“CARBON MONOXIDE POISONINGS: EXPLORING NEW APPROACHES FOR QUANTIFICATION AND EVALUATING MEASUREMENT ERRORS FROM AN ANALYTICAL AND EPIDEMIOLOGICAL POINT OF VIEW”

By Stefania Oliverio

Carbon monoxide poisonings: exploring new approaches for quantification and evaluating measurement errors from an analytical and epidemiological point of view

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By

Stefania Oliverio

College of Health and Life Sciences, Brunel University London

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Abstract

Thousands of deaths and hospital admissions worldwide are reported every year due to carbon monoxide poisoning. However, current biomarkers of exposure and quantification devices employed for detection and diagnosis lack sufficient specificity and accuracy, leading to frequent errors. This results in an underestimation of the true disease burden attributable to CO exposure, making it a topic of concern for the clinical and public health communities. The aim of this doctoral research was to investigate some of the factors leading to underestimation of the health risks associated with CO exposure and evaluate their margins of error, in order to propose improvements from an analytical and epidemiologic perspective.

The quantification of Total Blood CO (TBCO) via Airtight gas Syringe-Gas Chromtography-Mass Spectrometry (AGS-GC-MS) was identified as more accurate, specific and less error-prone than current biomarker carboxyhaemoglobin (COHb) and proposed as alternative biomarker for CO exposure. The method was validated for clinical and forensic applications and tested on real cases in both fields. Results for the comparison between the two biomarkers in the different settings (e.g. different storage conditions in clinical and postmortem cases) confirmed TBCO as more appropriate biomarker for CO exposure and highlighted the limitations of COHb. To determine the potential impact on CO exposure assessments of TBCO under controlled conditions and, consequently, its effect on the relative risk, the sources of measurement error in CO exposure assessment studies were determined and the improvement on measurement error was calculated on one exemplary study. The resulting relative risks were increased moderately, thus getting closer to the true risk of CO exposure in the population. This affirms the importance of even small improvements in one part of the error sources being able to achieve tangible changes with important public health implications. Despite COHb being a cheaper and more established biomarker, I currently recommend implementing TBCO for challenging cases and encourage further research in this area.

Keywords: carbon monoxide exposure, carboxyhaemoglobin, TBCO, blood biomarker, GC-MS, measurement error, improvement, relative risk.

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Abbreviations

ABI	Acquired brain injury
AGS	Airtight gas syringe
AGS-GC-MS	Airtight gas syringe-gas chromatography-mass spectrometry
AirCO	Ambient air carbon monoxide
ANFR	Accidental, non-fire related
ANOVA	Analysis of variance
APEX	Air pollutants exposure model
APPCOG	All-parliamentary party carbon monoxide group
β	Coefficient estimate
BGA	Blood gas analyser
Bl+IS	Bovine blood with internal standard
Bl-IS	Bovine blood without internal standard
^{13}CO	Isotopically labelled carbon monoxide
CB	Cardiac blood
CFK	Coburn, Foster and Kane
cGMP	cyclic guanosine monophosphate
CH_4	Methane
CI	Confidence interval
CNS	Central nervous system
CO	Carbon monoxide
CO_2	Carbon dioxide
COCGG	Carbon monoxide cross government group
COHb	Carboxyhaemoglobin
CV	Coefficient of variation
ED	Emergency department
EDTA	Ethylenediaminetetraacetic acid
EI	Electron ionization
EM	Exposure measurement
EMM	Exposure measurement method
EPA	Environmental protection agency
eV	Electron volt

ExCO	Exhaled breath carbon monoxide
FAAS	Flame atomic absorption spectroscopy
FID	Flame ionization detector
GC	Gas chromatography
GC-FID	Gas chromatography-flame ionization detection
GC-MS	Gas Chromatography-Mass Spectrometry
GC-RGA	Gas chromatography-Reduction gas analyser
GST	Gas Safety Trust
$H^{13}COOH$	Isotopically labelled formic acid
H_2SO_4	Sulphuric acid
Hb	Haemoglobin
HCl	Hydrochloric acid
HCN	Hydrogen cyanide
HCOOH	Formic acid
HHb	Reduced haemoglobin
HIM	Human inhalation model
HS	Headspace
HS-GC-MS	Headspace-Gas Chromatography-Mass Spectrometry
HUG	University Hospital of Geneva
ICC	Interclass correlation
ICORN	International carbon monoxide research network
ISO	International Organization for Standardization
$K_3Fe(CN)_6$	Potassium ferricyanide
LiH	Lithium heparin
LLOQ	Lowest limit of quantification
LOD	Limit of detection
Log_{10}	Base 10 logarithm
LOQ	Litmi of quantification
LUR	Land use regression
m/z	Mass-to-charge ratio
Mb	Myoglobin
MetHb	Methaemoglobin
MLR	Multiple linear regression
MS	Mass spectrometry

NAAQS	National ambient air quality standard
NaCit	Sodium citrate
NaF	Sodium fluoride
NO_2	Nitrogen dioxide
NO_x	Nitrogen oxides
O_2	Oxygen
O_3	Ozone
O_2Hb	Oxyhaemoglobin
PB	Peripheral blood
PBPK	Physiologically-based pharmacokinetic
pCO	Personal carbon monoxide
PE	Polyethylene
PHE	Public Health England
PLOT	Porous open layer tubular
PM	Postmortem
PMI	Postmortem interval
pNEM	probabilistic NAAQS exposure model
PP	Polypropylene
ppm	parts per million
PTFE	Polytetrafluoroethylene
QC	Quality control
R^2	Regression coefficient/correlation factor
RGA	Reduction gas analyser
RR	Relative risk
RT	Room temperature
RSD	Relative standard deviation
S100β	S100 calcium-binding protein B
SD	Standard deviation
SFSTP	French Society of Pharmaceutical Sciences and Techniques
SHAPE	Simulation of human activity and pollutant exposure model
SIM	Selected Ion Monitoring
SO_2	Sulphur dioxide
TBCO	Total blood carbon monoxide

TCD	Thermal conductivity detector
TIAFT	The international association of forensic toxicology
TI	Tolerance interval
UKIAFT	UK and Ireland association of forensic toxicology
UV	Ultraviolett
WHO	World Health Organization

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Chapter 1 - Introduction

1.1 Background – The 'Rap Sheet' of CO

Every year, approximately 50,000 emergency department (ED) visits and 2,741 deaths in the US are due to carbon monoxide (CO) poisoning [1]. In Europe, CO-related deaths average at 350 annually, while in non-European states such as Australia and Japan, this number reaches 250 and over 3,200, respectively [2]. It is thus not surprising, even for laymen, that the most common analogy used to describe CO is *the silent killer*. Mortality data shows that the majority of deaths are due to accidental, non-fire related (ANFR) CO poisoning. In the UK alone, around 200 hospital admissions and 25 deaths are attributed to ANFR CO poisoning annually [3]. The global burden of CO on morbidity and mortality seems to be still quite substantial, despite emissions of CO and other air pollutants being reduced by 87% in the last three decades [4]. Of the total global CO emissions, 60% are anthropogenic, while 40% originate from natural processes [5].

Generally, CO is generated during incomplete combustion of hydrocarbon-based materials, which is a phenomenon that occurs when oxygen(O_2)-availability is low, such as in poorly ventilated fires, exhausts of petrol-, wood- or oil-fuelled engines, motors, heating and cooking appliances as well as smoke from cigarettes, cigars and water pipes [6–11]. Since the majority of these sources are of anthropogenic nature, CO concentrations tend to be higher in urban areas where there is a high presence of industrial activities, motor vehicles (i.e. traffic), biomass burning sites, etc., with background CO levels in non-urban sites mainly deriving from natural biological processes such as oxidation of hydrocarbons [5]. Despite the high number of CO-generating processes, not all people living in highly populated regions are exposed to outdoor CO levels that have toxic effects. As the father of toxicology, Paracelsus, once said, "*All things are poisons, for there is nothing without poisonous qualities. It is only the dose which makes a thing poison.*", the toxicity of CO (the *poison*) depends on its concentration (the *dose*). CO concentrations tend to reach higher levels at a faster rate in indoor environments, where CO is 'trapped' in an enclosed space, compared to outdoors, where it can dissipate into the air [12].

CO is a gaseous, diatomic molecule with no taste, colour or odour – at first 'sight', not a menacing substance. However, despite its toxicokinetic and toxicodynamic mechanisms still

not being sufficiently understood, CO is known to have the potential of causing severe adverse health effects involving the respiratory, cardio-circulatory and neurological systems, which can, in the worst cases, lead to death [13,14]. The main route of exposure to CO is through inhalation (**Figure 1.1**). CO enters the bloodstream through the lungs during the normal breathing process, where its high affinity for haemoglobin (Hb) (~250 times higher than that of oxygen), the oxygen-carrying protein in erythrocytes, causes it to competitively replace O_2 to form carboxyhaemoglobin (COHb) [15].

While the mechanism and kinetics of CO to Hb binding are not fully elucidated yet, it is thought to be somewhat similar to the binding of O_2 to Hb, which has been thoroughly described in the past. Hb is a hetero-tetramer, consisting of two alpha and two beta subunits each containing a coordinated iron (Fe(II)) ion, which are the binding sites of the protein. The binding mechanism of O_2 to Hb has been described as a cooperative binding, meaning that the subunits cannot be seen as independent binding sites. In fact, with the binding of one O_2 molecule, the conformation of the haeme protein changes from a tense state (T-state) to a more stable, relaxed state (R-state), resulting in the increased affinity of the two proximal binding sites for O_2. After binding of the additional subunits, the affinity of the last remaining site is increased even more. For the oxygen to be released in the tissues, a change in the partial pressure is necessary, with oxygen release increasing with reduced pressure [16].

For CO, the binding mechanism is still under discussion, with one plausible hypothesis being a mixture between electrostatic forces and a coordination of the carbon to the iron due to the favourable position of their orbitals [17]. Assumption were made the kinetics are similar to those of the O_2-binding to Hb, with an increased affinity for CO after the first binding. Differently is the situation though for their dissociation, which is based on partial pressure gradients: while the oxygen release curve has a sigmoidal shape, the CO curve shows a hyperbolic shape [18]. This means that oxygen is released at higher partial pressures than CO, with CO being bound stronger at higher pressures and released only at lower pressures. Considering that O_2 is released differently to the tissues based on their partial pressure gradient, the same difference in pressures should be valid for the release of CO. Furthermore, while O_2 is generally present even at low concentrations in tissues, CO is not. Therefore, when CO is inhaled and crosses the alveoli-blood barrier, a pressure gradient between blood and tissues automatically results, which is higher compared to the one for O_2. This should lead to easier transfer of CO to tissues [17]. As a result of the inhibition of O_2 transport to the tissues, also the release of CO_2 from tissues is affected, mainly leading to hypoxia of vital organs such as

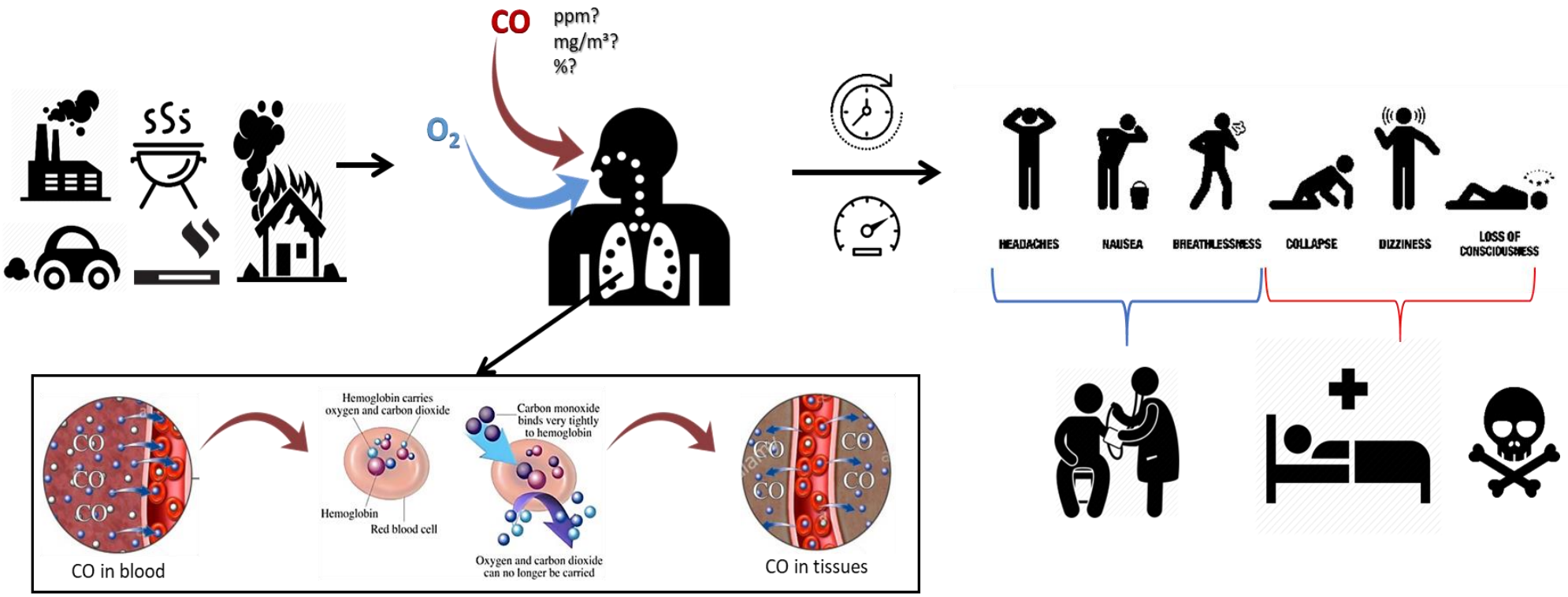

Figure 1.1: Mechanism of exposure to carbon monoxide (CO). CO is emitted from sources that use hydrocarbon-based fuels into the air and is then inhaled through the lungs and transferred into the blood circulation, where CO binds to haemoglobin and is transported to the tissues. Depending on the concentration of CO in the inhaled air and the time of exposure, a variety of health effects with different degrees of severity can be experienced by an individual, which can lead to seeking medical attention or, in worst cases, death. The image was created by Stefania Oliverio, with some parts adapted from external sources (https://preparednessadvice.com/medical/family-eight-died-carbon-monoxide-poisoning/, https://doctorstock.photoshelter.com/image/I0000SMQdj5CeN.w); icons were purchased from 'Iconfinder'.

brain and heart. Depending on the amount of time and concentration levels of exposure to CO, exhibited symptoms include headache, dizziness and nausea, which can exacerbate to loss of consciousness, coma and, ultimately, death [19,20].

1.2 New Issues for an Old Poison

One of the major issues with CO poisonings is the non-specificity of the reported symptoms, which often lead to misdiagnosis by both the patient and the clinician, who attribute the signs to other more common illnesses, such as the flu, general stress or fatigue and gastrointestinal issues. Due to the lack of suspicion, a test to measure the CO levels is not performed in these cases, which can have fatal outcomes: the exposed individual might return to the CO-generating source and continue being exposed to the gas for a prolonged period without being aware of the developing poisoning until it reaches a critical point where either irreversible neurological damage or death occur [21–23].

In correctly diagnosed, acute intoxication cases, the most common therapeutic measure is to administer normo- or hyperbaric O_2, which helps flush out CO and restore physiological O_2-levels in the body of the individual [24]. However, this treatment is not possible in instances where low-level, chronic exposures occur. On one hand, this is due to the mentioned lack of suspicion and specific symptoms, leading to misdiagnosis. On the other hand, when the clinician or patient might be aware of potential exposure to CO and, thus, correctly diagnose it, the measurement method used for detection has insufficient accuracy and the reported levels might be underestimated, leading to a false negative [25].

Supporting this hypothesis are the numerous cases describing inconsistencies between reported symptoms and measured CO levels, where people exposed to the same amounts of CO had different manifestations and different levels measured in their blood [19]. While it is possible that a part of these differences are related to distinct inter-personal characteristics, such as lung volume, ventilation rate, age, metabolic rate or various pre-existing morbidities [7,11], the reliability and accuracy of the methods used for CO measurement is also a highly important factor, since it is these values that are used to correlate CO exposure to their potential health effects. The choice of the measurement technique employed depends on the biomarker selected for the exposure measurement. In CO poisonings, this choice has fallen onto COHb, due to the very tight bond between the molecule and the protein, and the assumption that most CO entering the blood circulation binds to Hb [7,26]. However, various studies have debated the

percentage of CO bound to Hb, with around 10-15% of the absorbed CO being previously documented to bind to proteins other than Hb [20,24], some studies suggested that the percentage might be as high as 50% [27]. These studies also showed that CO also to other haemoproteins, including globins such as myoglobin (Mb), neuroglobin (Nb) and cytoglobin (Cb) and various other compounds such as cytochromes (cytochrome c oxidase, cytochrome P450, etc.) and guanylyl cyclase (**Figure 1.2**) [28–32].

Binding to myoglobin reduces the availability of oxygen in the heart and may lead to arrhythmias and cardiac dysfunctions as well as causing direct toxicity of skeletal muscle [28]. CO binding to mitochondrial cytochrome oxidase, which was reported for *in vitro* studies [29,33], impairs cellular respiration by inactivation of mitochondrial enzymes and also initiates an oxidative metabolism by generating oxygen free radicals [20,28,34]. Even though the affinity of CO for cytochrome oxidase is relatively low, the dissociation is very slow, leading to a prolonged impairment of oxidative metabolism, which may help explain the tardive and sustained effects of CO toxicity even after COHb has been cleared from the system through hyper- or normobaric oxygen therapy [20,24]. The loss of consciousness associated with CO poisoning may be caused by the stimulation of guanylyl cyclase since increases in cyclic guanosine monophosphate (cGMP) result in cerebral vasodilation [35,36].

In general, the role of direct cellular toxicity seems to be underestimated in the literature. Binding to these other proteins does not only explain the variety of physiological and neurological outcomes associated with CO but could potentially be associated with the misdiagnosed and underestimated cases when using COHb as a sole biomarker. Therefore, it is important to be able to determine the total amount of CO in blood and not only COHb in CO poisoning cases. These misdiagnoses and underestimations do not only affect the immediate health status of an exposed individual, but they also have implications into the health status of the population. These misdiagnoses and underestimations do not only affect the immediate health status of an exposed individual, but they also have implications into the health status of the population. Air quality guidelines and required occupational air pollutant levels are set up based on risk and exposure assessment studies. Exposure assessment studies typically sample a representative amount of people in a population subgroup, measure their exposure and with the aid of air pollution monitoring and environmental models, extrapolate the results to the population [29, 30].

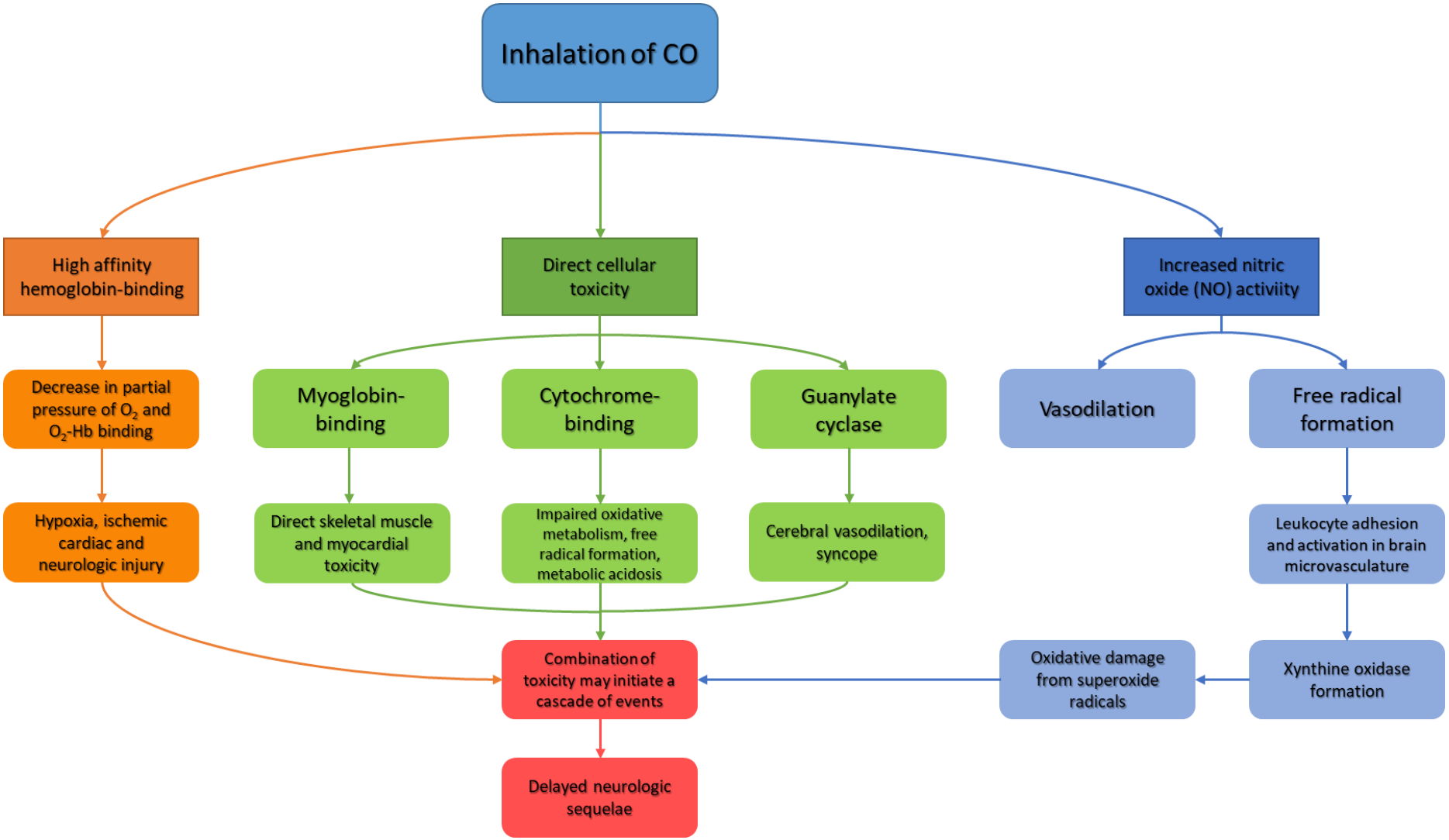

Figure 1.2: Adverse-outcome pathway for CO poisoning. Apadted from Kao and Nañagas [20].

1.3 CO and Public Health

It has been over 50 years that air pollution and exposure to certain environmental contaminants have been attributed as the underlying causes for a large portion of the global death burden worldwide and also associated with adverse health outcomes, including several pulmonary, cardiovascular, immune system and neurologic diseases [37–39]. Air pollution is defined as a heterogeneous mixture of gases and particulate matter (PM), the main components of which are ozone (O_3), nitrogen oxides (NO_x), carbon monoxide (CO) and sulphur dioxide (SO_2) [40]. Due to their high toxicity and occurrence, especially in outdoor environments, and severe impact on the global disease burden, recent studies have mainly focused on O_3, NO_x and PM [41–45].

CO is also a highly toxic gas, even though present in lower quantities in outdoor environments. It is in enclosed spaces that CO poses the greatest health risks, mainly due to the presence of CO-generating sources.

In indoor environments, CO levels tend to increase at a higher rate due to generally limited ventilation and low oxygenation, thus posing a higher risk of acute CO poisoning. Occupational exposure to indoor CO also constitutes an important part of the total human CO exposure, even though exposure levels are usually lower than during acute exposures due to high emission sources, such as during a fire. Toxicity in occupational settings is usually due to chronic exposure to lower CO levels but is more difficult to associate with pathological outcomes [46,47]. Therefore, interest for low levels of indoor CO with the potential for chronic toxicity was only recently raised, with several studies finding a link between low-level chronic CO exposure and neurocognitive impairments [18,48,49]. Exposure to low concentrations of CO for prolonged periods of time showed symptoms of acquired brain injury (ABI), which in most cases were permanent [23,50–52]. Despite known health consequences following low-level chronic CO exposures, there are only a limited number of clinical or epidemiological studies, and thus, evidence, looking at how to recognise, diagnose and prevent low-level chronic CO exposures. These chronic exposures, even though at low levels, also constitute an essential part of the global burden of CO exposure.

These are clearly of relevance for public health and should be given more importance by public health authorities than they are currently. The UK, driven by the Cross Government CO Group (COCGG), which includes all major institutions such as Public Health England (PHE),

PolicyConnect, the All-Parliamentary Party Carbon Monoxide Group (APPCOG) and the Gas Safety Trust (GST), together with institutions in Canada and the US are pioneering in the funds put into research for deeper understanding of low-level chronic CO exposures. Gaining more access to the scientific background behind CO exposures but also to their true incidence in the population is pivotal to inform policymakers and help them improve their decision-making process. For this purpose, epidemiologic studies provide an essential and unique tool to obtain the necessary evidence [53]. However, current knowledge is still far from being satisfactory for CO exposures, especially at low levels.

1.4 CO exposure assessments

To be able to determine the global burden of CO exposure on the population, it is important to determine the magnitude and role played by the part attributed to indoor CO levels. Epidemiologic studies that determine the distribution of indoor CO exposure and its health effects in the population are necessary. The part of epidemiology that allows us to obtain this kind of information is exposure assessments. Exposure assessment is defined as the study of the distribution and the determinants of compounds or factors affecting human health and aims at optimizing the exposure estimate and exposure-response relation by taking into account factors such as the distribution of the variance of exposure estimates [54]. It comprises the measurement of exposure and of other factors that determine the distribution and exposure levels relevant to human health in different population groups, environments and countries (e.g. elderly vs children, urban vs rural areas, underdeveloped vs developed countries) [53].
Exposure assessments are essential from a public health perspective since they provide the epidemiologic evidence that from exposure estimates leads to the determination of risk estimates in the population, which is what public health authorities need to set up indoor and outdoor air quality guidelines and reference levels [53]. Improved exposure assessments, therefore, result in improved epidemiologic evidence that leads to a better quantification of the risk associated with the exposure. Consequently, it promotes evidence-based decision-making, which drives the setting of standards by public health authorities and can help improve management and prevention strategies. As a result, improved standards have the potential to reduce morbidity and mortality associated with exposure to the assessed pollutants.

Therefore, exposure assessments are necessary to determine concentrations of CO in indoor and outdoor spaces and derive the evidence required to set up guidelines for safe living and

working environments for the population. The World Health Organization (WHO), United States Environmental Protection Agency (EPA) and authorities from other countries published reports from exposure assessments and guidelines on air quality criteria for most pollutants, including CO [55–57]. An example of these guidelines is given by the limits proposed by the WHO for CO levels in ppm based on the amount of exposure time [5], which were also correlated to COHb levels in % and typical symptoms (**Figure 1.3**).

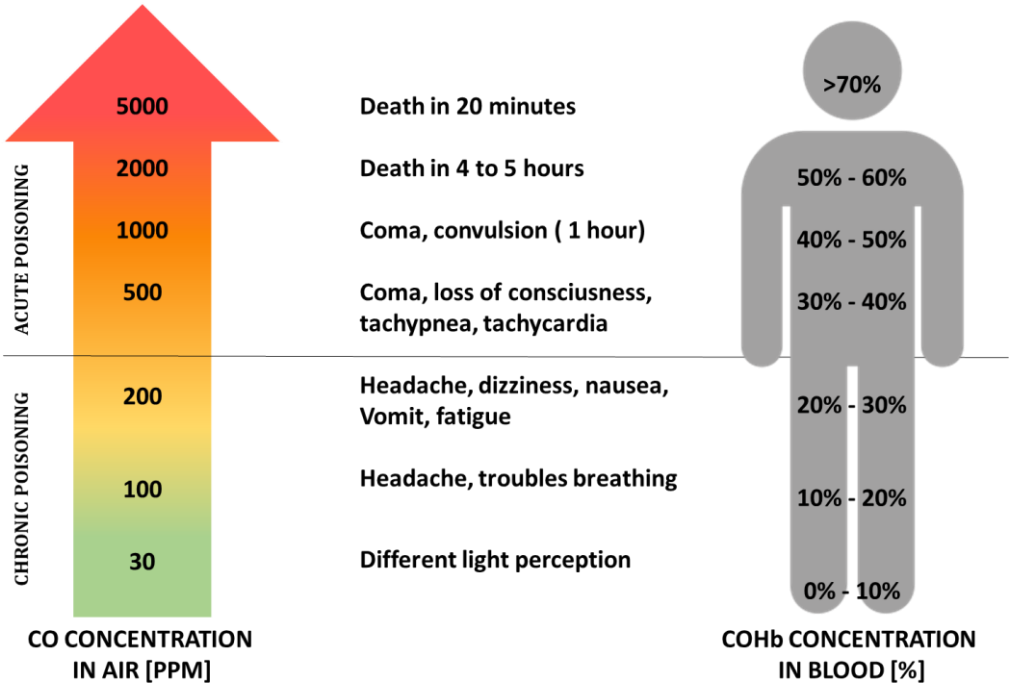

Figure 1.3: Symptomatology associated with reference levels for CO in parts per million (ppm) and carboxyhaemoglobin (COHb) in %. (Adapted from WHO guidelines [31]).

However, recent developments regarding the toxicity of low-level CO exposures were either not discussed or updated in these reports. While methods to assess exposures to acute, high-level CO concentrations are described in detail, little information is available on methods to assess exposures to low concentrations of CO over a prolonged time interval. Especially for indoor CO exposures, only brief descriptions can be found in these reports, despite the high complexity but also the importance of indoor environments. Factors that affect average and peak indoor concentrations include the type, quantity and frequency of use of pollutant-emitting sources, the availability, frequency and rate of ventilation of the indoor environment as well as the human activity pattern (people tend to spend different amounts of time in different indoor spaces) [58].

These guidelines are globally applicable and employed with similar values by numerous public health institutes, industries and policymakers worldwide as well as being a reference in clinical and forensic settings.

1.5 Investigation Goals

Despite the general public and medical aim of preventing death and treating injury cases, it is also an essential task from a legal and ethical point of view to determine the cause of death correctly and accurately. Apart from the already mentioned issues in clinical cases, CO poisoning detection poses additional challenges during the analysis of post-mortem (PM) samples. These are due to biochemical phenomena occurring in the human body during and after death, the interval between time of death and body retrieval (postmortem interval – PMI) and time and methods for sampling and storage, causing alterations of samples biasing CO levels and thus affecting the interpretation of the results, which has significant juridical implications [59,60]. Therefore, mistakes in exposure measurements have a widespread relevance, ultimately having an impact on the population morbidity, mortality and risk rates derived from CO.

Currently, public and medical awareness of CO exposure and the health dangers it poses are being improved through increased training of clinicians by experts in the field and advertisement in local communities as well as through broad media coverage in recognizing the signs of a CO poisoning. This study aims at tackling the raised issues from a scientific standpoint by following a path that links the toxicological to the epidemiological investigation.

i. One of the objectives is to develop and validate an alternative analytical method for more accurate CO poisoning determination for clinical and forensic applications, to help decrease misdiagnosis due to the inconsistencies between symptoms and COHb levels.
ii. Moreover, this research intends to determine and quantify the influence of storage parameters on changes in COHb/CO concentrations over time and aid in creating a model that allows incorporation of these alterations during the interpretation of results from CO poisoning determinations in individual measurements. This should also decrease the number of cases erroneously not attributed to CO as a cause of morbidity or mortality.
iii. An additional aim is to identify gaps in CO exposure assessments, with a focus on determining and quantifying measurement error arising from recent methodological and toxicological advances, including part of the work conducted in this study. This will

enable the development of an overview of current practices and frequent errors in CO exposure assessment as well as generating an approach to correct for a part of these errors.

iv. Finally, this study wants to determine the magnitude of the impact that improvements in methodological and analytical measurement and exposure assessment methods can have on the population estimates and, thus, on the relative risk related to CO. This is the essential step that can directly link the analytical improvements with population health.

1.6 Structure of the thesis

The thesis includes a total of six chapters.

The first chapter is the introductory chapter, which describes the main chemical, physical and toxicological properties of CO and the problems associated with CO exposure and its diagnosis in clinical and forensic cases, but also the relation and relevance of these issues to the global CO burden on public health.

Chapter two is a review of past and current trends in CO measurement methods in biological matrices, highlighting pros and cons of available methods, challenges faced during CO analysis in clinical and forensic cases as well as pointing out the limitations of these methods and potential ways to circumvent them.

In chapter three, I describe the development and validation of a novel measurement method for CO in blood based on gas chromatography-mass spectrometry (GC-MS) and propose a novel biomarker for CO poisoning diagnosis, Total Blood Carbon Monoxide (TBCO). I validated the method for use in clinical and PM concentration ranges and applied it to a series of real cases. All analyses were carried out with the newly developed method as well as the "golden standard" spectrophotometric measurement of COHb as a comparison.

The fourth chapter is dedicated to the investigation of several storage and sampling parameters employed in blood analyses for CO poisoning determination cases. I did not only determine the impact of these parameters on the results of blood analyses but also compared them in light of the measured biomarker and inherent measurement method, namely COHb via CO-oximetry and TBCO via GC-MS, to determine their differences both qualitatively and quantitatively.

Chapter 5 brings the results from the previous chapters together into the perspective of CO exposure in the population and public health-related issues, by identifying the gaps in CO exposure assessment methods and the influence an improved measurement method and improved sampling and storage parameters can have on the health risk associated with CO exposure. Calculations are carried out to quantify this impact in selected studies.

The final chapter, chapter six, concisely summarizes the major achievements of this study as well as indicating both pitfalls/limitations of the approaches described and further work required in the field.

All the toxicological *in vitro* analyses were carried out at the University Centre of Legal Medicine. These analyses were performed under routine activity at the University Centre of Legal Medicine, which confirms with the Swiss Federal Council Act on Research.

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Chapter 2 - What are the limitations of methods to measure carbon monoxide in biological samples?

2.1 Introduction

Carbon monoxide (CO) concentrations may be measured in exhaled breath, ambient air, or in blood. Due to the high affinity of CO to haemoglobin (Hb), it has been assumed that the majority, if not all, CO binds with Hb when introduced into the blood circulation. This has resulted in carboxyhemoglobin (COHb) being considered the most appropriate clinical marker of exposure for CO poisoning [1]. However, COHb does not represent the only reservoir of CO in the human body because CO may be found dissolved in blood at free state and can bind to other heme-containing respiratory globins, such as myoglobin in muscle, neuroglobin in the nervous system and, to a lesser extent, cytoglobin [2]. CO dissolved in blood in free form is known to have a role in the pathophysiology of CO poisonings [3], but might be more substantial than what studies revealed so far. This would result in under- or misestimation of the true CO level present in the analyzed blood sample, potentially elucidating some of the cases where inconsistencies between measured COHb level and reported symptoms were found. However, currently, there is not much data on free CO available.

COHb in blood is measured directly or indirectly by using either optical methods (CO-oximetry, UV-spectrophotometry or pulse-oximetry) or gas chromatographic methods in combination with a variety of detectors (flame ionization detector, mass spectrometer). In clinical cases, the “gold standard” is the measurement of COHb in blood is by CO-oximetry (or pulse-oximetry), either as a separate instrument or integrated into what is commonly known as a blood gas analyzer (BGA) or radiometer [5]. Although ultraviolet (UV)-spectrophotometry remains the most frequently used method in forensic cases, CO-oximetry and gas chromatographic methods are also widely employed in this field.

Like any biomarker, the quantitative measurement of COHb is subject to a variety of factors that influence the measurement. Measurement error in analytical studies is defined as “uncertainty” or “bias”. Uncertainty originates when several predictable, but not always controllable factors affect the measured values and may potentially alter the obtained value,

resulting in a deviation from the true value due to these factors. In medical practice and especially for toxicologists, it is crucial to correctly and accurately determine a biomarker, in order to make the correct diagnosis and initiate the proper treatment in clinical cases and to determine the correct cause of death in forensic cases. Shortcomings in doing so can have severe clinical and legal consequences. Therefore, in this chapter, I aim to review the accuracy of current methods to measure CO and to determine their potential sources of error and their effects in the interpretation process.

2.2 Methods

PubMed was searched in November 2018 using the keywords (“carbon monoxide” OR “carboxyhemoglobin”) AND (“poisoning”) AND ("measurement" OR "determination" OR "quantification" OR "analysis" OR "breath" OR "blood" OR "oximet*" OR "spectro*" OR "gas chromatography" OR "storage"); this gave 191 hits. Systematic reviews, meta-analyses and general review articles, retrospective, prospective, observational and clinical cohort studies were excluded as well as case reports, limiting included articles to those which were focused specifically on describing a method for analysis of CO or COHb in various tissues and those describing issues related to analysis of samples (storage, sample pretreatment, etc.). This left 49 relevant articles on measurement methods and sources of errors.

2.3 Measurement of CO in breath

2.3.1 Analytical techniques

Analysis of CO in exhaled breath was evaluated as a measurement method for clinical cases since a good correlation between alveolar breath CO and COHb was found by several research groups [6–9]. Portable devices, called MicroCOmeters or CO monitors, are often used in smoking cessation programs [8, 10] and may be useful when a rapid on-site assessment in multiple casualties is necessary, enabling the most severe cases to be identified [11]. This measurement is based on an electrochemical fuel cell sensor, which works through the reaction of CO with an electrolyte on one electrode and oxygen (from ambient air) on the other. This reaction generates an electrical current proportional to a CO concentration. The output from the sensor is monitored by a microprocessor, which detects a peak at expired concentrations of CO in the alveolar gas [12]. These are then converted to COHb% using the mathematical

relationships described by Jarvis et al. [8] for concentrations below 90 parts per million (ppm) and by Stewart et al. [13] for higher levels.

2.3.2 Sources of errors

Measurement of CO in breath cannot account for the total CO concentration present in the blood at the time of exposure. It is a very susceptible method and affected by a variety of factors that can easily alter the result into under- or overestimating the true concentration (**Figure 2.1**). A major aspect is the variation among the subjects' abilities of breath-holding. To obtain the alveolar gas, it was found that the breath needs to be held for 20 s and then only the end-tidal expired air is used for CO measurement. Given the interpersonal differences in pulmonary function, capillary diffusion surface and inspiration and expiration rates, as well as the inability to fully control whether a subject is properly holding the breath, the portion of expired alveolar gas sampled and the results obtained can have a high degree of variability [6, 8, 13]. This can also pose an issue in susceptible groups of the population, such as elderly, children or subjects with respiratory diseases. Furthermore, since they were initially designed for smoking cessation programs, the accuracy of CO monitors is better in lower CO concentrations (0-20 ppm) and might therefore not be sufficiently accurate for acute intoxications [14]. Nevertheless, CO monitors have high usefulness on sites of mass casualties or for first responders. They are portable and can give an indication of the gravity of the case, which can allow the appropriate treatment of the patient as well as proper precautions to be taken by first responders.

2.4 Measurement of CO in blood: optical techniques CO-oximetry and spectrophotometry

2.4.1 Analytical techniques

Spectrophotometric or optical methods measure the concentration of COHb based on the quantity of absorbance of light when the compound is exposed to light of different wavelengths. In the past, single-beam UV-spectrophotometry or double wavelength spectrophotometry was first developed due to the spectral absorbance of the Hb structures and due to the distinct spectral differences between oxyhemoglobin (O_2Hb) and COHb [15–17]. A similar method involves the measurement in the visible spectra of the differences in absorbance between

reduced Hb (HHb) and COHb, where a reducing agent is added to the blood sample that reduces O_2Hb, but not COHb [18, 19].

However, double wavelength spectrophotometry was not a very accurate and specific method [16], since results were based on the measurement of only two wavelengths. Automated differential spectrophotometry was later developed, which uses double-laser beams to determine the difference in absorbance of a sample compared to a negative sample, thus with this method, matrix effects are accounted for, resulting in better accuracy.

CO-oximetry is a measurement technique based on multiple wavelength spectrophotometry, which uses the multiple wavelengths up to the full range of wavelengths for analysis, allowing for more accurate measurement of COHb [20–22]. They are currently the standard analytical technique used for measurement of COHb, either with a separate instrument or, for hospital cases, integrated into a BGA [18, 23, 24].

Despite the advantages of CO-oximetry, due to cost-efficiency UV and double wavelength spectrophotometers are currently still used in many developing countries and are also listed in the International Organization for Standardization (ISO) 27368:2008 'Analysis of blood for asphyxiant toxicants – carbon monoxide and hydrogen cyanide' standards [25].

2.4.2 Sources of error

Several issues can alter the measurement results from optical methods, mainly due to the susceptibility of these methods to changes in sample quality in the light of poor choice of sample handling techniques and storage conditions (e.g., temperature, preservative, etc.) as well as biochemical alterations that occur over time [26]. Some of the most important potential errors for COHb determinations include:

1) Type of preservative: the type of preservative used in the blood tube used to store the sample can alter the results due to biochemical reactions that can take place, which can either increase or decrease the concentration of CO[27, 28].
2) Storage temperature: the use of different storage temperatures was shown to alter the results; storage over prolonged periods of time can lead to degradation of the sample, which can lead to in vitro CO production, resulting in overestimation of the concentration; storage at room or hot temperatures leads to faster degradation as compared to storage in the fridge or freezer [26, 28, 29].
3) Dead volume: the different amounts of volume of headspace (HS) in the sampling tube (which is known as dead volume) can alter the results because of the reversibility of the

bond between CO and Hb; the more dead volume in the tube, the more likely there is dissociation of CO from Hb and release into the HS [30].

4) Freeze-and-thaw cycles: whether a sample has been frozen and then thawed one or more times can also alter the resulting measurement, due to the breakdown of the erythrocytes [28].
5) Reopening of the sampling tubes: the repeated opening of the tube can lead to substance loss (in gaseous state when CO is not bound to Hb) with increasing number and time of reopening as well as increased exposure of the sample to oxygen [23, 28].
6) Postmortem (PM) changes: thermo-coagulation, putrefaction and PM CO production are all known sources of error, but they cannot be quantified due to their biological unpredictable nature [27, 31, 32].
7) Instrument and personal error: errors due to the instrument or the operator are random, but they can be corrected by using an internal standard when possible, which minimizes the error [33].

These altering factors are applicable not only to optical measurements of COHb but also to gas chromatographic measurements of CO. Specifically for spectrophotometric methods, several of the factors listed in **Figure 2.1** have been investigated and are described in more detail as follows.

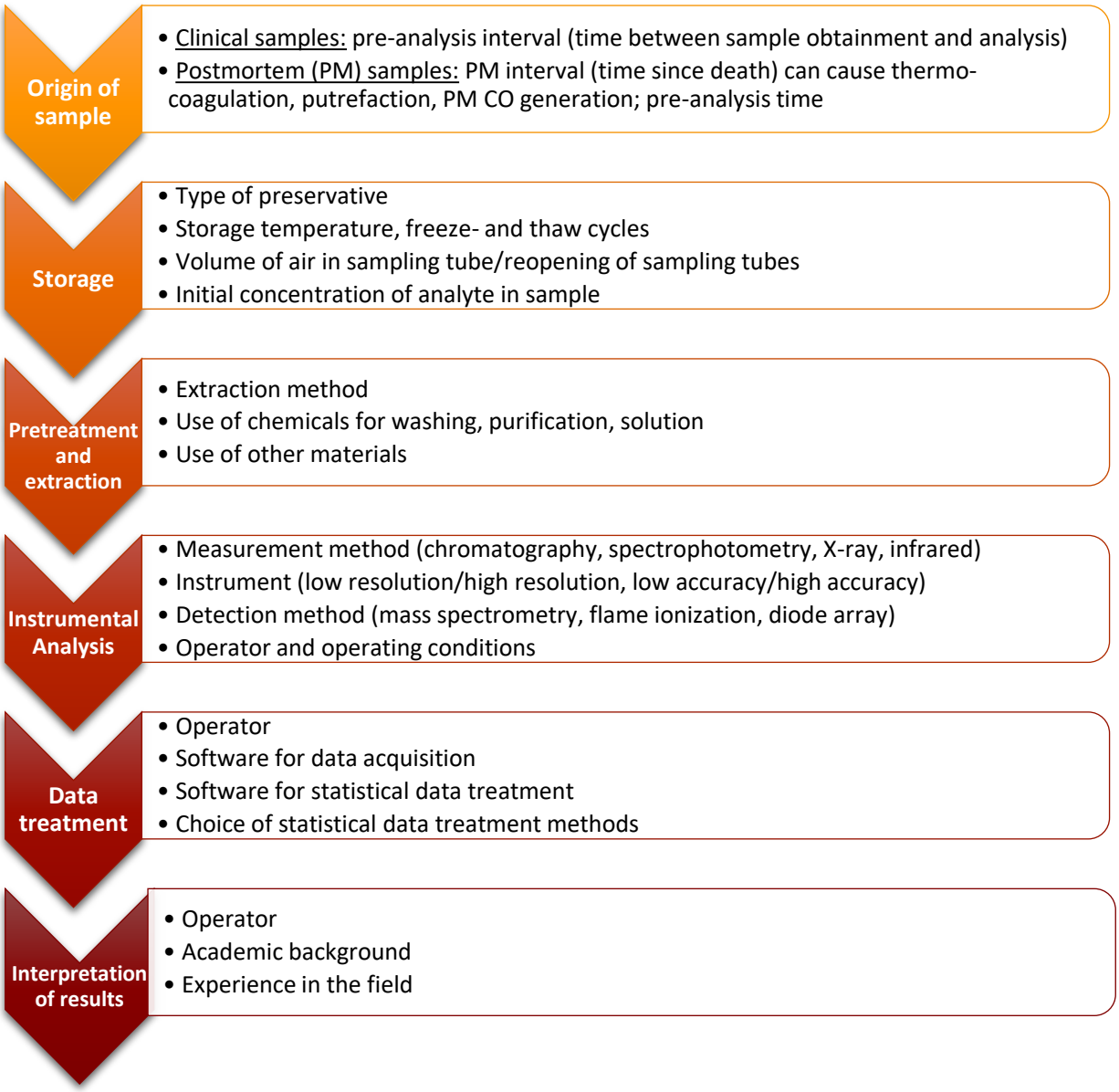

Figure 2.1: General steps for quantitative laboratory analysis and their respective potential sources of error for CO determinations.

Storage studies performed earlier by Chace et al. [28] and later by Kunsman et al. [27] evaluated a number of storage conditions, including the amount of air present in the sampling tube (known as dead volume, which can alter the results because of the reversibility of the bond between CO and Hb and potential dissociation of the gas into the HS of the tube), storage temperatures, preservatives and initial COHb saturation levels. They observed decreased COHb levels that were related to the ratio of exposed surface area to the volume of blood (the higher the exposed surface area, the greater the loss), the storage temperature (the higher the temperature, the greater the loss) as well as initial COHb% saturation levels (the higher the COHb levels, the greater the loss). The hypothesis of the formation of an equilibrium between

CO in blood and air above the blood sample in the tube was proposed to explain the influence of the HS in the sampling tube [28]. Storage at room or hot temperatures of blood leads to faster degradation and lower sample stability, affecting the spectrophotometric measurement of CO, which was also confirmed by other research groups [26, 34]. Additionally, they found no effect from the preservative used, even though it was tested on an insufficient number of preservatives (only two, namely sodium fluoride (NaF) and ethylenediaminetetraacetic acid (EDTA)), which were compared to samples with no preservative, and only on samples stored frozen right after sampling over a period of two years [39]. Analysis of the samples on only two significantly distant time points might fail to notice changes in short-term storage due to preservative use, which is more relevant than long-term storage since samples are in the majority of cases analyzed within a few hours to days. Nevertheless, these findings are especially relevant for forensic or legal cases, where retrospective analyses can still provide sufficiently reliable information. The resulting lack of impact from the preservative might, however, be biased because the measurements were performed with optical methods only, which are known to be influenced by the blood state. Therefore smaller changes due to the preservatives might not have been picked up by this less sensitive measurement methods. However, Vreman et al. [35] were able to find that using EDTA as preservative led to falsely increased COHb values when measured by CO-oximetry. Nevertheless, a stronger significance of these findings would have been achieved with confirmation by another measurement method, such as gas chromatography (GC).

Furthermore, these conditions may not only influence the CO levels present in the blood, but also the blood quality [28]. For samples that cannot be readily analyzed and are not stored under optimal conditions, a degradation of the sample occurs, which was confirmed to trouble the optical measurement methods used to determine COHb levels [36]. This can be a major issue for many laboratories where optical techniques are routinely used for sample analysis.

Additional factors influencing the measurement of COHb-levels that have been reported in the literature include the presence and amount of oxygen in air [23] and, in PM samples, thermo-coagulation in fire victims [34], putrefaction during a prolonged PM interval (PMI) [37], contamination due to hemolysis, high lipid concentrations or thrombocytosis, all of which result in turbidity of the sample troubling the measurement performed with optical techniques.

Another recurring and significant phenomenon to be considered during the evaluation of the results is the PM production of CO in the organism [32, 38]. CO was found to be produced in significant quantities in cases that were not related to fire- or CO exposure. However, the cases in which this occurs are mostly cases of putrefied bodies. It was confirmed that CO is formed

due to the decomposition of various substances present in the body, such as erythrocytes catabolism, a phenomenon that occurs also in living organisms [32]. Therefore, it is important to differentiate those cases from the real CO intoxication cases, which can be done with the help of the cause of death determined with an autopsy, even though it is not always a simple task to completely exclude the possibility of the role played by CO in these cases [23]. As a result, PM decomposition currently constitutes a field with open questions that require further investigation.

2.5 Antemortem COHb measurement by pulse CO-oximetry

2.5.1 Analytical techniques

In clinical settings and generally, for living patients, a noninvasive alternative to venous or arterial blood COHb measurement by a BGA or CO-oximetry that has been widely investigated is pulse CO-oximetry [39–43]. Similarly to standard CO-oximetry, pulse CO-oximetry is a spectrophotometric method that quantifies multiple types of haemoglobin, including COHb, based on the absorbance of light after exposure to different wavelengths [43]. As opposed to regular CO-oximeters, pulse CO-oximeters have the ability to measure COHb continuously and without the need of blood sampling, thus allowing the monitoring of COHb levels in real-time and simultaneously to the administration of treatment.

2.5.2 Sources of error

Noninvasiveness and cost- and time-efficiency are some evident advantages of using pulse CO-oximeters. However, for CO poisoning diagnosis, there are factors of higher importance from a medical perspective, such as accuracy, precision and reliability. Being able to diagnose a CO poisoning case quickly is necessary, but if the results obtained over- or underestimate the true COHb levels, this can have severe and potentially fatal consequences. Several studies have reported low precision and accuracy as well as an elevated false positive and negative rate, as opposed to regular blood measurements [5, 39–42]. Especially for COHb levels above 10%, pulse CO-oximeters significantly underestimated the COHb levels [39].

Furthermore, factors such as blood pressure, oxygen saturation and body temperature also seem to affect the accuracy of pulse CO-oximeters [42]. Feiner et al. [40] reported low signal quality or no report of CO saturation levels when the oxygen saturation decreased below 85%, which

is indicative of hypoxia. Considering that hypoxia is one of the main effects of a CO poisoning, it is a severe disadvantage not to be able to measure COHb accurately in hypoxic states. However, a more recent study by Kulcke et al. [43] found good accuracy levels in measuring COHb during hypoxemia, even though a slightly higher underestimation of COHb levels is reported for COHb concentrations above 10%. This confirms that pulse CO-oximeters can be useful for monitoring exposures to low CO levels, but accuracy and precision are not guaranteed for more severe poisonings as well as for smokers, who generally have baseline COHb levels that can range from 3-8% in normal smokers but can easily reach 10-15% in heavy smokers [1, 2].

In comparison to postmortem CO-oximetry, antemortem COHb measurement by pulse CO-oximetry is not affected by storage or sampling parameters, which reduces the sources of error. Additionally, no laborious and time-consuming calibration of the device seems to be needed based on what is reported in the literature, leading to a more simplified routine analysis, even though there is scarce information regarding device maintenance. Similarly to general CO-oximetry and despite good accuracy and precision, measurement of only CO bound to Hb can lead to underestimation of the total CO burden and thus lead to misdiagnosis. Another relevant point from a judicial perspective is that pulse CO-oximetry does not provide samples that can be used for confirmation or counter expertise in legal disputes.

2.6 Measurement of CO in blood: gas chromatography

2.6.1 Analytical techniques

The principle behind gas chromatographic CO detection is based on the measurement of the released CO dissolved in blood as well as the one bound to Hb through a liberating agent (after red cell lysis). Therefore, the sample is firstly treated with a hemolytic agent, such as saponin, Triton X-100 or other detergents, and subsequently acidified to liberate the CO in blood [34, 44–47]. The reaction of COHb with a powerful acid/oxidizing agent was found to efficiently release CO and water as products. The releasing agents commonly used are sulfuric acid (H_2SO_4), hydrochloric acid (HCl) and potassium ferricyanide ($K_3Fe(CN)_6$). Other acids such as lactic acid [48], citric acid [48, 49] or phosphoric acid [49] have also been tested.

In the studies performed in earlier years (the 70s, 80s and 90s), potassium ferricyanide was introduced for the release of CO and became very popular due to the availability, since it was already used in spectrophotometric methods as a hemolytic agent. It was also found to be

efficient in liberating the CO and its extent of reaction was not influenced by the presence of O_2 or O_2Hb at a wide pH range, as compared to other acids tested [30, 46, 48, 50, 51]. However, in more recent studies, sulfuric acid has been preferred, mostly because, as compared to other acids of the same efficiency, it is more readily available, cheaper and allows the simultaneous liberation of CO and production of ^{13}CO used as internal standard [4, 30, 31, 47, 49, 52–54]. After successful liberation, CO is into by GC and then detected with one of the above-mentioned detectors.

For the GC separation, a capillary column with a 5Å molecular sieve has been found to be specific for the separation of CO from other interfering gases such as nitrogen (N_2), oxygen (O_2) and methane (CH_4) [51]. Various packed columns were used previously, but have been substituted by the capillary columns due to their significantly reduced size.

To enhance sensitivity and accuracy and increase the range of analysis, GC methods were studied with various types of detection, such as thermal conductivity detection (TCD), flame ionization detection (FID), mass spectrometry (MS) and reduction gas analyzer (RGA) [55-66]. The most commonly used and investigated detector was FID, firstly reported in relation to CO determination in 1968 [51]. After GC separation, the CO is chemically reduced to methane (CH_4) with a methanizer and subsequently analyzed via FID.

2.6.2 Sources of error

The most important sources of error for GC techniques are found in the process of calibration before analysis and the methods of correlating measured CO concentrations to COHb levels that have previously been linked to the symptomatology. Generally, calibration of the instrument is performed either with pure CO gas, which was diluted to obtain the desired CO concentrations, or with fortification of blood with CO to reach different COHb% saturation levels. Additionally, excess CO was removed through the performing of a “flushing” step, in which the calibrators were flushed with a stream of inert gas (usually N_2). This step enabled the removal of unbound CO from the sample, thus leaving only CO bound to Hb to be analyzed, but thereby deliberately neglecting the potential toxicity of free CO.

First changes in the calibration method were made in 1993 when Cardeal et al. [49] firstly took advantage of the reaction of formic acid with sulfuric acid to form CO for calibration. However, no detail was given on how the analyzed blood was saturated with CO, nor was it explained how the formula used to back-calculate the measured CO concentration to a COHb level was created.

Czogala and Goniewicz [67] proposed a GC–FID based method which directly correlated the CO levels in air to COHb in blood through back-calculation and extrapolated it to the other factors assessed (exposure time, smoking frequency, number of smoked cigarettes and ventilation conditions). The technique was designed to ensure complete release of CO from the blood samples by performing the reaction and subsequent analysis in an airtight reactor. Similarly, the air samples were directly transferred from the room to the analysis instrument, which avoided time delays and possible losses of CO and allowed for direct correlation of the results to the other measurements. However, the details about the procedure to obtain 100% CO saturated blood used for calibration were not described, which is necessary to assess whether the method is reliable and reproducible. Furthermore, the formula used to back-calculate the COHb saturation levels from the measured CO concentrations contained the Hüfner factor of 1.51, which differed from the factor reported by other studies [30, 46]. The Hüfner factor expresses the maximum amount of CO that can be bound to 1 g of Hb [68, 69]. A detailed list of additional pitfalls of GC methods is found in **Table 2.1**.

2.7 Measurement of CO in blood: mass spectrometry, GC-MS and HS-GC-MS

2.7.1 Analytical techniques

MS is the method of choice to detect CO because the identification is not only based on the retention time but also the mass spectrum. Middleberg et al. [31] developed a method, which combined GC–MS with flame atomic absorption spectroscopy (FAAS). Hereby CO was determined by GC–MS after release with sulfuric acid and heating, while FAAS was used to determine the total iron content of the blood, which is used to calculate a more precise total amount of available Hb. By using this assay, it was assumed that all the iron present in blood was part of the heme protein and was capable of binding CO, even though it needs to be taken into account that this is not completely true and depends on the state of the organs, tissues and possible present diseases. Therefore, the obtained values might not accurately reflect the real CO levels.

2.7.2 Sources of error

Similarly to other GC methods, also in MS, main errors derive from the calibration of the methods, the subsequent back-calculation of COHb from CO and extrapolation of already existent COHb% saturation-symptom correlation (**Table 2.1**).

Hao et al. [37] published an approach built on an HS-GC–MS method for analysis of CO in putrefied PM blood. Hereby, the standard curve was constructed from putrefied blood, which was saturated by CO-bubbling to reach 100% COHb and then flushed to remove excess CO. COHb% levels were then calculated from the ratio of saturated to untreated blood. In PM cases, to prevent the variation of Hb levels to affect the results, direct blood saturation was performed. It was stated that 30 min of pure CO exposure of the blood was necessary to fully saturate blood, even though the procedure applied to assess complete saturation, putrefied blood state and PMI were not described [37]. Furthermore, according to their results of the storage condition tests (possible loss of sealing parts of the HS vial, water bath temperature, stability, interval and temperature), the storage temperature did not affect the COHb% levels. However, this appears in contradiction with the majority of previously published studies, even though they were obtained with the use of other approaches, such as optical methods and other GC-detectors.

Varlet et al. [52] were able to develop and validate a new method, which used isotopically labelled formic acid ($H^{13}COOH$) to produce ^{13}CO as internal standard for an HS-GC–MS method. This is very advantageous since formic acid (HCOOH) was already used for the calibration, and sulfuric acid could be used to react with both types of formic acid, forming a mixture of CO and ^{13}CO, from which the CO concentration could be derived mathematically and correlated to the COHb levels through the use of formulae previously published by other authors [46, 49]. However, these formulae describing back-calculation of COHb from CO concentrations measured by GC could be debatable due to the random finding of good correlation between the spectrophotometrically measured COHb levels and the CO levels measured by GC–MS [52]. Varlet et al. [36] improved their method and compared it with results obtained through CO-oximeter. They were able to obtain cut-off values for different categories of back-calculated COHb% levels as compared to the ones directly measured by the CO-oximeter. However, even if this approach seems to show reliability for both clinical and forensic cases, only a limited number was tested.

Table 2.1: Overview of analytical methods used for carboxyhaemoglobin/carbon monoxide analysis, their main properties and limitations and reference examples; CO: carbon monoxide, COHb: carboxyhemoglobin, PM: post-mortem, PMI: post-mortem interval, GC–RGA: gas chromatography–reduction gas analyser, GC-TCD: gas chromatography–thermal conductivity detector, GC–FID: gas chromatography–flame ionization detection, GC–MS: gas chromatography-mass spectrometry.

Specimen/method	Technique	Main characteristics	Pitfalls	References
Breath	**Electrochemical sensor**	• Easy to use • Non-invasive • Rapid (multiple determinations in short time period – useful in mass accidents) • Low cost • Portable • Alveolar breath CO correlated to COHb • Used in smoking cessation programs and to detect hemolytic diseases	• Only fraction of CO exhaled is measured • Not able to determine total amount of CO in blood circulation • No correlation to CO in tissues • Not sufficiently sensitive for low-level CO exposures • Only approximate diagnosis can be made • Correlation between exhaled CO and COHb still debatable • Not suitable for all patients (elderly, diseased) – requires sufficient exhaled airflow	Ogilvie et al. 1957 [6] Jarvis et al. 1980 [7] Jarvis et al. 1986 [8] Vreman et al. 1994 [14] Middleton et al. 2000 [9] Macintyre et al. 2005 [10] Penney 2007 [11]
Blood	**Double wavelength (DW)/automated differential/ultraviolet (UV) spectrophotometry**	• Use of multiple wavelengths • Rapid • Easy to use • Fairly accurate • Small sample size	• DW: not precise, accurate and specific • Sensitive to alteration of sample quality • Not optimal especially for PM samples with long/unknown PMI and/or storage conditions • Risk of misdiagnosis due to artefacts • Not able to determine total amount of CO in blood circulation • No correlation to CO in tissues • Focus only on COHb • Time-consuming sample preparation (COHb reduction) • Often observed inconsistency between measured levels and reported symptoms	Ramieri et al. 1974 [16] Winek et al. 1981 [17] Fukui et al. 1984 [19] Vreman et al. 1984 [46] Lewis et al. 2004 [55] Luchini et al. 2009 [56] Olson et al. 2010 [22] Varlet et al. 2012 [52] Hao et al. 2013 [37] Varlet et al. 2013 [36]

Specimen/method	Technique	Main characteristics	Pitfalls	References
Blood	**CO-oximetry**	• Easy to use • Rapid • Low cost • Accurate • Precise • COHb saturation correlated to the severity of poisoning and symptoms reported by patients	• Limit of accuracy: >5% COHb • Not applicable to low-level CO exposures • Invasive • Only CO bound to Hb taken into account • Often observed inconsistency between measured levels and reported symptoms • Susceptible to alterations due to sample quality • Difficult interpretation for PM samples with long/unknown PMI and/or storage conditions	Dubowski and Lu 1973 [57] Costantino et al. 1986 [58] Mahoney et al.1993 [24] Oritani et al. 1996 [65] Levine et al. 1997 [59] Bailey et al. 1997 [21] Widdop 2002 [23] Lee et al. 2002 [60] Lee et al. 2003 [61] Brehmer and Iten 2003 [62] Boumba and Vougiouklakis 2005 [18] Penney 2008 [11] Piatkowski et al. 2009 [5] Olson et al. 2010 [22] Fujihara et al. 2013 [20]
Attachment to the finger	**Pulse CO-oximetry**	• Measurement of COHb% saturation in circulation • Continuous measurement • Non-invasive • Rapid • Cheap • Applicable in clinical setting • No laborious calibration needed	• Not applicable in PM setting • Only CO bound to Hb taken into account • No correlation to CO in tissues • No blood sample available for confirmation/counter expertise • Low precision and accuracy for COHb >10% • Scarce information on device maintenance	Piatkowski et al. 2009 [5] Zaouter and Zavorsky 2012 [39] Feiner et al. 2013 [40] Weaver et al. 2013 [41] Wilcox and Richards 2013 [42] Kulcke et al. 2016 [43]
Blood, tissue	**GC–RGA**	• Measurement of CO in tissues • No dependency on blood quality • Automation possible	• Use of highly toxic mercury vapours • Time-consuming sample preparation • Invasive	Coburn et al. 1964 [44] Vreman et al. 1984 [46] Mahoney et al. 1993 [24] Marks et al. 2002 [63] Vreman et al. 2006 [30]
Blood	**GC–TCD**	• Accuracy for low COHb% • Precise • Specificity • Measurement of CO released into HS of tube possible • No dependency on blood quality • Automation possible	• Time-consuming sample preparation • High cost • Invasive	Ayres 1966 [40] Dubowski and Lu 1973 [57] Fukui et al. 1984 [19] Van Dam and Daenens 1994 [66] Oritani et al. 1996 [57] Lewis et al. 2002 [55] Brehmer and Iten 2003 [64]

Specimen/method	Technique	Main characteristics	Pitfalls	References
Blood, tissue	**GC–FID**	• Rapid • Best sensitivity for CO • Specificity • Lowest LOD and LOQ • Assessment of different sample preparation and storage conditions (liberating agent, heating time, heating temperature, etc.) • Application to CO in tissues (PM) • Automation possible • Measurement of CO in tissues • No dependency on blood quality	• Instrument specific for CO due to necessity of methanizer • Not applicable to analysis of other substances • Time-consuming sample preparation • Invasive • Back calculations of COHb from measured CO • Flushing of calibrators → removal of dissolved CO	Collison et al. 1968 [51] Rodkey and Collison 1970 [48] Guillot et al. 1981 [58] Vreman et al. 1984 [46] Costantino et al. 1986 [60] Cardeal et al. 1993 [49] Levine et al. 1997 [61] Penney 2000 [69] Sundin and Larsson 2002 [53] Czogala and Goniewicz 2005 [67] Boumba and Vougiouklakis 2005 [18] Vreman et al. 2006 [30] Walch et al. 2010 [47]
Blood, tissue	**GC–MS**	• Versatile • Simple • Rapid • Accurate • Reproducible • High power of identification (retention time + mass spectrum) • Automation possible • Application to clinical and PM samples • No dependency on blood quality • Use of isotopically labelled formic acid for calibration and internal standard • Measurement of total amount of CO	• Back calculations of COHb from measured CO • Debatable correlation between CO and COHb% → often inconsistency between COHb% and reported symptoms • Flushing of calibrators → removal of dissolved CO • Invasive • Time-consuming sample preparation	Middleberg et al. 1993 [31] Oritani et al. 2000 [50] Marks et al. 2002 [63] Varlet et al. 2012 [52] Hao et al. 2013 [37] Varlet et al. 2013 [36]

2.8 Interpretation of results and choice of biomarker

After analysis of the samples, an important and challenging aspect for CO determination is the interpretation of the results. There is not a consensual agreement on the cutoff values for the different levels of exposure and severity of poisonings. According to the World Health Organization (WHO), COHb levels in blood of the healthy non-smoking population should not exceed 2.5-3%, while for smokers, levels above 10% are considered to be abnormal [11, 71–73]. Values of 30-35% COHb are the upper extreme values reportedly found in clinical poisoning cases. Above this limit, irreversible damages to the organs are expected, thus resulting in a cascade of events that eventually leads to death.

However, these values are interpreted differently according to the cases. Various parameters can affect the perimortem COHb% levels and in the agonal period before death, which include the presence of oxidative smokes or other gases that can interfere and/or compete with the absorption mechanism of CO such as nitrogen dioxide (NO_2) (increased MetHb), or the formation of other toxic gases like hydrogen cyanide (HCN) [74]. Pre-existing cardiovascular, hemolytic and respiratory diseases also can alter the mechanism and magnitude of CO absorption, with the potential to both decreases and increase the resulting COHb% levels [11, 23]. Therefore, each case needs to be analyzed and interpreted individually, based on all the relevant information available. For example, a COHb level of 25% in a PM case may be considered as a contributing factor to the cause of death but should not be considered exclusively as the cause of death. Similarly, in clinical cases, 15% COHb can be considered as a poisoning case, but in heavy smokers, levels up to 18% have been found [72] in individuals that did not show any symptom of CO poisoning. Overall, there seem to be some significant discrepancies between COHb values and reported symptoms, which make the correct diagnosis of CO poisonings in clinical cases and the determination of the cause of death in forensic cases challenging.

A possible explanation for these phenomena is that basing the diagnosis of a CO poisoning only on COHb% levels might actually underestimate the real CO burden. There might be an unknown amount of CO that on the one hand dissociates back from COHb, and on the other hand is dissolved in the blood without being bound to Hb, resulting in a higher total CO content than the one determined by CO-oximetry. The conventional assumption that the part of CO bound to Hb causes the most significant adverse health effects was repeatedly debated [3, 4, 75–78]. Free CO in blood could constitute a toxic reservoir of CO for the organism and additionally fuel the major implications on the central nervous system (CNS) by the known

binding to other globins such as myoglobin, neuroglobin and cytoglobin [79, 80]. The ratio of COHb to CO dissolved and dissociated probably is also subject to interpersonal variability, which includes all factors such as e.g., metabolic rate and age [11] and needs to be taken into account when interpreting the results obtained by CO-oximetry.

Another issue is that the majority of GC assays, with the exception of Varlet et al. [36, 52], includes the "flushing" step in their sample-preparation procedure. The CO in excess, which is not bound to Hb, is flushed away with inert gas, allowing the determination of only CO bound to Hb. This procedure is done under the assumption that only CO bound to Hb is relevant and responsible for the adverse effects of a CO poisoning. However, this assumption has been widely debated, leaving the possibility of additional CO found in blood and not bound to Hb to be able to have an effect on an intoxicated individual. Furthermore, in clinical routine COHb analyses, blood samples are not flushed, because it does not comply with the pathophysiology of CO poisoning. In general, the use of formulae to back-calculate CO measured with GC methods to COHb might be prone to additional errors and could lead to a misestimation of the true amount of CO present in the blood of an individual.

All these issues raise the doubt whether the measurement of COHb is the most appropriate method for CO poisoning determinations. It seems plausible to propose a more accurate biomarker for CO poisonings. Several alternative biomarkers have been proposed in the past, such as lactate [81–83], bilirubin [84], S100β [85] and troponin concentrations in blood. Some of these gave positive and good correlations with COHb and were reported to be potentially helpful in diagnosing CO poisonings. However, none of these biomarkers are specific to CO poisonings but are rather indirect biomarkers derived from toxicity caused by CO in the cardiovascular, nervous system and cellular levels, which can be attributed also to other diseases.

The development of an alternative biomarker specific to CO should be derived from the investigation of a novel measurement approach that does not only focus on the CO bound to Hb but also takes into consideration the role and toxicity of CO at cellular level, by measuring the total amount of CO present in the analyzed sample. Mainly due to the dependency of spectrophotometric methods from the good quality of the sample, which especially in forensic cases is not always available, it seems that GC methods are currently the most suitable techniques to be further explored. As detector, the mass spectrometer is the most versatile, accurate, user-friendly and nowadays routinely present in the majority of laboratories. Being able to determine the true CO exposure and correlating this to the symptoms reported by

patients would allow a more conclusive and comprehensive CO poisoning determination, diminishing the number of misdiagnosed cases and falsely determined causes of death.

2.9 Conclusions

Even though COHb is routinely measured by spectrophotometric methods, several issues concerning sample stability and the dependency of optical methods from the sample quality have led to the search for an alternative way for measuring CO, such as GC. In addition, there is raised discussion about a significant amount of CO present in blood in free form. Free CO has major toxic effects at a cellular level, affecting not only the respiratory system but also especially the CNS. However, it is not quantified with current methods focusing only on COHb; hence the back-calculation of COHb from CO leads to misestimations. Therefore, an alternative approach to quantifying the total amount of CO in blood directly instead of using CO in breath or COHb in blood should be used for CO poisoning determinations. Even though blood CO concentration cut-offs and their correlation with symptomatology are not yet available and GC–MS is more time-consuming, I recommend toxicologists especially for doubtful or very challenging cases to use GC–MS methods to verify the results obtained by CO-oximetry or spectrophotometry. This leads to results closer to the true CO burden, reducing the underestimation caused by COHb measurement and thus the risk and number of misdiagnoses. Especially if the analysis is delayed from sampling requiring storage, I further recommend toxicologists to document and indicate information about sampling time, analysis time and storage conditions because they can significantly influence the final interpretation.

2.10 References

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Chapter 3 - Development and validation of a novel method for carbon monoxide poisoning determination

3.1 Aims

3.1.1 Postmortem (PM) range

As thoroughly discussed in chapters 1 and 2, a main issue in the diagnosis of a CO poisoning involves the currently existing correlation between the COHb% levels and the symptoms developed by patients, which do not always agree: patients were found to have an elevated COHb% saturation level, but showed no signs of CO-intoxication, while other patients with a low COHb% -level lost consciousness or suffered severe delayed consequences. There seems to be a great fallacy in the understanding of the true role played by CO in poisoning cases. This might be due to an underestimation of the CO measured with the current techniques, which is based on spectrophotometric determination of COHb, and the neglect of the possible presence of CO in dissolved state and not bound to Hb, which can have major implications in the role of CO in the pathophysiology of a CO-poisoning. Gas chromatography (GC) coupled to mass spectrometry (GC-MS) was shown to have the potential to measure CO with higher accuracy.

Therefore, an improved approach for CO determination by airtight gas syringe (AGS) GC-MS is hereby presented, which not only shows improved sensitivity and lower costs but also takes into account the total amount of CO present in blood by analysing the CO in blood and in the headspace (HS) of the blood tube used to store the sample, with high importance from both an analytical and clinical point of view. This constitutes the first step to acknowledge the significance of total CO in blood as an alternative biomarker for CO exposure.

3.1.2 Clinical range

Another important issue that was also discussed in the previous chapter is the "flushing" of the calibrator with a stream of inert gas prior to GC-MS analysis in order to target only CO bound to Hb. This approach does not consider the amount of excess, free CO as relevant, which could lead to mis- or underestimation of the total load of CO in blood. For this reason, in the second part of this study, I aim to test the hypothesis of the presence of CO in free form in blood, by comparing analysis results of flushed and unflushed blood samples obtained at bedside from individuals that were exposed to CO. This represents the first step in expanding the knowledge of the true CO burden. Furthermore, I want to validate AGS-GC-MS technique for CO measurement in blood for a concentration range applicable also in clinical settings.

3.2 Materials and Methods

3.2.1 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 (13C, 99%) was ordered from Cambridge Isotope Laboratories (Cambridge, UK). Sulfuric acid (≥97.5%) was purchased from Fluka (Buchs, Switzerland). For the validation in PM range and *in vitro* study of CO fortified blood, bovine blood was obtained freshly 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). Ethylenediaminetetraacetic acid (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). Human blood samples were obtained from volunteers participating in a study at the Department of Nephrology of the University Hospital of Geneva (HUG) in Switzerland. Blood of non-smokers before exposure to CO was used as blank blood for the validation in clinical range.

3.2.2 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 (Ethylenediaminetetraaceticacid, EDTA), 3mL 9NC (sodium citrate, NaCit), 2.7mL FE (sodium fluoride, NaF), 2.6mL KH (lithium heparin, LiH), 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). Aluminium caps were from Milian (Vernier, Switzerland). All extractions were carried out in 20mL headspace vials from Agilent Technologies (Santa Clara, CA, USA).

3.2.3 Instruments and GC-MS conditions

For spectrophotometric analysis, AVOXimeter 4000 Whole Blood CO-Oximeter from ITC was used. The instrument uses five wavelengths for quantitative analysis, namely 520.1, 562.4, 585.2, 597.5 and 671.7 nm. Following parameters can be measured: total haemoglobin (tHb), oxyhemoglobin (O_2Hb), COHb and Methemoglobin (MetHb). Manufacturer guidelines were followed to obtain COHb analyses.

For gas chromatographic analysis, Agilent 6890 N GC (Palo Alto, USA) equipped with an HP Molecular Sieve 5 Å PLOT capillary column (30 m x 0.32 mm x 30µm) obtained from Restek (Bellefonte, USA) was used. Following temperature programme was used: 50°C, held for 4 minutes; the injector was set at 180°C, used in splitless mode, and the MS interface at 230°C. The employed carrier gas was helium at a flow rate of 40.0 mL/min. A solvent delay of 1.8 minutes was used.

For detection, Agilent 5973 mass spectrometer (Palo Alto, USA) was used, 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, both at the same retention time of 3.5 min.

3.2.4 Sample preparation

3.2.4.1 Method development (not in published paper)

The method was developed on the basis of previous studies that showed the generation of CO through a combination of sulphuric acid and formic acid and the use of sulfuric acid as a releasing agent for CO bound to Hb. Sulfuric acid was hence employed not only as a liberating agent for CO bound to Hb but also to generate the internal standard ^{13}CO from isotopically labelled formic acid ($H^{13}COOH$) as well as CO from formic acid for the calibrators. During the method development phase, several parameters were investigated to optimize the sample preparation and analysis procedures compared to previous studies (mentioned in Chapter 2), including the following:

- Amount of blood sample required: 25µL, 50µL, 100µL
- Amount of reagent (H_2SO_4) required: 50µL, 100µL, 200µL
- Size of sample preparation vial: 20mL GC-vial, 2mL GC-vial
- Order of sample input in vial: sample in aluminium cap/polypropylene (PP) pipette tip and sulfuric acid in the vial, sulfuric acid in aluminium cap/PP pipette tip and sample in the vial
- Temperature of pre-heating: 50°C, 80°C, 100°C
- Pre-heating time: 0 min, 30 min, 45 min, 60 min
- Temperature of GC oven: 30°C, 40°C, 50°C
- Injection volume: 500µL, 1000µL

The different combinations tested are listed in **Table 3.1**. The detailed protocol for sample, calibration solution and reagent solution preparation and analysis can be found in **Appendix 3, A3.1.1 – A3.1.8 and Tables A3.1 and A3.2**.

Table 3.1: List of combinations of sample preparation and analysis parameters for the measurement of CO via Gas Chromatography-Mass Spectrometry (GC-MS) tested during the method development phase.

Test	Amount of matrix [µL]	Amount of H_2SO_4 [µL]	Size of GC-vial	Order of sample input	Pre-heating temperature [°C]	Pre-heating time [min]	GC oven Temperature [°C]	Injection volume [µL]
1	50	200	2	1	80	45	40	500
2	50	200	2	1	0	0	40	500
3	50	200	2	1	80	30	40	500
4	25	50	2	1	50	30	30	500
5	50	200	2	2	80	30	30	500
6	50	200	2	2	80	45	40	500
7	100	200	2	2	50	60	40	500
8	100	200	20	2	50	45	50	1000
9	100	100	20	2	80	45	40	1000
10	100	100	20	2	100	45	40	1000
11	100	100	20	2	80	60	50	1000
12	200	200	20	2	80	60	50	1000
13	200	200	20	2	80	45	50	1000
14	200	200	20	2	80	30	50	1000
15	100	100	20	2	100	60	50	1000
16	100	100	20	2	100	45	50	1000
17	100	100	20	2	80	60	50	500
18	100	100	20	2	80	45	50	500

Results of the different combinations are shown in **Appendix 3, Figures A3.1-A3.18** as plots with linear regression lines (N.B. some plots have missing values, which are due to either instrument malfunction, sample preparation or injection error or being below the limit of detection). While nowadays studies aim at reducing the amounts of samples required for analysis, especially in forensic toxicology, where sample availability might be limited, in case of gas analyses, no reproducible results were obtained with the reduced sample and reagent sizes. This might be due to a non-completed reaction when using small volumes. Additionally, the volume of gas produced in a small 2mL GC-vial compared to the 20mL GC-vial is relatively

low and, thus, might not be sufficient to guarantee reproducibility. When adding the sample and reagents to the GC-vial, more accurate results were obtained when adding the sample, internal standard and calibration solutions into the vial first, followed by the sulfuric acid in the aluminium cap, which then reacted after sealing the vial and vortexing. Adding the sample into the aluminium cap lead to incomplete reactions. Similarly, no sufficient pre-heating, as well as a not sufficiently high GC oven temperature, did not guarantee a complete reaction of the reagents and release of CO. More reproducible results were obtained when injecting 1mL of gas volume rather than 0.5mL. The best results (best linearity/highest R^2) were obtained in test 15 (**Appendix A3.15**). Therefore, the parameters employed in test 15 were selected as the parameters to be validated (**Table 3.2**):

Table 3 2: Selected parameters for preparation of samples to be analysed and instrumental analysis.

Parameters	
Amount of H_2SO_4 [µL]	100
Amount of blood sample [µL]	100
Vial size [mL]	20
Order of input	Sample in vial, H2SO4 in aluminium cap
Pre-heating temperature [°C]	100
Pre-heating time [min]	60
GC oven temperature [°C]	50
Injection volume [mL]	1

The concentration range for validation was determined based on the analysis of some test samples (real postmortem blood samples of CO-poisoned individuals with known COHb concentrations) and their placement across the CO concentration range.

3.2.4.2 Fortification of blood

To obtain CO-fortified blood samples to be used for the validation and *in vitro* storage study, pure CO gas was bubbled in blank bovine blood-containing tubes for a specified amount of time. The COHb% saturation levels were checked in 10-minute-intervals with the CO-oximeter until the desired initial COHb% level was reached (10-20% for low saturation, 30-40% for medium saturation and 50-70% for high saturation). To ensure homogenization, the bottles were agitated for 20 minutes after fortification and the final COHb%-concentration was subsequently determined by CO-oximetry.

3.2.4.3 Calibration standards (PM range)

An aliquot of freshly sampled bovine blood, which was previously analysed with CO-oximeter to guarantee the absence of CO before use, is used as matrix for calibration. Fresh solutions of the calibration standard formic acid (87 nmol/µL) and internal standard (IS) isotopically labelled formic acid (84 nmol/µL) were prepared daily with deionised water to prevent degradation. Calibration points were set in a working range between 0-208 nmol/mL HS, congruent with CO-saturation in a range relevant for postmortem samples (based on the results obtained from available real postmortem samples), with points at 6.5, 13, 26, 52, 104, 156 and 208 nmol/mL HS (equivalent to 2.6, 5.2, 10.4, 20.8, 41.6, 62.4 and 83.2 µmol/mL in blood). Matrix effects were evaluated by preparing a blank sample with the matrix (i.e. blood) without any reagent. 10 µL of the IS solution were added to each calibration sample before extraction, leading to a final concentration of 42 nmol of ^{13}CO/mL HS. All standards were stored at +4°C when not in use.

3.2.4.4 Calibration standards (clinical range)

An aliquot of human blood from non-smokers, which was previously controlled by CO-oximetry and found at 0% COHb, is used as matrix for calibration. Calibration standard solutions of formic acid (43 µmol/mL) and IS solution of 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 0-104 nmol/mL HS, with points at 1.63, 3.25, 6.5, 13, 26, 52 and 104 nmol/mL HS (equivalent to 0.65, 1.3, 2.6, 5.2, 10.4, 20.8 and 41.6 µmol/mL in blood). Matrix effects were evaluated by preparing a blank sample with the matrix without any reagent.

3.2.4.5 Quality controls (QC)

QC samples were prepared daily with formic acid obtained from a different lot than used for calibration solutions. For PM range, five QC solutions at concentrations of 10, 25, 80, 150 and 200 nmol/mL HS (2, 5, 16, 30 and 40 µmol/mL blood), and for clinical range, five QC solutions at concentrations of 2, 5, 10, 30 and 60 nmol/mL HS (0.8, 2, 4, 12 and 24 µmol/mL blood) were prepared daily from formic acid diluted with deionised water.
Additionally, the validity of the methods were tested with external controls, which were prepared by dilution of pure CO gas at two concentration levels, low and high, respectively 20 and 150 nmol/mL HS for PM range and 5 and 60 nmol/mL HS for clinical range.

3.2.4.6 Extraction procedure

100µL of blood was introduced in a 20mL HS-vial, followed by aliquots of the various formic acid solutions for each respective calibrator and 10µL IS solution. Subsequently, an aluminium cap of 11mm (i.d.) was first filled with 100µL of sulphuric acid and then carefully introduced into the HS-vial. The vial was immediately hermetically sealed with magnetic PTFE/silicone septum caps of 20 mm (i.d.) and afterwards vigorously shaken and vortexed, in order to ensure complete mixing of the liquids contained in the vial. After preparation of all vials, extraction was completed by heating at 100°C for 60 minutes.
Concerning real PM and clinical samples as well as *in vitro* storage study samples, two types of analysis were performed per tube: CO analysis in the HS before tube opening in order to measure an eventual CO release in tube HS during storage and CO analysis in blood after tube opening and blood sampling. For CO in HS-analysis, only IS and sulphuric acid were inserted into the HS-vial (no matrix).

3.2.5 Analysis procedure

3.2.5.1 CO-oximeter

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

3.2.5.2 CO in HS

250µL HS were sampled from the closed blood tube and inserted into a previously prepared 20 mL HS-vial with IS. Subsequently, 1mL was sampled and injected in the GC-MS for analysis.

3.2.5.3 CO in blood

1mL HS was sampled from the 20mL HS-vial containing the extract and injected in the GC-MS for analysis.

To ensure that no contamination from CO contained in the air affected the measurements, a sample of air in the analysis-room was also analysed. Air samples were collected with the AGS and analysed by GC-MS with the same conditions as the blood samples.

3.2.6 Validation procedure

The validation was performed according to the guidelines of the "French Society of Pharmaceutical Sciences and Techniques" (SFSTP) and included following validation parameters: response function (calibration curve), linearity, selectivity, trueness, precision (repeatability and intermediate precision), accuracy, limit of detection (LOD) and limit of quantification (LOQ).

The response function, also defined as the calibration curve, is described as the relationship between the concentration of the analyte in the sample and the corresponding instrument response.

The linearity of the method is assessed by fitting back calculated concentrations of control samples against the theoretical concentrations through the application of the linear regression model and evaluating the resulting regression coefficient.

Trueness, also defined as bias, describes the closeness between the average of the experimental value and the calculated target value. It is expressed as percent deviation from the calculated target value.

Precision is defined as the closeness of agreement (degree of scattering) between a measurement series obtained from multiple sampling of the same homogenous sample under the prescribed conditions and is determined by calculating the repeatability (intra-day precision) and intermediate precision (inter-day precision) for each control sample. The repeatability is determined by calculating the intra-day variance (S^2r) and the intermediate precision through the sum of intra- and inter-day variances (S^2_{IP}).

Accuracy describes the closeness of agreement between the conventional true value or an accepted reference value and the value experimentally found. It is expressed as the sum of trueness (systematic error) and precision (random error).

Calibrators and QCs were used for the validation experiments performed on three non-consecutive days (p=3) not within the same week. The validation approach is based on the use of a β-expectation interval tolerance of 80%, indicating that the intervals for each experimental point include an average of 80% of the total values. The tolerance intervals (TI) were defined as $TI = X \pm k \times \sqrt{(S^2_r + S^2_R)}$, where S^2_r is the standard deviation of repeatability and S^2_R is the standard deviation of reproducibility. In the β-expectation interval tolerance approach, $k = t_v \times \sqrt{(1 + [1 / (I \times J \times B^2)])}$, where I is the number of series, J is the number of repetitions, and B^2 is a coefficient. This coefficient is given as $B^2 = (R + 1) / [J \times (R + 1)]$ with $R = S^2_r / S^2_R$. t_v is Student's coefficient with degrees of freedom v defined as $v = (R + 1)^2 / \{[(R + 1 / J)^2 / (I - 1)] + [(1 - 1/J) / (I \times J)]\}$.

3.2.7 PM samples

A set of three samples of both cardiac and peripheral blood from CO positive cases were analysed (**Table 3.3**). The samples obtained during autopsy were all with a PM interval (PMI) of less than 40 hours and analysed immediately after collection at the toxicology lab. Measurement with CO-oximeter and GC-MS were performed with the above-mentioned conditions. The samples were then subdivided into sampling tubes with four different preservatives, namely ethylenediaminetetraacetic acid (EDTA), sodium fluoride (NaF), trisodium citrate (Cit) and lithium heparin (LiH), and stored at -20°C for a period between 4 to 7 weeks. Subsequently, for all samples, the COHb and CO concentrations were determined with CO-oximetry and GC-MS respectively, where GC-MS was used to determine the CO in both the headspace and the blood of the sample. Additionally, the validity of the proposed approach was tested by comparison with approaches previously published by Cardeal *et al.* [1] and Sundin *et al.* [2] by back calculating the COHb%-levels from the obtained CO concentrations. These methods were found to give similar results to other methods found in the literature [3,4].

Table 3 3: Summary of relevant information on a set of real PM cases.

Sample	Sample type	Age	Sex	Manner of Death
1	Cardiac and Peripheral Blood	22	F	Fire victim
2	Cardiac and Peripheral Blood	67	M	Fire victim
3	Cardiac and Peripheral Blood	44	M	Suicide by CO intoxication

3.2.8 Samples from volunteers

3.2.8.1 CO-rebreathing method

The CO-rebreathing method consists of a closed-circuit breathing system containing oxygen mixed with a certain amount of carbon monoxide. Patients breathe in and out through a mouthpiece linked to the circuit. In the study from which I acquired the blood samples, patients were lying in a horizontal position. After insertion of the mouthpiece, an adjustment period of 2 minutes was used to deliver O_2 only, before starting the delivery of the O_2-CO mixture for a period of 10 minutes [62]. The volumes of CO delivered in O_2 were between 57 and 105 mL, calculated according to the body mass index of each volunteer, in order to reach a target COHb value of approximately 10%.

3.2.8.2 Blood collection and preparation

Blood samples were obtained from a cohort of 13 former patients (9 men, 4 women) of the Nephrology Department of the University Hospital in Geneva (HUG), Switzerland.
Three blood samples were taken from volunteers, one before and two directly after exposure to CO. Samples were immediately analysed by CO-oximetry. Half of the samples taken after exposure of the individual to CO were flushed with a nitrogen stream for 2 minutes, with a flow rate below 5 mL/min. Two needles were inserted in the rubber septum of the blood tube. One needle was plunged in blood and provided the nitrogen stream whereas the other was placed in the HS of the blood tube in order to relieve the pressure in the blood tube built with the release of CO and the nitrogen flush. After flushing, the samples were analysed by CO-oximetry. Simultaneously, all samples (before exposure, flushed and not flushed after exposure) were prepared for analysis with AGS-GC-MS and analysed in triplicates. All

sampling and testing performed on volunteers were approved by an ethical committee under the study number CCER-2017-00421.

3.2.9 Statistical analyses

Mean, standard deviation and interquartile ranges were determined for all measured variables. Paired student *t*-test with an α-error of 0.05 was performed for comparison of the group means. All data treatment and statistical analyses were performed with R (version 3.3.1, 2016-06-21).

3.3 Results (PM range)

3.3.1 Selectivity

The selectivity of the method was assessed with the measurement of samples obtained by the mixture of various intracadaveric gases with CO. These analyses were evaluated for co-eluting chromatographic peaks with possible interferences with either the CO or ^{13}CO detection. No interference peaks for any of the other gases were observed at CO *m/z* ratio of 28 or ^{13}CO *m/z* ratio of 29 (see **Figure 3.1**), which indicates that the method is sufficiently selective for determination of CO.

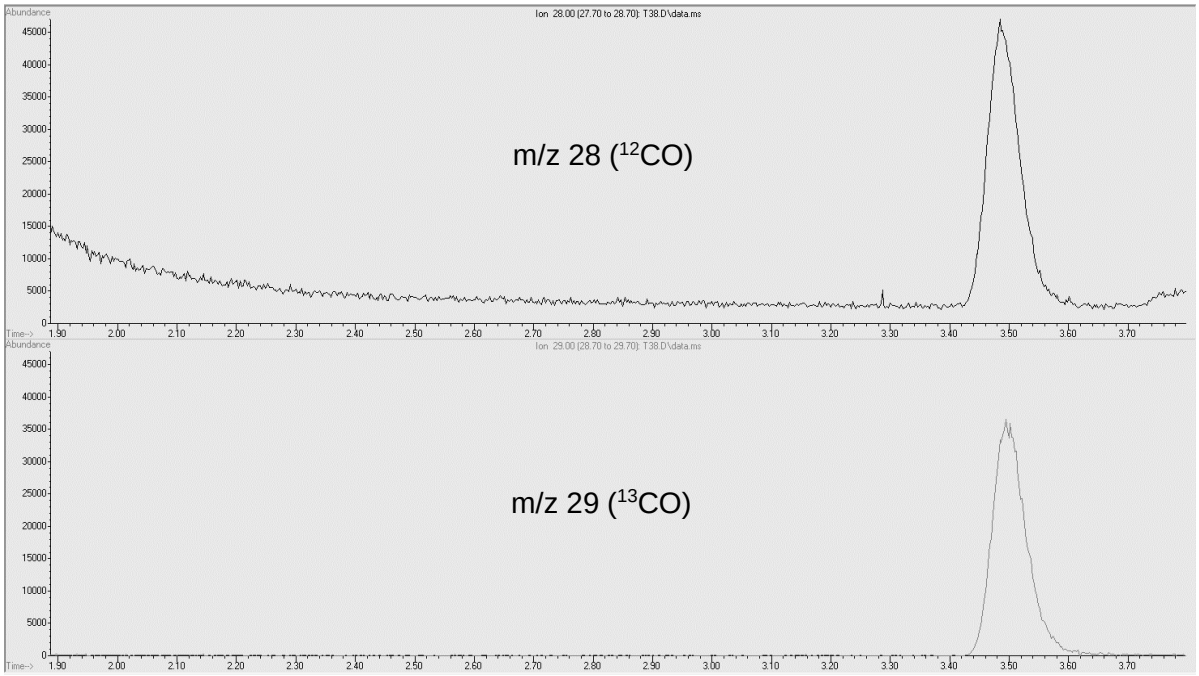

Figure 3.1: Extracted ion chromatograms for m/z ratios 28 (upper) and 29 (lower), corresponding to CO and 13CO respectively, for selectivity tests.

3.3.2 Response function (calibration curve)

The response function, also known as the calibration curve, is defined as the relationship between the concentration of the analyte in the sample and the corresponding response. An assay of calibration curves was performed for CO determination by using bovine blood as blank matrix and each point of the curve was defined as the area ratio of CO to ^{13}CO. The calibration curves were prepared on three non-consecutive days (p = 3), in triplicates (n = 3) and at seven concentration levels (k = 7): 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). The calculated concentrations for each calibration point were compared to the target values and found to be within ± 20%. A linear relationship between the CO concentration from samples spiked with formic acid and the measured response was determined. **Table 3.4a** shows the validation results for the calibration curves.

3.3.3 Linearity

The linearity of the model was evaluated by fitting back-calculated concentrations of control samples against the theoretical concentrations through the application of the linear regression model. On each non-consecutive day (p = 3), control samples at five different concentrations (k = 5) , namely 10, 25, 80, 150 and 200 nmol/mL HS (2, 5, 16, 30 and 40 µmol/mL blood), were measured in triplicates (n = 3). The concentrations of the control samples were calculated by using the calibration curve determined for each analysis day. As represented in **Table 3.4b**, satisfactory linearity was obtained, with a slope of 0.9887 and a regression coefficient of 0.989 in the range of 10 to 200 nmol/mL HS (2-40 µmol/mL blood).

3.3.4 Trueness

The trueness, also known as bias, expresses the closeness between the experimental average value and the calculated target value and is expressed as the percent deviation from the calculated target value. Trueness was found to be lower than the acceptance criteria (within ±15 of the accepted reference value and within 20% of the LOQ), as can be seen in **Table 3.4c**, and hence defined as satisfactory for CO determination.

Table 3.4: Validation results for CO determination in blood by AGS-GC-MS. (**a**) represents the mean coefficients of the response functions obtained from analysis of the calibrators; (**b**) represents the coefficients of the linear regression function obtained from analysis of the QCs against their theoretical value; (**c**) represents the trueness obtained from the QC analyses expressed in %; (**d**) represents the repeatability and intermediate precision obtained from QC analyses expressed in relative standard deviation % (RSD%).

a) Response function [6.5-208 nmol/mL HS] (k = 7, n= 3, p = 3)			
	Day 1	**Day 2**	**Day 3**
Slope	0.0252	0.0219	0.0214
Intercept	0.4698	0.5803	0.4528
r^2	0.9892	0.9864	0.9920

k: number of concentration levels; *n*: number of repetitions for each level; *p*: number of non-consecutive

b) Linearity [10-200 nmol/mL HS] (k = 5, n = 3, p = 3)	
Slope	0.9887
Intercept	-0.5322
r^2	0.9962

c) Trueness (relative bias %) (k = 5, n = 3, p = 3)	
Levels [nmol/mL HS]	**Trueness (%)**
10	-12
25	0
80	-2
150	-3
200	-5

d) Precision (RSD%) (k = 5, n = 3, p = 3)		
Levels [nmol/mL HS]	**Repeatability**	**Intermediate Precision**
10	0.951	0.952
25	4.001	4.326
80	5.980	5.980
150	8.364	11.347
200	4.046	8.630

3.3.5 Precision: repeatability and intermediate precision

Precision is designed to detect random errors and is defined as the closeness of agreement (degree of scattering) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. It is assessed by calculating the repeatability (intra-day precision) and intermediate precision (inter-day precision) for each control sample. The repeatability variance was established by calculating the intra-day variance (S^2r) and the intermediate precision was determined through the sum of intra- and inter-day

variances (S^2_{IP}). As can be seen in **Table 3.4d**, the RSD for repeatability and intermediate precision are in a range between 0.95% and 11.95%.

3.3.6 Accuracy and limit of quantification

Accuracy expresses the total error defined by the sum of trueness (systematic error) and precision (random error). It is defined as the closeness of agreement between the conventional true value or an accepted reference value and the value found. The accuracy profile for CO, depicted in **Figure 3.2**, expresses the method's ability to provide analytical results by using both systematic and random errors, with a risk set at $\alpha = 5\%$ for each concentration level. The mean bias (%) confidence interval limits for the control samples were within the acceptability limits of ±30%. Taking into consideration the acceptability limits of ±30%, the limit of quantification within validation criteria was found at below 10 nmol/mL HS (2 µmol/mL blood). Thus, the method is confirmed to be accurate within the range of 10 and 200 nmol/mL HS (2-40 µmol/mL blood), considered as the relevant range for postmortem analyses. The analytical LOQ was later determined at 0.9 nmol/mL HS (0.18 µmol/mL blood).

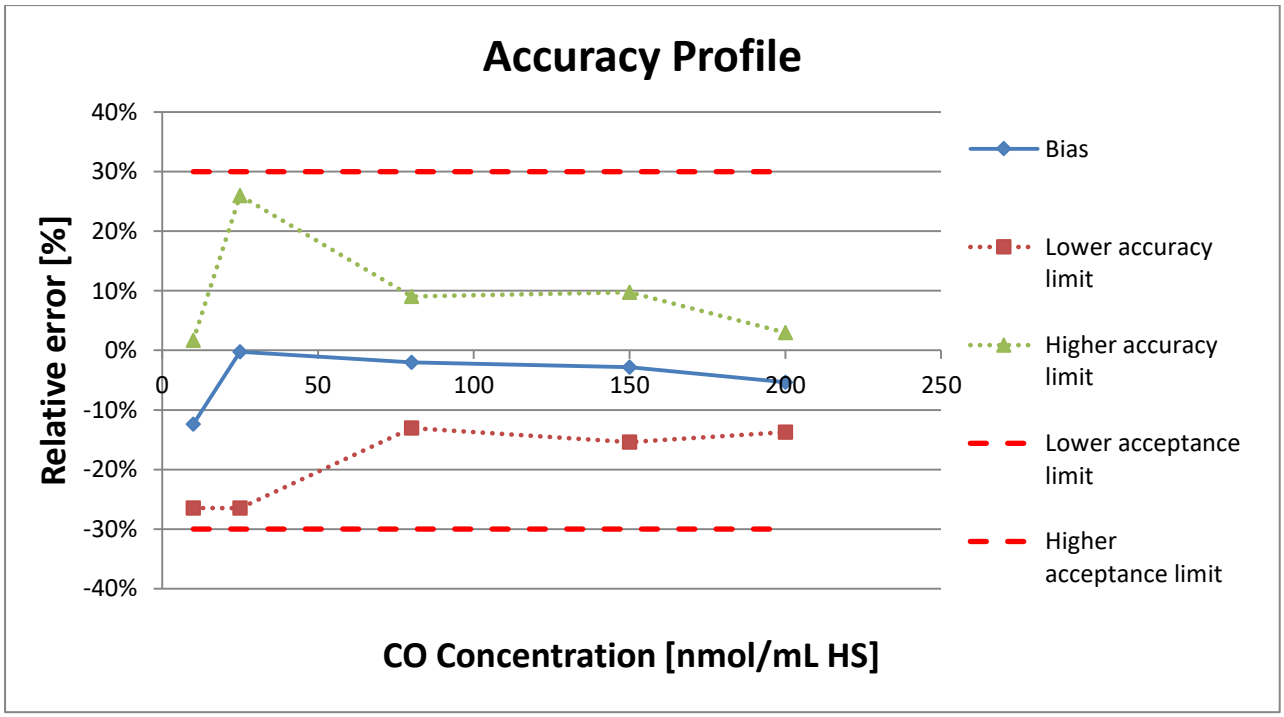

Figure 3 2: Accuracy profile for CO determination using a simple linear regression model within the range of 10-200 nmol/mL HS (2-40 µmol/mL blood). The continuous line represents the trueness (bias), the dashed lines represent the acceptance limits set at ± 30% and the dotted lines are the relative lower and upper accuracy limits.

3.3.7 Limit of detection

The LOD was determined by analysis of samples containing sulphuric acid and decreasing amounts of formic acid and assessed by using a signal-to-noise ratio of S/N >3. The noise was estimated by measuring 15 blank samples. The resulting LOD for CO quantification was found at 0.1 nmol/mL gas.

3.3.8 Matrix effects

The possible presence of matrix effects was evaluated by comparing the results obtained from the analyses of blank samples containing only bovine blood (Bl-IS) and sulphuric acid and samples containing bovine blood, sulphuric acid and internal standard (Bl+IS). The Bl-IS samples show the generation of low amounts of CO, which is most likely due to the acidic conditions the reaction takes place as well as heat, which lead to decomposition and degradation of proteins contained in blood. This confirms what was previously reported by Varlet *et al.* in 2012 [5].

The Bl+IS samples allow for quantification of the matrix effects through interpolation of the calibration curves. The matrix effects were quantified as a mean concentration of 21,8 nmol/mL HS (4.5 µmol/mL blood), with a standard deviation (SD) of ± 4.3 nmol/mL HS (0.9 µmol/mL blood). With the use of an internal standard, this matrix effect is taken into account equally for all samples analysed.

3.3.9 Analyses of postmortem samples

Three postmortem blood samples were analysed in order to assess the performance of the method and its applicability on real human blood samples of people subjected to fatal CO intoxication. Changes due to storage were investigated by re-analysing each sample after a period of one month, in which the sample was stored at -20°C with different preservatives (EDTA, NaF, Heparinate, Citrate). The results are presented in **Table 3.5**. The results obtained for the three postmortem cases were also compared to the results obtained from the fortification of blank blood with CO in the range of 60%-80% COHb, as obtained by CO-oximetry analysis (**Figure 3.3**).

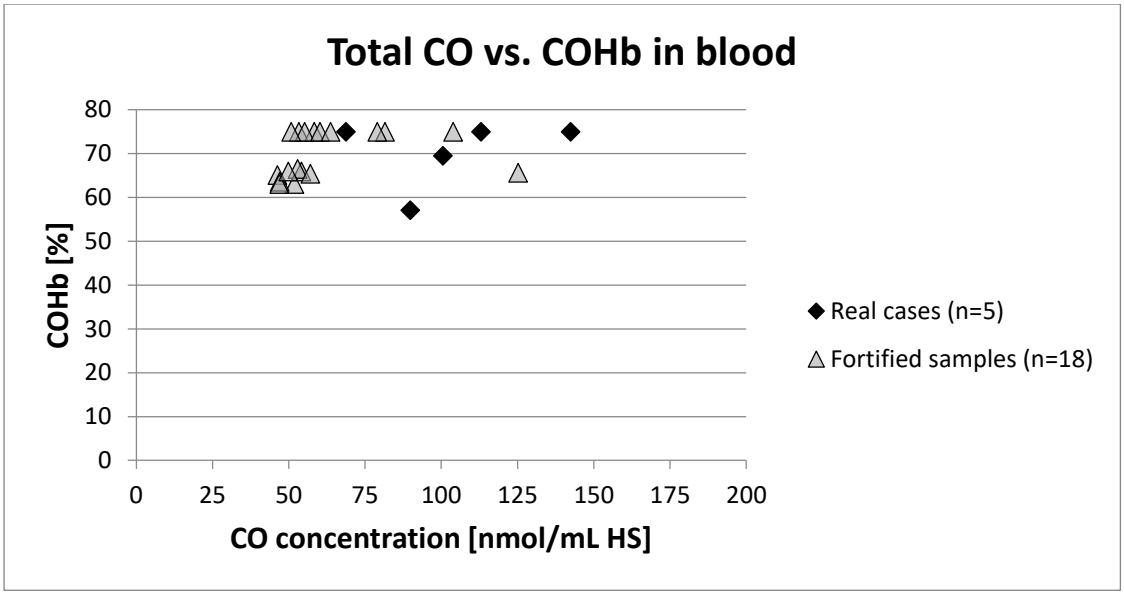

Figure 3.3: Correlation plot between the total CO concentration in nmol/mL HS (measured by AGS-GC-MS) vs. the COHb saturation in % (measured through CO-oximetry), measured in blood for two groups of samples: real case samples (n=5) and samples fortified with CO stored in blood (n=18). Analyses were carried out in triplicates, but for illustration purposes, error bars are not displayed here.

3.4. Discussion (PM range)

3.4.1 Determination of CO through AGS-GC-MS

CO content of three cardiac blood and three peripheral blood samples was determined through AGS-GC-MS, showing significant amounts of CO. Measurements by CO-oximetry, when possible, also result in high amounts of COHb determined (all >50%), indicating that CO intoxication was most likely the cause of death.

For cardiac blood of case #3, no measurement was possible with the CO-oximeter due to the poor quality of the blood sample, leaving peripheral blood as the only available sample for COHb%-determination. It was, however, possible to determine the CO content of cardiac blood through AGS-GC-MS, which resulted in a concentration of 50.5 nmol/mL HS (10.1 µmol/mL blood). Cardiac blood of samples 1 and 2, which when analysed by CO-oximetry revealed a COHb concentration of >75%, resulted in CO concentrations of 142.5 nmol/mL HS (28.5 µmol/mL blood) and 68.8 nmol/mL HS (13.2 µmol/mL blood) respectively. Therefore, if compared with the results from cardiac blood of samples 1 and 2, the cardiac blood concentration in sample 3, with a concentration of 50.5 nmol/mL HS (10.1 µmol/mL blood), is significant and could be indicative of CO poisoning. This was further confirmed by the

peripheral blood sample, which showed a COHb% level of >75% and a CO concentration of 113.1 nmol/mL HS (22.6 µmol/mL blood).

Nevertheless, this case confirms the significant drawbacks of optical measurement methods. Without the peripheral blood sample, accurate determination of COHb levels would have not been possible, leaving the case unsolved, whereas total CO concentration by GC-MS was still possible, allowing interpretation. Furthermore, the importance of the development of an alternative method for CO determination, such as AGS-GC-MS, is highlighted, which might have the potential to also be of use in routine CO-poisoning determinations.
However, it is not yet possible to fully interpret the results obtained through AGS-GC-MS measurements from a diagnostic point of view, since correlations between the symptoms and COHb% levels are the only associations available in the literature. Until now, no correlation between total blood CO and the symptoms has been developed. To obtain that, a complete assay to study the link between total CO in blood, independent of Hb, and the symptoms is required.

Figure 3.3 illustrates that the CO concentration range associable to lethal doses (above 55% COHb) is hereby found to be above 45 nmol/mL HS (9 µmol/mL blood). This result is consistent with the CO concentration of 3 µmol/mL blood previously proposed to define CO as an actor in the cause of death [6].

3.4.2 Back calculation of COHb% from CO

To assess the validity of the proposed approach, the results obtained for CO concentrations were back calculated to COHb% saturation levels with formulae already published and compared to the values obtained through CO-oximetry. In fact, the correlation between the CO measured with GC and COHb% levels and the use of formulae to back calculate the CO to COHb% is still under discussion. Several formulae have been published to calculate the equivalent COHb% from the values obtained through the analyses with gas chromatographic methods [1–3,7,8]. The application of these formulae is mainly due to the apparent satisfactory correlation between the spectrophotometrically measured COHb% levels and CO levels obtained through the back calculation from the GC-analyses. However, these correlations were obtained with different experimental designs. While CO levels were measured with GC-MS or GC-FID, COHb ranges did not cover the full range of expected COHb saturations: for example, Cardeal *et al.* [1] obtained their correlation formula with a dataset that included a CO

concentration range of 0.005-16.85 nmol/mL HS (0.001-3.35 µmol/mL blood), which is equal to back calculated COHb% levels of 0.01%-16.1%, therefore being exclusively in a low level, clinical range; Sundin *et al.* [2] have a range that covers even lower levels, between 0.5% and 5% COHb, which are within normal clinical levels for not intoxicated people.

Another difference concerns the calibrators, which were artificially generated *in situ*, either diluted from pure gaseous CO or prepared after fortification of blood with CO followed by flushing with an inert gas, intended for the removal of residual dissolved CO. However, through this flushing step, the presence at the time of analysis of CO dissolved in blood and not bound to Hb is neglected, which is not in accordance with the pathophysiology of CO intoxications [4]. Nevertheless, despite these alterations, a majority of coherent results were confirmed with the use of several formulae by Varlet *et al.* [5].

In the present study, the back calculated COHb% values obtained by applying the formulae by Cardeal *et al.* [1] and Sundin *et al.* [2] were found to be in a range between 83% and 274% and 82% and 285% respectively, which is prevalently outside the physiologically relevant range and not consistent with the results obtained with the analyses by CO-oximetry. While the lower limits are in conformity with the expected high COHb amount, the highest back calculated COHb saturations are not relevant. Despite the fact that the number of samples analysed in this study is not statistically significant, the results cannot be disregarded and legitimate the investigation to identify the source of variation for higher back calculated COHb results:

(i) PM changes can lead to CO production through microbial metabolism and/or erythrocyte catabolism. However, all samples were obtained with minimal PMI, no PM changes could have occurred with our samples. Storage of the body and sampling was performed according to the regulations of Swiss forensic laboratories, therefore no degradation of the sample occurred due to poor storage conditions. However, no detailed information about the origin of the samples, PMI and storage conditions were given by Cardeal *et al.* [1], Sundin *et al.* [2] or Vreman *et al.* [3], even though these are important factors that need to be controlled to guarantee reproducibility and specificity of the results. Thus, this is a possible cause for the incongruence between approaches.

(ii) A part of the blood CO burden is not bound to Hb in the tubes at the time of analysis. This might be explained by COHb dissociation during storage in tubes and/or during PMI in the body, or an existing, variable amount of CO dissolved at the time of sampling. The hypothesis of the presence in blood of CO dissolved during intoxications can be formulated because even

though CO dissolved in blood binds preferably to Hb, an unknown part can remain dissolved until binding with tissue proteins (Mb) and intracellular distribution (mitochondrial distribution) occurs. So far, it was assumed that once in the blood system, CO binds almost completely to Hb, resulting in COHb as the used biomarker for CO intoxications. But the results obtained here provide some doubts in whether there is more CO present in blood than just COHb and that the CO dissolved might play a more important role than expected [31]. This can result in a highly relevant role from a physiological perspective, with possible pathophysiological effects caused by the amount of CO dissolved in blood that have not yet been investigated, but might be relevant for both clinical and forensic cases. This could also help to find an explanation for the disagreement between the symptoms reported and COHb% levels measured in individuals. For this purpose, the correlation between total CO in blood and reported symptoms could be investigated.

Furthermore, in PM blood samples, CO might also originate by dissociation from Hb, Mb or cytochromes, since binding of CO to these proteins is reversible with time. COHb measured optically in this type of sample would show only a part of CO present in blood and would not take into account this variable and unknown part of CO dissolved.

(iii) Finally, a part of the CO in blood not bound to Hb in the tubes at the time of analysis can originate from COHb dissociation during storage. When blood showing an important COHb saturation is exposed to air for a significant time, the COHb measured at the end of exposure by optical methods was found to be lowered [10]. As time goes by during storage, CO dissociation from Hb can be hypothesized because the CO-Hb bond is reversible. Many studies have been led to investigate the influence of storage conditions on COHb measured by optical methods and an important diversity of results was obtained. However, to our knowledge, few data is available concerning the influence of storage conditions on CO measured by GC-MS. Moreover, the part of CO dissociated from COHb during storage could only partly explain the discrepancy between the results from the optical measurement methods and the back calculated results – for values as high as 75% COHb, even if completely dissociated, theoretically the CO dissolved could not reach values that back calculated are higher than 100%. Furthermore, COHb decreases did not exceed 20-25%.

Preliminary tests for the evaluation of the role played by preservatives in the stability of the sample were performed with two sets of samples from two cases. The COHb and CO concentrations obtained for the measurements with different preservatives (**Table 3.5**) show the same trend for case 1 (decrease of CO in both cardiac and peripheral blood). For case 2,

increased CO concentrations were observed for two of the preservatives (EDTA and NaF) and decrease for the other two preservatives (LiH and NaCit), while COHb generally decreases slightly. Case 3 shows a fairly stable cardiac blood sample, while CO in peripheral blood decreases.

Table 3.5: Summary of data from the analyses of three real PM cases with suspicion of CO intoxication, analysed by CO-oximetry and AGS-GC-MS on day of sample collection and after storage in the freezer for 30 days. *for Sample 3, second analyses were carried out after 52 days; Samples: cardiac blood (CB), peripheral blood (PB); preservatives: ethylenediaminetetraacetic acid (EDTA), sodium fluoride (NaF), lithium heparin (LiH), trisodium citrate (Cit).

Sample	Preservative	COHb [%] CO-oximetry		CO in Blood [nmol/mL HS] (µmol/mL blood) AGS-GC-MS	
		Day 0	Day 30	Day 0	Day 30
1 CB	EDTA	>75	>75	142.45 (28.49)	80.00 (16.00)
	NaF		>75		78.04 (15.61)
	LiH		>75		93.15 (18.63)
	Cit		>75		77.73 (15.55)
1 PB	EDTA	57,1	49.7	89.92 (17.98)	68.80 (13.76)
	NaF		44.9		87.33 (17.47)
	LiH		61.8		62.38 (12.48)
	Cit		55.3		47.57 (9.51)
2 CB	EDTA	>75	70.7	68.77 (13.75)	95.55 (19.11)
	NaF		64.8		108.10 (21.62)
	LiH		70.5		79.75 (15.95)
	Cit		71		57.10 (11.42)
2 PB	EDTA	69.5	65.1	100.62 (20.12)	119.50 (23.90)
	NaF		47.5		136.21 (27.24)
	LiH		64.7		81.94 (16.39)
	Cit		64.8		52.15 (10.43)
3* CB	NaF	NA	NA	50.48 (10.10)	47.60 (9.52)
3* PB	NaF	>75	70.8	113.09 (22.62)	64.15 (12.83)

Previous studies suggested that no significant effects or differences were observed when using EDTA or NaF tubes [11], which contradicts our results for case 2, where increases are observed

in both cardiac and peripheral blood. No antecedent research was found on the other two preservatives LiH and NaCit. Therefore, our results do not give clear indications on whether preservatives play a relevant role in the stability of samples and given the limited number of samples, no significant conclusions can currently be drawn from our data. The role of preservatives might be of relevance, but further investigation is required. In order to be able to determine the significance of the influence with a strong statistical relevance, a higher number of samples need to be tested and evaluated.

Additionally to the preservatives, there are several other factors that could influence the measurement of total CO in blood and have not yet been investigated. Storage conditions, such as temperature and volume of air in sampling container, are known to be of relevance in the measurement of samples in forensic and clinical cases and have specifically been studied for the measurement of COHb through spectrophotometric methods and gas chromatographic methods, which used calibrators with flushing of CO in excess prior to the analysis [10–13]. However, no data is available on storage conditions for the measurement of total CO in blood.

In general, the increased CO of PM samples can be due to enzymatic or bacterial action that occur when a sample is not analysed immediately after death (long PM interval). It is possible that after the first analysis on the day of sampling, bacteria or enzymes still present in the blood lead to increased CO concentrations, even though storage in the freezer should significantly reduce their activity. Therefore, it is very important to minimize PMI, whenever possible, and perform all analysis in a timely manner, to avoid the effects of PM-induced concentration changes.

Decreased CO concentrations could be derived from the fact that the aliquots of blood transferred to the tubes with different preservatives came from the same blood tube sampled during autopsy and that homogenization of CO within that blood tube had not yet occurred, thus resulting in higher CO levels during the first measurement than in the aliquots after storage. This could have been avoided by aliquoting the blood and then measuring CO levels at day 0, rather than using only one blood tube to determine baseline CO at the time after sampling. Another potential reason for reduced CO concentrations during storage can be the transfer of CO from the blood into the HS. In the present study, a measurement of the amount of CO in the HS of the samples was thus also performed. However, the amounts detected were as low as 0.001% of total CO, indicating that no significant amounts of CO are released into the HS during frozen storage over one month. This implies that CO would remain under

dissolved form in blood during storage. But given the limited number of samples, this assumption is not definitive and needs to be analysed further for confirmation.

Nevertheless, in the present study, the discrepancy between optical measurements of COHb% by CO-oximetry and back calculated COHb% from total CO measured by GC-MS is noticeable even in samples immediately analysed without any storage. As a result, the important back calculated COHb saturations might, in fact, derive from a variable amount of CO dissolved in blood, not bound to Hb at time of sampling. The CO-oximeter is only capable of quantifying the amount of CO bound to Hb. With this AGS-GC-MS approach, the total amount of CO present in the blood sample is analysed, which is a sum of COHb and dissolved CO.

3.5 Conclusion (PM range)

An AGS-GC-MS method for the quantification of the total amount of CO in blood from CO-poisoning cases, validated according to the 'β-expectation tolerance interval' accuracy profile as recommended by the SFSTP, was hereby exposed. The method presents improved sensitivity (lower LOD and LOQ) and lower costs due to reduced quantities of reagents compared to the previously published study by Varlet *et al.* from 2012 [5]. Moreover, the method is accurate and reliable (±30%) for measurements of CO concentrations in a range from 10-200 nmol/mL HS (2-40 µmol/mL blood).

The main novelty of this study is the consideration of the totality of CO present in the blood, which includes CO bound to Hb as well as CO dissolved in blood and the CO released into the HS of the sampling tube. No flushing of the calibrators is hereby performed, which is in accordance with physiological principles. The results reported show a significant difference of the CO concentrations if compared to results from previously published works, suggesting that the AGS-GC-MS method might be a valid alternative to the use of COHb as a biomarker for CO exposures, with the latter possibly underestimating the true role played by CO in such an intoxication.

Additionally, this method could be of high importance in explaining numerous cases in which the reported COHb% levels did not correlate to the symptoms shown by intoxicated patients, with applications in both the clinical and forensic field, even though further research into this approach needs to be performed for confirmation. Furthermore, for applicability and validity in clinical cases, the validation of this method with a lower calibration range and with real clinical samples also needs to be completed.

3.6 Results (clinical range)

3.6.1 Validation of the method in a clinical range

Results of the validation for all criteria are summarized in **Table 3.6**.

Calibration curves for CO determination were obtained by using CO negative human blood as blank matrix. Calibration curves, which represent the response function, were acquired on three non-consecutive days (p = 3), in triplicates (n = 3) and at seven concentration levels (k = 7): 1.63, 3.25, 6.5, 13, 26, 52 and 104 nmol/mL HS (equivalent to 0.65, 1.3, 2.6, 5.2, 10.4, 20.8 and 41.6 µmol/mL blood). For each calibration point, calculated concentrations were compared to the target values and found to be within ± 20%. The relationship between the CO concentration from samples spiked with formic acid and the measured response was found to be linear. Validation coefficients for the calibration curves are shown in **Table 3.6a**.

On each non-consecutive day (p = 3), control samples at five different concentrations (k = 5), namely 2, 5, 10, 30 and 60 nmol/mL HS (0.8, 2, 4, 12 and 24 µmol/mL blood), were measured in triplicates (n = 3). The concentrations of the control samples were calculated by using the calibration curve determined for each analysis day. As shown in **Table 3.6b**, satisfactory linearity was obtained, with a slope of 1.05 and a regression coefficient of 0.99 in the range of 2 to 60 nmol/mL HS (0.8-24 µmol/mL blood).

The selectivity of the AGS-GC-MS method was previously confirmed in section 3.3.1.

Trueness was found to be lower than the acceptance criteria (within ±20% of the accepted reference value and within 20% at the LOQ), as is shown in **Table 3.6c,** which is satisfactory for validation according to SFSTP guidelines [14].

Table 3.6d shows that the relative standard deviation (RSD) for repeatability and intermediate precision are in a range between 0.50% and 3.55%.

Figure 3.4 represents the accuracy profile for CO. The mean bias (%) confidence interval limits for the control samples were within the acceptability limits of ±30%. Taking into consideration the acceptability limits of ±30%, the limit of quantification within validation criteria was found between 2 and 5 nmol/mL HS (0,8 and 2 µmol/mL blood). Thus, the method is confirmed to be accurate within the range of 5 and 60 nmol/mL HS (2-24 µmol/mL blood) according to the β-interval tolerance accuracy profile.

In addition, the external controls gave excellent accuracy, with an RSD below 15% (**Table 3.6e**).

Table 3.6: Validation results for CO determination in blood by AGS-GC-MS - (**a**) represents the mean coefficients of the calibration functions obtained from analysis of the calibrators; (**b**) represents the coefficients of the linear regression function obtained from analysis of the QCs against their theoretical value; (**c**) represents the trueness obtained from the QC analyses expressed in %; (**d**) represents the precision obtained from QC analyses expressed in relative standard deviation % (RSD%); (**e**) shows mean and RSD% of the external controls.

a) Response function [1.63-104 nmol/mL HS] (k = 7, n= 3, p = 3)			
	Day 1	**Day 2**	**Day 3**
Slope	0.03	0.02	0.03
Intercept	0.59	0.63	0.61
r^2	0.98	0.99	0.98

k: number of concentration levels; *n*: number of repetitions for each level; *p*: number of non-consecutive days

b) Linearity [2-60 nmol/mL HS] (k = 5, n = 3, p = 3)	
Slope	1.05
Intercept	-0.48
r^2	0.99

c) Trueness (relative bias) (k = 5, n = 3, p = 3)	
Levels [nmol/mL HS]	**Trueness (%)**
2.0	-11
5.0	-10
10	1
30	-18
60	-19

d) Precision (RSD%) (k = 5, n = 3, p = 3)	
Levels [nmol/mL HS]	**Repeatability and Intermediate Precision***
2.0	0.5
5.0	0.6
10	1.7
30	1.6
60	3.6

*Inter-series variance (s^2g) is negligible for all levels, resulting in same values for repeatability and intermediate precision

e) External controls (k = 2, n = 3, p = 1)		
Levels [nmol/mL HS]	**Mean (Confidence Interval)**	**RSD [%]**
5	4.49 (3.93, 5.06)	12.0
60	65.5 (60.0, 73.9)	6.57

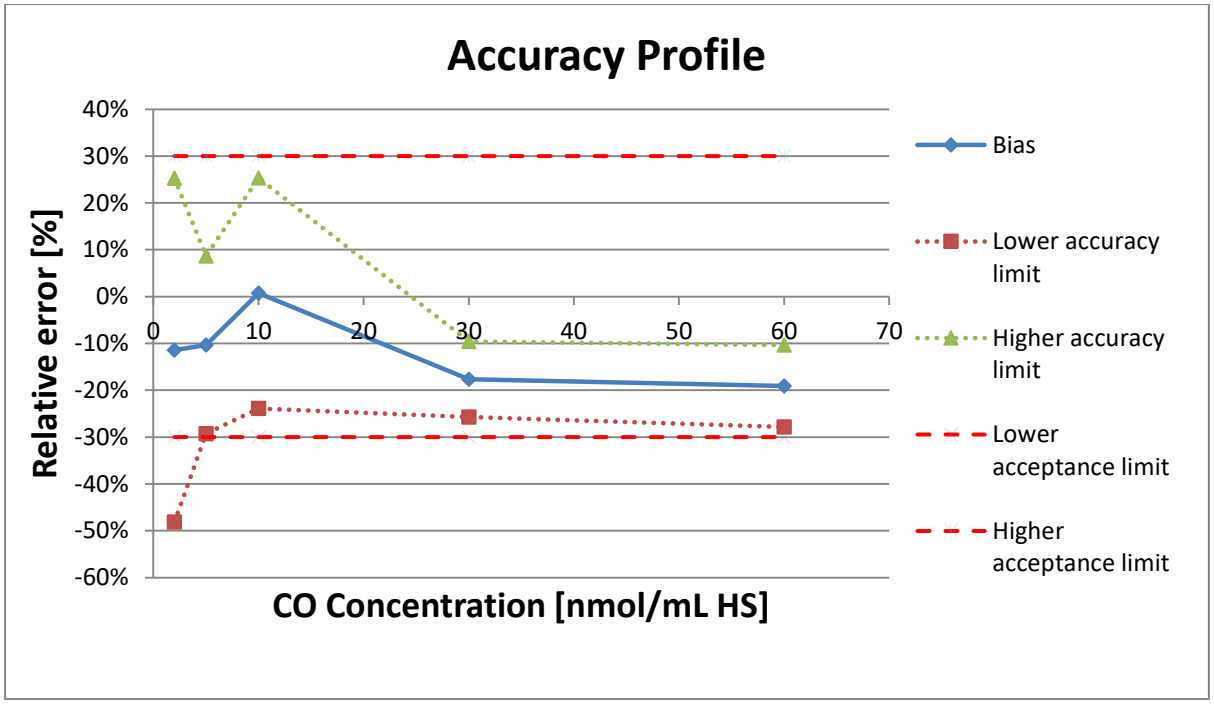

Figure 3.4: Accuracy profile for CO determination using a simple linear regression model within the range of 2-60 nmol/mL HS (0.8-24 µmol/mL blood). The continuous line represents the trueness (bias), the dashed lines represent the acceptance limits set at ± 30% and the dotted lines are the relative lower and upper accuracy limits.

3.6.2 Analyses of flushed samples

Blood samples of 13 patients were analysed before and after exposure to CO as well as with and without a flushing step. Triplicates were acquired for analyses with CO-oximetry and AGS-GC-MS. Results of all analyses are found in **Table 3.7** and represented in **Figure 3.5**.

For all samples analysed, a relative increase of both TBCO and COHb can be observed when comparing the values before and after CO exposure (**Figure 3.5** and **Table 3.7**). Increases were confirmed and found to be significant by comparing the means with Student t-Test (**Table 3.8**). Important observations can be made when comparing the COHb and TBCO levels before and after a flushing step (**Table 3.7 and 3.8**). For the levels of COHb, there is no consistent trend that can be observed, with a COHb increase in some patients and a decrease in others. The 25th and 75th interquartile range (**Table 3.8**) for COHb before and after flushing (range includes 0), as well as the results of the t-Test (p-Value > 0.05), confirm this result. This behaviour could be explained by the known analytical variability of CO-oximeters at such low COHb saturations [15,16].

Table 3.7: Differences for before and after CO exposure and before and after flushing, measurement results for COHb [%] and TBCO [µmol/mL].

Patient ID	Sex	Volume CO [mL]	COHb [%]		TBCO [µmol/mL]	
			Difference After-Before CO exposure	Difference Not flushed-flushed	Difference After-Before CO exposure	Difference Not flushed-flushed
1	M	84.3	1.90	-2.20	0.11	0.11
2	M	89.0	7.30	-1.50	1.95	0.81
3	F	87.9	7.30	-1.30	2.20	0.30
4	M	59.3	6.80	-0.90	1.84	0.75
5	F	48.3	5.20	0.60	0.75	0.34
6	M	104	6.10	-0.90	0.93	0.67
7	M	86.7	7.70	-2.60	0.74	0.33
8	M	89.1	8.10	-0.90	1.06	0.31
9	F	71.2	10.90	1.20	1.99	0.30
10	M	57.4	6.80	1.50	0.78	0.66
11	F	99.5	6.90	2.50	1.51	0.49
12	M	60.8	6.20	2.10	1.63	0.23
13	M	65.3	8.70	0.10	3.05	0.57

Conversely, TBCO shows a consistent trend: for all samples analysed, TBCO before flushing is higher than after flushing. Statistical significance was confirmed by performing a paired Student t-Test of the means (p-Value = 8.955e-06). Mean and interquartile range for TBCO differences before and after flushing additionally confirm the positive relationship (**Table 3.8**).

Table 3.8: Summary statistics and results of paired t-Test with 95% confidence interval (CI) for the differences for COHb and TBCO before (B) and after (A) exposure and before (NF) and after flushing (F); p-Values in **bold** are significant (below 0.05).

	COHb Diff A/B [%]	COHb Diff NF/F [%]	TBCO Diff A/B [µmol/mL]	TBCO Diff NF/F [µmol/mL]
Mean	6.92	-0.18	1.43	0.45
SD	2.06	1.64	0.79	0.22
1st Quartile	6.20	-1.30	0.78	0.30
3rd Quartile	7.70	1.20	1.95	0.66
p-Value	**6.02*e-07**	0.71	**2.93*e-05**	**8.96*e-06**
95% CI	5.98; 9.53	-1.17; 0.81	0.95; 1.90	0.32; 0.58

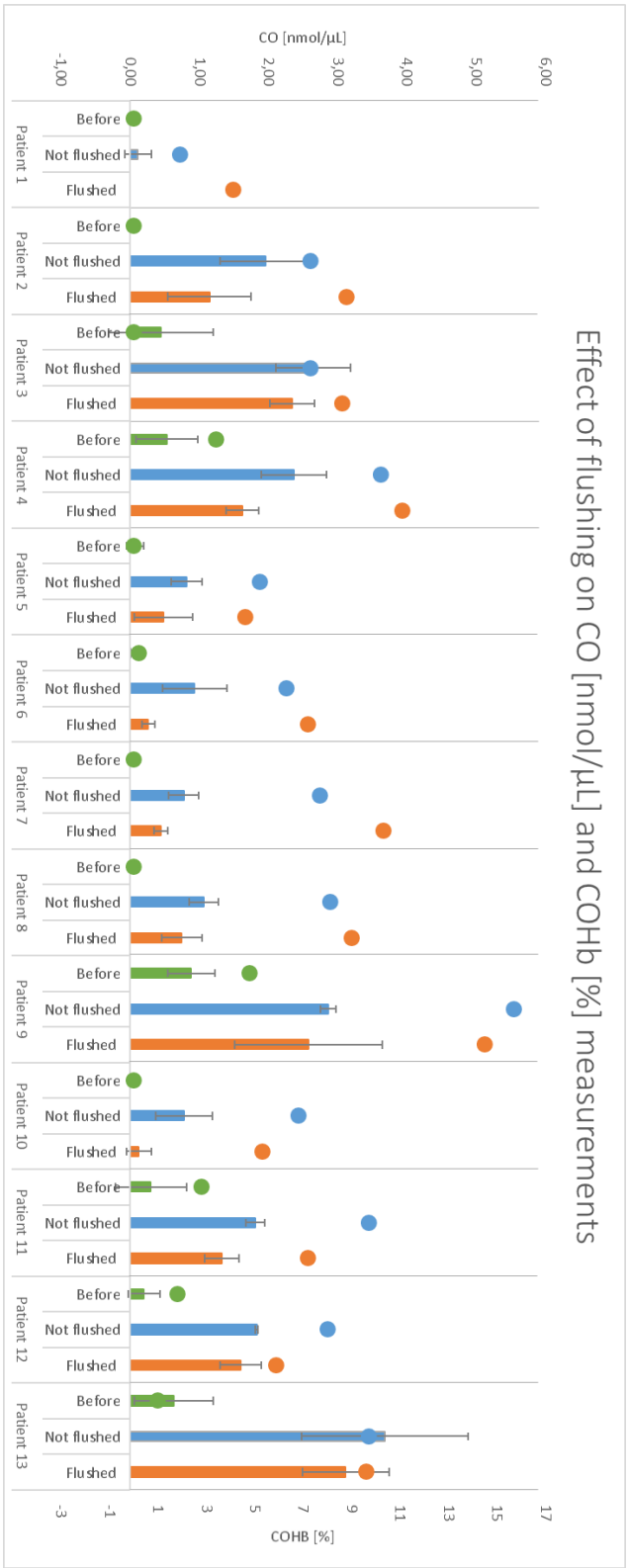

Figure 3.5: Results for the total CO concentration in blood (TBCO) in µmol/mL (bars, left axis) measured by AGS-GC-MS and the COHb saturation in % (dots, right axis) measured through CO-oximetry in blood for 13 patients before (green) and after (blue) CO exposure and after a flushing step (orange).

3.7 Discussion (clinical range)

An improved AGS-GC-MS method for CO determination in blood was validated for a clinical concentration range (1.63-104 nmol/mL HS / 0.65-41.6 µmol/mL blood) and was successfully applied to the analysis of blood samples coming from individuals with controlled CO exposures. The observed increase of both COHb and TBCO before and after CO exposure was to be expected, since all individuals were breathing in a mixture of O_2 and CO. Patients with a CO burden before exposure, namely patients with ID number 3, 4, 9, 11, 12 and 13, admitted to being smokers. Smokers are known to have a higher baseline CO level, which varies depending on the frequency [17–19], hence it explains the presence of CO in several patients before exposure.

Significant variability of CO burden after exposure is found between individuals. Even though the volumes of CO administered were adapted to the weight and height of the patients, other factors involving the respiratory system and blood circulation, such as ventilation rate, tidal volume, inspiratory and expiratory reserve volume, alveolar ventilation, cardiac rhythm and cardiac output, influence the net amount of CO that enters the circulation [20]. In addition, malfunctioning of the rebreathing system can lead to altered amounts of CO effectively being administered, resulting in the observed inter-patient variations.

The samples were all subject to the same storage and sample treatment conditions (immediately after blood collection) and analysed with the same parameters and measurement method (within 48 hours after blood samplings). It is therefore not very likely that the differences in detected concentrations are due to any error in the measurement technique or used parameters, but mainly to the removal of CO through a constant nitrogen stream. This legitimizes the hypothesis that there is a significant amount of CO present in free form in blood from an individual, who was subject to CO exposure. The amount of free CO on average ranges between 10 and 60% compared to the initial TBCO burden.

Additionally, when plotting the results of the 13 clinical samples with the results obtained from *in vitro* CO-fortified samples (**Figure 3.6**), it can be seen that the clinical samples all comply with the *in vitro* measurements, further diminishing the probability of errors in the measurement and strengthening the assumption of CO being eliminated through flushing.

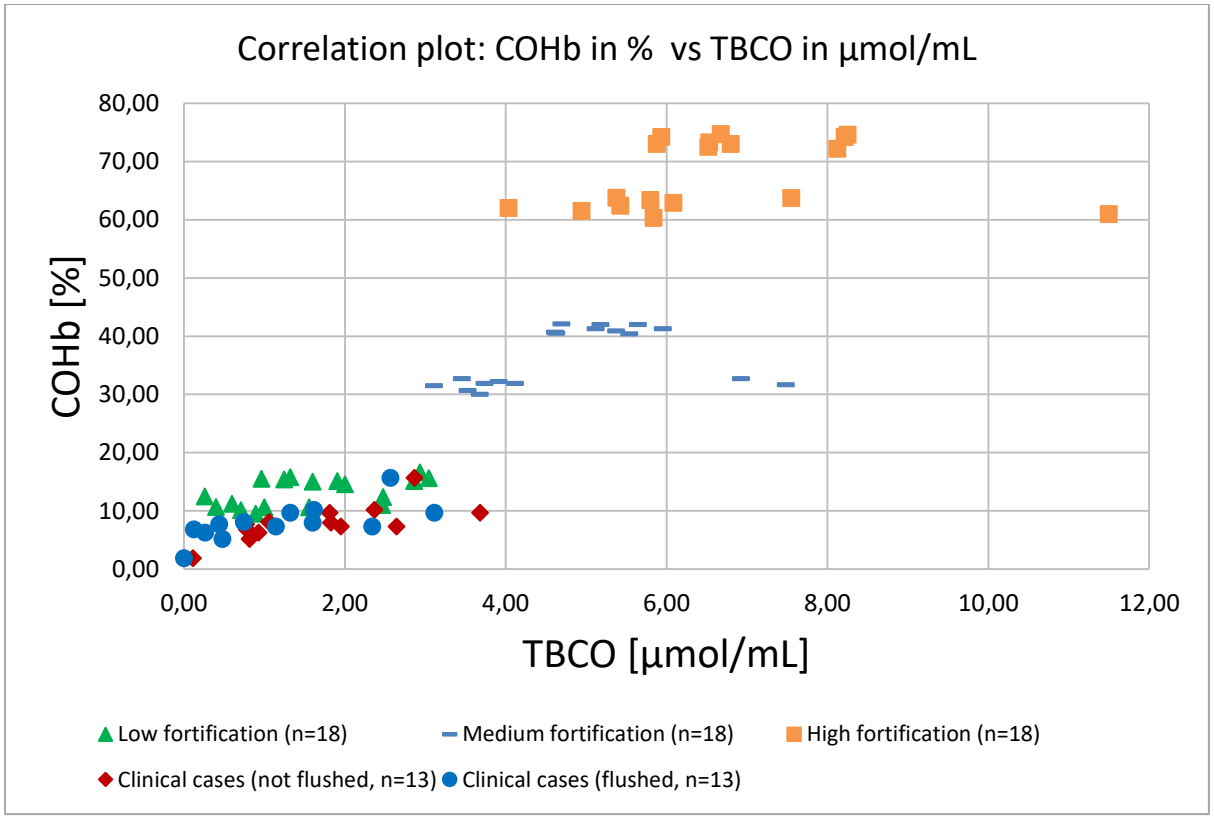

Figure 3.6: Correlation plot between TBCO in µmol/mL measured by AGS-GC-MS vs. the COHb saturation in % measured through CO-oximetry for two groups of samples: stored blood samples fortified with CO (squares for high fortification level 60-70%, lines for medium fortification level 30-40% and triangles for low fortification level 10-20%, n=54) and real case samples (circles for flushed and diamonds for not flushed samples, n=26). Analyses were carried out in triplicates, but for illustrative purposes, error bars were not displayed.

This suggests that with the execution of a flushing step a significant amount of CO is removed from the analysed sample. Thus, the result is biased not only from an analytical but also from a clinical point of view: the excess amount of CO may have a more significant pathophysiological activity than previously suspected. The direct cellular toxicity of molecular CO through impairment of cellular respiration and generation of free radicals, which are known to be tumour-cells promoters, was reported in previous studies [21–27]. Yet, the importance given to its implications in the direct adverse effects in CO poisonings was held to a minimum. Most likely this was because the presence of CO dissolved in blood in free form was never clearly demonstrated before.

Furthermore, the acknowledgement of dissolved CO represents one possible argument for explaining the discrepancy between reported symptoms and measured COHb. Considering only the amount of CO bound to Hb when determining a CO poisoning may lead to underestimation of the true burden CO poses, explaining why in many cases the measurements are inconsistent with the symptoms a patient is showing and also why several patients show symptoms with a certain time-delay. The amount of CO that is dissolved and not bound to Hb

may be the missing quantity that gets closer to the true CO concentration in blood and burden on the body of an exposed individual. CO toxicity at cellular level may not only explain some of the symptoms of acute CO poisonings but, due to the slow dissociation rate from cellular proteins, it may also elucidate the reasons behind the delayed neurological effects reported hours or days after COHb was removed from the system through oxygen therapy and after low-level chronic exposures.

3.8 Limitations

This study constitutes a preliminary study that aims to demonstrate the existence of CO dissolved not bound to Hb. This hypothesis has been tested with a cohort of 13 individuals through bedside blood collection. However, these findings have to be verified on a higher number of volunteers, even if the experimental design is complex to perform due to time-dependent analysis. Moreover, there is no clear evidence about a constant amount of CO dissolved, since in each patient the difference between TBCO before and after flushing varies. These variations are most likely due to interindividual variability: several factors such as pre-existing cardiovascular or respiratory conditions, metabolic rate, ventilation rate and volumes, sex and age can play a role in the behaviour and amount of CO in blood. Additional measurements with more individuals will lead to higher statistical significance and will reduce the interindividual variability. In addition, analytical parameters affecting the storage as well as biological phenomena taking place after sampling of blood are known to potentially alter the measurement results, even if I reduced them to a minimum in this study. Further investigations into these TBCO pharmacodynamics and pharmacokinetics are needed to account for this behaviour.

3.9 Conclusion (clinical range)

This study presents the validation of an improved carbon monoxide analysis method in human blood, based on AGS-GC-MS, for a range of 1.63 to 104 nmol/mL HS (0.65-41.6 µmol/mL blood), which is applicable to clinical CO exposure cases. The method was applied to a cohort of 13 patients, who were exposed to controlled amounts of CO, and results compared to measurements by CO-oximetry. Furthermore, a flushing step was performed on samples after CO administration. Results seem to support the hypothesis that TBCO may be an alternative to

COHb as biomarker for determination of CO poisoning, since consideration of only CO bound to Hb may underestimate the total burden of CO in blood. By comparing flushed and unflushed samples, it was determined that there is a significant amount of CO present in blood in free form (10-60%) at the sampling time post-exposure.

This represents an important finding for the understanding of the true role played by CO in poisoning cases and for the explanation of the discrepancy often encountered by clinicians between symptoms and results and the onset of delayed neurological sequelae, even after complete removal of COHb from the system after normo- or hyperbaric oxygen therapy, possibly leading to a decrease in the number of misdiagnoses. Nevertheless, before the application of the method in clinical settings, this hypothesis needs to be verified by more numerous cohorts and in-depth statistical analyses, to increase statistical power. Additionally, further investigation into the biochemical mechanisms behind the distribution and behaviour of dissolved CO in human blood is required.

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Appendix 3

A3.1 Protocol for sample preparation and analysis of blood samples for CO determination via CO-oximetry and GC-MS

A3.1.1 Preparation of blank matrix (i.e. unfortified bovine blood)

1) Prepare a 1 L polyethylene (PE)
2) Retrieve 1 L of bovine blood per preservative for fortification and 250mL of blood per preservative to use for the calibration curve.
3) Analyse a sample of the fresh blood via CO-oximetry to determine the baseline COHb% content – only use for subsequent analyses if at <1% COHb.

A3.1.2 Preparation of calibration standards, internal standard (IS) solution and quality controls

Calibration standard: formic acid (M_{HCOOH} = 46 g/mol, ρ = 1.22 g/mL) solution at 87 µmol/mL (for post-mortem (PM) range)

1) Insert 10 mL of distilled water in a 20mL headspace (HS)-vial.
2) Pipette 32.8 µL of formic acid into the HS-vial.
3) Seal vial with a septum and vortex upside down for several seconds to allow homogenization.
4) When not in use, store refrigerated at 4°C for a maximum of one week.

Calibration standard: formic acid (M_{HCOOH} = 46 g/mol, ρ = 1.22 g/mL) solution at 43 µmol/mL (for clinical range)

1) Insert 10 mL of distilled water in a 20mL HS-vial.
2) Pipette 16.4 µL of formic acid into the HS-vial.
3) Seal vial with a septum and vortex upside down for several seconds to allow homogenization.
4) When not in use, store refrigerated at 4°C for a maximum of one week.

IS solution: isotopically labelled formic acid ($M_{H^{13}COOH}$ =47 g/mol, ρ = 1.246 g/mL) solution at 84 µmol/mL)

1) Insert 10mL of distilled water in a 20mL HS-vial.
2) Pipette 32.0 µL of isotopically labelled formic acid into the HS-vial.
3) Seal with a septum and vortex upside down for several seconds to allow homogenization.
4) When not in use, store refrigerated at 4°C for a maximum of one week.

Quality control: pure CO gas

1) Fill one 20mL HS-vial completely with distilled water and seal it with a septum.
2) Turn the vial upside down.
3) Insert one needle for injection.
4) Insert another needle for release of excess water.
5) Transfer pure CO (99%) gas into the HS-vial through the injection needle until the vial is half-filled with gas.
6) Depending on the concentration of the quality control (QC) required, transfer the required amount of CO gas into another 10mL HS-vial that was previously sealed.
7) Dilute into another sealed HS-vial if necessary (for low QC concentrations).

A3.1.3 Preparation of calibration curve for PM range

1) Insert 100µL of blank blood into a 20mL HS-vial.
2) For each calibration solution, insert the volumes of the calibration and IS solutions into the HS-vial according to the following table:

Table A3 1: List of amounts of calibration and internal standard solutions required for each calibration point in analyses of PM samples.

Calibration points [nmol/mL HS]	0	6.5	13	26	52	104	156	208
Amount of Formic acid solution [µL]	-	1.49	2.99	5.98	11.96	23.92	35.88	47.84
Amount of isotopically labelled formic acid solution [µL]	10	10	10	10	10	10	10	10

3) Insert 100µL of sulphuric acid in an aluminium cap that is then carefully inserted into the vial.
4) Seal the vial immediately and vortex upside down for several seconds.

A3.1.4 Preparation of calibration curve for clinical range

1) Insert 100µL of blank blood into a 20mL HS-vial.
2) For each calibration solution, insert the volumes of the formic acid and isotopically labelled formic acid solutions into the HS-vial according to the following table:

Table A3.2: List of amounts of calibration and internal standard solutions required for each calibration point in analyses of clinical samples.

Calibration points [nmol/mL HS]	0	1.63	3.25	6.5	13	26	52	108
Amount of Formic acid solution [µL]	-	0.75	1.5	3.0	6	12	24	48
Amount of isotopically labelled formic acid solution [µL]	10	10	10	10	10	10	10	10

3) Insert 100µL of sulphuric acid in an aluminium cap that is then carefully inserted into the vial.
4) Seal the vial immediately and vortex upside down for several seconds.

A3.1.5 Preparation of blood samples for analysis

1) Insert 100µL of blood sample into a 20mL HS-vial.
2) Add 10µL of isotopically labelled formic acid solution.
3) Insert 100µL of sulphuric acid in an aluminium cap that is then carefully inserted into the vial.
4) Seal the vial immediately and vortex upside down for several seconds.

A3.1.6 Analysis of samples via GC-MS

1) Insert prepared samples into an oven at 100°C for 60 minutes.
2) Take samples out of the oven and let it cool down to room temperature.
3) With an airtight gas syringe (AGS), sample 1mL of the samples' HS.
4) Inject it into the GC-MS instrument.
5) To perform triplicate analyses, perform the sample preparation and analysis steps again.

A3.1.7 Analysis of samples via CO-oximetry

1) Calibrate instrument prior to analysis with calibrators provided by the manufacturer.
2) With a syringe provided by the CO-oximeter manufacturer, samples approximately 50-100µL of blood from the sample.
3) Insert blood into cuvettes provided by the manufacturer.
4) Insert cuvette with blood sample into CO-oximeter for analysis.
5) Once the sample is analysed, discard of the cuvettes and syringe.

A3.1.8 Protocol for flushing of samples

1) Sample 2 blood tubes per patient, which have been administered CO through the CO-re-breathing-system, into EDTA tubes (2.3 mL).
2) Analysis:

NO FLUSHING

a. Prepare and analyse samples according to the protocol for blood samples

FLUSHING

a. Adjust nitrogen (N2) flow rate to reach the required maximum of 5mL/min.
b. Once blood sample is obtained, insert one needle into septum to release excess pressure.
c. Insert the needle with N_2 flow into the blood tube, making sure it is at the bottom of the tube.
d. Flush sample for 3 minutes, then remove N_2 flow and additional needle.
e. Vortex (slowly) for 30 sec to ensure homogenization, then let rest for 1 min
f. Prepare and analyse samples according to the protocol for blood samples.
g. If analysis not possible immediately, storage in the fridge (4°C) – record time between sampling and analysis.

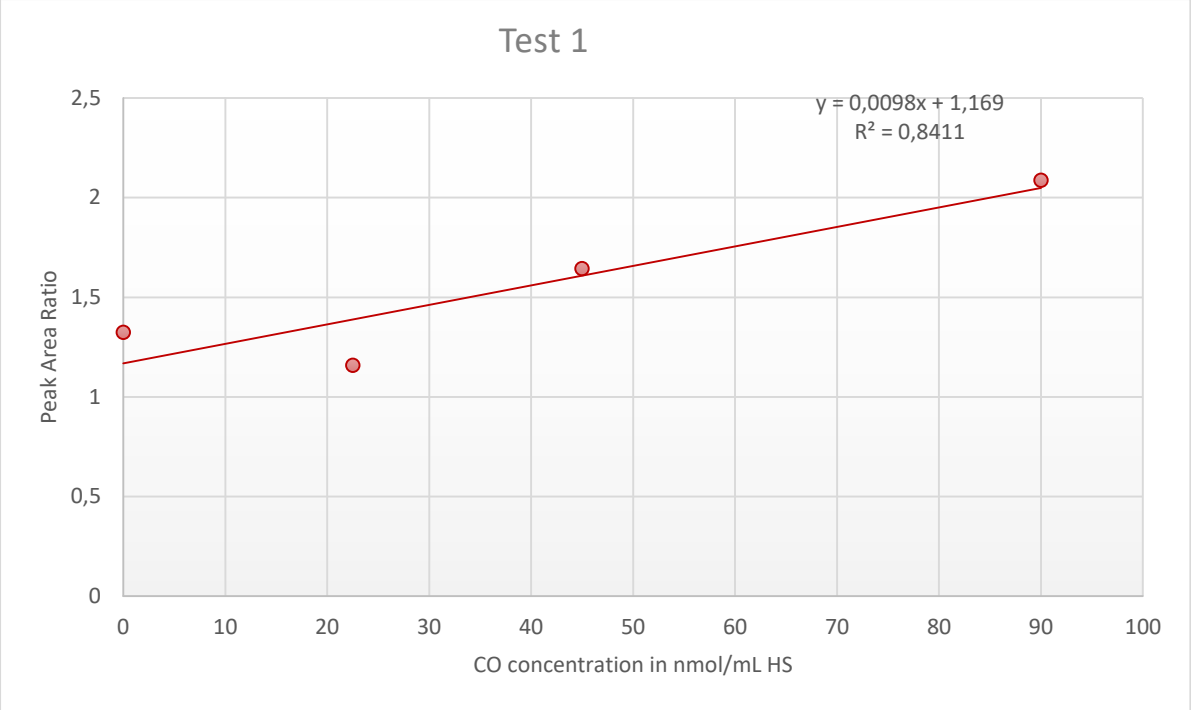

Figure A3.1: Peak Area Ratio of carbon monoxide (CO) from the blood sample over ^{13}CO from the internal standard (IS) plotted against the CO concentration in the calibration standards, with addition of linear trendline, linear regression equation and regression coefficient (R^2) for Test 1.

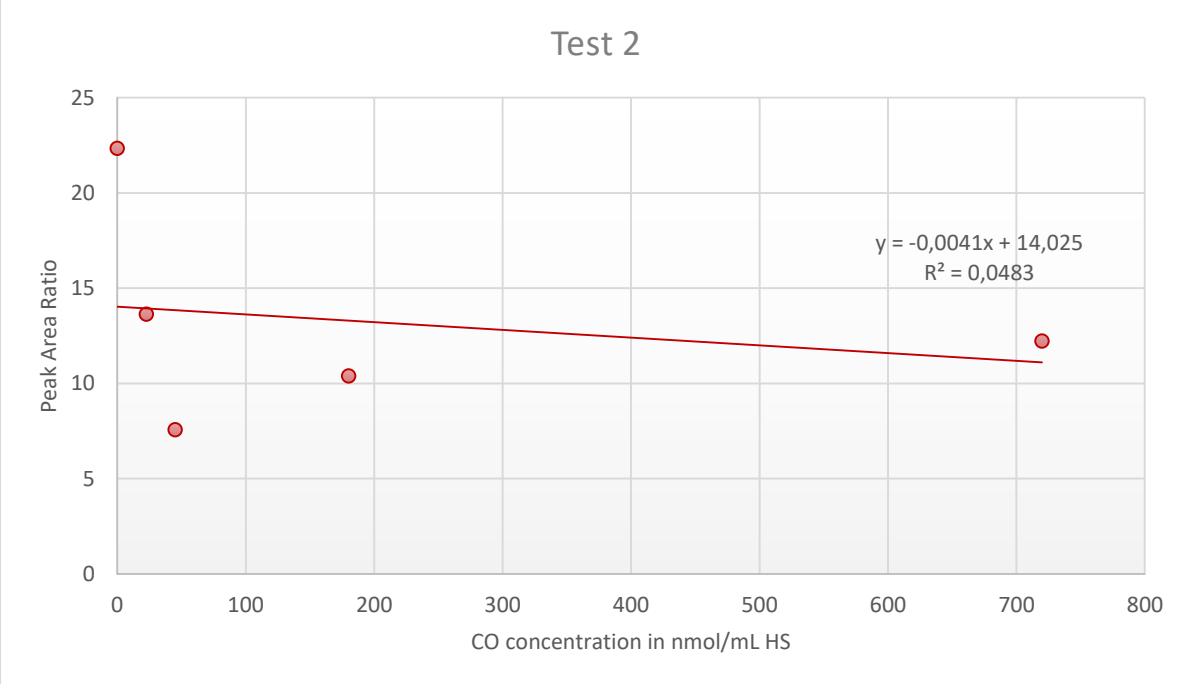

Figure A3.2: Peak Area Ratio of carbon monoxide (CO) from the blood sample over ^{13}CO from the internal standard (IS) plotted against the CO concentration in the calibration standards, with addition of linear trendline, linear regression equation and regression coefficient (R^2) for Test 2.

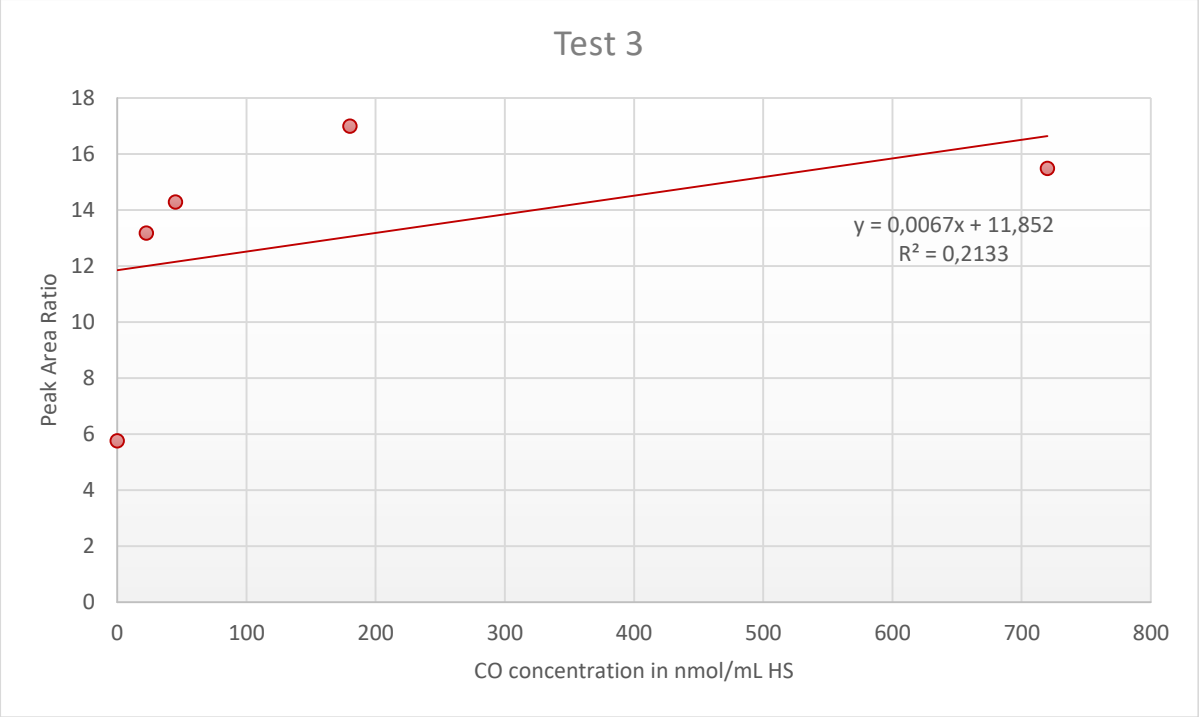

Figure A3.3: Peak Area Ratio of carbon monoxide (CO) from the blood sample over ^{13}CO from the internal standard (IS) plotted against the CO concentration in the calibration standards, with addition of linear trendline, linear regression equation and regression coefficient (R^2) for Test 3.

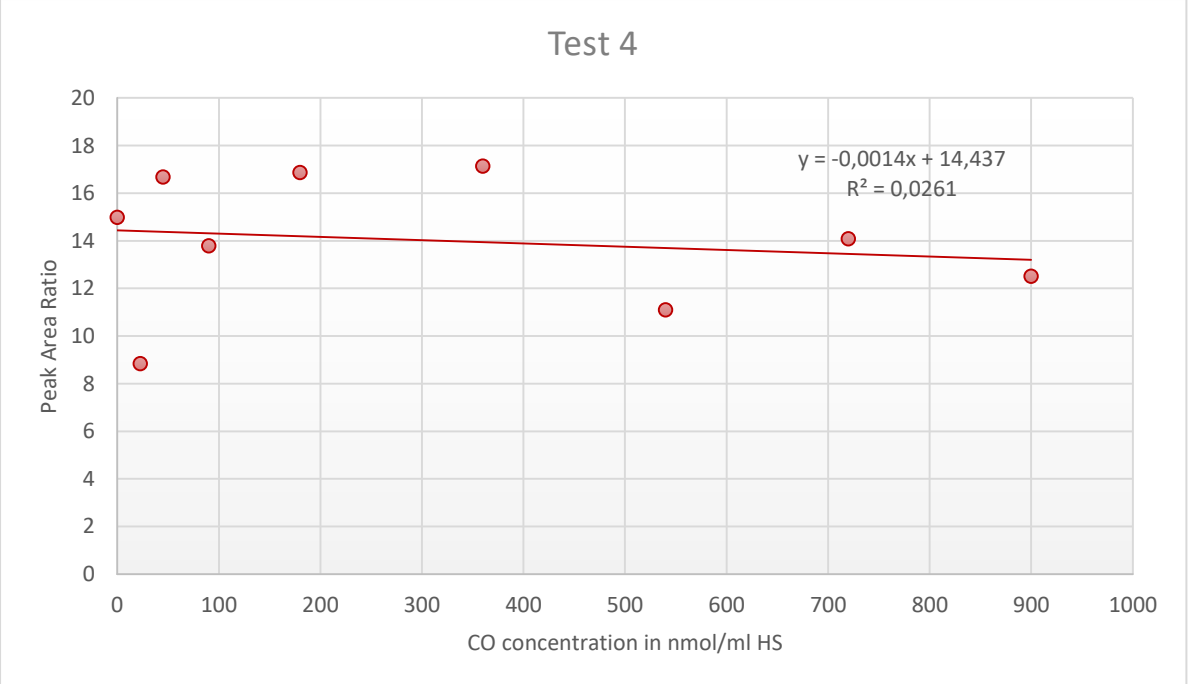

Figure A3.4: Peak Area Ratio of carbon monoxide (CO) from the blood sample over ^{13}CO from the internal standard (IS) plotted against the CO concentration in the calibration standards, with addition of linear trendline, linear regression equation and regression coefficient (R^2) for Test 4.

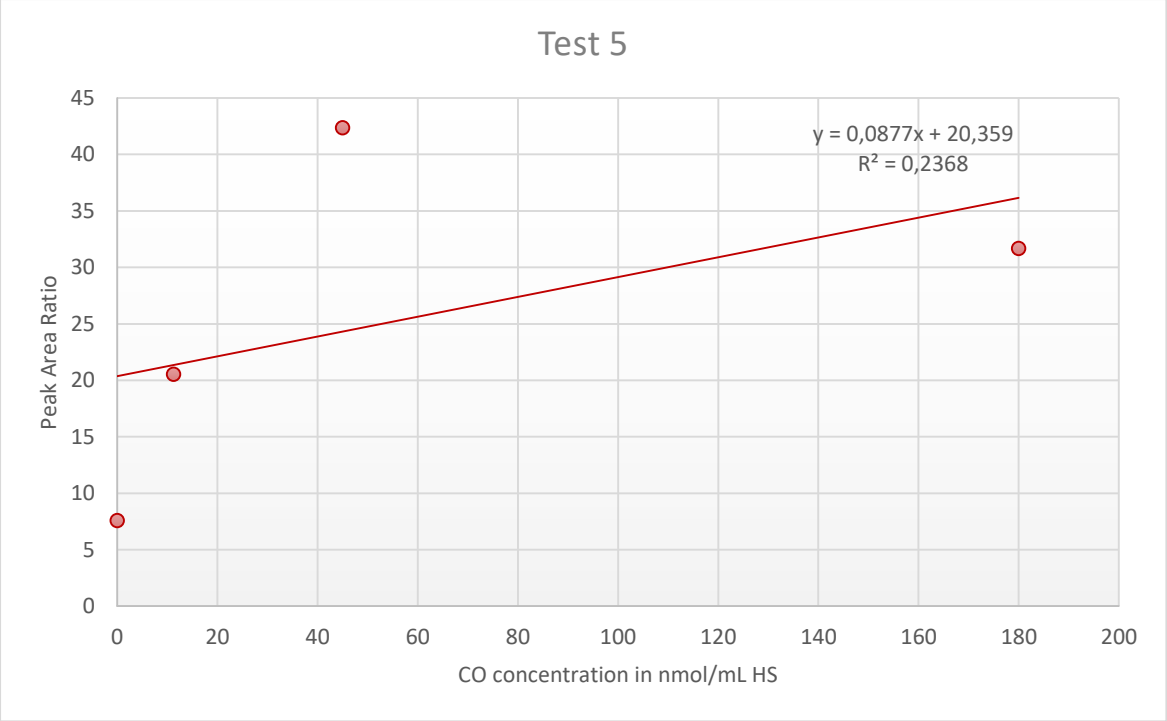

Figure A3.5: Peak Area Ratio of carbon monoxide (CO) from the blood sample over ^{13}CO from the internal standard (IS) plotted against the CO concentration in the calibration standards, with addition of linear trendline, linear regression equation and regression coefficient (R^2) for Test 5.

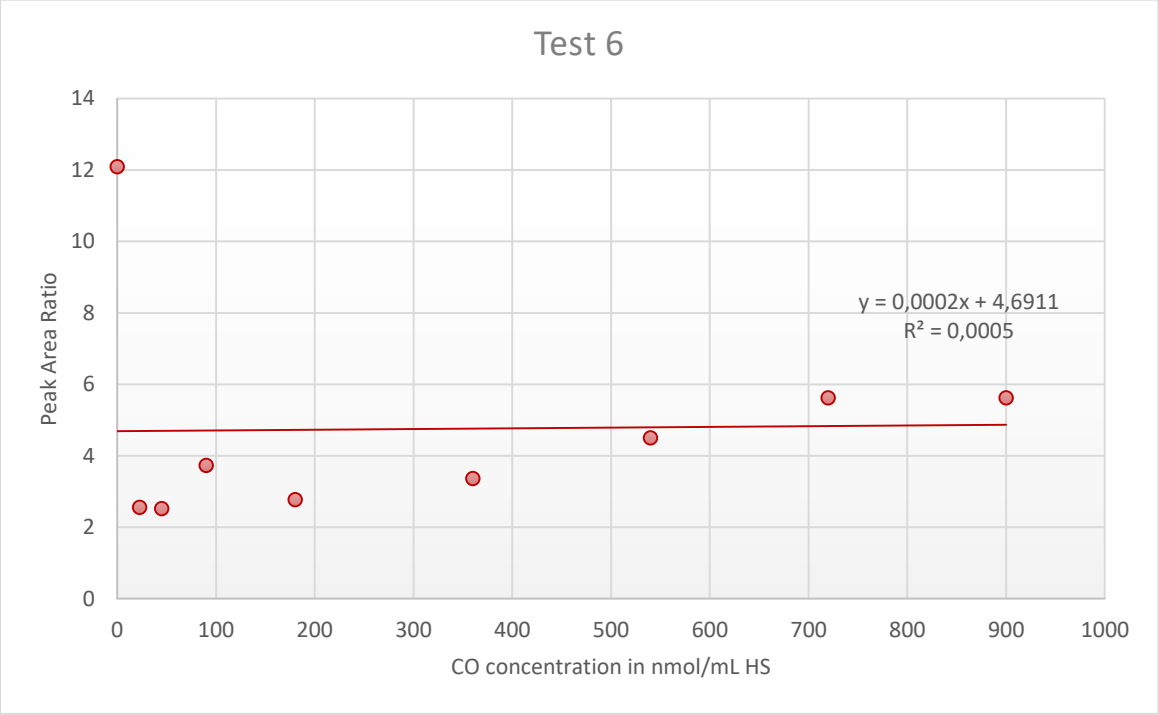

Figure A3.6: Peak Area Ratio of carbon monoxide (CO) from the blood sample over ^{13}CO from the internal standard (IS) plotted against the CO concentration in the calibration standards, with addition of linear trendline, linear regression equation and regression coefficient (R^2) for Test 6.

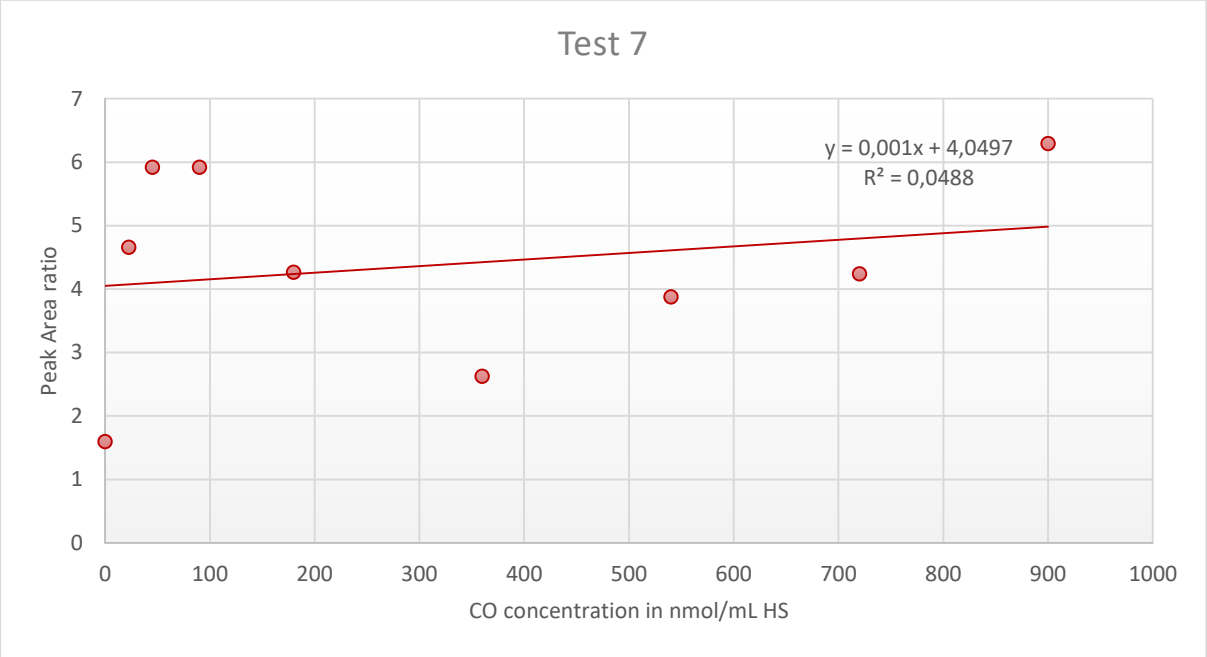

Figure A3.7: Peak Area Ratio of carbon monoxide (CO) from the blood sample over ^{13}CO from the internal standard (IS) plotted against the CO concentration in the calibration standards, with addition of linear trendline, linear regression equation and regression coefficient (R^2) for Test 7.

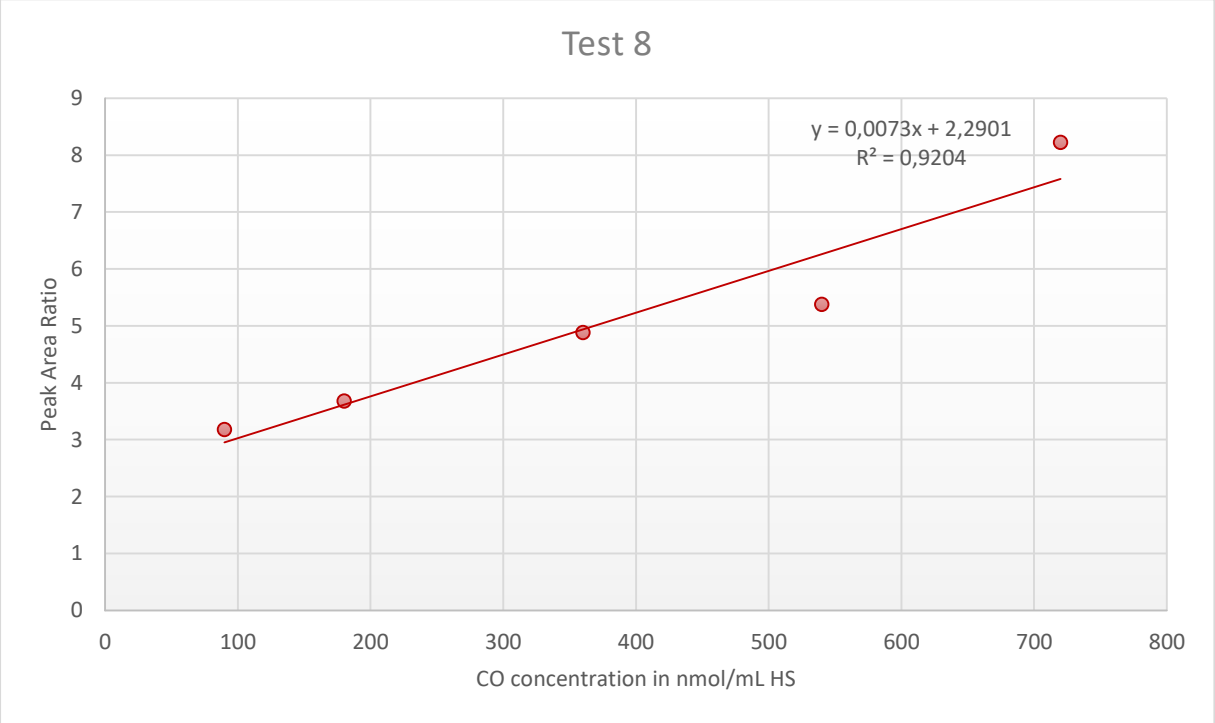

Figure A3.8: Peak Area Ratio of carbon monoxide (CO) from the blood sample over ^{13}CO from the internal standard (IS) plotted against the CO concentration in the calibration standards, with addition of linear trendline, linear regression equation and regression coefficient (R^2) for Test 8.

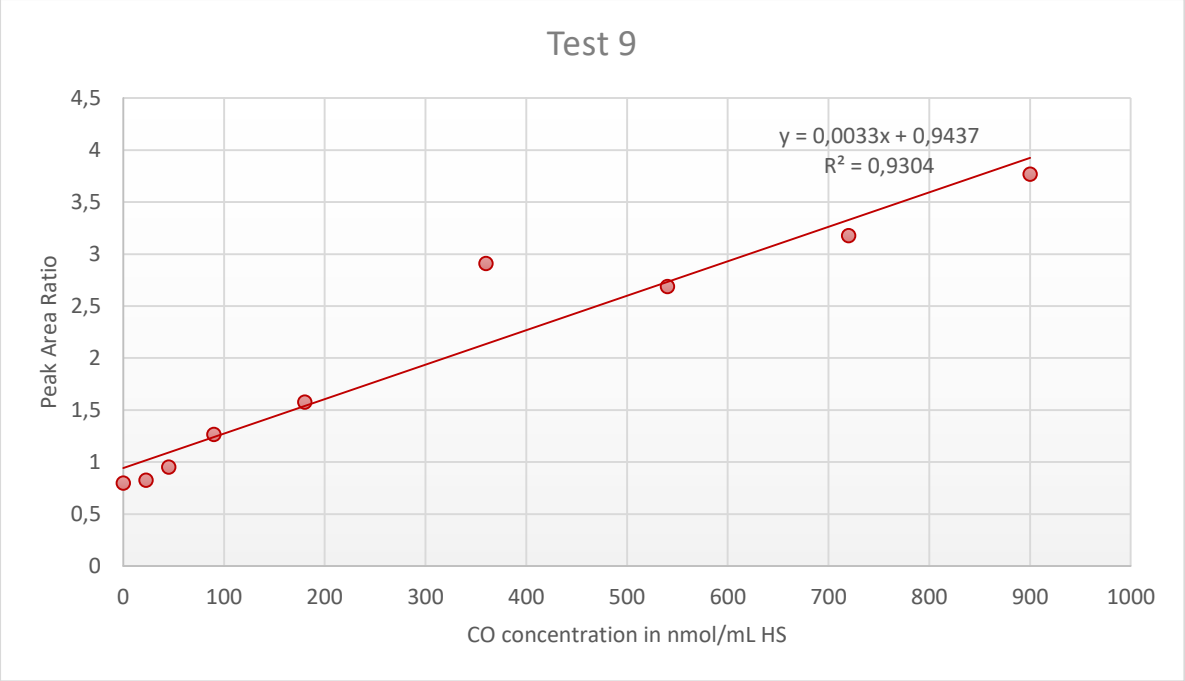

Figure A3.9: Peak Area Ratio of carbon monoxide (CO) from the blood sample over ^{13}CO from the internal standard (IS) plotted against the CO concentration in the calibration standards, with addition of linear trendline, linear regression equation and regression coefficient (R^2) for Test 9.

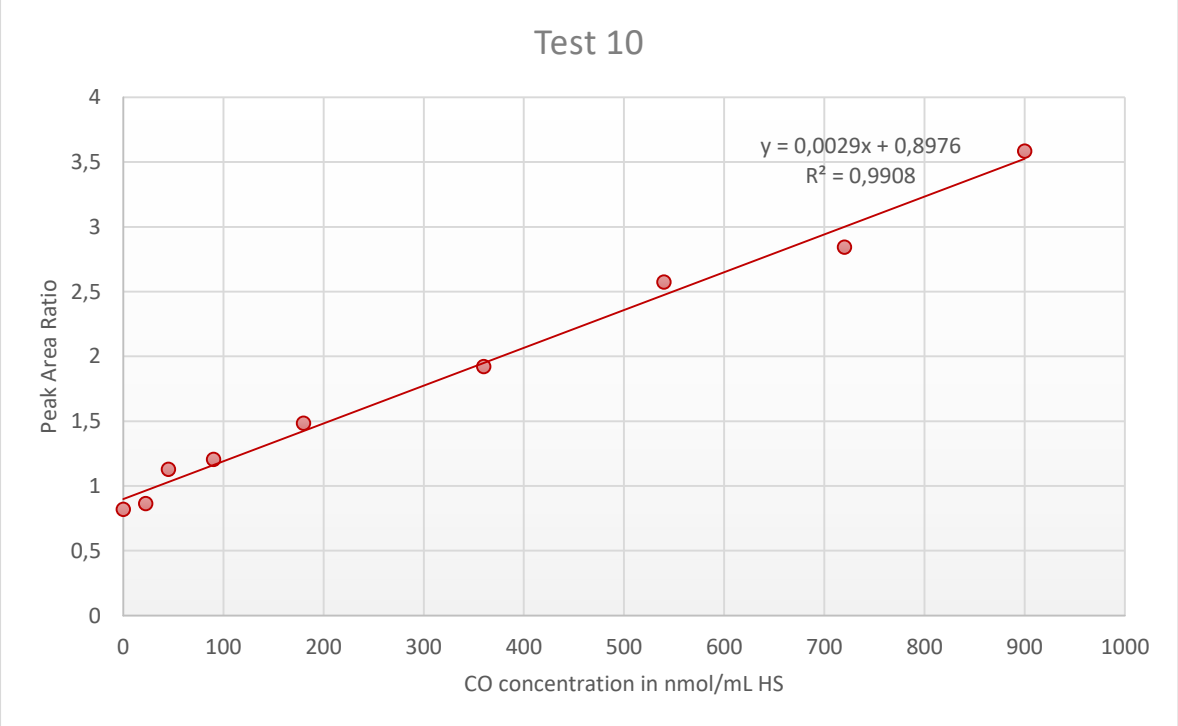

Figure A3.10: Peak Area Ratio of carbon monoxide (CO) from the blood sample over ^{13}CO from the internal standard (IS) plotted against the CO concentration in the calibration standards, with addition of linear trendline, linear regression equation and regression coefficient (R^2) for Test 10.

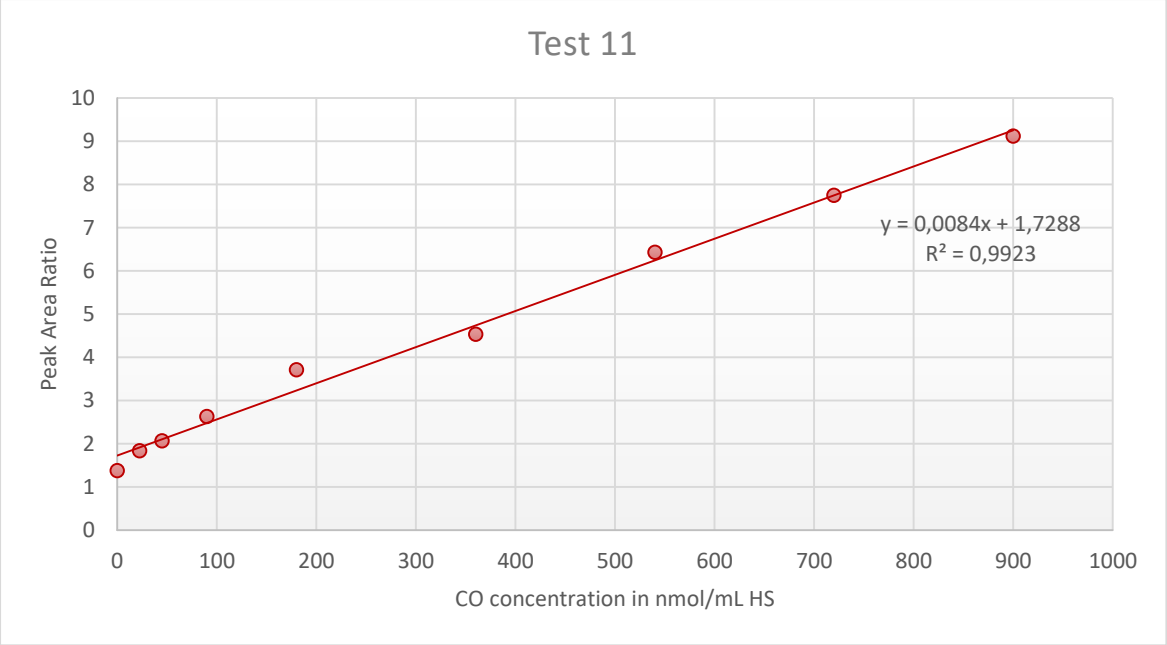

Figure A3.11: Peak Area Ratio of carbon monoxide (CO) from the blood sample over ^{13}CO from the internal standard (IS) plotted against the CO concentration in the calibration standards, with addition of linear trendline, linear regression equation and regression coefficient (R^2) for Test 11.

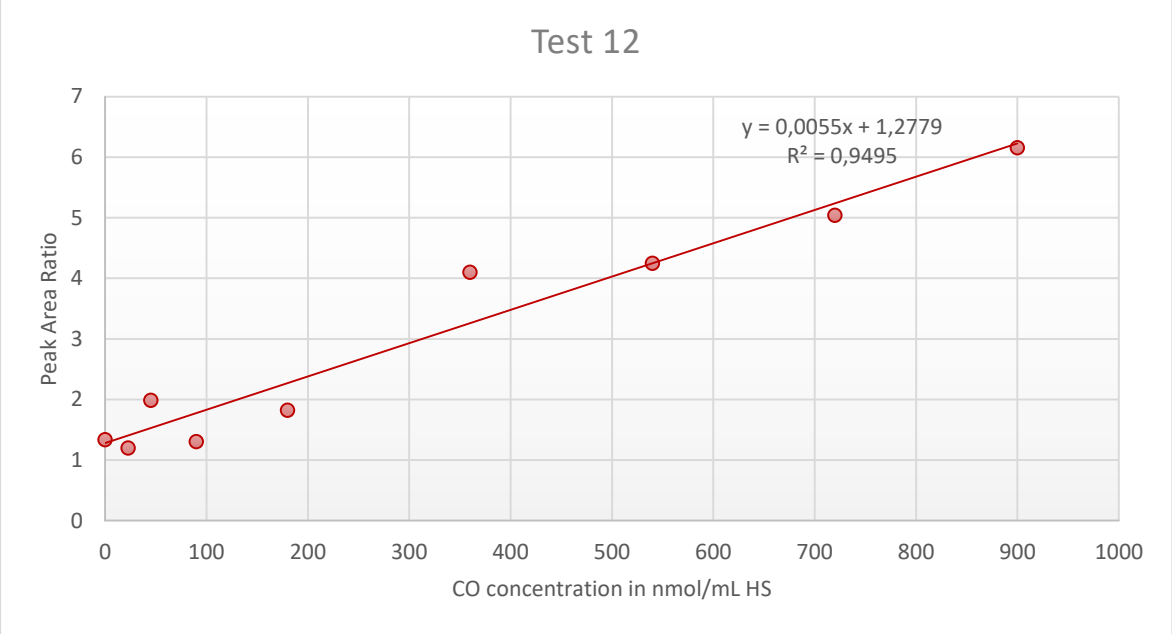

Figure A3.12: Peak Area Ratio of carbon monoxide (CO) from the blood sample over ^{13}CO from the internal standard (IS) plotted against the CO concentration in the calibration standards, with addition of linear trendline, linear regression equation and regression coefficient (R^2) for Test 12.

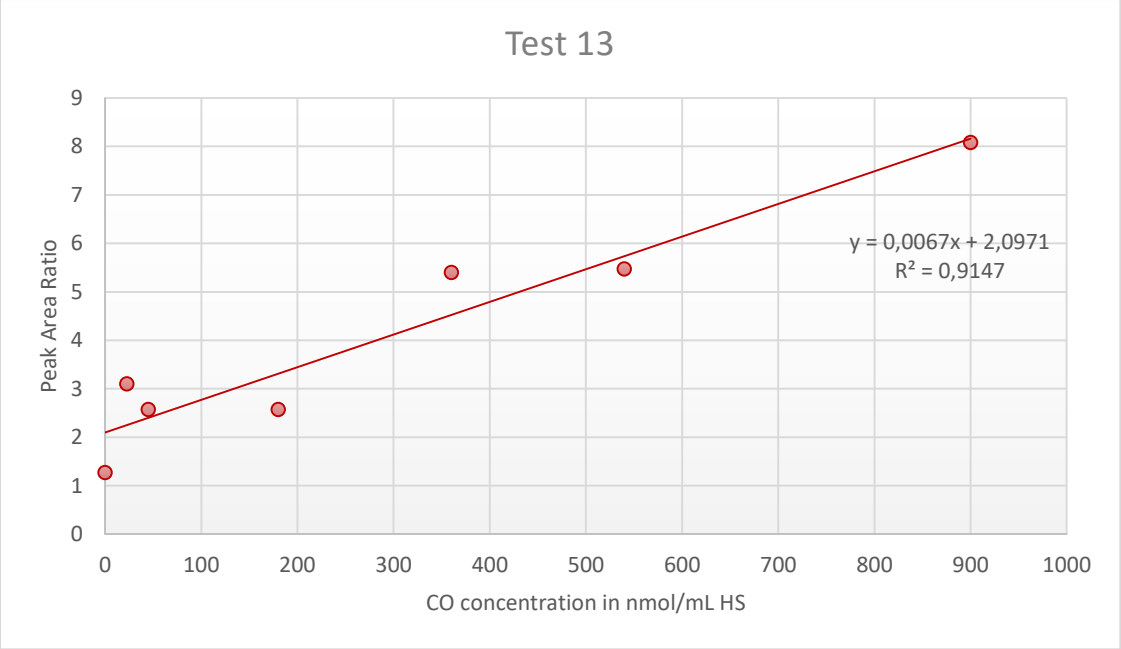

Figure A3.13: Peak Area Ratio of carbon monoxide (CO) from the blood sample over ^{13}CO from the internal standard (IS) plotted against the CO concentration in the calibration standards, with addition of linear trendline, linear regression equation and regression coefficient (R^2) for Test 13.

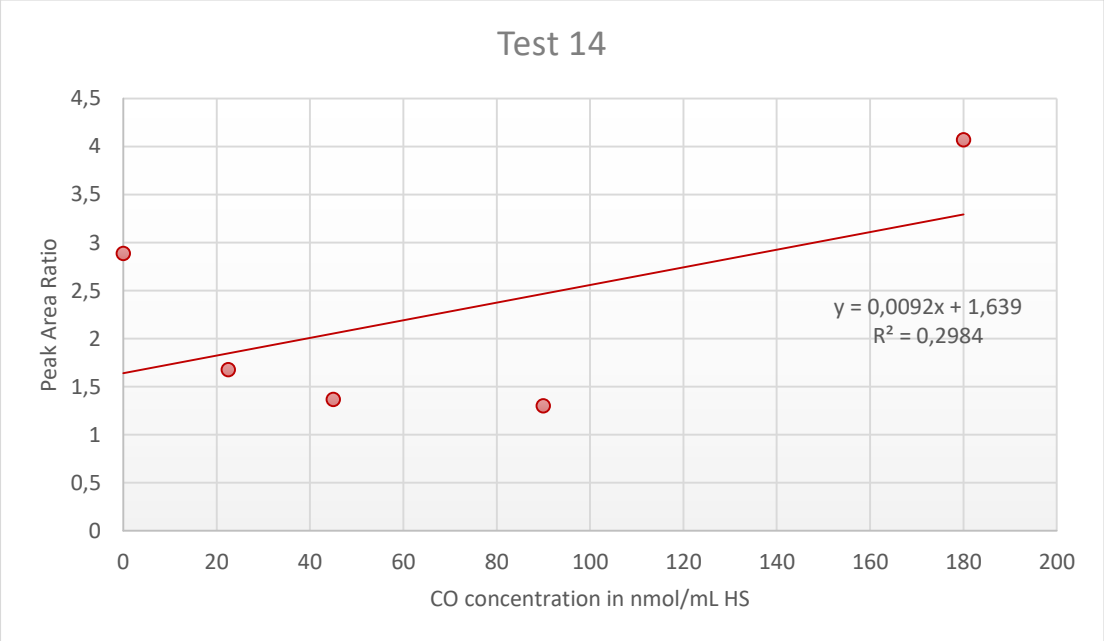

Figure A3.14: Peak Area Ratio of carbon monoxide (CO) from the blood sample over ^{13}CO from the internal standard (IS) plotted against the CO concentration in the calibration standards, with addition of linear trendline, linear regression equation and regression coefficient (R^2) for Test 14.

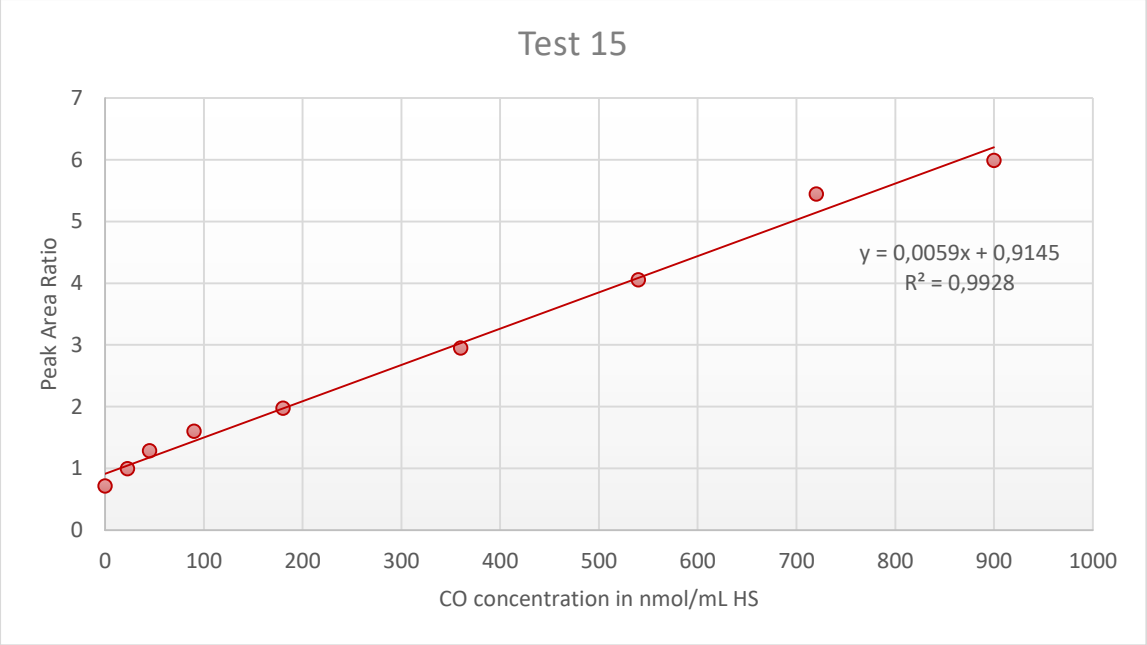

Figure A3.15: Peak Area Ratio of carbon monoxide (CO) from the blood sample over ^{13}CO from the internal standard (IS) plotted against the CO concentration in the calibration standards, with addition of linear trendline, linear regression equation and regression coefficient (R^2) for Test 15.

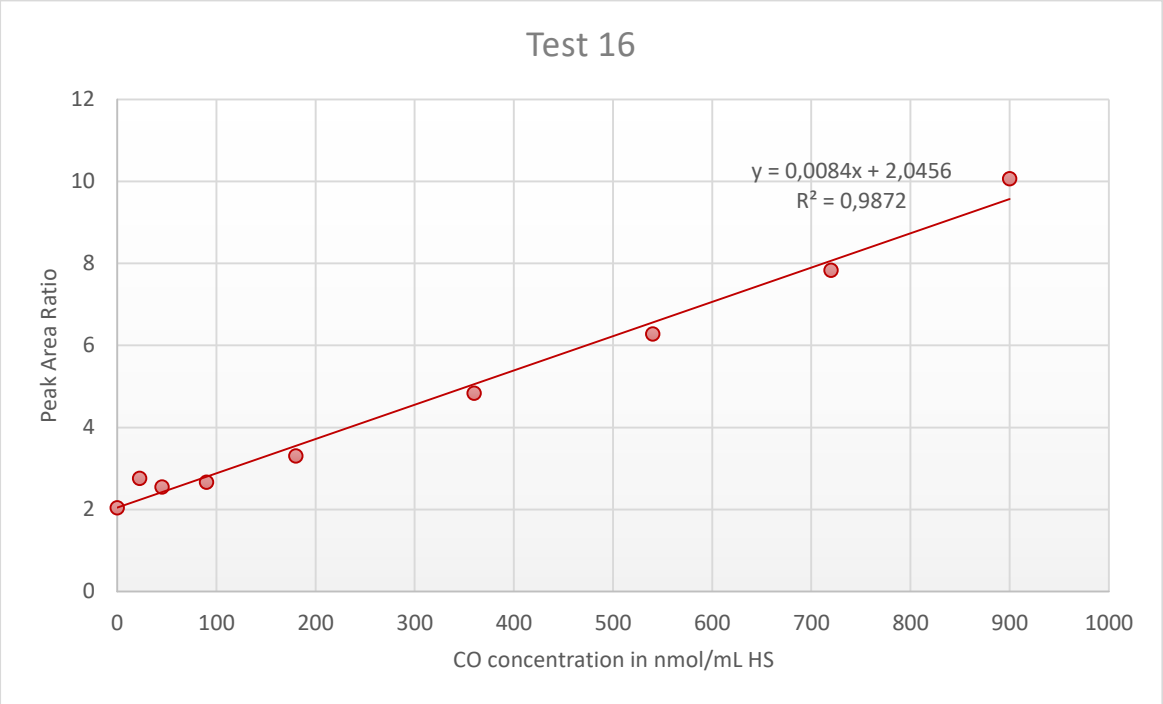

Figure A3.16: Peak Area Ratio of carbon monoxide (CO) from the blood sample over ^{13}CO from the internal standard (IS) plotted against the CO concentration in the calibration standards, with addition of linear trendline, linear regression equation and regression coefficient (R^2) for Test 16.

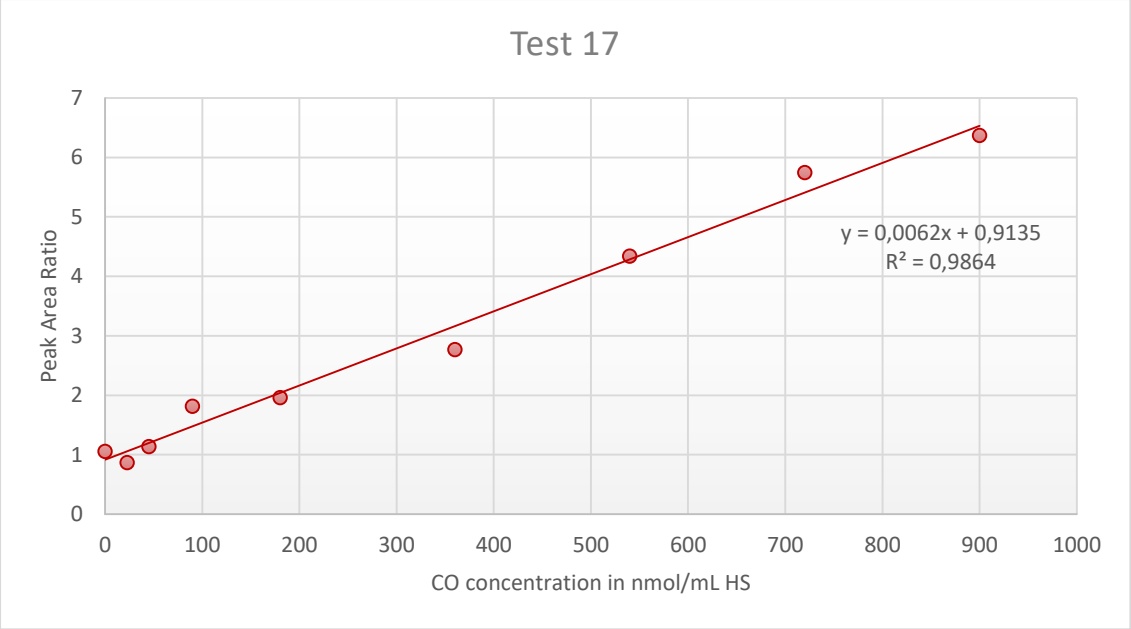

Figure A3.17: Peak Area Ratio of carbon monoxide (CO) from the blood sample over ^{13}CO from the internal standard (IS) plotted against the CO concentration in the calibration standards, with addition of linear trendline, linear regression equation and regression coefficient (R^2) for Test 17.

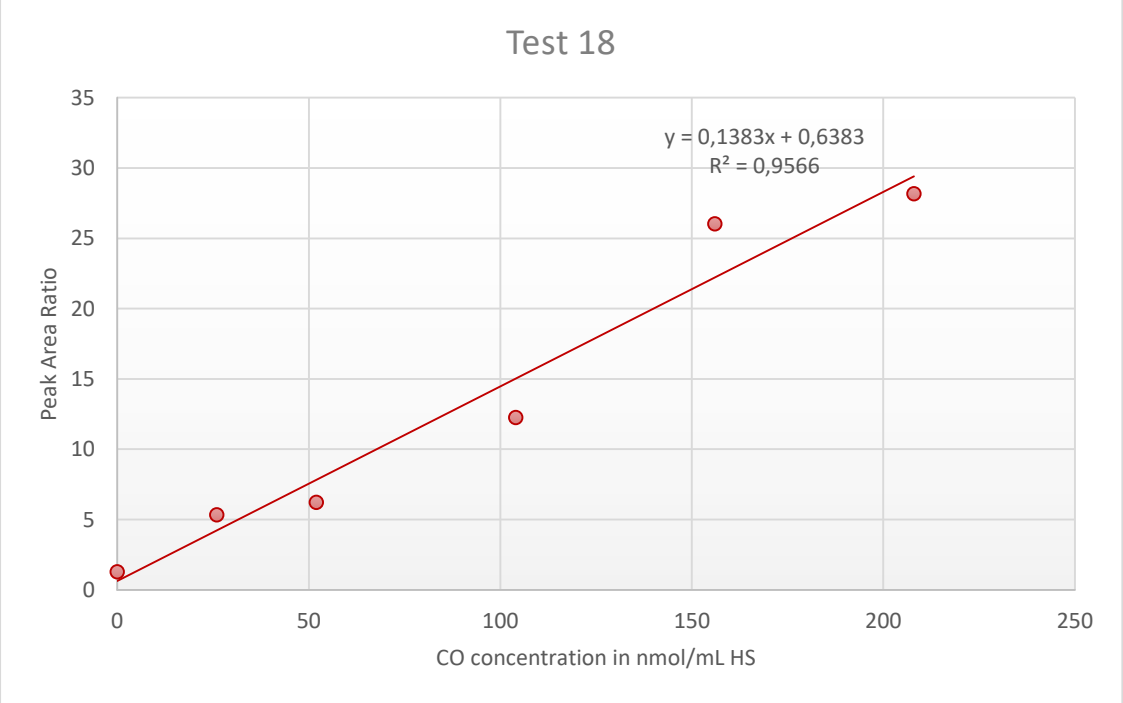

Figure A3.18: Peak Area Ratio of carbon monoxide (CO) from the blood sample over ^{13}CO from the internal standard (IS) plotted against the CO concentration in the calibration standards, with addition of linear trendline, linear regression equation and regression coefficient (R^2) for Test 18.

Chapter 4 - New strategy for carbon monoxide poisoning diagnosis: Carboxyhemoglobin (COHb) vs Total Blood Carbon Monoxide (TBCO)

4.1 Introduction

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].

A common practice is to store blood samples possibly frozen or at least refrigerated, with the 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 headspace (HS) or air volume above the sample 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 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 a 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, I 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, I 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.

4.2 Materials and Methods

4.2.1 *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 the following parameters at various levels (Detailed protocol is found in **Appendix 4.1**):

- 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 the 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. Analyses were carried out in triplicates.

4.2.2 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]. I 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.

4.2.3 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 modelling. Data was then checked for normal distribution and transformed accordingly (**Table 4.1**). 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 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 be, however, problematic, since the software returns estimated values that can exceed 100, which is the physiological limit for COHb saturation.

Table 4.1: List of transformations employed for data according to the 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

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 the 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

(COHb and TBCO) as well as multiple linear regression (MLR) and comparisons via Student t-test were used to assess the 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).

See Materials and Methods section 3.2 for description of all other relevant details.

4.3 Results

4.3.1 Correlation between COHb and TBCO

Figure 4.1 shows the plot of results obtained for COHb vs results of the same samples for TBCO. 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, I have used it to back calculate 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.

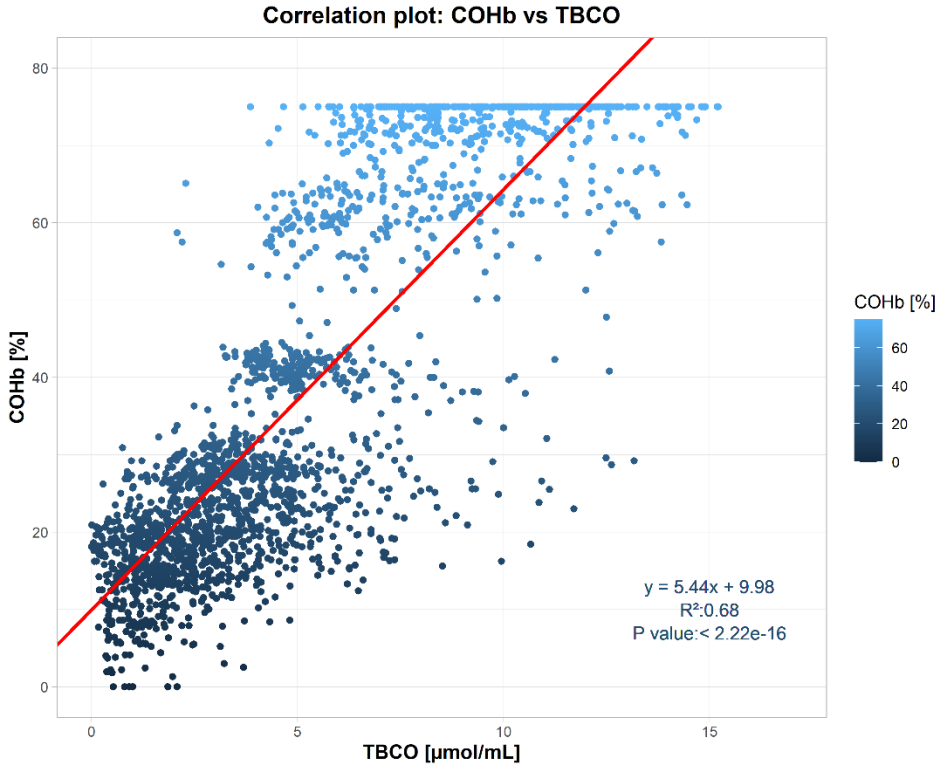

Figure 4.1: Correlation plot for COHb [%] vs TBCO [µmol/mL] from the storage study results, with correlation formula, correlation factor (R^2) and p-value.

4.3.2 Influence of storage parameters

4.3.2.1 Relevance of CO in HS

To determine whether there was a significant amount of CO released into the HS of the sampling tube, 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 4.2**).

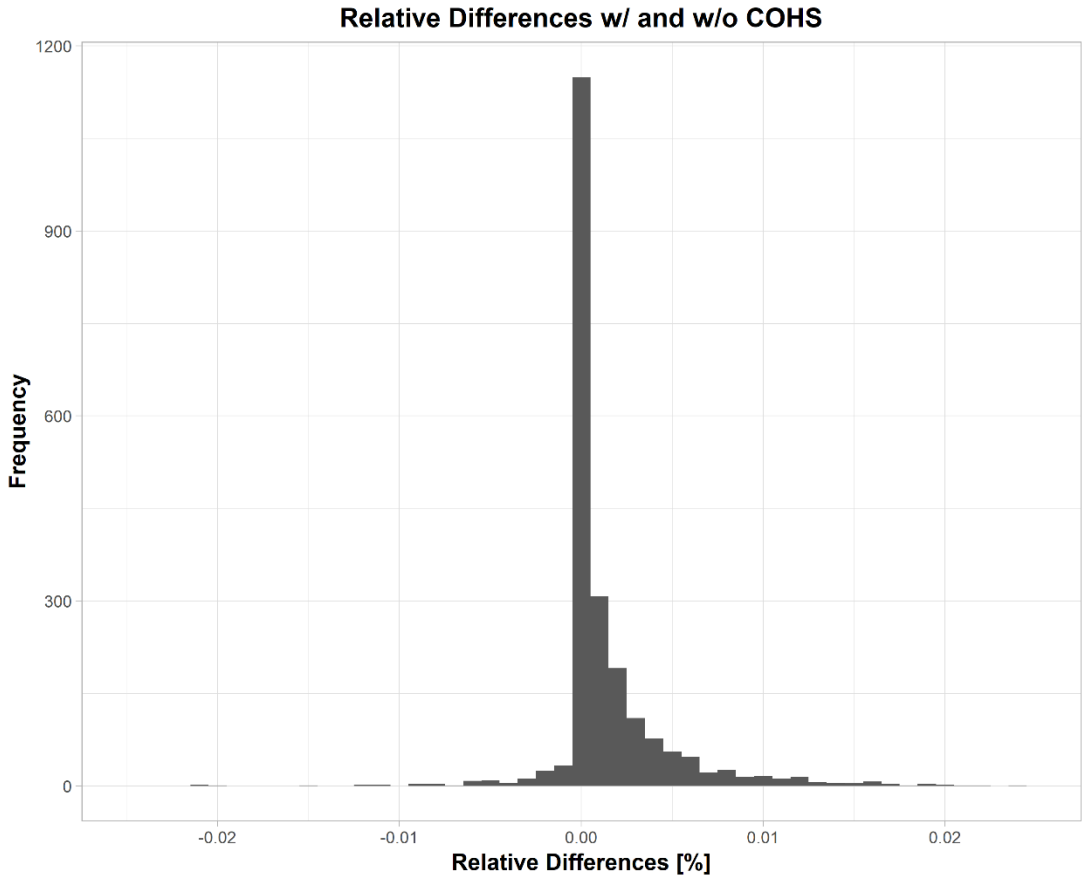

Figure 4.2: Histogram of relative differences in % of CO concentrations with and without taking into account the CO in the headspace (HS).

4.3.2.2 Reopening and freeze- and thaw-cycles

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.

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, I 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.

4.3.2.3 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. I was 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 4.2**.

Table 4.2: Results of ANOVA for single parameters and combination of parameters for high, medium and low saturation level; COHb: carboxyhaemoglobin, 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	#	–	**	–	–	–

4.3.3 Correction model

To be able to generate a correction model applicable to COHb or TBCO measurements based on this dataset, first, the behaviour of the response variables over time for each saturation level needed to be identified. Therefore, time plots for COHb and TBCO were produced (**Figures 4.3a-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 4.3a and 4.3b**), a weakly decreasing trend can be observed, whereas for low COHb saturation (**Figure 4.3c**) there is a slight decrease in the initial phase, followed by a plateauing towards the second half of the month. For high TBCO levels (**Figure 4.3d**), 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 4.3e**) 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 4.3f**) 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 behaviour, 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 the 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 4.3**. 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).

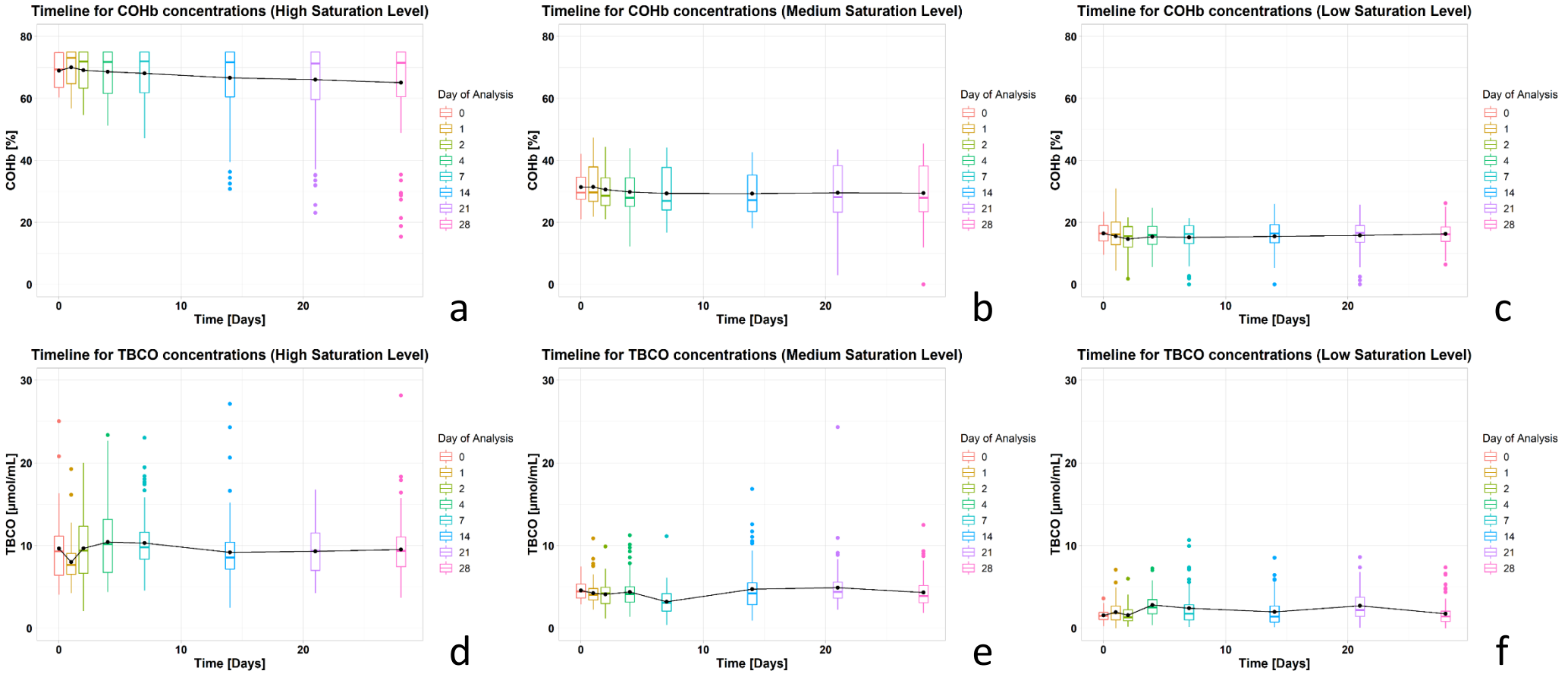

Figure 4.3a-f: Boxplots with error bars for COHb concentrations in % (a-c) and TBCO concentrations in µmol/mL (normalized) (d-f) over time for high, medium and low saturation levels, black dot: mean COHb/TBCO concentration for the day of analysis, line in box: median.

Table 4.3: 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 4.1**).

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)

4.3.3.1 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.

4.3.4 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 modelling set and a testing set. The testing set was obtained by extracting the data of one repetition for each analysis. The modelling 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 4.4**. This model was then used to predict the COHb concentrations based on the TBCO values and storage parameters form the training set.

Table 4.4: 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. To evaluate the 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 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**.

	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

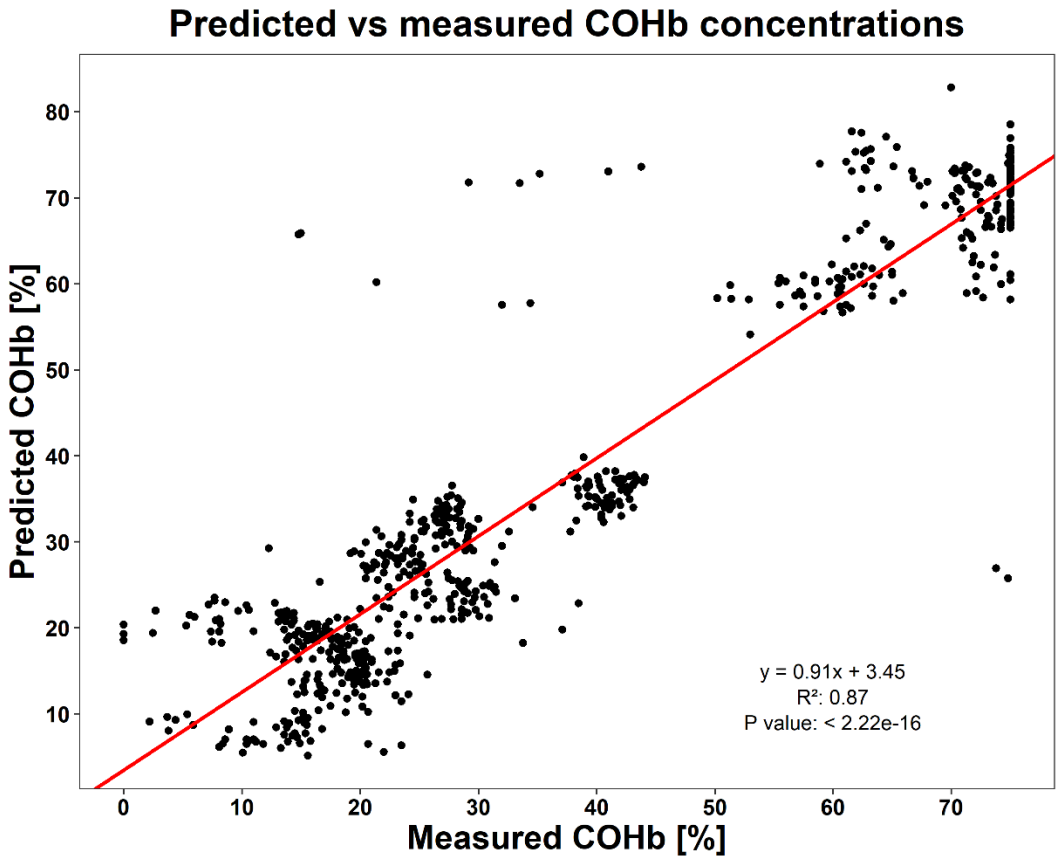

Figure 4.4: Correlation plot for measured COHb vs predicted COHb concentrations in %. Complete dataset was subdivided in training (2/3) and test (1/3) set, prediction model was generated based on training set and applied to test set and plotted to determine applicability and accuracy of prediction model.

4.4 Discussion

4.4.1 Correlation between COHb and TBCO

Before assessing each storage parameter and their potential impact on the measurement results, I first needed to determine the direction and magnitude of the 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 reduction 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 4.1**) 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 [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, I 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].

4.4.2 Influence of storage parameters

4.4.2.1 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 4.2**, 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, I conclude that there is not a significant amount of CO that is released into the HS of the blood tube during storage.

4.4.2.2 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 in 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 behaviour is confirmed in this study.

4.4.2.3 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 4.2, 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 behaviour 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 4.3**), 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 in 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 a 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 is 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 behaviour

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 a 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 a preservative should not be used for forensic drug analyses [55–57]. Therefore, I 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 4.3**). However, this behaviour is not shown with TBCO, for which HS volume, time and storage in the fridge or freezer do not play an influential role. Only the choice of the preservative and storage at RT has a significant impact on TBCO measurements.

This supports the hypothesis that TBCO appears as 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 blood constituents 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 long PMI, the blood quality is often altered, potentially leading Hb levels higher or lower to the instrument's limits [29]. The majority of these factors are, however, not relevant for TBCO measurements. Optical blood changes, blood component 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 gaseous molecules, thus reducing the potential interference of compounds present in the sample. However, TBCO measurements are impacted by PM generation of CO, similarly to COHb measurements, 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.

4.4.3 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 the 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, I 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:

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

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, the 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.

4.4.4 Prediction model

Going a step further to obtain the most accurate and reliable CO poisoning determinations possible, I 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 4.4**) into equation (2), COHb concentrations can be predicted. The 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.

4.5 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, I 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, I 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 a cause of death), this is not a significant limitation.

4.6 Conclusion

In this study, I 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 a reanalysis of supportive evidence. Therefore, I recommend the use of TBCO as an 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, I generally recommend collection of samples for CO analysis in EDTA tubes for short storage periods (up to one month), 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 the 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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Appendix 4

A4.1 Protocol for storage study

Day 0

1) Prepare polyethylene (PE) containers for blood retrieval by adding the required amount of preservative – 1.6g/L of Ethylenediaminetetraacetic acid (EDTA), 1g/L of sodium fluoride (NaF) (+ 1.2 g/L of EDTA), 4 vials of lithium heparin (20,000 IU/L), 29.98g/L of trisodium citrate (NaCit).
2) Retrieve 1 L of bovine blood per preservative for fortification and ~250mL of blood per preservative to use for calibration curve into the PE containers.
3) Put containers on a shaker for homogenization for 30 minutes.
4) Analyse blood from each container via CO-oximetry to determine baseline COHb% content.
5) Fortify blood with pure CO gas (various amounts, based on desired initial COHb% saturation, which is determined with analysis of a sample with CO-Oximeter at regular intervals of 10 minutes),
6) Waiting for homogenization of blood (sample is put on a shaker for 30 minutes).
7) Filling of the previously labelled and stacked blood tubes (monovettes).
8) Analyse samples of Day 0 via CO-oximetry and GC-MS.
9) Storage of the remaining samples according to their intended storage condition.

Day X

1) Retrieve samples from storage location – for frozen samples, make sure that samples are thawed at least 30 minutes prior to analysis to allow complete thawing.
2) Prepare samples and analyse them according to protocol.
3) After analysis, store samples that need to continue storage into its allocated storage place, discard the other blood tubes.

Chapter 5 - Improvement of measurement error in carbon monoxide exposure assessments: review and calculations

5.1 Introduction

Recently there have been significant developments in methods to measure CO and determine individual CO exposure estimates [29–32], which can consequently have repercussions on the health risk estimates and on the strength and direction of association between CO exposure and certain health outcomes for different exposure groups. This association is described numerically by the relative risk (RR) (often also reported as odds ratio or risk ratio) and can be significantly affected by errors in the exposure estimation especially in low-level CO exposures, where changes in lower concentration ranges can make a difference in the classification of the case into either exposed or non-exposed group [33].

An aspect that all exposure assessment studies in environmental epidemiology have in common is the unavoidable disposition for uncertainty. According to Blair *et al.* [34], the two main methodological issues in epidemiological studies are confounding and exposure misclassification, of which the second is of greater concern than the first. In the case of a continuous or measured variable, exposure misclassification is called *measurement error* and can cause substantial bias in exposure estimates [35,36]. Measurement error in the assessment of individual exposure levels often leads to bias towards the null in estimates of health effects and to an underestimation of the variability of the estimates, which can have severe consequences [37,38]. It is, therefore, crucial to determine exposure measurement error and correct for it. Generally, methods to correct for measurement error are available, but a specific model for the assessed exposure is required and often different results are obtained based on a different type of error [35]. In case of CO exposure, it is of particular interest to assess the effects new methodological developments have on the individual measurement of CO, quantify the resulting differences and errors and use these to evaluate the magnitude of their effect on exposure estimates and health risks in a population.

Therefore, the aim of this study is to critically explore current trends in CO exposure assessments and identify potential methodological gaps, with special focus on low-level indoor CO exposures, as well as determine their sources of error and quantify measurement errors arising from recent methodological and toxicological advances. For this purpose, a conceptual map based on the human exposure assessment graph proposed by Mark Nieuwenhuijsen in his book "Exposure assessment in Environmental Epidemiology" (Figure 1.6) [24], but adapted to indoor CO exposure and extended according to information found in the investigated literature will be created. The final goal is to create an updated and clear overview of the type and magnitude of errors in CO exposure measurements and assessments, which is then employed to correct exposure estimates and relative risks. This will help get closer to the true global burden of CO exposure and ideally will be used by public health institutes, occupational and environmental authorities when evaluating health risks from indoor CO levels and creating new regulations and guidelines.

5.2 Methods

5.2.1 Literature Search

To be able to determine and characterize measurement errors in CO exposure assessments, a thorough literature search to identify studies that performed CO exposure assessments through measurement of CO exposures was necessary. A literature search was conducted from November 2018 to May 2019 in databases PubMed, Web of Science and Scopus (**Table 5.1**). A first search with the keywords ("carbon monoxide" AND (exposure OR measurement OR assessment)) gave 9088, 15695 and 26577 hits. I narrowed down the search by changing the search string to ("carbon monoxide" AND exposure AND (assessment OR measurement)) in order to include all studies investigating carbon monoxide and exposures related to either assessments or measurements. This resulted, however, in a higher number of hits for all databases. Therefore, I further modified the search term by using ("carbon monoxide" AND ("exposure assessment" OR "exposure measurement")). This gave us 78 hits in PubMed, 148 in Web of Science and 234 in Scopus. A title and abstract search of these studies was then carried out to include all relevant studies. Since the scope of this review is to focus on indoor CO levels, all studies investigating outdoor air pollution and outdoor CO levels were excluded. Conference papers were also excluded due to insufficient methodological details. Studies focusing on the biological and physiological mechanisms of CO exposures or not with CO as

the primary scope and using animal- or cell-based approaches were also excluded since the main scope of this study is to evaluate human CO exposure (**Table 5.2**). After removal of duplicates, a total of 92 papers were selected.

Table 5.1: Search strings used in the literature search for CO exposure assessment studies.

Keywords	Hits on Pubmed	Hits on Web of Science	Hits on Scopus	# of relevant articles
"carbon monoxide" AND (exposure OR measurement OR assessment)	9097	15727	26578	Nd
"carbon monoxide" AND exposure AND (assessment OR measurement)	675	1423	2572	Nd
"carbon monoxide" AND ("exposure assessment" OR "exposure measurement")	78	148	234	Nd
"carbon monoxide" AND ("exposure assessment" OR "exposure measurement") WITH EXCLUSION CRITERIA	**44**	**59**	**42**	**92**

Table 5.2: Inclusion and exclusion criteria for literature search.

Inclusion criteria	Exclusion criteria
Indoor CO exposure assessment studies	Outdoor air pollution papers
Human CO exposure measurement studies	Outdoor CO exposure assessment papers
Biological monitoring, personal monitoring and microenvironment monitoring and modelling studies	Conference papers
	Biological/physiological mechanism-focused papers
	Studies with animal-/cell-based approaches
	Number of study subjects <50

These were categorized according to the type of study: observational (**Table A5.1a**), experimental (**Table A5.1b**) and review articles (**Table A5.1c**). To be able to evaluate low-level CO exposures over time, longitudinal studies provide the required detailed methodological information and time data, as opposed to cross-sectional studies, which generally focus on time point measurements [39]. Therefore, the observational studies were

further divided into longitudinal and cross-sectional studies and only the 48 longitudinal studies were further evaluated. Another important parameter that defines a high-quality epidemiological study is study power and the related sample size. Identifying the sample size needed to be able to determine the smallest clinically important effects and also reduce costs and resources is an essential step of every epidemiological study. Sample size mainly depends on the accepted significance level, the statistical power, the expected effect size and variability of the sample [23]. Significance level (α) and statistical power (1-β) are conventionally at 0.05 and 0.80, respectively [40]. Effect size and variability are usually determined specifically for each type of exposure based on previously reported studies, but generally, in order for 2-group comparisons to obtain statistically valid results for exposure estimates, a sample size greater than 50 was proven necessary. Consequently, studies with >50 study subjects were retained, while studies that had between 40 and 50 subjects were individually evaluated. Furthermore, for an exposure measure to be considered valid and accurate, especially when involving the use of a biomarker, measurements should be obtained with a minimum of two different measurement techniques [41,42]. The remaining 22 studies were thus scanned for the number of measures used for the exposure assessment, which left 7 studies using 2 or more exposure measures (**Figure 5.1**).

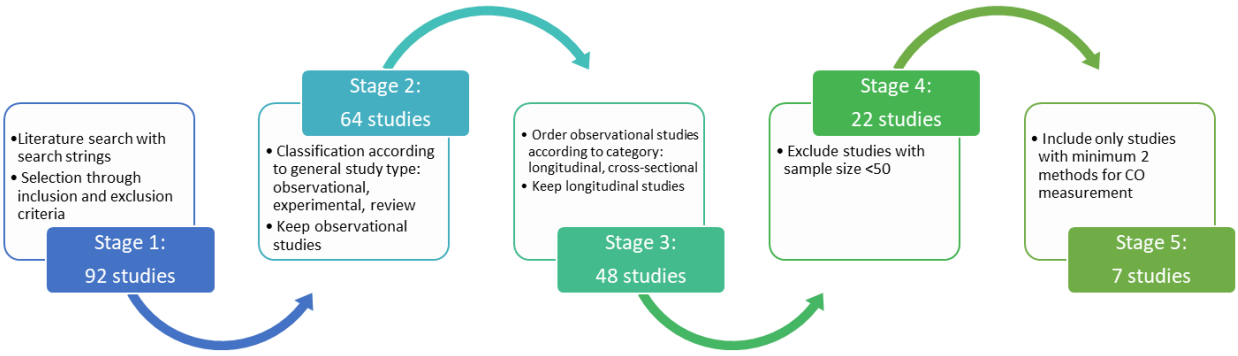

Figure 5.1: Schematic overview of the study selection process.

5.2.2 Classification of CO exposure assessment and exposure measurement methods

Classification of the methods used for CO exposure assessment in these papers revealed that the most employed methods are the direct ones, with 35.9% of the total studies, including only biological monitoring (19.6%), only personal monitoring (13%), and a combination of both

(3.3%). Indirect methods were only used in 19.7% of the studies. However, the main method for CO exposure assessment was a combination of direct and indirect methods, which made out 44.4% of the total studies under investigation (**Figure A5.1**).

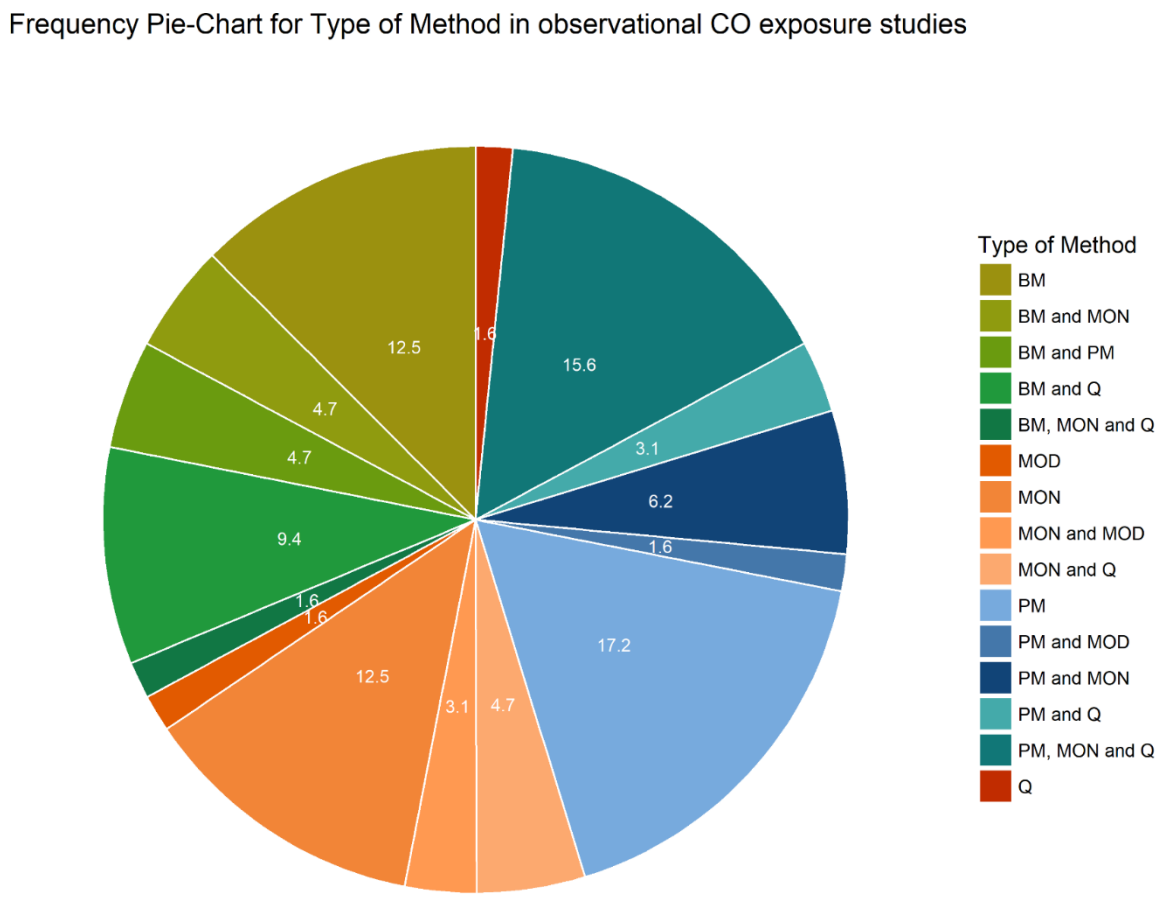

Figure 5.2: Summary of frequencies for exposure assessment methods in observational CO exposure studies based on literature review; BM: biological monitoring, MON: environmental monitoring, PM: personal monitoring, Q: questionnaire, MOD: environmental modelling.

Figure 5.2 shows the frequencies for each combination of exposure assessment method for the selected observational studies, which reflects the results when including all types of studies. Detailed information about the type of exposure assessment method, the measure of exposure, the measurement devices, the number of study subjects, measurements over time and replicates and the length of the observation period were extracted for the 7 selected studies and are summarized in **Table 5.3.**

Table 5.3: Detailed study information on selected CO exposure assessment studies; BM: biomonitoring; PM: personal monitoring; MON: environmental monitoring; MOD: environmental modelling.

Article	Type of study	Exposure assessment method	Measure of exposure	Measurement device	Number of subjects	Number of measurements in time (pi)	Number of repetitions per measurement	Number of total measurements	Time interval of measurements	Validation (Y/N)
[43]	cohort	BM, PM and Q	ExCO and pCO	MicroCO monitor and HOBO CO data logger	288 subjects in 72 homes	ExCO: 2 per individual (before and after temazcal use); pCO: 1 per individual during use	ExCO: duplicates; pCO: continuous at 10s intervals	288	4 weeks	Y*
[44]	cohort	BM, MON and Q	COHb, exCO, air CO	CO-oximetry, breath CO analyser and ambient monitors	36 adults, 30 children	2 measurement rounds (before and after temezcal use, not applicable to all participants)	airCO: continuous during temezcal use at 2s logging interval; SpCO: 2-4 min intervals (central 40s averaged); exCO: triplicates with 1-2 min breaks in between trials	34 before and 51 after exposure measurements	3-4 hour prior to temazcal use and within 1 h after	N
[45]	cohort	PM, MON and Q	pCO and airCO	Portable real-time EL-USB-CO Logger and CO monitors	104 households (48 traditional stove: 56 RMS)	2 simultaneous measurements per stove type	Continuous	104	48h	N
[46]	cohort	PM, MON and Q	pCO and airCO	HOBO CO logger	60 homes	pCO: 2 per household (before and 1 month after stove installation); airCO: 8 per household (4 locations in house (close to stove, center of kitchen, just outside of kitchen and bedroom) and before and after stove installation)	pCO: semicontinuous (1min averages); airCO: continuous real-time; repeated on 5 days for 25% of homes	pCO: 24; airCO: 32	pCO: 24h; airCO:48h	N
[47]	cohort	PM, MON and Q	pCO and airCO	Langan T15 high-resolution personal monitor and fixed-site monitors	401	1 per participants	Continuous	359	pCO:48h; airCO from fixed-site stations for same period	N
[48]	cohort	PM and MON	pCO and airCO	Passive electrochemical CO monitor and non-dispersive infrared monitors (CO 10-M and 11-M)	194	1-4 times per child	Continuous	449	20-24h	N
[49]	cross-sectional case control	PM and MON	pCO and airCO	Draeger diffusion tubes	182 intervention, 197 control households; 161 intervention, 154 control; CO personal tubes: 43 intervention and 50 controls)	1 per individual and 3 per household	2-3 for 13 intervention and 12 control households	Not specified	40-57 hours(~48) (June-August 2009)	N

To give an overview of the type and frequency of exposure measurement methods selected in CO exposure assessment studies, cohort and cross-sectional studies with enough study power and one or more methods for exposure measurement (n=26) were assigned to each assessment method and device used for CO exposure measurement (**Table 5.4**). Details of exposure assessment and measurement methods are described in the following paragraphs and displayed in **Figure A5.2**.

Table 5.4: Overview of exposure assessment methods and associated exposure measurement devices identified in the literature review with respective references for observational and cross-sectional studies**.** *: cross-sectional studies, **: includes pCO monitors and handheld/portable CO monitors.

Type of exposure assessment method	CO exposure assessment method	Exposure measurement device	References
Direct	*Personal monitoring*	Personal CO monitor**	[48,50–53]
		CO passive diffusion tubes	[49,54–56]
		Real-time gas monitors	[57]* [45,46]
	Biological monitoring	Pulse CO-oximeter	[44,58–60]
		Blood Gas Analyser	[58], [57,61,62]*
		Breath CO monitor	[43,44,63–66], [67]*
		Gas chromatography	*Only studies with <50 study subjects use GC methods*
Indirect	*Microenvironmental monitoring and modelling*	CO data logger	[17,43–46]
		CO passive diffusion tubes	[49]
		Modelling	[68]

5.2.2.1 Personal monitoring

The measure of exposure in personal monitoring is CO in parts per million (ppm) or milligrams per cubic meter (mg/m^3) and was mainly determined by four types of devices:

1) Personal CO monitors, which were fixed on the individual for the whole observation period and readings were taken on the selected measurement times [69];

2) Handheld/portable CO monitors, which are similar to personal CO monitors but carried by the individual in the observation location and for the whole duration of the measurement [70];
3) CO passive diffusion tubes, which were attached to the individual or the immediate environment of the individual for the observation period; readings were taken by measuring the length of the tube where a colour change can be observed [49];
4) Real-time gas monitors, which measure the concentration in continuous mode, data was directly saved or sent to a computer for analysis [71].

Measurements were generally performed over a set monitoring interval, which ranged from 24h to 72h, with either continuous measurements or repeated measurements in intervals ranging from 30 minutes to full working shifts of 8h. The complete monitoring period was found to range between a few days or weeks to months or several seasons during a year (See **Table 5.3**).

5.2.2.2 Biological monitoring

For biological monitoring, measurement methods for COHb include:

1) Pulse CO-oximetry, a non-invasive method to measure the peripheral blood CO concentration [75];
2) Blood Gas Analyser (BGA) or CO-oximetry (which is part of a BGA), a multiwavelength photometer that measures the concentrations of blood gases based on their spectral absorbance when exposed to light, including CO, which is reported in COHb% [76];
3) Breath CO monitors, a CO detector with an electrochemical sensor where the CO levels in the end-tidal volume of expired air are measured; COHb levels are then obtained through calculation with a reported empirical relationship formula [73,74].
4) Gas chromatography (GC) in combination with different detectors (Flame-Ionization Detector, Mass Spectrometer, etc.), which measures the CO released from a blood sample after having undergone sample treatment; CO concentrations are then correlated to COHb levels through formulae published in the literature [77];

While personal monitors were used for prolonged monitoring intervals, biomonitoring usually occurred on specific time points, but included several replicates (duplicates or triplicates) on each analysis day as well as repeated measurements on several times of the day or different

days after exposure. Methods 1-3 were all used in observational studies, while GC methods were only used in studies with a limited sample size (<50) and usually of experimental nature.

5.2.2.3 Environmental monitoring and modelling

CO in environmental settings is measured as ambient air CO in either ppm or $\mu g/m^3$, similarly to personal monitoring. The difference hereby is that while personal monitors measure the individual CO exposure, with environmental monitoring/modelling an average CO concentration is determined for a defined time period (e.g. daily, monthly, annually) in a specific (micro)environment (urban/rural area of delimited surface) for a specific population group and assigned equally to all individuals in the exposure group [78]. Outdoor CO monitoring mainly occurs through fixed/site monitors (passive sampling), which are positioned throughout a country with usually a population-based distribution (higher density in urban areas than rural areas) [6,79]. Averages are reported for daily, weekly, monthly or annual intervals and used to estimate the population exposure to CO [80,81]. For indoor CO monitoring, ambient CO monitors and CO alarms but also active sampling through CO gas analysers are used to obtain CO concentrations in specific microenvironments, such as households [17,45,46,49,81–83], motor vehicles [51,84,85] or occupational settings [12,51,56,57,86–88].

Exposure modelling is often used in combination with personal or environmental monitoring (hybrid models) to validate the developed models and consequently allow these models to reflect the true human exposure to air pollutants over time in order to assess the health outcomes of air pollution exposure in the population [78]. Main categories of exposure models include proximity models, dispersion models, land use regression (LUR) models, human inhalation models (HIM) and hybrid models [89]. While these models are generally employed and more suitable for the assessment of outdoor CO levels, a few have been found to be employed also for indoor CO, such as the HIM (see **Appendix A5.2**).

HIM are a type of probabilistic model that estimate the individual exposure level by taking into account the time spent in a microenvironment as well as the human activity (e.g. inhalation rates, microenvironments, etc.), physiological parameters (e.g. sex, age, height, etc.) and chemical and environmental conditions [90,91]. By incorporating environmental and human activity and physiology data, human inhalation models can provide more accurate exposure estimates than other types of models and have the potential to better support associations

between air pollution and adverse health outcomes. Examples of such models for CO exposure include the probabilistic National Ambient Air Quality Standard (NAAQS) Exposure Model (pNEM) for CO (pNEM/CO) developed by the EPA in 1992 and revised in 2000 [92], the Simulation of Human Activity and Pollutant Exposure (SHAPE) model [16] and the Air Pollutants Exposure Model (APEX) [93], the latter being derived from pNEM/CO. Predictions from the pNEM/CO model were evaluated against observed data for subjects from two study areas, Denver, Colorado, and Washington, D.C. CO measurements were performed with CO data loggers, personal diaries and data from fixed-site monitors. The model resulted in overestimating the 8h exposure of people with low-level exposures and underestimating the 8h exposure of people with high-level exposures. Furthermore, over 10% of the daily maximum exposures in Denver and 4% in Washington exceeded the NAAQS of 9ppm. Additionally, estimates of breath CO levels from fixed-site monitors were in excess of 10 ppm, which is equivalent to around 2% COHb [16,92,94]. Considering that the NAAQS for CO were designed to guarantee blood COHb levels of the population and especially high-risk groups below 2%, this study confirmed the doubts of the inability of fixed-site monitors alone to represent the total CO exposure.

5.2.3 Errors in CO exposure assessments

In epidemiologic studies, there are three main classes of errors that can cause bias in the exposure-outcome associations and need to be considered when assessing the validity of a study: random errors, systematic errors and confounding (**Figure A5.3**). Random errors represent the variability in estimates due to unknown or uncontrollable causes. These can normally be adjusted for through repeated readings, adjusted surroundings and adequate sample size. Systematic errors arise when some aspect in the study design, execution or interpretation phase has not been performed or selected correctly. Confounding occurs when an observed association between an exposure and a health outcome is due to a third factor that was not considered but is associated with the exposure and independently affects the risk for the health outcome [95]. According to Sackett [96], there are 35 biases that can arise in an epidemiologic research study that involves the measurement of exposure, the two main ones being selection bias and information/misclassification bias.

Selection bias can alter the study results through an incorrect selection of the study participants, which can falsely direct the study course towards the desired outcome since the study sample selected might not represent the general population [95].

Information/misclassification bias includes for example recall bias, which occurs when individuals with a particular health outcome or characteristic remember and report their previous experiences incorrectly. Observer bias arises when the researcher performing the study has knowledge of the disease status and, as a consequence, asks more specific questions [96]. This can usually be avoided by performing a blinded study. Thus, the most significant errors tend to occur due to misclassification/measurement error [35].

5.3 Results

5.3.1 Measurement error in CO exposure assessment

Even though CO exposure studies all are subject to random errors, systematic errors such as selection bias and confounding, given that the majority of CO exposure assessment studies involve personal or biomonitoring, the focus is on the evaluation of errors in measurement of CO estimates in epidemiologic studies. Based on the investigated literature, an overview of the identified sources of measurement error in CO exposure assessments is presented in **Figure 5.3** and described in more detail in the following section.

5.3.1.1 Biological monitoring

For CO exposures, the main biomarker of exposure is COHb concentrations in blood, even though alternative biomarkers have been investigated, such as total blood CO (TBCO) [97], lactate [98], bilirubin [99], S100β [100] and troponin [101]. To obtain an accurate estimate, a reliable and valid biomarker is necessary [41]. Therefore, an important part of exposure assessment is the choice of the biomarker (**Figure 5.3**). The aim is to achieve the most accurate relationship between *in vivo* concentrations of the selected biomarker, the health outcome and the true exposure levels, which is evidently affected by the biomarker selection.

CO in breath is being used to estimate CO exposure levels, which are obtained from the correlation of CO measured in exhaled breath to COHb blood concentrations [102]. However, measurement of CO in breath cannot account for the total CO concentration present in an exposed individual at the time of exposure, since it is a method that is very susceptible to intra-subject and inter-subject variability [42]. Main sources of variability include the subject's breath-holding abilities, pulmonary capacity, ventilation rate and comorbidities as well as random instrumental errors [102–104]. These can lead to variability between subjects, but also

intra-subject variability that can occur due to measurements performed on different times of the day, with biological variation in the body due to sleeping, eating, exercise and general lifestyle changes within the measurement period. Repeated measurements are usually performed to account for the intra-subject variability [42].

Apart from TBCO, all other alternative biomarkers are not specific for CO, but rather indirectly related to the toxicodynamic process of CO in a human body, hence have been found unsuitable for CO exposure determination due to potential confounding from other types of exposure or health status [21,99,105]. Estimates can be significantly over- or underestimated and misclassification can occur, with potentially fatal consequences.

For specific biomarkers such as COHb and TBCO, errors can arise from various sources within the analytical process, including the origin of the sample, storage conditions, sample extraction procedures, instrumental analysis, data analysis and interpretation of the results [35,41,42,106] (**Figure 5.3**).

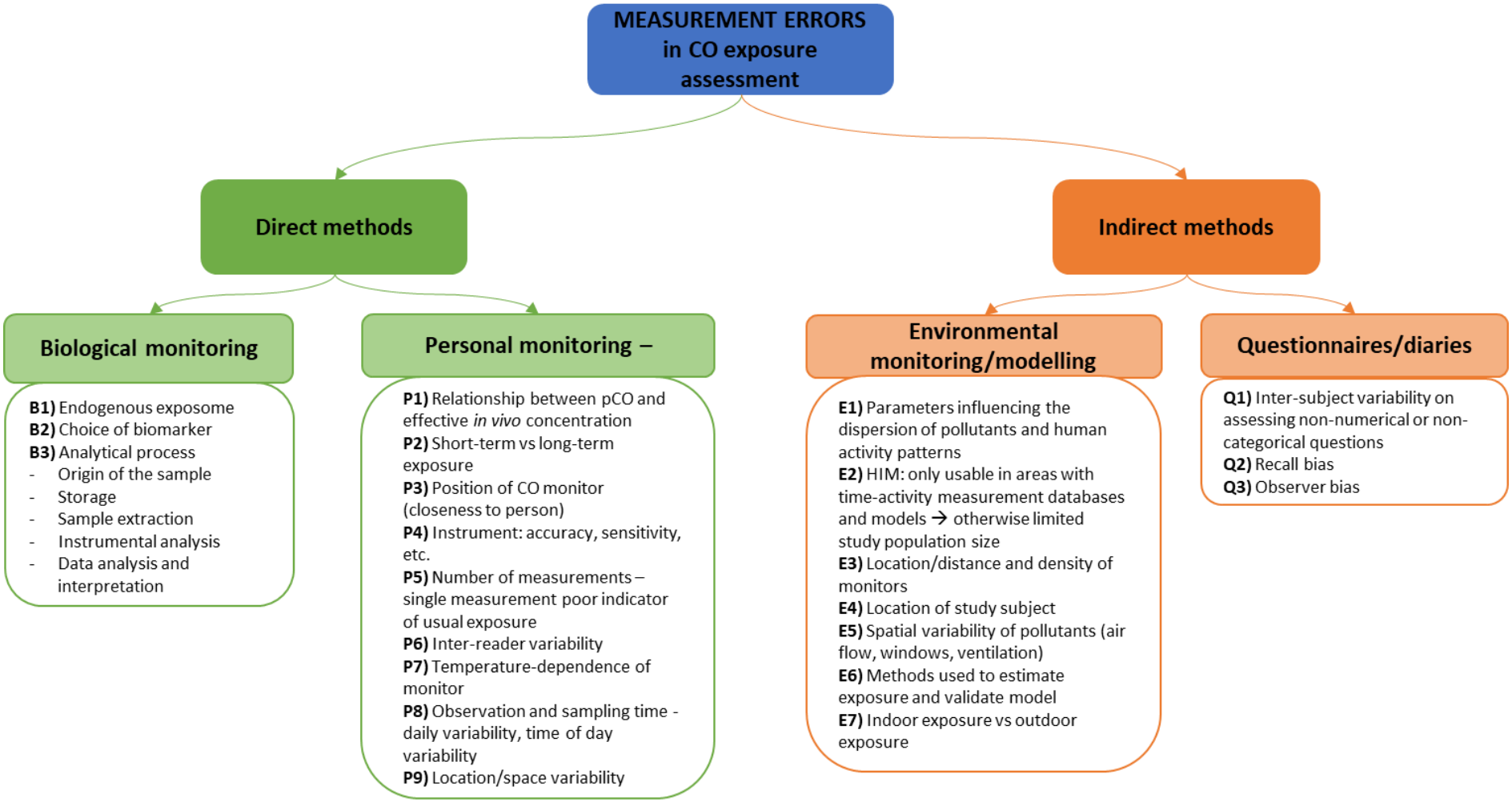

Figure 5.3: Overview for conceptual map of sources of measurement error in CO exposure assessments, based on investigated literature.

5.3.1.2 Origin of the sample

The choice of the tissue for biomarker extraction and analysis is of relevance. Blood is the most commonly selected tissue for CO analysis, but breath and, in post-mortem cases, solid tissues such as skeletal muscle or spleen have also been tested [107,108]. However, representativeness of the biomarker in the selected tissue must be evaluated: distribution of a gaseous substance like CO is heterogeneous in solid compared to liquid specimens, therefore significant inter-specimen variability can occur, leading to systematic errors in their estimation.

One major contributing aspect to measurement error is time. Once xenobiotics enter the body, they undergo several metabolic steps (absorption, distribution, metabolism, elimination – ADME), which take different amounts of time. An important measure that needs to be known is the 'half-life' of a substance, which is the time needed for the concentration of the substance to be reduced to half the initial concentration. The shorter the half-life, the more difficult it is to detect a substance in human tissues and the more important it is to time the sampling appropriately [109]. The half-life of COHb is stated to be between 2-4 hours, depending on individual characteristics (e.g. ventilation rate, metabolic rate, etc.), exposure duration and concentration [110,111]. For clinical samples, where exposure most likely occurred within the last hours or days, variations can be due to time differences down to minutes between samplings. These differences are due to the toxicokinetic processes (blood flow can cause concentration differences between arterial and venous blood samples) as well as the biochemical behaviour of CO, such as dissociation over time (CO binding to Hb is a reversible reaction) or equilibration (steady-state). A small part of CO is also produced endogenously during enzymatic activity causing haem-degradation. [72,112,113]. For post-mortem samples, where most likely an acute and fatal exposure occurred, timing is significant due to several phenomena that occur after death during decomposition. Thermo-coagulation, putrefaction and post-mortem elevation or generation of CO can alter the measurement results [114–118].

5.3.1.3 Storage

Biological specimen storage is another known and major source of error when using biomarkers [106]. Factors that can alter CO concentrations during storage include time, type of preservative, temperature, freeze- and thaw-cycles, volume of air in sampling container, tube reopening and initial saturation [115,119–121]. Over time, degradation of the sample can occur due to biochemical phenomena (e.g. bacterial degradation), which can cause both CO

generation and CO loss through chemical transformation to other substances [119,122]. Different preservatives and temperatures have been reported to cause both increased and decreased CO levels. A higher temperature can speed-up the degradation process or catalyse enzymatic reactions that chemically modify CO [115,120,123]. Also oxygenation of the sample and repeated opening of the sampling tube can cause significant CO loss [120].

5.3.1.4 Pre-treatment and extraction

Errors in biomarker monitoring can arise also due to specimen processing [106]. Selection of the extraction method is important to be able to guarantee complete recovery. For COHb, analysis via CO-oximetry is specific for CO and does not require extraction of the analyte, since it is measured directly in the blood sample, thus no error is introduced, while UV-spectrophotometry requires the additional analysis of a matrix to obtain the full spectrum and, thus, introduces additional potential error sources. For TBCO, the full release of CO from the blood sample was established and validated through the use of a strong acid (sulphuric acid). Other releasing agents have been tested, however with less recovery. Another aspect important during the processing and analytical phase is the operator performing the steps. If multiple people are handling the sample, random errors may be introduced due to variations between laboratory technicians following the protocol differently. Therefore, it is suggested that either the same operator performs all the steps or that the protocol is described in specific detail [42].

5.3.1.5 Instrumental analysis

After proper collection, storage and processing, the sample is ready to be analysed. For analysis, there are several choices that need to be made to optimize the outcome, such as the measurement method, the instrument for analysis and instrumental setup [41,106,113,124]. Random errors can be introduced by instrumental or methodological errors. These are not known but can be controlled and accounted for through repeated measurements, daily calibration of the instrument and regular quality controls (QCs). Calculation of the coefficient of variation (CV) and interclass correlation (ICC) are some examples of how to describe and quantify laboratory errors. The CV can be used to determine intra- and inter-assay variability. ICC is an alternative to CV and measures the stability or reliability within groups or individuals, with the advantage of considering the impact of the intra-assay variation compared to the total variation [106].

Another aspect to consider is that to make sure that no selection bias occurs, the timing of analysis between cases and controls needs to be the same.

The analysis method selected is required to have the capability, reliability and validity of measuring the biomarker concentration with optimal accuracy and precision [41]. COHb is measured spectrophotometrically with a CO-oximeter, either as a separate instrument or as part of a blood gas analyser (BGA) [58,125,126], TBCO is measured gas chromatographically via GC-MS [29,127]. While COHb measures only the quantity of CO bound to Hb, TBCO is able to measure the total amount of CO in blood, including CO bound to Hb and free CO. Tests on clinical samples have shown that the amount of free CO can vary from 10 to 80% between subjects [127], which can have a significant impact on individual exposure estimates. However, these analyses were performed on a limited number of subjects, therefore further tests are needed to confirm the magnitude of these variations.

5.3.1.6 Data treatment and interpretation of results

A final, but nonetheless important potential source of errors is the data treatment and interpretation of results part. Analysing and interpreting the results correctly can have a crucial impact on the subsequent use of these results for group estimates or risk or incidence rates, which are then employed by public health and environmental institutions to set-up strategies for disease prevention and control. The choice of the software, as well as the statistical methods and protocols for data treatment, can cause substantial misclassification bias. A simple error as choosing a paired instead of an unpaired Student t test can cause a change in the significance of the p-value or confidence interval (CI), thus classifying the measured concentrations into the wrong group (disease vs control). Therefore, it is very important that the laboratory personnel performing the data analysis is well trained, with sufficient and appropriate experience and background in the topic. Another aspect that might influence the interpretation of the results is observer bias. If the person performing the analyses is aware of the subject's health status and the study aim, he might direct the data treatment towards the desired outcome, thus biasing the results [96]. This can be minimized by blinding a study.

5.3.1.7 Personal monitoring

As opposed to biomonitoring, in personal monitoring, exposure to CO is not measured as a *dose* in the body, but as concentrations of CO in the immediate environment of an individual,

which should represent the closest estimates of the true personal exposure. Therefore, it is important that the relationship between personal CO (pCO), the effective *in vivo* concentration and the true exposure are accurately determined. These then are correlated to the true exposure and can be extrapolated to the general population through probabilistic modelling techniques. The errors introduced during the estimation and extrapolation process cannot be completely removed, but it can be minimized through appropriate exposure assessment and error correction methods [5,38]. The first step is the relationship between personal exposure and biological dose measurements. Correlation between pCO and COHb in blood has been determined by performing studies that simultaneously employed CO monitors and blood COHb measurements [128,129]. The first study investigating an empirical relationship between CO exposure and COHb levels was by Coburn, Foster and Kane (CFK), who are known to have developed the CFK-model describing the physiologically-based pharmacokinetic (PBPK) model for CO [130]. The model has since been expanded by other study groups [131–134] but the CFK-model remains the preferred mathematical PBPK model for pCO/COHb calculations and predictions. However, this model not only relies on a study cohort with a low number of participants, but it also does not take into consideration the magnitude and effects of free CO in blood and extravascular tissues, which has been the focus of extensive debates recently [14,29,127,135,136]. Changes in the effective amounts of CO in blood (total CO, not only COHb) can invalidate the equations and models used so far for CO estimations and predictions if the changes are too significant.

Another aspect to consider is the differences in short-term vs long-term and low-level vs high-level CO exposures. CO monitors usually are employed by measuring the CO exposure for limited amounts of time and for only limited repeated measurements, which are then averaged, therefore potentially being unable to register sudden, short-term increased exposures. Also, averaged measurements tend to attenuate the results, not reflecting extreme increases or decreases in the CO concentrations, which are however important for diagnostic purposes. The number of repeated measurements is also important since a single or a low number of measurements are a poor indicator of usual exposure [55,137,138].

The distance of the monitor to the person is an additional factor to be considered. To be accurate and truly reflect the exposure of the individual to CO, the monitor should be located right next to the nose or mouth, however, due to practical infeasibility, they are usually carried in a backpack or suitcase or, especially for children, allocated beneath an individual's face, such as

on the back or chest [45,48,57,87]. This could potentially lead to underestimated measurements since CO is a gas lighter than air and tends to rise [139].

Regarding the analytical process and the instrumental characteristics, measurement error can arise similarly to biomonitors due to random instrumental errors, instrumental or methodological inadequacy and due to different personnel performing the analyses as well as the data treatment and interpretation. Inter- and intra-assay variation can occur also due to day-to-day variability or sampling times that differ between runs or cases and controls [38,42].

Furthermore, some CO monitors are temperature-dependent, resulting in differences in results based on the time of the year or the location the measurements were performed [140]. Spatial variability of CO can potentially bias the measurements, both increasing and decreasing CO concentrations, if a monitor is not placed on the individual, but rather in an indoor environment and used as a surrogate for personal CO exposure (e.g. kitchen) [6,80].

5.3.1.8 Environmental monitoring/modelling

Following the correlation of pCO and blood COHb levels, which results in estimates for individual CO exposure, correlations between pCO and environmental CO concentrations lead to group estimates, which can be used to obtain population CO exposure and risk levels. pCO and environmental CO levels have been linked by application of models such as pNEM/CO and APEX to environmental or personal exposure studies, such as in the Denver and Washington population [92,94,141]. Issues with these studies have been highlighted previously, where exposures were both over- and underestimated, based on the levels of exposure (high vs low) [94,135]. Alterations to the correlation of measures of exposure and individual estimates can consequently also significantly affect this type of environmental modelling in population studies. Measurement error in environmental CO studies have been investigated and thoroughly discussed by various research groups, however, the impact of individual exposure measurement error was never directly related to the group estimates [33,35–38,55,80,142–145].

5.3.1.9 Combination of methods

The findings of the model prediction evaluations (e.g. from the pNEM/CO study) indicate that models alone cannot accurately determine personal exposure to a pollutant such as CO. A problem associated with indirect exposure assessment methods is that they generally result in

exposure estimates for outdoor sources and/or (micro)environments since this is where most monitoring sites and satellites are located. However, for the association with health outcomes, it is important to take into account the total/cumulative human exposure that represents not only the outdoor sources of exposure but most of all the indoor sources of exposure. Indoor exposure accounts for the major exposure pathway since people generally spend more time indoors than outdoors. Personal exposure/ambient exposure concentration ratios tend to vary substantially by location and microenvironment. Even though ambient exposure estimates might have been validated, personal exposure errors, which are difficult to eliminate, can still exist and thus affect the overall relationship between exposure and health outcome. Therefore, it is reasonable that the majority of studies assessing CO exposure use a combination of direct and indirect methods to obtain their results. Relevant studies in our review showed that the most common approach is to use a combination of three methods: personal or biological monitoring together with environmental monitoring or modelling and questionnaires (17.2%, **Figure 5.1**) are used to determine duration and frequency of exposure to different types of CO sources, the number of cooking and heating appliances as well as typical living and working patterns. Some studies employed a combination of personal monitoring with environmental monitoring/modelling (8.6%). Other common combinations include biological monitoring aided by the use of questionnaires, which make up 8.6% of the total studies under investigation. The majority of the studies in **Figure 5.1** were usually limited to a low number of study subjects (<50), resulting in a lower power for epidemiologic studies. Only seven studies were identified that met the criteria for high-quality CO exposure assessments with a sufficient study power and that used a minimum of two methods to measure CO [43–46,49–51,53].

Out of these seven studies, only the study by Thompson et al. [43] performed a validation study (see **Table 5.3**). Validation is, however, a crucial step in an epidemiologic study to guarantee an accurate estimate of the exposure-outcome association. With a validation study (or, in absence of the true exposure, a reliability study) an important part of the error burden on exposure assessments relying on exposure measurement is taken into account and corrected for. This is particularly important for exposures that rely on biomarkers, such as in the case of CO [37,41,42].

5.3.2 Calculation of measurement error improvement based on an example

Every individual is exposed to CO from background levels present in the atmosphere. These are usually not toxic, but create a baseline CO level that is above zero [146]. The baseline level

changes from person to person, not only due to inter-person variability but also to confounders such as smoking. Smokers have a CO baseline level that is significantly higher than for non-smokers in the population. As already mentioned in a chapter 2 (section 2.5.2), baseline COHb levels for smokers can range from 3-8% for normal smokers (15-25 ppm), while for heavy smokers levels of 10-15% are reached (30-40 ppm) [107,147]. To measure whether population groups are exposed to indoor CO levels, biomarker cut-offs are common to differentiate between exposed and non-exposed individuals, taking into account their CO baseline level [10].

When improving the measurement method for CO exposure, the measurement error affected is non-differential, since samples and the measurement device do not vary between exposed and non-exposed groups and, thus, neither do bias and error variance [148]. Since individual measurements are improved, which are part of a population sample that is then averaged to represent the population, we are improving classical measurement error [37].

Improvement of the method for CO measurement involves a biomarker that not only measures CO bound to Hb but also the amount of free/dissolved CO in blood. This means that the method gives more accurate results, with values approximating the true value better. Therefore, the sensitivity of the method is improved by reducing the systematic bias coming from an inaccurate biomarker.

Precision of the measurement is defined as the measure of variation in the measurement error in the population, which is independent of the measurement accuracy, and described by the variance (square of standard deviation) [148]. By having an improved biomarker and measurement method, not only is accuracy increased, but also precision, since we decrease measurement error in the measure, which is reflected in the population estimate as well.
The range of detection of the biomarker is also expanded, thus potentially increasing the variability of the exposure estimate in the population. Nevertheless, better accuracy should also lead to a narrower confidence interval.

Furthermore, from an exposure group perspective, this also means that sensitivity (probability of correctly classifying a truly exposed subject as exposed) and specificity (probability of correctly classifying a non-exposed subject as non-exposed) of the exposure were improved, since more accurate results lead to reduced misclassification in exposed/non-exposed groups. Therefore, this should change the proportion of the exposure in the population and affect the relative risk.

These improvements result in more accurate CO estimates when comparing the improved biomarker total blood carbon monoxide (TBCO) to COHb measurements, thus decreasing classical measurement error [97]. The general aim is to determine the potential impact that measurement error for CO exposure measurement has on the RR of CO exposures.

5.3.2.1 Study protocol

For continuous measures, a simple model of measurement error is described by the following equation[42]:

$$X_i = T_i + b + E_i \qquad (1)$$

With X: observed/measured exposure; T: true exposure; b: bias (systematic error that occurs for all subjects in population); E: inter-subject error (from sources of error such as choice of measurement method, omissions or variations in execution of protocol, etc.) differs in each subject *i*.

To be able to quantify the impact, an observational study based on the results of the critical review of the relevant literature, which identified the most valid CO exposure assessment studies available so far, was selected.

Out of these, the study by Thompson *et al.* [43], who reports results of the comparison between personal CO monitor (pCO) and exhaled breath CO (exCO) measurements (which were back calculated to COHb with the use of formulae), was selected and the data reported in the study employed to quantify the effect improved COHb levels would have on the RR.

5.3.2.2 Calculation steps

1. To perform the calculations, two main scenarios are taken into consideration, one for each exposure measurement (EM) method. In this study, the first scenario is COHb measured via pCO, scenario two is COHb measured via exCO. Each scenario differs from each other by their observed standard deviation (SD), which is inherent to the EM method and obtained by the data reported in the study [43].

2. Furthermore, each scenario has a different set of parameters, with variables tested over a certain range (see descriptions below).

3. σ_T is the true standard deviation (SD – square root of true variance) of the exposure estimate, which is unknown; however, it is known that the variance of the observed measure

X is greater than the variance of the true measure T in the population since the observed measure is affected by measurement error [2]. Mathematically, this is also true for the SD, which justifies the assumption of σ_T being smaller than σ_X. As in the study at hand we have observed SDs of between 7 and 9 for the two proxy methods, we have selected a "dummy" true SD that is smaller than the observed ones. For mathematical simplicity, we have set σ_T was at 5. It is, however, possible indeed that the true SD is even smaller than that, which would make the results of the calculations more substantial, or also the other way around. This assumption can be changed numerically based on the case under investigation.

4. σ_X (the observed SD) is calculated from the data in the selected study [43] from the given data of minimum, maximum, median, sample size and 25^{th} and 95^{th} percentile (with assumption of normal distribution), based on the equation (13) given in the study by Wan *et al.* [149] for estimation of the standard deviation when it is not given in the original study.

5. The difference between true and observed measure and, thus, true and observed variance, is the amount of variance that is due to measurement error [37]. σ^2_E is the variance of the error and is calculated by subtracting the variance of the true exposure (square of true SD) from the observed exposure (square of observed SD). The SD of the error is then calculated as the square root of the error variance.

6. There are several sources of error that contribute differently to the total error (see critical review for details). The relative error contribution represents the relative amount of the observed error that can be attributed to a source of error; different scenarios are tested, which are based on error estimates either mentioned in the study or obtained from other studies using the same method/device/parameters. This range is variable and adaptable to different studies.

7. The next step involves assumptions to be made on the relative improvement made on the error contribution of the error by improving certain sources of errors. I hereby selected an improvement range of 20%-80%, based on the results obtained from the clinical study with comparison of COHb and TBCO as biomarkers for CO poisoning determination [97].

8. To calculate the amount of improvement on the observed exposure that these improved errors would have, first, the net remaining fraction of error SD in the improved exposure estimate is determined, which is calculated by multiplying the relative error contribution with the relative improvement of the error contribution and subtracting that from 1.

$$\sigma_{ENetImp} = 1 - (\sigma_{ECR} * \sigma_{ECRImp}) \quad (2)$$

9. To obtain the variance of this net fraction, $\sigma_{ENetImp}$ is squared.

10. The absolute improved error variance can be calculated by multiplying the net fraction $\sigma^2_{ENetImp}$ with the original error variance.

11. From this, the improved exposure estimate is calculated by adding the true variance with the improved error variance.

12. The average magnitude of errors (both classical or Berkson type) for continuous measures is described by the standard deviations (σ_X) or variances (σ_X^2). Classical measurement error is described by the coefficient of reliability ρ_{XX}, which is equal to the square of the validity coefficient, which is the correlation between the true and observed/measured exposure variance, ρ_{XT} [37,42]. In linear regression, the reliability coefficient is represented by the attenuation of the slope [150].

$$\rho_{XX} = \rho_{XT}^2 = \frac{\sigma_T^2}{\sigma_X^2} \quad (3)$$

13. An improved reliability coefficient ρ_{XXImp} is calculated by using the improved σ^2_{XImp} compared to the true variance. The validity coefficient ρ_{XT} can range between zero and one, with a value of one meaning that *X* is a perfectly precise measure of *T*. It can be estimated in a validity study by the Pearson correlation coefficient of *X* with *T* (for linear regressions). Thus, the square of the validity coefficient, which is equal to the reliability coefficient ρ_{XX}, represents the proportion of *X* explained by T, with the remaining part being variance due to error [42].

14. Since the RR was not reported in the selected study, a literature search for a study with similar study area, population and parameters was performed, resulting in a study by Hubbell *et al.* to be the most suitable in providing RR_X [151].

15. The improved RR is calculated by using the observed RR and taking the fraction of improved to originally observed exposure estimate as exponent.

$$RR_{XImp} = RR_X^{\left(\frac{\sigma_{XImp}}{\sigma_X}\right)} \qquad (4)$$

16. The relative improvement of the RR is calculated as a percentage relative to the observed RR.

$$\%RR_{Imp} = \left(\frac{RR_{XImp}}{RR_X}\right) - 1 \qquad (5)$$

5.3.2.3 Calculation output

It is clear from the review of the literature that there is a substantial gap in indoor CO exposure assessments, with a lack of sufficiently high-quality CO exposure assessment studies fully considering and correcting for measurement error. Therefore, out of the seven studies that were identified as performing high quality indoor CO exposure assessments, the one by Thompson *et al.* [43] was selected as an example, since it was the only one that performed a validation study. Having in mind the sources of measurement error overview (**Figure 5.3**), the sources of error in the study were determined and summarized in **Table 5.5**. Measurement error improvement calculations were then carried out and used to calculate the effect on relative risks. **Table 5.6** summarizes the results of the calculations of measurement error improvements and the effect on the relative risks for two different exposure measurement methods (EMM), COHb measured with a personal monitor and COHb measured through an exhaled breath device. Detailed results of the calculations are found in **Table A5.2**. **Table A5.3** provides a description of the abbreviations and acronyms used in the calculation process.

It can be observed that for sources of error that contribute from 5 to 70% to the total measurement error, by using an improved measurement method that improves the measurement from 20-80%, the RR is increased by <1% to a maximum of 18.28% for exCO

and from <1% up to 28.96% for pCO. The higher the error contribution, the more influential the exposure measurement improvements, and, thus, the closer we get to the true RR. These results also show that for pCO the improved RR is higher than for exCO.

Table 5.5: Identified sources of measurement error in the study by Thompson et al. based on the measurement errors in CO exposures overview (Figure 5.3) with references; BM: biological monitoring, PM: personal monitoring; B1: Error resulting from the endogenous CO levels, B2: error deriving from the choice of a less accurate biomarker such as exhaled CO (exCO), B3: error deriving from the analytical process, which in this case is the use of the exhaled breath CO monitor and is given in the study, P1: error deriving from the use of the CFK equation to relate pCO to the true CO exposure estimate, P2: error deriving from the use of averages for short-term and long-term exposures, P3: error deriving from the position of the monitor, in this case, non is present since the monitor is located in the optimal position, P4: instrument accuracy is reported in the study for the personal CO monitor, P5: there is no error deriving from an insufficient number of readings since continuous measurements are performed, P6: there is no inter-reader variability since the data is logged automatically, P7: error deriving from temperature dependence, P8: no error from the daily or time of day variability since monitoring is performed for 24h intervals over 4 weeks, P9: no error deriving from location or space variability since the spaces are on average the same size

Error class	Error type	Error description	Error estimate [%]	Reference
BM	B1	Endogenous exposome	1.0	[151]
	B2	Choice of biomarker – ExCO	8.5	[109]
	B3	Analytical Process – Instrumental Analysis	1.0	[61]
PM	P1	pCO-total CO relationship (use of CFK equation)	8.5	[139]
	P2	Short-term vs long-term exposure – use of averages	1.2	[152]
	P3	Position of CO monitor	0	[61]
	P4	Instrument accuracy/sensitivity	7.0	[61]
	P5	Number of measurements – continuous readings	0	[61]
	P6	Inter-reader variability – automated data-logging	0	[61]
	P7	Temperature dependence	5.0	[61]
	P8	Daily variability/time of day variability – 24h measurements over 4 weeks	0	[61]
	P9	Location/space variability – same size for ovens	0	[61]

Table 5.6: Summarized results of measurement error improvement calculations on data from the study by Thompson et al. [127];]; σT: standard deviation of true estimate; σ_X: standard deviation of observed estimates; σ^2_X: variance of observed estimate; σ^2_E: variance of error; σ_{ECR}c modifiable fraction of relative contribution to standard deviation of error; σ_{ECRImp}: Fraction of σ_{ECR} reduced in improved x; $\sigma^2_{ENetImp}$: Net remaining fraction of error standard deviation in improved x; σ^2_{EImp}: improved error variance; σ^2_{XImp}: improved variance of x; RR_X: observed relative risk; RR_{XImp}: improved relative risk; $\%RR_{Imp}$: Relative improvement of RR.

EMM	σ_T	σ_X	σ^2_X	σ^2_E	σ_{ECR}	σ_{ECRImp}	$\sigma^2_{ENetImp}$	σ^2_{Eimp}	σ^2_{Ximp}	RR_X	RR_{XImp}	%ImpRR
COHb via pCO	5	9.07	82.23	57.23	5%-30%	20%-80%	0.58-0.98	30.25-56.09	58.6-81.09	1.22	1.22-1.36	0.28%-8.63%
COHb via exCO	5	7.60	57.82	32.82	5%-30%	20%-80%	0.58-0.98	18.96-32.17	43.96-57.17	1.22	1.22-1.30	0.23%-6.47%

5.4 Discussion

This study aimed at critically evaluating current CO exposure assessment studies, in order to determine their advantages, identify potential pitfalls and create a better understanding of how different sources of error can affect exposure estimates and relative risks, with a special focus on low-level indoor CO exposures, which were identified as an undervalued exposure risk. After a thorough examination of the relevant literature, a conceptual map of sources of error and measurement error in CO exposure assessment studies, which was based on the model described by Mark Nieuwenhuijsen [24], was developed. This was then applied to a selected study to help determine and quantify the sources of measurement error and employed to calculate how alterations in the error contributions influence exposure and risk estimates.

Exposure measurement error, or misclassification of exposure, is a significant limitation of epidemiologic studies, due to the fact that exposures occur in different locations over varying periods of time and due to feasibility, cost and efficiency matters of obtaining accurate individual exposure estimations [5,34]. Large uncertainty in exposure measurement leads to a large error in relative risk estimates, thus it is paramount to have valid and reliable measurements of exposure, which includes the correct determination and interpretation of measurement errors.

Based on this critical review, the exposure assessment method selected (e.g. biological monitoring, personal monitoring), there are different types and amounts of error sources that can affect the exposure measurement and, thus, the exposure estimate. For an exposure assessment to achieve robustness and validity, it is important to take all the relevant potential sources of error into account when designing the study. Clearly, not all listed errors are relevant for every CO exposure study. It depends on the type of exposure investigated (e.g. occupational vs recreational), the exposure group that is investigated (e.g. pregnant women vs. bus drivers), the area of interest (e.g. school vs kitchen) as well as the main aim of the study (e.g. association to certain health outcomes vs comparison between exposure groups) [24]. The choice of these parameters in a study delineates the types and amounts of potential errors arising, there is no fixed rule generally applicable to all epidemiological exposure studies, but for an exposure assessment to be valid, measurement error needs to be minimized, in order to get closer to the true exposure estimate [154]. Classical measurement error usually biases effect measures towards the null value (indicating no association). This can have serious adverse effects [37],

for example in cases where potentially harmful levels of CO are not correctly detected and, as a consequence, proper treatment is not administered, leading to adverse health effects.

The ideal situation is where a method can estimate the true exposure. This is, however, usually not the case in epidemiological studies. As an alternative, proxies are used in combination with measurement error correction methods that have been proposed, such as regression calibration [36], simulation extrapolation, bootstrapping [47] or moment reconstruction [155]. In all these measurement error correction methods, the assumption is that there is a validity or reliability study available. A validity study is a study in which the exposure is measured for a subset of individuals with two methods, one giving high accuracy, a "golden standard", and one "proxy". This is why often a combination of biomonitoring and personal monitoring or personal monitoring and environmental monitoring are used. The alternative to a validity study is a reliability study, where in the subset of individuals the exposure is measured with the same method, but repeatedly and independently [148,155].

In the selected example study by Thompson and colleagues [43], two methods are used to estimate CO exposure, biological monitoring (exhaled breath CO monitor) and personal monitoring (personal CO monitor). However, neither of these methods measure the true exposure. Therefore, in a previous study, a more accurate biomarker that could improve the measurement of CO and get closer to the true value of CO exposure was developed. The developed biomarker is called TBCO and measures the total amount of CO present in blood, both the free fraction and the one bound to Hb. Results showed that for clinical cases, differences between 10 to 80% were found between COHb and TBCO measurements [97]. I then wanted to use this new method to see the effects that improving the measurement method could have on measurement error and, as a consequence, on the relative risk of CO exposure in the given example. As a general trend, it is observed that with reduced measurement error variance, an improved reliability coefficient and consequently a higher RR are obtained. Depending on the size of the error contribution (σ_{ECR}) and the relative improvement of that error source (σ_{ECRImp}), the improvements on the RR vary from very small (<1%) to relatively substantial (29%). Similar relative improvements are obtained in other studies looking at measurement error correction in outdoor CO exposures [156] as well as other air pollutants [47,142]. Even though these studies look at outdoor and not indoor air pollution, it is reassuring to know that the values obtained in our simulation are in the same range as reported by other research groups. From an epidemiological point of view, improving the RR from 1.22 to 1.57 is relatively moderate, especially considering that a source of error is unlikely to have a

contribution of 70% (such as in the most extreme case displayed), but more in the range of 5-30%, thus having an improved RR of ~1.32. However, if we consider that CO exposure generally has a rather small RR and in light of the fact that measurement error contributes one part of the total error sources in epidemiological studies, it can be seen as a significant achievement to improve the RR by approximately 1-30%, solely by improving the measurement method. Additionally, when looking at other types of exposures investigated in epidemiologic studies, for example, where one group might be exposed to a certain chemical due to an occupational hazard and another group is not, the RRs in these cases vary substantially from group to group, due to the absence of the evaluated exposure in one of the groups. In the case of CO exposure, it is important to bear in mind that CO is ubiquitous and everyone is exposed to it, even if at different levels. The RRs obtained in these cases do not differ greatly from one group to another, making a difference from 1.22 to 1.32 a significant improvement.

Having a closer look at the improvements for the different methods, it can be seen that the highest relative improvements are obtained for the method with the higher observed SD (here pCO). This is in congruence with what was reported by Armstrong in his 1998 study [37] as well as his more recent chapter with Basagaña [155], where they showed that measurement error will have less effect if the true exposures are more spread out. They stated that measurement error needs to be relatively important to cause significant bias, which is obviously very reassuring but should not be underestimated.

Another aspect that is affected by classical measurement error is study power. Increased measurement error reduces study power. According to a study by Lagakos [157], measurement error in a study should be reduced by improving accuracy, but only when the proportional increase of the reliability coefficient is less than the proportional increase in the total study cost per individual that is needed to achieve it. As an example, if the reliability coefficient is increased by 50% with spending 40% more money per individual, it is worth it, but if the cost in this example would be 70%, the increased accuracy would not be worth the additional cost [155,157]. In our study example, the increases in reliability coefficient for error contributions of 5-30% range from 1-30%. The additional cost per study subject is unknown since no data is available on the cost for the measurement devices used in the study under investigation. Assumptions can be made on the cost of using a portable electrochemical sensor (the principle behind personal monitors and exhaled breath CO monitors) compared to a GC-MS instrument, with portable electrochemical sensors known to be cheaper than GC-MS. Based on the size of

the error contribution, the probability is higher for higher contributions that the cost might be worth it, whereas for only small error contributions, it is more recommendable to keep employing the less accurate method. Nevertheless, study power is also achieved with an increased sample size. With an increased coefficient of reliability, study power increases and lower sample size is required to be able to find the same statistically significant association, thus reducing the cost of the study [149,155]. This should also be taken into account when deciding whether improving the accuracy of a measurement method is worth the cost.

An additional element to consider in our improvement calculations is the portability of the coefficients, such as the reliability coefficient or the RR, from one study to another. In our example, data for the SD and variances from one study (Thompson *et al.* [43]) were used, the RR from a similar, but slightly distinct study (Hubbell *et al.* [151]), with a different study country (Guatemala vs Haiti) and measurement device (pCO/exCO vs CO-oximetry). The reliability coefficient depends not only on the distribution of the error variance but also on the variance of the true exposures [155]. In our example, equal true variances were assumed, therefore the reliability coefficient is applicable to both exposure measurement methods. However, the 'real' observed RR might be different for the Thompson [43] study compared to the Hubbell [151] study. The study countries are different but located in a geographically and socio-economically very similar area, hence it is plausible that the RRs are similar. When looking at the measurement devices, all three methods were compared by using COHb as biomarker. With CO-oximetry known to be more accurate then exCO, but pCO being a more complete marker for the true exposure, it is possible that the RR for the Thomson study might be higher [158]. A higher initially observed RR might result in a more substantial increase in the relative improvements of the RR, which increases their importance from a public health perspective, potentially shifting the decision of using the improved measurement method vs cost efficiency towards the improved method.

5.5 Conclusion

What beomes clear in this study is the complexity but also the importance of exposure assessments for CO exposures. There are numerous factors to consider when designing a study aimed at assessing the exposure to a pollutant, of which a major part is played by the assessment and reduction of potential error sources (e.g. bias, confounding, etc.). Out of these, an important one in exposure assessments in environmental epidemiology is measurement error. Each exposure type can have a variety of errors arising from different parameters inherent to the

exposure itself, the measurement method, the instrument used, the study group as well as the general aim of the study, which is what makes the study design phase of an exposure assessment such as complex but crucial step. To help identify the sources of measurement error for CO exposures, I have generated a conceptual map for sources of measurement error in CO exposures, which represents a very useful tool helpful in simplifying CO exposure assessments and creating more robust, valid and accurate studies. The importance of these assessments is not only in identifying the sources of error but also in minimizing them. This can be done in several ways, including the selection of appropriate study size and study population as well as a suitable exposure measurement method, which combined need to give satisfactory study power to be able to detect an exposure that is the closest to the true exposure. In the example given in this study, the effect of improving the exposure measurement method on varying amounts of measurement error contributions was determined in a selected study and found that relatively moderate increases of the RR were obtained. Despite this being a single example, it can be used as a model applicable to other studies, showing that even small improvements in the study design of an exposure assessment can have a significant impact on exposure estimates and relative risks. Therefore, I recommend researchers wanting to perform exposure assessment studies for CO exposures to make use of the conceptual map and calculation model as part of their study design phase. I also want to highlight the importance not only of accurate and valid exposure assessments but also of their crucial role in providing public health institutes with the epidemiologic evidence for the generation of prevention, treatment and training plans, protocols, guidelines and reports, which ultimately leads to improving global health.

5.6 References

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Appendix 5

Table A5.1a: List of observational carbon monoxide (CO)-related exposure studies with description of the used type of exposure assessment method, measure of exposure, measurement device and detailed assessment method; BM: biological monitoring, PM: personal monitoring, MON: environmental monitoring, MOD: environmental modelling, Q: questionnaires, pCO: personal CO monitor, airCO: air CO monitor, exCO: exhaled breath CO monitor, COHb: carboxyhaemoglobin, PM2.5: particulate matter with a diameter of 2.5μm, Na: not applicable or reported in the study.

Article	Type of exposure assessment method	Measure of exposure	Measurement device	Assessment method
[43]	BM. PM, Q	ExCO, pCO	MicroCO monitor and HOBO CO data logger	Questionnaire
[159]	MON, Q	COHb, symptoms	Measurement device not specified	Syndromic surveillance and surveys, based on expert classification of symptoms and CO levels
[18]	MON	Number of CO alarm activations	Na	Incidents of CO alarm activation
[58]	BM	COHb	Pulse-oximeter and Blood Gas Analyser	Na
[126]	BM, MON	COHb	CO-oximetry	Nonlinear mixed-effects modelling program to create prediction method
[54]	PM, MON, Q	pCO (and PM 2.5)	Draeger CO passive diffusion tubes (ppm/h)	Questionnaires about cooking appliances, GM and SD calculations
[160]	MON	AirCO	Monitoring stations	ED admission data

Article	Type of exposure assessment method	Measure of exposure	Measurement device	Assessment method
[161]	Q	COHb, symptoms, number of HBO therapy recipients	Measurement of COHb not specified (but performed in hospitals)	Detailed patient level data
[162]	PM	pCO	Portable gas detector	Na
[163]	BM, PM	COHb, pCO	CO-oximeter, CO passive sampler (then analysed via GC-FID)	Na
[164]	PM	pCO	Personal Monitor	Na
[82]	MON	AirCO	HOBO CO logger	Na
[165]	MON	AirCO	Multigas detectors	Na
[70]	PM, Q	pCO	CO monitors (Draeger PAC)	Questionnaires, mixed-effect model for statistical analysis
[50]	PM	pCO and AirCO	Personal portable CO monitors	Na
[166]	PB	COHb	Pulse CO-oximeter (arterial), BGA (venous)	Na
[61]	BM, Q	COHb	BGA (arterial)	Questionnaire
[55]	PM, MOD	pCO	Passive color stain diffusion tubes	Na
[67]	BM	ExCO	Breath CO monitor	Na
[167]	PM, MON	AirCO and pCO	CO monitor (installed in home) and portable personal monitor	Na
[168]	BM	ExCO	BreathCO monitor	Na

Article	Type of exposure assessment method	Measure of exposure	Measurement device	Assessment method
[64]	BM	ExCO	Breath CO monitor	Na
[62]	BM	COHb	CO-oximetry	Na
[169]	BM	COHb	UV-Spectrophotometer	Na
[170]	MON	AirCO	Ambient monitors	According to microenvironment categories
[171]	MON, MOD	AirCO	Fixed ambient air quality monitoring station	Scenario assessment method (average and continuous exposure for 24h and annual exposure for different age groups)
[65]	BM, Q	ExCO	CO breath analyser	Questionnaire about smoking status and SHS exposure
[172]	MON	AirCO	Air monitoring stations	Na
[128]	BM, PM	ExCO/COHb and pCO	microCO monitor and personal CO monitoring with EasyLog CO Monitor	Results input into CFK-equation to predict COHb
[68]	MOD	AirCO	Regional atmospheric modelling system	Na
[173]	BM	pCO	Personal Monitor	Na
[174]	PM, MON	pCO, air CO, exCO	personal monitors (48h monitoring), ambient monitors in kitchen and microCO monitors	Na
[6]	MON, MOD	AirCO	Fixed ambient monitors	Estimated daily CO ambient concentrations, based on four different estimation approaches: (1)

				average across values from all monitors in the city, (2) use of the value at the monitor nearest to the study subject's residence, (3) inverse distance weighting of monitors' values, and (4) kriging
Article	**Type of exposure assessment method**	**Measure of exposure**	**Measurement device**	**Assessment method**
[17]	MON, Q	AirCO	CO data logger (and PM2.5)	Questionnaires
[175]	PM, MON, Q	pCO, airCO	Portable passive diffusion data-logger and ambient monitors	Questionnaires
[176]	MON	AirCO	Micrenvironments and ambient monitor	Also other volatile toxics measured
[177]	BM, MON	ExCO, airCO	Breath CO and ambient monitor	Na
[66]	BM, Q	ExCO	piCO Smokerlyzer	Questionnaires
[71]	PM	pCO	Portable multiple gas monitor	In different microenvironments
[178]	MON, Q	AirCO	CO sensors in air quality monitoring platforms	Quesionnaires
[51]	BM, MON, Q	pCO, airCO	Portable personal CO monitors and fixed monitoring stations	Personal diaries
[129]	BM, PM	ExCO, COHb, tHb, pCO	CoVITa electrochemical sensor, HemoNIR and Draeger PAC III	Na
[179]	PM, MON	AirCO, pCO	Draeger CO electrochemical sensor and CO passive diffusion tube	Na

Article	Type of exposure assessment method	Measure of exposure	Measurement device	Assessment method
[52]	PM	pCO	Draeger confined space monitor	Na
[44]	BM, MON, Q	COHb, exCO, airCO	CO-oximetry, breath CO analyser and ambient monitors	Estimation of COHb by measuring ME concentrations with CO monitor and time-diaries
[60]	BM	COHb	pulse CO-oximeter in blood (Masimo RAD-57)	Na
[86]	PM, MON, Q	pCO, airCO	LOCD and Draeger CO diffusion tubes	Questionnaires
[57]	BM, MON, Q	COHb and airCO	real-time CO detector and UV/vis-spectrophotometry	Questionnaires
[87]	PM, MON, Q	pCO and airCO	Q-trak CO monitor and Multigas monitors	Questionnaires
[56]	PM	pCO	Drager CO Diffsion Tubes	-
[83]	MON	AirCO	Indoor Air Quality Meter	-
[45]	PM, MON, Q	pCO and airCO	Portable real-time CO Logger and CO monitors	Questionnaires
[46]	PM, MON, Q	pCO and airCO	HOBO CO logger	Questionnaires
[53]	PM, MON, Q	pCO and airCO	Personal Monitor (Langan) and fixed-site monitors	Questionnaires
[48]	PM, MON	pCO and airCO	Passive electrochemical CO monitor and non-dispersive infrared monitors (CO 10-M and 11-M)	-

Article	Type of exposure assessment method	Measure of exposure	Measurement device	Assessment method
[88]	PM	pCO	Draeger CO Monitor	-
[59]	BM	COHb	Pulse CO-oximeter	Na
[180]	BM, Q	COHb	Handheld pulse CO-oximeter	Questionnaires
[181]	PM	pCO	Personal monitor	Na
[182]	PM	pCO	Real time gas monitor	Na
[63]	PM, Q	End respiratory CO	Handheld breath analyser	Questionnaires
[49]	PM, MON	AirCO and pCO	Passive diffusion CO tubes (3 per household, 2 personal (mother and child) and 1 ambient)	Na
[183]	PM	pCO	Personal monitors	Na
[184]	PM, MON, Q	pCO and airCO	Draeger Pac gas monitor and real-time measurement	In kitchen

Table A5.b: List of experimental carbon monoxide (CO)-related exposure studies with description of the used type of exposure assessment method, measure of exposure, measurement device and detailed assessment method; BM: biological monitoring, PM: personal monitoring, MON: environmental monitoring, MOD: environmental modelling, Q: questionnaires, pCO: personal CO monitor, airCO: air CO monitor, exCO: exhaled breath CO monitor, COHb: carboxyhaemoglobin, PM2.5: particulate matter with a diameter of 2.5µm, TBCO: total blood CO, Na: not applicable or reported in the study.

Article	Type of exposure assessment method	Measure of exposure	Measurement device	Assessment method
[80]	MON, MOD	AirCO	Central Site Monitors	AERMOD simulated concentrations, local- and regional-scale modeling, APEX and SHEDS model to estimate population exposure to CO
[134]	PM, MOD	COHb	Not specified	Model for individual COHb exposure estimation
[185]	BM, Q	ExCO, respiratory symptoms	Breath CO analyser	Questionnaires on sociodemographic info, respiratory symptoms and exposure to second hand smoke
[186]	BM, Q	COHb	CO-oximetry	Questionnaires
[187]	BM, MON, Q	ExCO, airCO	Breath CO analyser and portable CO data logger	Questionnaires
[63]	PM, Q	End respiratory CO	Handheld breath analyser	Questionnaires
[188]	PM, MOD	pCO	Personal monitors (shadowing for kids)	Modeling of monitoring in microenvironment (personal vs static concentrations)
[84]	BM	COHb and air CO (in car)	Method for COHb in blood measurement not specified, CO monitor for air CO	Na

Article	Type of exposure assessment method	Measure of exposure	Measurement device	Assessment method
[189]	BM	Plasma lipids, lipoproteins and apolipoproteins, COHb	CO-oximeter and GC-RGA	Na
[190]	BM	Endothelin-1 mRNA levels	Fluorescence detection after PCR and electrophoresis separation	Na
[191]	BM	COHb	CO-oximeter	Na
[192]	BM	COHb	PdCl2-method	Na
[94]	PM, MOD	pCO	Personal monitors	Comparison of computer-simulated exposure distributions to distributions of the population through personal exposure monitoring study
[193]	PM	pCO and blood pressure	Personal CO monitors and BP measurement	Na
[81]	PM, MON, Q	pCO and airCO	Personal CO diffusion tubes and fixed monitoring at different locations	Questionnaires
[194]	MON	AirCO, heart rate	Ambient CO monitors and heart rate variability	Na
[137]	PM, MOD	pCO	Passive diffusion tubes	Prediction model based on validation study
[97]	BM	COHb, TBCO	CO-oximetry and GC-MS	Na

Article	Type of exposure assessment method	Measure of exposure	Measurement device	Assessment method
[195]	BM	AirCO, CO, COHb	IR CO analyser, GC, CO-oximeter	Na
[196]	BM	COHb	GC-TCD	Na

Table A5.1c: List of reviews on carbon monoxide (CO)-related exposure studies with description of the used type of exposure assessment method, measure of exposure, measurement device and detailed assessment method; BM: biological monitoring, PM: personal monitoring, MON: environmental monitoring, MOD: environmental modelling, Q: questionnaires, pCO: personal CO monitor, airCO: air CO monitor, exCO: exhaled breath CO monitor, COHb: carboxyhaemoglobin, PM2.5: particulate matter with a diameter of 2.5µm, TBCO: total blood CO, Na: not applicable or reported in the study.

Article	Type of exposure assessment method	Measure of exposure	Measurement device	Assessment method
[197]	BM, Q	COHb, symptoms	CO-oximetry, hospital data	Categorize patients CO exposures based on their characteristics
[198]	BM	COHb	Mainly spectrophotometric methods (CO-oximetry, pulse CO-oximetry) but also breath CO	Na
[79]	PM, MON	pCO and air CO	Personal CO monitors and fixed site monitoring	Comparison between two measures in different microenvironments
[12]	BM	AirCO, exCO, COHb, symptoms	Ambient monitors, breath CO monitors, CO-oximetry and questionnaires	Data for occupational CO intoxications from surveillance database
[199]	BM	COHb	Hopcalite method of Linderholm and Sjöstrand, Van Slyke method, and others	Fetal and maternal COHb in blood
[200]	MON	Number of CO exposure cases	Exposure cases from NYC Poison Control Center and Syndromic Surveillance Unit	Na
[201]	BM, MON	AirCO and COHb	Measurement device not specified	Na
[85]	PM, MON	pCO and airCO	CO Loggers, Infrared Spectrometric Monitors and Passive diffusion tubes	Critical Review
[202]	PM, MON	pCO and airCO	Personal CO monitors and fixed-site monitors	Na

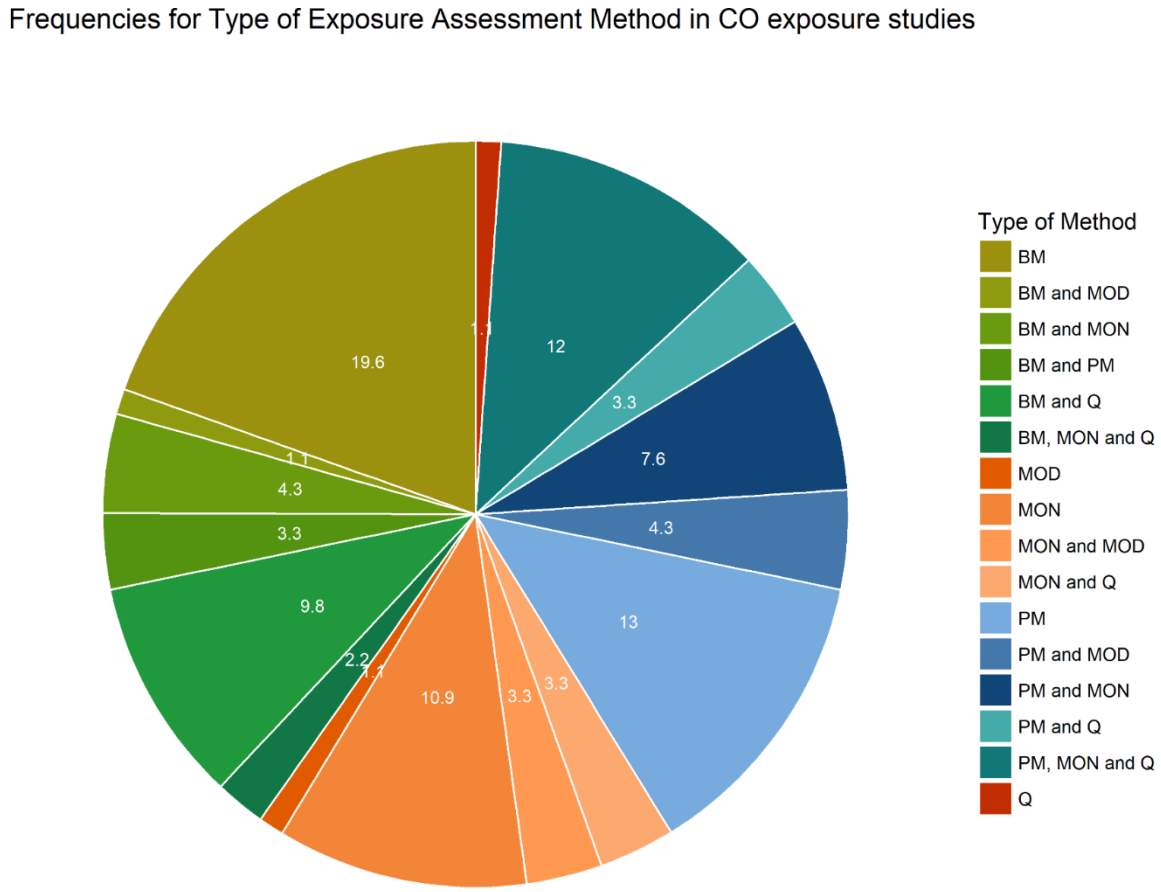

Figure A5.1: Summary of frequencies for exposure assessment methods in CO exposure studies identified in literature review; BM: biological monitoring, MON: environmental monitoring, PM: personal monitoring, Q: questionnaire, MOD: environmental modelling.

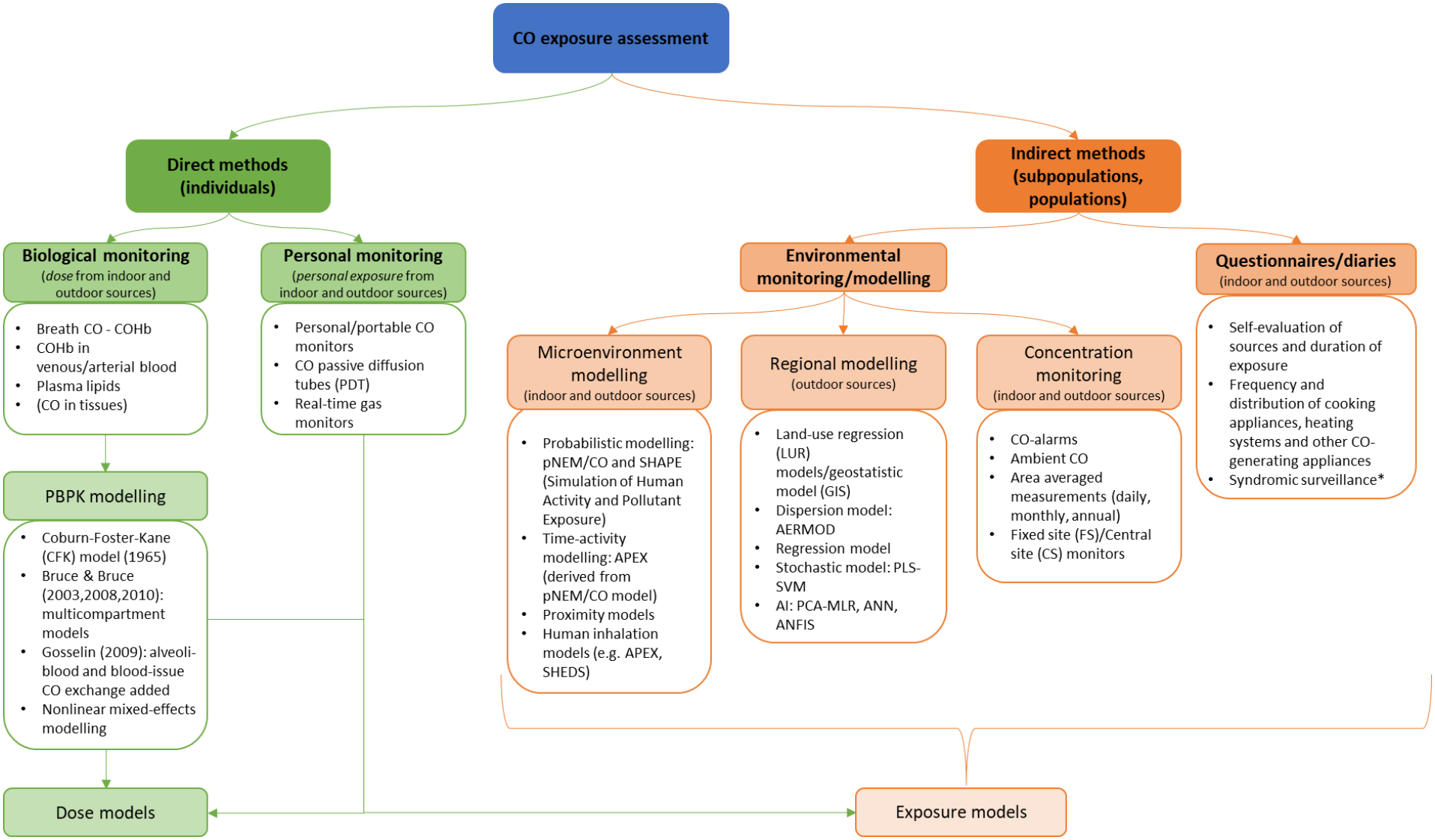

Figure A5.2: Overview for conceptual map of methods used in CO exposure assessment studies, based on investigated literature.

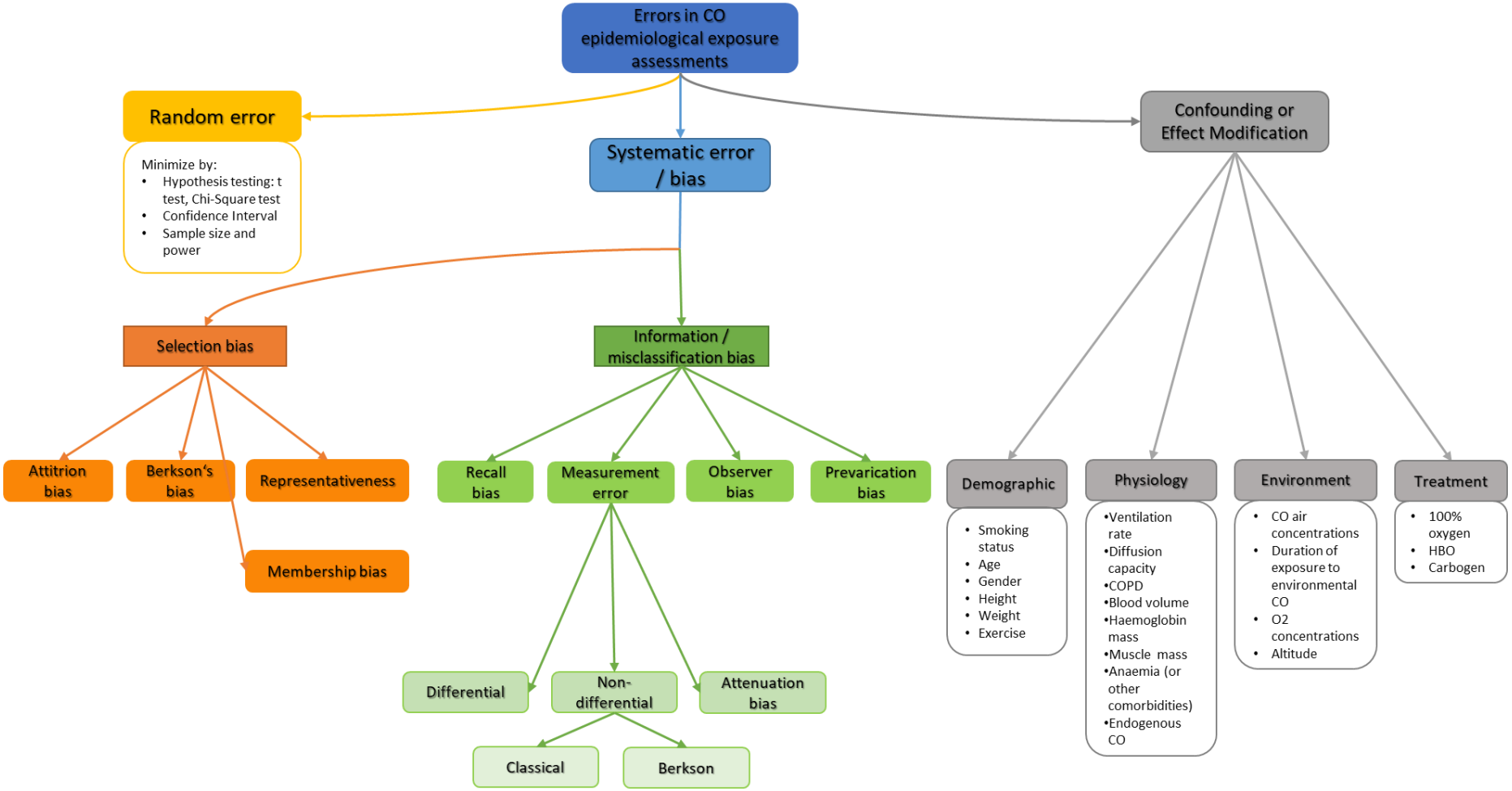

Figure 5.3: Overview conceptual map of errors in CO exposure assessments.

Table A5.2: Overview of results for calculation process to determine the impact of classical measurement error improvement on the relative risk (RR). See protocol in chapter 5 for detailed description of variables and how they are derived/calculated.

EMM	Set	σ_T	σ_X	σ^2_X	σ^2_E	σ_E	σ_{ECR}	σ_{ECRImp}	$\sigma_{ENetImp}$	$\sigma^2_{ENetImp}$	σ^2_{Eimp}	σ^2_{Ximp}	ρ_{XX}	ρ_{XXimp}	$\%\rho_{XXImp}$	RR_X	RR_{Ximp}	$\%RR_{Imp}$
COHb via pCO	1	5	9.07	82.23	57.23	7.56	0.05	0.80	0.96	0.92	52.74	77.74	0.30	0.32	5.46%	1.22	1.234	1.15%
	2	5	9.07	82.23	57.23	7.56	0.05	0.50	0.98	0.95	54.40	79.40	0.30	0.31	3.44%	1.22	1.229	0.71%
	3	5	9.07	82.23	57.23	7.56	0.05	0.20	0.99	0.98	56.09	81.09	0.30	0.31	1.38%	1.22	1.223	0.28%
	4	5	9.07	82.23	57.23	7.56	0.10	0.80	0.92	0.85	48.44	73.44	0.30	0.34	10.69%	1.22	1.249	2.41%
	5	5	9.07	82.23	57.23	7.56	0.10	0.50	0.95	0.90	51.65	76.65	0.30	0.33	6.79%	1.22	1.238	1.46%
	6	5	9.07	82.23	57.23	7.56	0.10	0.20	0.98	0.96	54.96	79.96	0.30	0.31	2.76%	1.22	1.227	0.57%
	7	5	9.07	82.23	57.23	7.56	0.20	0.80	0.84	0.71	40.38	65.38	0.30	0.38	20.49%	1.22	1.284	5.26%
	8	5	9.07	82.23	57.23	7.56	0.20	0.50	0.90	0.81	46.36	71.36	0.30	0.35	13.22%	1.22	1.258	3.08%
	9	5	9.07	82.23	57.23	7.56	0.20	0.20	0.96	0.92	52.74	77.74	0.30	0.32	5.46%	1.22	1.234	1.15%
	10	5	9.07	82.23	57.23	7.56	0.30	0.80	0.76	0.58	33.06	58.06	0.30	0.43	29.40%	1.22	1.325	8.63%
	11	5	9.07	82.23	57.23	7.56	0.30	0.50	0.85	0.72	41.35	66.35	0.30	0.38	19.31%	1.22	1.279	4.87%
	12	5	9.07	82.23	57.23	7.56	0.30	0.20	0.94	0.88	50.57	75.57	0.30	0.33	8.10%	1.22	1.242	1.77%
	13	5	9.07	82.23	57.23	7.56	0.50	0.80	0.60	0.36	20.60	45.60	0.30	0.55	44.54%	1.22	1.431	17.32%
	14	5	9.07	82.23	57.23	7.56	0.50	0.50	0.75	0.56	32.19	57.19	0.30	0.44	30.45%	1.22	1.331	9.10%
	15	5	9.07	82.23	57.23	7.56	0.50	0.20	0.90	0.81	46.36	71.36	0.30	0.35	13.22%	1.22	1.258	3.08%
	16	5	9.07	82.23	57.23	7.56	0.70	0.80	0.44	0.19	11.08	36.08	0.30	0.69	56.12%	1.22	1.573	28.96%
	17	5	9.07	82.23	57.23	7.56	0.70	0.50	0.65	0.42	24.18	49.18	0.30	0.51	40.19%	1.22	1.394	14.30%
	18	5	9.07	82.23	57.23	7.56	0.70	0.20	0.86	0.74	42.33	67.33	0.30	0.37	18.12%	1.22	1.275	4.50%
COHb via exCO	19	5	7.60	57.82	32.82	5.73	0.05	0.80	0.96	0.92	30.25	55.25	0.43	0.45	4.45%	1.22	1.231	0.93%
	20	5	7.60	57.82	32.82	5.73	0.05	0.50	0.98	0.95	31.20	56.20	0.43	0.44	2.80%	1.22	1.227	0.58%
	21	5	7.60	57.82	32.82	5.73	0.05	0.20	0.99	0.98	32.17	57.17	0.43	0.44	1.13%	1.22	1.223	0.23%
	22	5	7.60	57.82	32.82	5.73	0.10	0.80	0.92	0.85	27.78	52.78	0.43	0.47	8.72%	1.22	1.243	1.92%
	23	5	7.60	57.82	32.82	5.73	0.10	0.50	0.95	0.90	29.62	54.62	0.43	0.46	5.53%	1.22	1.234	1.17%
	24	5	7.60	57.82	32.82	5.73	0.10	0.20	0.98	0.96	31.52	56.52	0.43	0.44	2.25%	1.22	1.226	0.46%
	25	5	7.60	57.82	32.82	5.73	0.20	0.80	0.84	0.71	23.16	48.16	0.43	0.52	16.71%	1.22	1.270	4.07%
	26	5	7.60	57.82	32.82	5.73	0.20	0.50	0.90	0.81	26.58	51.58	0.43	0.48	10.78%	1.22	1.250	2.43%
	27	5	7.60	57.82	32.82	5.73	0.20	0.20	0.96	0.92	30.25	55.25	0.43	0.45	4.45%	1.22	1.231	0.93%
	28	5	7.60	57.82	32.82	5.73	0.30	0.80	0.76	0.58	18.96	43.96	0.43	0.57	23.98%	1.22	1.299	6.47%
	29	5	7.60	57.82	32.82	5.73	0.30	0.50	0.85	0.72	23.71	48.71	0.43	0.51	15.75%	1.22	1.266	3.79%
	30	5	7.60	57.82	32.82	5.73	0.30	0.20	0.94	0.88	29.00	54.00	0.43	0.46	6.61%	1.22	1.237	1.42%
	31	5	7.60	57.82	32.82	5.73	0.50	0.80	0.60	0.36	11.82	36.82	0.43	0.68	36.33%	1.22	1.367	12.01%
	32	5	7.60	57.82	32.82	5.73	0.50	0.50	0.75	0.56	18.46	43.46	0.43	0.58	24.83%	1.22	1.303	6.79%
	33	5	7.60	57.82	32.82	5.73	0.50	0.20	0.90	0.81	26.58	51.58	0.43	0.48	10.78%	1.22	1.250	2.43%
	34	5	7.60	57.82	32.82	5.73	0.70	0.80	0.44	0.19	6.35	31.35	0.43	0.80	45.77%	1.22	1.443	18.28%
	35	5	7.60	57.82	32.82	5.73	0.70	0.50	0.65	0.42	13.87	38.87	0.43	0.64	32.78%	1.22	1.344	10.18%
	36	5	7.60	57.82	32.82	5.73	0.70	0.20	0.86	0.74	24.27	49.27	0.43	0.51	14.78%	1.22	1.263	3.51%

Table A5.3: List of abbreviations and descriptions of parameters used for calculation of measurement error improvement.

Abbreviation	Name
EMM	Exposure measurement method
Set	Combination of variables over different ranges
σ_T	True standard deviation
σ_X	Total observed standard deviation
σ^2_X	Total observed variance
σ^2_E	Variance of the error
σ_E	SD of the error
$\sigma_{Econrel}$	Modifiable fraction of relative contribution to error SD
$\sigma_{Econrelimp}$	Fraction of $\sigma_{Econrel}$ reduced in improved x
$\sigma_{ENetImp}$	Net remaining fraction of error SD in improved x
$\sigma^2_{ENetImp}$	Net remaining fraction of error variance in improved x
σ^2_{Eimp}	Improved error variance
σ^2_{Ximp}	Improved variance of x
ρ_{XX}	Reliability coefficient
ρ_{XXimp}	Improved reliability coefficient
RR_X	Observed relative risk
RR_{XImp}	Improved relative risk
$\%RR_{Imp}$	Relative improvement of RR

Chapter 6 - General Discussion and Conclusions

6.1 Discussion

With this doctoral research, some of the most pressing concerns related to CO poisonings were addressed, which include frequent misdiagnoses and unsuitability of current measurement methods, especially for low-level CO exposures, that lead to underreported morbidity and mortality rates as well as errors in the measurement. Consequently, these lead to underestimating the true risk that CO poses on the population and the part it contributes to the global burden of disease (for further details, see Chapters 1 and 5).

The main approach was to first investigate the literature on CO exposures and the methods adopted for quantification, in order to obtain an overview of the current state-of-the-art and identify issues related to these methods from an analytical point of view (Publication 1[1]). The main and most commonly employed biomarker of exposure is COHb [2, 3]. This is due to the high-affinity Hb has for CO as well as the simplicity in measuring it in an individual, which is through a spectrophotometric technique [4]. The use of a spectrophotometer is more simple, easy and cheap compared to GC methods, and, thus, the current method of choice to determine levels of CO exposure in a subject (Publication 1 [1]). However, several issues were identified related to the use of an optical quantification method. One main problem when measuring a biological sample based on its optical appearance is that biochemical alterations tend to occur as soon as the biospecimen leaves the *in vivo* status and becomes *in vitro* and also during transport and prolonged storage of the sample [5, 6]. This alters the optical sample quality, affecting the results obtained by measurement with a spectrophotometer, which is the main instrument used in the majority of EDs (BGA) and forensic laboratories (CO-oximeter/UV-spectrophotometer). Therefore, these results might give an inaccurate account of the real COHb concentration present in the sample analysed. When looking at a sample from a patient in the ED, this could lead the main clinician to a misdiagnosis or to the wrong necessary therapeutic action, potentially putting the patient at risk of further poisoning. In forensic cases, inaccurate results can have legal consequences, since different levels of COHb can determine whether CO

poisoning was the main cause of death, whether it contributed to death or whether it was unrelated to the fatality [7, 8].

Another crucial point in question of the use of COHb as the main biomarker of CO exposure that was identified is the fact that the assumptions behind its use as a biomarker have not been fully elucidated and might be lacking some important information. Several studies have shown that CO binds to Hb as well as to many other hemoproteins in the body; therefore, the main toxicity mechanism of CO is not only through reduced oxygen-transport but also through other pathways (see Figure 1.2 in Chapter 1 for more details). This has raised several discussions regarding the amount of CO bound to Hb versus the amount of CO in free form in blood that is transported directly to the tissues and their relative contribution to CO toxicity. Some research groups have suggested that the amount of free CO might be higher than previously reported and, thus, might contribute to a higher extent than assumed so far to CO toxicity [9–11]. Groups lead by Bruce and Bruce [12, 13] and Gosselin *et al.* [14] have been working on determining these amounts by setting up a toxicokinetic compartment-based model for the absorption, distribution, metabolization and elimination of CO in the body, which was also intended to improve the main model introduced in the 1960s by Coburn, Foster and Kane [15]. No successful quantifications of free CO were reported, even though several groups have proposed CO measurement through the use of GC methods, such as Collison *et al.* [16], Vreman *et al.* [17], Cardeal *et al.* [18], and Varlet *et al.* [19]. In these studies, CO was quantified through GC-RGA, GC-FID or GC-MS and back calculated to equivalent reference COHb levels, which solved the problems related to sample quality, but not the one regarding misdiagnoses and underestimations. One conceptual error that I identified in them is their intention to relate the measured CO to COHb levels, which on the one hand is sensible considering that COHb is the main biomarker of CO exposure, but on the other hand, does not get closer to the true CO exposure levels. The main issue in the majority of these studies is that to obtain a correlation between CO and COHb, they flush the calibrators prior to analysis to remove the excess amount of CO in the blood sample. Since we are interested in exactly this 'excess' amount of CO, I have investigated alternative biomarker and measurement methods for the determination of CO in biological samples that would be able to determine not only CO bound to Hb but also free CO.

For this purpose, I have taken the GC-based studies as a starting point for the development of an alternative measurement method. During this phase, several factors were taken into consideration, including the quantities of sample and reagents (liberating agent, calibration

solution, internal standard solution, etc.) required to obtain sufficient accuracy and reliability, sample preparation steps (heating time, heating temperature, type of releasing agent, type of calibrator, type of internal standard, etc.) and instrumental conditions (GC temperature, run time, etc.). The analytical method developed (**Figure 6.1**) is based on AGS-GC-MS and was optimized in the initial phase of this research project and then validated according to the guidelines of a scientific society (SFSTP) (see Chapter 3).

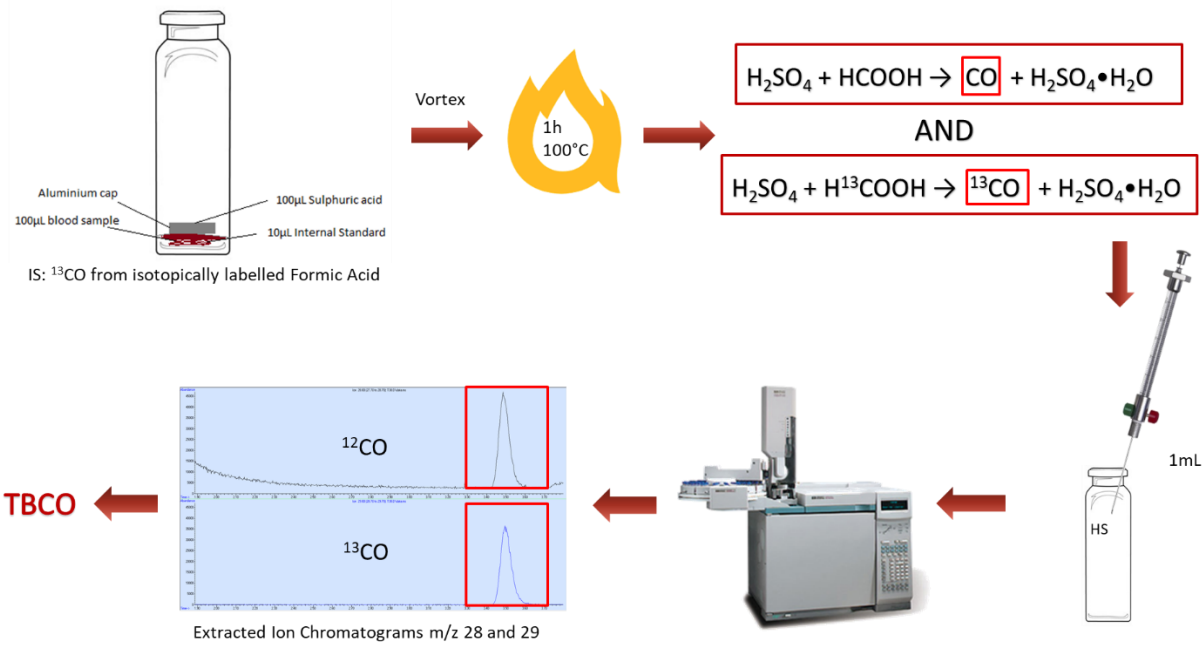

Figure 6.1: Overview of developed analytical method to determine TBCO in blood via AGS-GC-MS. Procedure: 100µL of the blood sample to be analysed are inserted into a 20mL HS vial, followed by 10µL of IS solution (isotopically labelled formic acid; for calibration samples, formic acid solution is also added in required concentrations); 100µL of sulphuric acid are inserted into an aluminium cap, which is then carefully inserted into the HS vial and hermetically sealed; the vial is vigorouslyy vortexed prior to heating for 1h at 100°C; during this time, the reaction taking place is that the sulphuric acid not only liberates the CO from the blood (both bound to Hb and unbound), but also generates ^{13}CO from the IS solution, which is used for quantification; 1mL of the HS is sampled with an AGS and analysed via GC-MS; quantification of TBCO occurs through the ratio of CO to ^{13}CO.

Given the broad concentration spectrum of CO, which can extend from clinically relevant to fatally toxic concentrations, and the general difficulty of quantification methods to be linear over a long concentration range, the method was validated separately for clinical (2-60 nmol/mL HS/0.5-15µmol/mL blood) and postmortem (PM) (10-200 nmol/mL HS/2-40µmol/mL blood) ranges. Similar to real-life cases, where CO concentrations found in some clinical cases were fatal in other ones and vice versa, the ranges overlap. Compared to previous GC-based studies, this quantification method for CO has improved the method's sensitivity (lower LOD and LOQ) and lowered the cost through reduction of reagents required for analysis (100µL of H_2SO_4 used as liberating agent and reagent to generate the IS) as well as the quantity

of sample required (200µL to 100µL). The latter is especially relevant for forensic cases, where sample availability might be limited. The main novelty of this method is the introduction of a novel biomarker, TBCO, which is derived from the method's ability to measure the total amount of CO in blood, as opposed to only CO bound to Hb. This is an essential step since it provides a valuable tool with the potential to get closer to the true CO levels in a blood specimen, which to some extent should reflect the subject's exposure levels.

To determine whether the validated methods are applicable to real cases, a number of PM and clinical samples were obtained and their CO concentrations determined via the newly developed AGS-GC-MS method and compared to CO-oximetry (Publication 2 [20]). Results confirmed the successful application of the novel approach to real cases. However, a concern that might arise when comparing the two methods is the different units they are measured in; while COHb is measured as a percentage, TBCO is measured as a concentration in µmol/mL. For this purpose, several of the previously mentioned studies developed and proposed formulae to back calculate the obtained CO concentration into COHb% levels [17, 18, 21]. As an example, I have tested the formula proposed by Cardeal *et al.* [18] by inputting the TBCO concentrations obtained for some PM cases with the novel method and comparing the results to the spectrophotometric measurements. Results showed that for all cases, the equivalent COHb% concentrations obtained were significantly above 100%, which from a pathophysiological perspective is not very sensible. The problem with these formulae is that flushed calibrators were used for their development and, thus, are not applicable to our measurements that quantify the total CO amounts. Nevertheless, what these tests proved is that the quantity of CO measured with the novel approach is higher than when measuring COHb only.

The subsequent step was to quantify this difference, which corresponds to the free CO quantity. This was achieved by obtaining access to samples from a study in which the subjects were exposed to a known amount of CO. Blood samples were obtained before and after the exposure, to determine the baseline CO levels in the study individuals and then confirm the total increase of CO through the exposure. After the exposure, two samples were collected, of which one was directly analysed, while the other was flushed with a N_2 stream prior to analysis. With this approach, I was able to measure the amount of CO bound to Hb (with flushing) and the amount of TBCO (no flushing) and determine the free CO part by calculating the difference of the two (Publication 3 [22]). The results confirmed that in all study subjects, significant amounts of CO were present in free form, which ranged from 10% in some individuals to most extreme values

such as 80%. This is the first successful quantification of free CO, which can have significant impacts on the diagnosis of CO poisonings when supported by instrumental analysis.

However, I must recognize that the number of samples investigated was limited for both clinical and PM cases; it can be argued that sufficient study power was not achieved. Considering that CO poisoning cases in a small country such as Switzerland are not very frequent, and less frequent in only a part of the country (canton Vaud), it is not surprising that higher sample size was not achieved. A longer study period could have been an option to increase sample size, but it is recommendable to perform this kind of study in other countries and world areas where the population and CO poisoning incidence are higher, such as Mediterranean countries in Europe and developing or less developed countries in Latin-America, Asia or Africa. In these countries, the number of CO poisoning cases is higher not only due to the larger population size but also due to the more widespread usage of gas-, wood- and fuel-based cooking stoves and heating systems, less access and popularity of motor vehicles with a gas filter as well as less ventilated facilities, such as underground parking lots, where CO is accumulated very rapidly [23]. The major steps into improving these issues on a more global level would include the implementation of air quality guidelines, more frequent and controlled security and maintenance regulations and more substantial support into generating prevention strategies. Since these take more time and involve more cost, a first step could be represented by improving the diagnosis and appropriate treatment of CO poisoning cases, which makes this study highly valuable for these countries. Another research group in Colombia has employed our GC-MS method for a group of bus drivers and confirmed the concentration range I found in my clinical samples, further substantiating the applicability and importance of measuring TBCO (*unpublished data*).

Another aspect to contemplate is the feasibility from a technical and cost efficiency perspective of a GC-MS instrument as part of an ED or forensic laboratory. Most hospitals do not have the manpower and resources to buy a GC-MS instrument and train personnel on how to use it. Additionally, it is much faster and cheaper to obtain results from a BGA than a GC-MS analysis (see Publication 1 [1]). Currently, I do not have evidence that is strong enough to support a permanent switch from COHb to TBCO, but it is recommendable to use confirmation by GC-MS in doubtful cases, for example when results reported by the BGA and symptoms shown by the patient are in disagreement. Many hospitals have collaborations with universities and other laboratories, where the samples can be sent to for confirmatory analysis. From a forensic perspective, GC-MS and gas analysis are routinely part of most forensic laboratories and,

therefore, there are only minimal costs associated with the addition of CO quantification, which is highly recommended.

To further investigate another concerning point identified in the literature review, the effects that changes originating from storage under different conditions and for prolonged periods have on the results obtained for the newly developed method and the routine spectrophotometric one were determined (Publication 4 [24]). The aim hereby was to determine the changes that can be associated to certain storage conditions and storage periods, in order to compare the two methods but also allow the formulation of guidelines to propose for routine usage in laboratories. The parameters tested included different temperatures, preservatives, volumes of tube HS, freeze- and thaw-cycles, reopening cycles and time. Results of this study showed that generally, TBCO is less affected by storage than COHb, with TBCO being more stable during storage over a period of one month. This represents a significant result especially for clinical analyses, where storage if required, does usually not exceed a few days, but in this case, can be supported for at least one month. From a forensic perspective, taking into account that trials have the tendency to be long-lasting, storage might be required for longer periods, such as for one year or more. Therefore, this type of study should be extended to longer periods to confirm their validity in medico-legal circumstances.

Additionally, this study generated correction and prediction formulae with the available dataset. The first is to be used in cases where optimal storage conditions cannot be guaranteed either due to cost or the circumstances surrounding the sampling. The latter is a proposition to back calculate the measured TBCO into COHb, in order to obtain a value that potentially could be used for comparison and understanding purposes. It is clear that both models represent a tentative study based solely on the dataset collected in our study and, therefore, needs to be tested and corroborated by other research groups and in real clinical and PM cases, in order to confirm its validity and robustness. However, this should not undermine the importance of this part of the work, which can be used as a basis for other scientists to continue working in this line of research. Based on the results of this study, I recommend and propose as a guideline for all laboratories performing CO analyses the storage of blood samples for CO analysis in EDTA blood tubes that are at least half-filled and stored in the freezer. In addition, these guidelines should also include a standard operating procedure for CO analysis by GC-MS, which should complement and update the International Organization for Standardization (ISO) standard 27368 from 2008 for CO and hydrogen cyanide that still lists UV-spectrophotometry as standard method for CO determination. This will enable a unified and standardized approach

to CO analysis via GC-MS, as opposed to the variety of approaches presented in the past, thus potentially further reducing the quantification errors deriving from instrumental differences.

A major aspect that the clinical and PM analyses as well as the study of the storage conditions highlight is that there is a significant difference in CO quantifications obtained between the measurement methods but also based on different storage parameters. This means that if using non-optimal storage conditions or using a spectrophotometric approach, errors in the measurement are bound to occur. These measurement errors affect the results of individuals by potentially under- or overestimating them, which leads to the frequently occurring misdiagnoses. However, these errors have implications not only from a clinical and forensic perspective (as shown in the previous studies) but also from a global public health viewpoint. Errors in the individual measurements lead to errors in the association of certain individuals into the exposure group (exposed vs non-exposed) and to errors in the estimation of exposures for groups of individuals (classical measurement error). This then leads to erroneous population exposure estimates, which affects the relative risks for morbidity and mortality caused by CO exposure [25–28]. In order to have an impact on public health, it is thus very important to determine the consequences of different sources of error, such as measurement error, on exposure estimates. Therefore, the issues identified in the experimental studies performed in this doctoral work were applied to data from epidemiologic studies, so that the effect that changes in the measurement method and conditions can have on measurement error could be evaluated. Measurement error is a major fraction of the total errors that affect exposure estimates in epidemiologic studies and, consequently, has the potential to significantly alter relative risks (Chapter 5). To approach this from a methodological perspective, I have first investigated the literature on types of exposure measurements, exposure measurement devices, and sources of error present in CO exposure studies. Considering the general aim of this doctoral thesis to tackle especially chronic low-level exposures to CO, which tend to occur in indoor environments and occupational settings, the focus was put on indoor and occupational CO exposure assessments. Indoor exposure assessments are very complex due to the high amount of factors that need to be accounted for during the study design phase and implemented into the execution of the study and interpretation of the results. Considering that most of the population spends the majority of their time indoors, it makes exposure assessments of indoor environments the more important. In the case of CO exposure, numerous sources of measurement error arising in the different exposure assessment types (biological monitoring, personal monitoring, etc.) were idenfitied, which have been extensively discussed in Chapter

5. Out of the studies identified in the literature review, only 7 studies were found to correspond to our criteria of a valid and robust epidemiological exposure study [29–34]. One of these studies was then selected to perform a follow-up step, which was to use the data from the method development studies (Chapters 3 and 4) and apply them to the sources of measurement error identified in this study [29]. For this purpose, a calculation model based on classical measurement error as described by Armstrong in his paper from 1998 [35] and later improved by Armstrong and Basagana [36] and Emily White [37] in their book chapters was developed. Results of the calculations performed with the developed model and the data from the method and literature review showed that for small error contributions, the relative risk was not altered significantly, but for higher error sources, the risk increased quite substantially. Since no "golden standard" exposure measurement method is available to determine true CO exposure, the calculations were a simulation based on the assumption of a true exposure estimate and do not correspond to exact data. However, this model can be used for future studies and act as a reference for researchers performing CO exposure assessments, since the relative changes observed are likely to be similar or higher for CO exposures. A similar approach can also be applied to exposures to other pollutants; after determining the sources of error and if data from validation or reliability studies are available, calculations of relative risk changes can be carried out on an epidemiological study. Relative risks are the fundamental tool that public health institutes and occupational and environmental safety authorities use to generate air quality guidelines and reference levels of air pollutants in different settings (indoor, outdoor, occupational, urban areas, areas with high density of children or elderly, etc.). Therefore, this study has valuable information that these institutions and authorities can and should make use of in their strategical plans to assess and improve the part that CO exposure poses on the global burden of disease.

6.2 Conclusion and future perspective

Exposure to CO in indoor and outdoor environments and the resulting CO poisonings pose a threat to public health both presently and for the foreseeable future, which is until the use of wood-, fuel- and gas-fired stoves, heating and motor vehicles is still in place. Even though there have been reductions in CO emissions in many countries, CO poisonings still occur and remain a challenging problem for public health authorities. It is therefore essential that research into improving CO exposure assessments and measurements continues, in order to help tackle the issue by reducing the sources and quantity of errors currently present and, thus getting closer to the true exposure estimate. Exposure assessments are the fundamental tool employed by

government bodies to assess the health risks associated with an exposure. They provide the evidence necessary to inform stakeholders and keep them up-to-date, but also to help them inform the population about the dangers posed by exposure to certain contaminants. Despite increasing research carried out in the field of CO exposures, awareness of the health risks associated with it continues to be relatively limited in the general population and, thus, needs to be increased. It is very important that both legislators and citizens are informed on how to handle possible cases of intoxication, how to recognize them and how to minimize the exposure by taking appropriate precautions and regular controls. The best way to obtain the information and data required to provide this evidence is given by performing studies that undertake the issues related to CO exposure from all sides, which involves looking at it from the point of view of the individual as well as from the population perspective.

The work carried out in this doctoral research provided a strategical pathway to approach the CO issue from both points of view: the individual one, in which analytical and methodological errors in the quantification of CO poisoning cases were identified, which have an impact for clinicians and forensic laboratories and can help improve the situation from the 'bottom-up'; the population one, where this study helped identify the errors that can mostly affect the relative risk of CO exposures in the population and quantify the effect that improving a part of these analytical errors has on the exposure estimates, thus ameliorating the problem from the 'top+down'. As one of the first in its kind, this research not only provided the necessary data and information on both the analytical/toxicological and epidemiological issues related to CO exposure, it also employed these findings to generate a linkage between the two fields, allowing us to directly observe the effects that changes in the laboratory can have on the population health.

A lot of work still needs to be carried out to improve CO exposure assessments and measurements even further and a lot more funding needs to go into research in this field to achieve results with even higher impact. Nevertheless, with this work I have established a foundation and provided some valuable tools to help future researchers to continue towards closing the knowledge gap on CO exposure and CO poisonings.

6.3 References

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List of publications

Chapter 2

Publication 1

Title of the publication – What are the limitations of methods to measure carbon monoxide in biological samples?

Authors – Stefania Oliverio[a,b] and Vincent Varlet[c]

Affiliation – [a]Department of Environmental Sciences, College of Health and Life Sciences, Brunel University London, United Kingdom; [b]Forensic Toxicology and Chemistry Unit, University Center of Legal Medicine Lausanne-Geneva, Switzerland; [c]Swiss Human Institute of Forensic Taphonomy, University Center of Legal Medicine Lausanne-Geneva, Switzerland

Journal – Forensic Toxicology (Impact factor: 2.48)

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Contribution – The project and study design were developed by Dr Vincent Varlet and me. The literature review was carried out by me. The manuscript was written by me and developed by me with major support from Dr Vincent Varlet. Figures and Tables in the article were designed and created by me.

Chapter 3

Publication 2

Title of the publication – Carbon monoxide analysis method in human blood by Airtight Gas Syringe – Gas Chromatography-Mass Spectrometry (AGS-GC-MS): relevance for postmortem poisoning diagnosis

Authors – Stefania Oliverio[a,b] and Vincent Varlet[c]

Affiliation – [a]Department of Environmental Sciences, College of Health and Life Sciences, Brunel University London, United Kingdom; [b]Forensic Toxicology and Chemistry Unit, University Center of Legal Medicine Lausanne-Geneva, Switzerland; [c]Swiss Human Institute of Forensic Taphonomy, University Center of Legal Medicine Lausanne-Geneva, Switzerland

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Contribution – The project was developed by Dr Vincent Varlet, Dr Ariana Zeka, Dr Giovanni Leonardi and me. The experimental design was developed by Dr Vincent Varlet and me. The blank matrix (bovine blood) was procured by Dr Vincent Varlet and me. All the analytical aspects, which include the sample preparation, CO-oximetry and AGS-GC-MS analyses, data extraction and data treatment were carried out by me, with support by Dr Vincent Varlet in the initial phase. The validation experiments were carried out by me. Postmortem samples were procured by Dr Vincent Varlet and analysed by me. The manuscript was written by me and developed by me with major support from Dr Vincent Varlet. Figures and Tables in the article were designed and created by me.

Publication 3

Title of the publication – Total Blood Carbon Monoxide: Alternative to Carboxyhemoglobin as Biological Marker for Carbon Monoxide Poisoning Determination

Authors – Stefania Oliverio[a,b] and Vincent Varlet[c]

Affiliation – [a]Department of Environmental Sciences, College of Health and Life Sciences, Brunel University London, United Kingdom; [b]Forensic Toxicology and Chemistry Unit, University Center of Legal Medicine Lausanne-Geneva, Switzerland; [c]Swiss Human Institute of Forensic Taphonomy, University Center of Legal Medicine Lausanne-Geneva, Switzerland

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Chapter 4

Publication 4

Title of the publication – New strategy for carbon monoxide poisoning diagnosis: Carboxyhemoglobin (COHb) vs Total Blood Carbon Monoxide (TBCO)

Authors – Stefania Oliverio[a,b] and Vincent Varlet[c]

Affiliation – [a]Department of Environmental Sciences, College of Health and Life Sciences, Brunel University London, United Kingdom; [b]Forensic Toxicology and Chemistry Unit, University Center of Legal Medicine Lausanne-Geneva, Switzerland; [c]Swiss Human Institute of Forensic Taphonomy, University Center of Legal Medicine Lausanne-Geneva, Switzerland

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