concentration of 5.00 µmol/mL, the coefficients need to be back transformed for use with the correction formula. In this case (medium saturation level), log transformation was performed, therefore the exponential function needs to be applied to the coefficients, giving us following corrected concentration:

$$c_{TBCO} = 5.00\ \mu mol/mL - 0 - \left(e^{(-0.21)}\right) - 0 - 0 = 4.19\ \mu\text{mol/mL}$$

This provides an important tool to be employed by laboratories and emergency departments that do not have the financial or logistical capacity to guarantee the best conditions for sampling and storage of specimens, such as in less developed countries where samples might need to be mailed to a laboratory with the appropriate equipment.It will enable them to obtain accurate and reliable determinations in CO poisoning cases, despite non optimal storage conditions. However, this formula cannot be applied if temperatures during transport exceed 20°C, as temperatures above were not investigated here. Generally, laboratory guidelines and best practice regulations may vary across countries, even though a lot of effort is being put into reaching a global consensus on clinical and forensic laboratory standards. However, differences in storage and sampling practice are still common and therefore a consensus should at least be reached regarding the accuracy of results, which is the main goal and, finally, achievement of this study. With this model, not only can correct diagnoses in suspected CO poisonings be obtained regardless of the sampling and storage conditions, but results can also be compared across laboratories and countries, allowing the creation and expansion of a collaboration network, which can be fruitful under other aspects as well.

4.4 Prediction model

Going a step further to obtain the most accurate and reliable CO poisoning determinations possible, we have integrated the storage conditions with the proposed alternative biomarker TBCO to be able to obtain COHb values that reflect with higher accuracy the levels present in blood specimen, even in cases where COHb cannot be measured due to degradation. By measuring TBCO and inputting the coefficient estimates (**Table 4**) into equation (2), COHb concentrations can be predicted. Efficiency of the prediction model was confirmed by testing it on a set of data with known COHb and TBCO concentrations and storage conditions, which gave a satisfactory correlation coefficient of 0.87. Therefore, this prediction model together with TBCO measurement can be employed by laboratories for cases where measurement with CO-oximetry is not possible, allowing CO poisoning determinations in all possible conditions. However, a limitation of this prediction model is that it can only be applied to samples with a short postmortem interval (PMI). PM degradation affects the concentrations of CO in ways that go beyond storage, such as PM CO production through bacterial activity in the body. This was not a factor investigated in this study, but would be an important aspect to research in order to further expand the potential application range of the proposed prediction model.

5. Limitations

In forensic cases, samples are usually stored for periods longer than 1 month, often for more than 1 year, since the timeline of court cases is very long and samples might be reanalyzed for cross-examination. Therefore, it is reasonable that the effects of time on COHb and TBCO are not very significant. Even though they are arithmetically significant for COHb, the differences over one month of less than 1% COHb will not affect the interpretation of toxicological findings. Studies with prolonged storage time should be carried out to examine the long-term effects. Another aspect that needs to be taken into consideration is that these tests were performed on non-human blood. Despite the similarities in blood density and Hb concentrations between bovine and human blood, it is possible that results might differ when using human blood. Nevertheless, we believe that these differences would not be very significant. Furthermore, this study focused on investigating storage parameters, not considering PM changes occurring when dealing with forensic cases. Therefore, the models generated here are applicable to clinical cases, but when dealing with forensic cases, PM changes need to be taken into consideration for interpretation of the results. Nevertheless, we

believe that the models can be used to assess the storage conditions and are to be added to the interpretation of potential PM changes. An additional aspect that might limit this study is the instrument's limit of 75% on COHb measurements. However, considering that from a toxicological perspective, the findings will not change based on whether the COHb concentration is at 75% or above (CO at 75% or above is considered as cause of death), this is not a significant limitation.

6. Conclusion

In this study, we have not only compared two biomarkers and detection methods (COHb measured via CO-oximetry and TBCO measured via GC-MS) for the application in CO poisoning determinations, but also investigated the nature and magnitude of effects caused by different storage conditions on the accuracy of the obtained measurement results by both biomarkers.

The significant discrepancy between TBCO and COHb is shown by the weaker correlation found between the two measures, as opposed to correlations of previously reported studies, who used to flush the calibrators prior to analysis. This affirms the importance of the measurement of free CO in addition to bound CO to obtain results that more closely correspond to the true pathophysiological levels.

Furthermore, TBCO appears to be more stable during storage for prolonged time intervals, with no significant alterations observed due to different HS volumes, storage in the fridge or freezer and several preservatives during this period. On the contrary, COHb is affected by all investigated parameters, even though at different extents. This confirms that optical measurement methods are more prone to deliver inaccurate results due to storage conditions. Conversely, TBCO measurement should be promoted, especially in forensic investigations, where trials can be delayed and last for long periods and often require reanalysis of supportive evidence. Therefore, we recommend the use of TBCO as alternative biomarker to COHb for CO poisoning determinations. Moreover, unlike general storage guidelines for clinical and forensic toxicology (e.g. TIAFT, UKIAFT, etc.), who suggest NaF as the preservative of choice [15,22,58], based on our results, we generally recommend collection of samples for CO analysis in EDTA tubes, stored possibly in the freezer or fridge. When COHb is analysed, it is

also important to fill the collection tube at more than 50% of its volume and to analyse the sample as soon as possible.

However, in laboratories or institutions where optimal storage is not possible, the use of the proposed correction formula provides an important tool to obtain more accurate measurements, even in non-optimal conditions. Additionally, in cases where spectrophotometric measurements are not possible due to degradation of the sample during storage, the provided prediction formula can be used to estimate the corresponding COHb concentration by measuring TBCO.

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Conflict of interest

The authors declare no conflict of interest.

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Tables and Figures

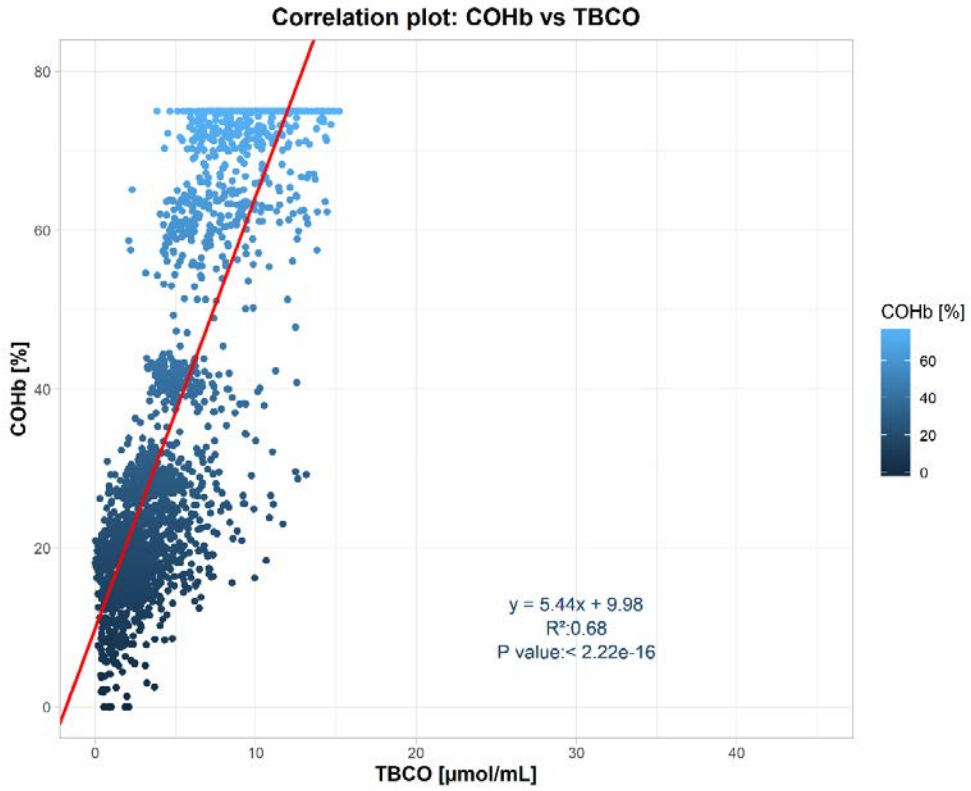

Figure 1: Correlation plot for COHb [%] vs TBCO [µmol/mL] from the storage study results, with correlation formula, correlation factor (R^2) and p-value.

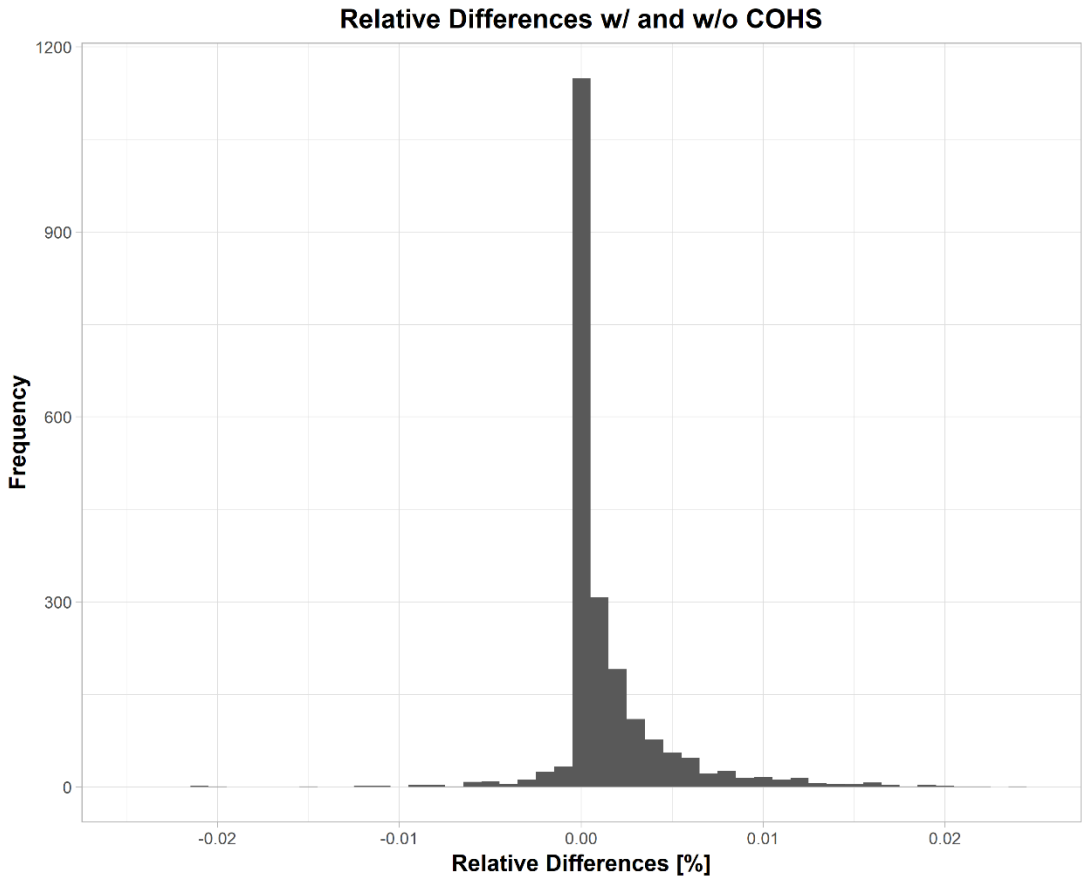

Figure 2: Histogram of relative differences in % of total blood CO (TBCO) concentrations with and without the CO in the headspace (HS).

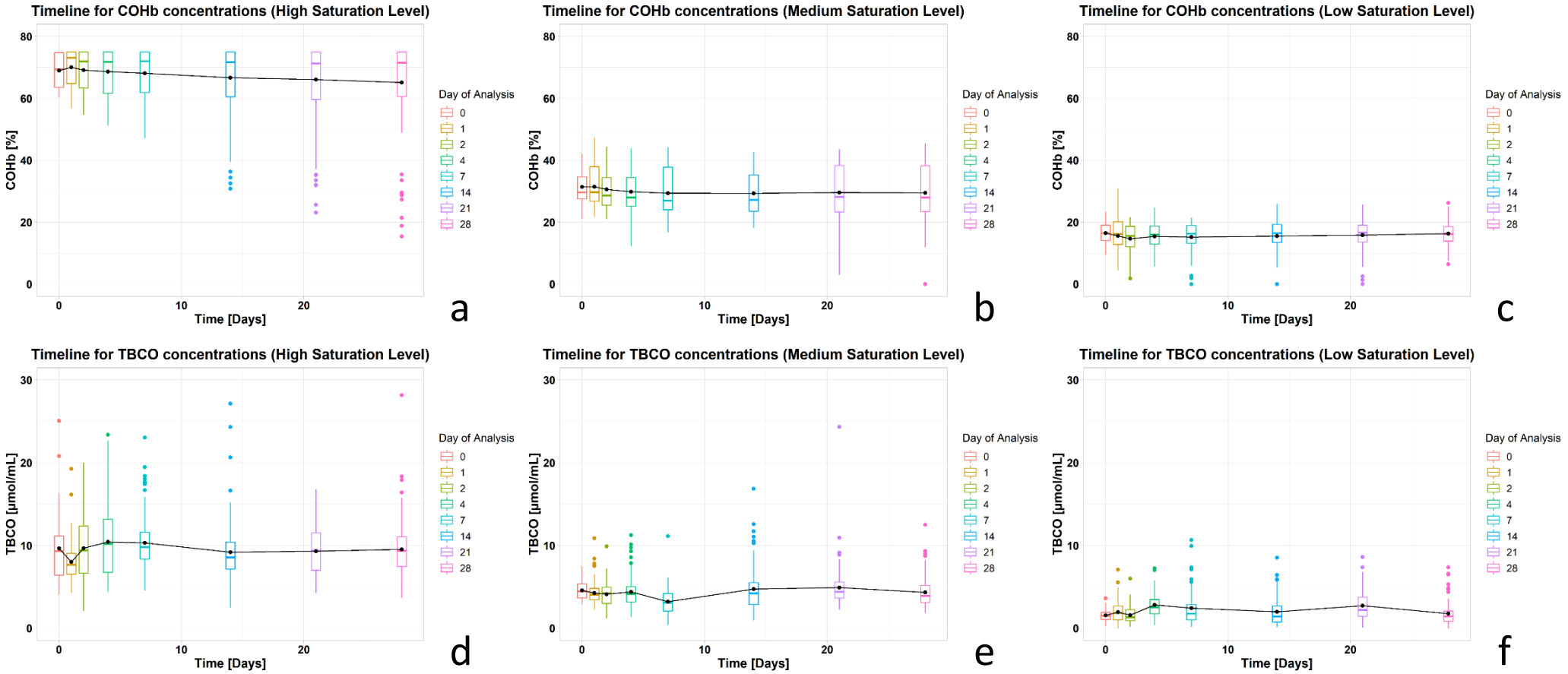

Figure 3a-f: Boxplots with error bars for COHb concentrations in % (a-c) and TBCO concentrations in µmol/mL (normalized) (d-f) over time for high, medium and low saturation levels, black dot: mean COHb/TBCO concentration for day of analysis, line in box: median.

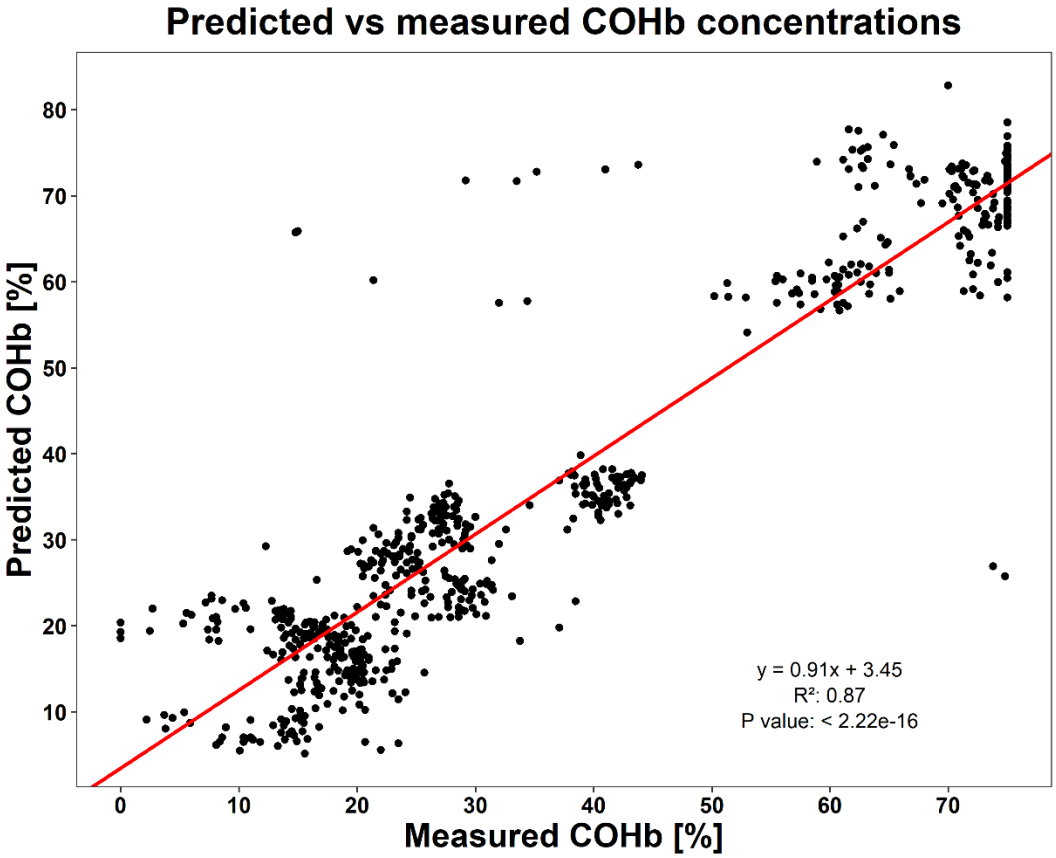

Figure 4: Correlation plot for measured vs predicted COHb concentrations in %

Table 1: List of transformations employed for data according to analyte of interest and saturation level, *: for high saturation level COHb, no normal distribution was obtained, thus non-parametric tests were employed.

Saturation Level	COHb	TBCO
High* **(60-70%)**	-	Log_{10}
Medium **(30-40%)**	-	Log_{10}
Low **(10-20%)**	-	Cube root

Table 2: Results of ANOVA for single parameters and combination of parameters for high, medium and low saturation level; COHb: carboxyhemoglobin, TBCO: total blood carbon monoxide; **: $p < 0.01$, *: $0.05 \leq p \leq 0.01$, –: not significant parameter ($p > 0.05$), #: for high saturation COHb levels, non parametric Kruskal-Wallis test was performed for single variables only.

SATURATION LEVEL		HIGH (60-70%)		MEDIUM (30-40%)		LOW (10-20%)	
Model Number	Variables	**COHb#**	**TBCO**	**COHb**	**TBCO**	**COHb**	**TBCO**
1	Temperature	**	**	–	**	–	*
2	Preservative	**	**	**	**	**	**
3	Time	–	–	*	–	–	–
4	HS volume	–	–	–	–	–	–
5	Temperature	#	**	**	**	**	**
	Preservative	#	**	**	**	**	**
6	Temperature	#	**	**	**	**	**
	Preservative	#	**	**	**	**	**
	Time	#	–	**	*	*	*
7	Temperature	#	**	**	**	**	**
	Preservative	#	**	**	**	**	**
	Time	#	–	**	–	*	*
	HS volume	#	–	**	–	–	–

Table 3: Coefficient estimates (β) and 95% confidence intervals (CI) from Multiple Linear Regression (MLR) with storage parameters preservative, temperature, time and HS volume for measurement of COHb and TBCO for high, medium and low saturation levels. In **bold** the significant parameters ($p<0.05$). MLR was performed with normalized data for TBCO (see **Table 1**).

SATURATION LEVEL	HIGH (60-70%)		MEDIUM (30-40%)		LOW (10-20%)	
	COHb ($R^2 = 0.67$)	TBCO ($R^2 = 0.39$)	COHb ($R^2 = 0.81$)	TBCO ($R^2 = 0.22$)	COHb ($R^2 = 0.76$)	TBCO ($R^2 = 0.22$)
Parameter (Reference)	Coefficient estimate β (95% CI)	Coefficient estimate β (95% CI)	Coefficient estimate β (95% CI)	Coefficient estimate β (95% CI)	Coefficient estimate β (95% CI)	Coefficient estimate β (95% CI)
Preservative (EDTA)						
NaF	**-16.35 (-17.47, -15.24)**	**-0.24 (-0.29, -0.19)**	**-13.92 (-14.58, -13.26)**	**-0.34 (-0.41, -0.26)**	**-7.01 (-7.54, -6.48)**	**-0.20 (-0.26, -0.13)**
LiH	**2.39 (1.25, 3.53)**	**0.27 (0.21, 0.32)**	**-13.49 (-14.14, -12.83)**	**-0.51 (-0.59, -0.44)**	**5.30 (4.83, 5.77)**	**-0.10 (-0.15, -0.04)**
NaCit	**-5.96 (-7.20, -4.72)**	**0.18 (0.12, 0.23)**	**-17.63 (-18.30, -16.96)**	**-0.27 (-0.35, -0.19)**	**3.00 (2.53, 3.47)**	**0.21 (0.16, 0.27)**
Temperature (-20°C)						
+ 20°C	**5.63 (4.63, 6.64)**	**0.06 (0.01, 0.10)**	**-0.71 (-1.28, -0.13)**	**-0.20 (-0.26, -0.13)**	0.07 (-0.37, 0.51)	**-0.09 (-0.14, -0.04)**
+ 4°C	**2.60 (1.57, 3.64)**	-0.02 (-0.07, 0.02)	0.12 (-0.46, 0.69)	-0.04 (-0.10, 0.03)	**0.75 (0.33, 1.17)**	0.02 (-0.03, 0.07)
Time (Day 0)						
Day x	**-0.83 (-1.02, -0.63)**	0.01 (0.00, 0.02)	**-0.44 (-0.55, -0.33)**	0.00 (-0.01, 0.01)	**-0.16 (-0.24, -0.08)**	0.00 (-0.01, 0.01)
HS volume (<25%)						
25%-50%	-0.23 (-1.25, 0.78)	0.00 (-0.05, 0.04)	**-0.72 (-1.30, -0.14)**	0.05 (-0.02, 0.12)	0.04 (-0.39, 0.46)	0.00 (-0.05, 0.05)
>50%	**-1.32 (-2.33, -0.31)**	-0.03 (-0.07, 0.02)	**-1.14 (-1.72, -0.57)**	-0.04 (-0.11, 0.03)	-0.35 (-0.78, 0.07)	-0.03 (-0.08,0.02)

Table 4: Coefficient estimates (β) and standard error (SE) from Multiple Linear Regression (MLR) for prediction model ($R^2 = 0.94$), with storage parameters preservative, temperature, time and HS volume for measurement. In **bold** the significant parameters.

	COHb	
Parameter (Reference)	**Coefficient estimate (β)**	**Standard error (SE)**
Saturation level (Low, 10-20%)		
Medium (30-40%)	**13.75**	0.57
High (60-70%)	**48.79**	0.87
Preservative (EDTA)		
NaF	**-12.00**	0.63
LiH	**-2.04**	0.59
NaCit	**-7.53**	0.62
Temperature (-20°C)		
+ 4°C	**2.16**	0.53
+ 20°C	**2.87**	0.53
Time (Day 0)		
Day x	**-0.10**	0.02
HS volume (<25%)		
25%-50%	-0.43	0.53
>50%	-0.71	0.53