Investigating the use of CO-oximetry for simultaneous measurement of carboxyhaemoglobin and methaemoglobin in post-mortem blood

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DECLARATION

I, Jane Mahlatse Muleya, hereby declare that a study on "**Investigating the use of CO-oximetry for simultaneous measurement of carboxyhaemoglobin and methaemoglobin in post-mortem blood**" presented hereby to the University of Cape Town, for the Master of Philosophy in Biomedical Forensic Science, is based on my original work (except where acknowledgements may indicate otherwise). Neither the whole work or part thereof has been, is being or is to be submitted for a degree in this or any other university. It is my own work in design and execution, and that all the material contained herein has been duly recognised.

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LIST OF ABBREVIATIONS AND SYMBOLS

%	percentage
µL	microliter
CO	carbon monoxide
COHb	carboxyhaemoglobin
EDTA	ethylenediaminetetraacetic acid
FTU	forensic toxicology unit
Hb	haemoglobin
HHb	reduced haemoglobin
HREC	Human Research Ethics Committee
MetHb	methaemoglobin
mL	millilitre
$NADH_2$	nicotinamide adenine dinucleotide (reduced form)
$NADPH_2$	nicotinamide adenine dinucleotide phosphate (reduced form)
NR	no result
^0C	degrees Celsius
O_2Hb	oxyhaemoglobin
SHb	sulfhaemoglobin
SRM	Salt River Mortuary
tHb	total haemoglobin
UCT	University of Cape Town

ABSTRACT

Carbon monoxide (CO) is a gas that exerts its toxicity on humans, when inhaled, by bonding with haemoglobin (Hb) to produce carboxyhaemoglobin (COHb). This results in tissue hypoxia which can be fatal at high blood saturation levels. Carboxyhaemoglobin may be measured using a Radiometer ABL825 FLEX analyser, a spectrophotometric instrument that applies a technique called CO-oximetry to measure Hb derivatives such as COHb and methaemoglobin (MetHb). The latter is an oxidised form of Hb that can cause or contribute to mortality at high concentrations. Methaemoglobin is notorious for its instability *in vitro*. This study, therefore, sought to determine handling conditions best suited for the stability of MetHb in post-mortem blood such that the ABL825 FLEX analyser may be used for the simultaneous measurement of COHb and MetHb. To this end, blood samples collected from 15 cases of potential CO poisoning at Salt River Mortuary were aliquoted into red (no additive)-, green (containing lithium heparin)-, and purple (containing ethylenediaminetetraacetic acid)-top tubes. The samples were stored at 4°C or –80°C and retrieved from storage on days 0, 1, 4, 7, 14, and 30, for analysis. While COHb remained stable in all storage conditions over a 30-day period, this was not true for MetHb. When samples were stored at 4°C, a rapid increase followed by a gradual decline was observed for MetHb in all the tube types investigated. The MetHb content was at its lowest after two weeks of storage, which was consistent with COHb levels at the same time period and temperature. At –80°C, continuous increase of MetHb was observed, with the samples stored in green-top tubes showing the least amount of overall change from the reference (day zero) values. The study provided useful data regarding the stability of MetHb under the considered storage conditions, the investigators concluded that refrigerating blood samples in either red-, purple-, or green-top tube was suitable for the accurate simultaneous determination of both COHb and MetHb, if the analytes are measured immediately after collection or after two weeks of storage. Given that the nature of post-mortem forensic casework is such that suitable blood specimens are not always available for toxicological analyses, for the second aim of the study, the researchers sought to investigate the suitability of thoracic cavity fluid as an alternative specimen for the measurement of COHb. For this aim, thoracic cavity fluid collected into green-top tubes from the aforementioned cases

was analysed immediately after collection. The results were compared to those obtained from the analysis of blood collected into green-top tubes and analysed on day zero (before storage). The statistical analyses used for this assessment indicated that thoracic cavity fluid would be a suitable alternative to blood for the measurement of COHb using the ABL825 FLEX analyser.

1. CHAPTER ONE: INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction to literature review

A significant number of unintentional poisonings and fatalities reported each year worldwide are a result of carbon monoxide (CO) poisoning (Raub *et al*., 2000). Carbon monoxide is one of the most important hazardous gases in the environment because of its ability to bind to haemoglobin (Hb) with a high affinity. When carbon monoxide binds to Hb, it results in the formation of carboxyhaemoglobin (COHb). The COHb molecule does not bind to oxygen, and this may lead to an unnatural death as a result of hypoxia.

In South Africa, the law stipulates that all unnatural deaths should be investigated to determine the cause of death (Inquest Act 58 of 1959). This is achieved by medical practitioners who perform a post-mortem examination on the decedent's body. In cases of CO poisoning, blood is often collected during the post-mortem examination and submitted for toxicological analysis, to determine the role of gases (such as carbon monoxide), drugs, and other toxic chemicals in the cause of death (Lappas and Lappas, 2016).

The determination of COHb content, which is a biomarker of CO poisoning, is a routine procedure for forensic toxicologists when analysing blood samples from fire-related cases or cases of CO gas exposure (Widdop, 2002). The findings are used to interpret the extent of CO exposure in suspected cases which may include suicides, homicides, car accidents, fire-related fatalities, and industrial accidents (Kunsman, Presses and Rodriguez, 2000). The Radiometer ABL825 FLEX analyser is a spectrophotometric instrument that measures COHb and other Hb derivatives, including methaemoglobin (MetHb), using a technique known as CO-oximetry.

Several reports have found elevated levels of MetHb in addition to COHb in cases of CO exposure. Methaemoglobin is an oxidised form of Hb, which is formed due to exposure to nitrite/nitrate containing substances. Anoxia or, in severe circumstances,

death can occur when blood contains high quantities of MetHb, which cannot bind to oxygen. Acquired methaemoglobinemia is commonly caused by drugs and chemicals such as amyl nitrite, benzocaine, lidocaine and dapsone (Porter, 2008).

COHb stability in various storage circumstances has been thoroughly examined, and the studies all agreed that COHb is stable for a long-time in various storage situations (Ghanem, Rahman and Shabka, 2012; Kunsman, Presses and Rodriguez, 2000; Lim and Tan, 1999). However, research on MetHb preservation, even though the majority were in vitro or employed ante-mortem blood, yielded contradictory results. As a result, it's assumed that MetHb does not remain stable. As a result, the study's goal was to identify storage settings that were acceptable for measuring both analytes.

1.2 Bonds formed by haemoglobin

Haemoglobin (Hb) is a blood tetra-metric protein contained within erythrocytes (red-blood cells), which is responsible for transporting oxygen around the body. Haemoglobin transitions between its functional forms, deoxyhaemoglobin (HHb) and oxyhemoglobin (O_2Hb). Certain compounds (such as CO) can interact with Hb, chemically modifying their structure and compromising the functional and structural stability of Hb. Sulfhaemoglobin (SHb) is formed when the oxygen in the porphyrin ring of Hb is replaced by sulfur, whereas methaemoglobin (MetHb) is formed when certain oxidising compounds oxidate the Hb molecule (Fairbanks and Klee, 1986; Higgins *et al.*, 2008). Elevated levels of dysfunctional haemoglobin limit the oxygen-carrying capacity of arterial blood (Lim and Tan, 1999).

1.3 Carbon monoxide gas and its sources

Carbon monoxide is a colourless, non-irritative, tasteless, and odourless gas that is responsible for more than half of all fatal gaseous poisonings globally (McAllister *et al.*, 2020; Raub *et al.*, 2000). Carbon monoxide is liberated as a by-product into the atmosphere when the incomplete combustion of organic compounds (specifically hydrocarbons) occurs (Olson *et al.*, 2010). The most common source of exogenous CO exposure is smoke inhalation in fires (Thorn and Keim, 1989). Improperly

ventilated or maintained house heating systems, automobile exhaust leaks, faulty or poorly ventilated charcoal gas stoves are other sources of CO (Vevelstad and Morild, 2009; Widdop, 2002). All these sources of CO release varying amounts of CO into the atmosphere. Automobile exhaust fumes can contain up to 10% of CO, and purposeful self-exposure to these fumes in a confined environment is a widespread and highly effective form of suicide (Widdop, 2002).

Many casualties of fire perish not only from heat but from the inhalation of poisonous compounds (such as nitric oxide, cyanide, CO, and phosgene), as well as a reduction in ambient oxygen (Ferrari and Giannuzzi, 2015). Oxygen depletion, inhalation of smoke with black soot and the high temperatures impedes the escape of fire victims due to vision impairment and irritation of the upper respiratory tract (Ferrari and Giannuzzi, 2015).

1.3.1 Exposure to carbon monoxide

When CO gas is inhaled from the surrounding environment into the human body, it is easily absorbed from the lungs into the bloodstream where it binds with Hb to form carboxyhaemoglobin (COHb) (McAllister *et al*., 2020). Carbon monoxide binds to Hb with an affinity that is 200 or 300 times more than that of oxygen (McAllister *et al*., 2020). The binding of CO to Hb results in two sections of the same mechanism, firstly, the percentage of Hb that is already bound to CO is not available to bind and carry oxygen leading to low oxygen where required causing anoxia. Secondly, the presence of CO causes an increase in the affinity oxygen preventing the release of the bound oxygen to tissues, thereby intensifying anoxia (McAllister *et al*., 2020). The binding of CO also prevents Hb from absorbing carbon dioxide (CO_2) from tissues for elimination (Olson *et al*., 2010).

The heart and brain, which are areas of high metabolic activity and high oxygen demand, are particularly susceptible to the resulting hypoxia (Widdop, 2002; McAllister *et al*., 2020). Toxicity is a consequence of cellular hypoxia (Olson *et al*., 2007). This oxygen deprivation can lead to death, depending on exposure time and the

concentration of CO in the atmosphere (Klaassen, 2013). Carbon monoxide also has a high affinity for haemoproteins such as myoglobin in myocardium and skeletal muscle (Coburn, 1979). The gas competes with oxygen for myoglobin, where it causes cardiac dysfunction and hypotension, which result in hypoxia (Widdop, 2002), sometimes followed by death.

1.3.2 Carboxyhaemoglobin as a relevant biomarker in CO poisoning cases

Even though COHb is currently considered the most appropriate biomarker in CO poisonings, recent findings indicate that the COHb biomarker has its drawbacks. Focusing on COHb as a biomarker excludes CO that has been inhaled but is not bound to Hb. The high affinity of CO for haemoglobin has led to the assumption that when CO is absorbed into the bloodstream, most of it binds to the iron moiety in Hb (Oliverio and Varlet, 2020). However, COHb is not the only reservoir of CO; it can also be found in the blood in free form, where it can attach to other haem-containing globins such myoglobin within the muscle and heart (Raub *et al.*, 2000), which can have severe consequences on the central nervous system (Oliverio and Varlet, 2020). Carbon monoxide does not react quickly with Hb. As a result, much of the inhaled CO which is dissolved in plasma gets little time to combine with Hb before blood reaches other susceptible organs (Widdop, 2002).

Oliverio and Varlet (2020) raised issues as to whether the analysis of COHb is the most suitable method for the establishment of CO poisoning, because data suggests that a high percentage of CO is in the free state. In many cases, discrepancies between measured values and reported symptoms have been recorded, casting doubt on COHb's suitability as a biomarker and the reliability and/or accuracy of its measurement (Oliverio and Varlet, 2020). Carbon monoxide in its free state is recognised to play a part in the pathophysiology of CO poisoning, and its effects are more severe than previously thought and should be considered during interpretation (Oliverio and Varlet, 2020).

The COHb biomarker indirectly measures the amount of Hb that is unusable in the blood, whereas the abovementioned studies attempt to estimate the CO in the bloodstream. So, the COHb gives an indication of the amount of corrupted Hb an individual had due to exposure of CO, which in turn helps determine the contribution of COHb to cause of death. Which is why it was utilised in the current study.

In the event of a death, carboxyhaemoglobin measurement is critical for identifying CO as a factor in mortality caused by fires, automotive exhausts, airplane accidents, and home exposures (Olson *et al*., 2010). The high affinity of CO for Hb makes COHb the most appropriate biomarker in carbon monoxide poisoning cases (Oliverio and Varlet, 2020; Ghanem, Rahman and Shabka, 2012). The testing of COHb levels in blood is considered a useful approach for confirming CO exposure in forensic toxicology laboratories (Luchini *et al*., 2009).

1.3.3 Physiological effects at different carboxyhaemoglobin saturation levels in blood

Carboxyhaemoglobin saturation level in blood and the associated clinical effects have been well documented by Widdop (2002), Bleecker (2015) and Kinoshita *et al*. (2017). Carboxyhaemoglobin saturation should be related to the extent to which it affects oxygen supply to tissues (Widdop, 2002). Non-smokers usually have less than 2% of COHb from endogenous production due to haem catabolism, with no associated symptoms (Bleecker, 2015). Smokers may have levels between 10-15% of COHb in blood, owing to the cigarette smoke which contains approximately 4.5% of CO, with no associated symptoms (Raub *et al*., 2000). No statistical significance has been found between light smokers who have COHb levels between 3-8% and heavy smoker's levels, which go up to 15% (Raub *et al*., 2000). Individuals who are exposed to chronic levels of CO at the workplace also show elevated levels of COHb. In fire-related fatalities, COHb saturation levels below 10% generally suggest that the person died before the fire began or shortly following its ignition (Widdop, 2002).

Carboxyhaemoglobin levels greater than 10% are usually indicative of ante-mortem CO inhalation even for light smokers. It therefore provides evidence that the person

was breathing when the fire begun (Widdop, 2002). Except for heavy smokers, carboxyhaemoglobin saturation levels between 10% and 20% are associated with fatigue and headache (Widdop, 2002). At levels between 20% and 30%, blurred vision, severe headache, dizziness, nausea, fainting and vomiting are some of the symptoms observed. Between the levels of 30% and 40%, people experience higher respiratory and cardiac rates, as well as reduced neurological function. Convulsions, impaired neurological and cardiovascular function, and coma occur at levels between 40% and 60% (Kinoshita *et al.*, 2020; Widdop, 2002). When the levels of COHb are between 10% and 50%, CO may be a contributing cause of death in addition to thermal burns and/or traumatic injury.

Levels greater than 50% usually indicate fatal inhalation of CO, however there is great variation between cases and it is possible that levels below 50% may be fatal. Elderly individuals may demise at low levels due to co-morbidities and less tolerance for tissue hypoxia (Kinoshita *et al.*, 2020). Diseases, including respiratory insufficiency, coronary artery disease, and other disorders, may cause mortality, even when levels of COHb are relatively low (Boumba and Vougiouklakis, 2005). Carboxyhaemoglobin saturation levels greater than 70% generally lead to respiratory failure and cardiopulmonary depression, resulting in death (Kinoshita *et al.*, 2020; Widdop, 2002).

Reports suggest that the signs and symptoms of CO poisoning are non-specific (Hampson and Hauff, 2008), because CO exposure mimics many clinical disorders (Ghanem, Rahman and Shabka, 2012). This makes its diagnosis challenging and misinterpretation may have serious consequences (Ghanem, Rahman and Shabka, 2012). It has been reported that many cases are unsuspected and undiagnosed (Hampson and Hauff, 2008), and therefore under-reported, leading to a potentially higher number of deaths that should be attributed to CO (Oliverio and Varlet, 2020). Following an unnatural death in South Africa, forensic practitioners perform a post-mortem examination to determine the cause of death, and there are often signs noted in this process that suggest CO exposure or poisoning.

In fire-related fatalities, black soot may be noted in the mouth, nose, larynx, trachea, and bronchi of the decedent (Saukko and Knight., 2016; Ferrari and Giannuzzi, 2015; Olson *et al.*, 2010; Maeda *et al.*, 1996) in addition to thermal injuries. The presence of carbon particles (black soot/smoke) in the airways and elevated COHb in circulating blood indicates that the victim was breathing when the fire started, which is useful for forensic diagnosis (Saukko and Knight, 2016). Carboxyhaemoglobin production causes blood, organs, and muscle to turn into a cherry-red colour and produces generalised organ congestion (Saukko and Knight, 2016). Pulmonary oedema is also noted (Maeda *et al.*, 1996). To confirm that CO exposure caused or contributed to the fatality, blood is routinely collected and submitted to the toxicological laboratory where it is analysed for COHb saturation.

1.3.4 Stability of carboxyhaemoglobin in blood

The common practice is to store post-mortem blood samples either in a freezer or fridge with the addition of anticoagulant (Oliverio, 2020). Various storage conditions have been studied and reported as being able to stabilise the COHb saturations in blood samples. Kunsman and colleagues (2000) displayed that the type of preservative used (no additive, ethylenediaminetetraacetic acid (EDTA) or sodium fluoride), the initial percentage of COHb and the volume of headspace did not significantly impact COHb levels in post-mortem blood samples refrigerated at 4˚C for two years. This was in agreement with results from Widdop (2002), who demonstrated that the anticoagulants lithium heparin, EDTA, fluoride heparin and potassium oxalate, had no effect on the stability of COHb.

An *in vitro* study on post-mortem blood demonstrated that COHb saturation in blood collected in capped vials and stored either at room temperature or at 4˚C remained constant over a period of 4 months with changes below 8% (Ocak, Valentour and Blanke, 1985). A decrease in COHb saturation was observed when samples were stored in tubes that were not tightly sealed (Ocak, Valentour and Blanke, 1985). Lim and Tan (1999) investigated the stability of *in vitro* COHb in blood samples refrigerated (4˚C) with anticoagulants (lithium heparin, dipotassium EDTA and fluoride/heparin) for 18 hours. The COHb levels in all storage conditions did not show any appreciable

change over the 18-hour period. After 2 hours the COHb content dropped to 96-98% of the initial value and after 18 hours this dropped to 90-95% (Lim and Tan, 1999).

Hampson and Hauff (2008) explored the stability of COHb saturation in ante-mortem blood collected in standard sample tubes containing sodium heparin. The tubes were stored either at room temperature (22˚C) or refrigerated (4˚C) over a period of 28 days. The results obtained suggested that COHb levels remained stable in the storage conditions over the study period. Ghanem and colleagues (2012) demonstrated insignificant change in COHb saturation levels after refrigerating ante-mortem blood samples collected in heparinized tubes over a 3-year period. These results agreed with those obtained by Kunsman and colleagues (2000) and Hampson and Hauff (2008) where the second analysis was within the range of the first analysis, giving the same interpretation even though the studies conducted by Hampson and Ghanem employed ante-mortem blood.

1.3.5 Effect of anticoagulants on COHb

Ethylenediaminetetraacetic acid (EDTA) is an anticoagulant with the potential of lowering sample pH, altering the binding of CO to Hb and resulting in an alteration of COHb saturation levels (Hampson and Hauff, 2008). Heparin is a widely used anticoagulant despite its high cost and short-lasting action (Olivero and Varlet, 2020). Sodium fluoride is a weak anticoagulant with an effect that diminishes during prolonged storage, therefore increasing the chance of blood clotting and reducing its quality. This may result in a decrease in COHb as seen in the study conducted by Olivero and Varlet (2020), who investigated the effects of storage parameters (such as preservatives) and the level of initial COHb saturation over the period of one month. This study revealed that preservatives had greater effect in blood with higher COHb levels. For blood stored in lithium heparin and sodium citrate, no consistency was observed between saturation levels (low, mild, and high COHb levels) and the effect of the preservative (Olivero and Varlet, 2020). The greatest influence of lithium heparin on blood was observed at medium (30–40%) COHb levels while an increase was noted for higher and lower COHb saturations.

1.3.6 Additional factors that affect carboxyhaemoglobin saturation

In addition to storage parameters there are other factors such as putrefaction and thermocoagulation, that also affect the COHb saturation. This may be attributed to the breakdown of haem during decomposition. Carboxyhaemoglobin saturation levels are lower in victims of open-air petrol or flash fires (Saukko and Knight, 2016). Maeda and colleagues (1996) demonstrated that COHb levels in heart blood collected from victims of open-air fires tend to be below 30%; those of house-fire victims, on the other hand, varied from case to case, suggesting a varied contribution of heat and smoke inhalation. Thermocoagulation from samples that are subjected to heat results in decreased total soluble haemoglobin, leading to either an over- or under-estimation of COHb at the time of death. Elevated lipid concentrations and incomplete haemolysis influence the turbidity of blood samples, leading to inaccurate results. However, this can be reduced by using more refined instrumentation for analysis (Widdop, 2002).

If cases are reinvestigated, and re-analysis is required, or results are challenged, specimens collected for COHb saturation should be stored in conditions that maintain COHb saturation or result in minimal change that will not influence the interpretation of the result. It is important to be knowledgeable about the strengths and limitations of the technique utilised to quantify the COHb saturation levels to accurately interpret the toxicological results, as they may have major implications in judicial proceedings (Ghanem, Rahman and Shabka, 2012; Boumba and Vougiouklakis, 2005). The reliability of an analytical method is critical for proper interpretation of results (Luchini *et al.*, 2009).

1.4 Analytical techniques for COHb measurement

Conventional spectrophotometry and gas chromatography are some of the methods used for identifying and quantifying CO or COHb (World Health Organisation,1999). Spectrophotometry, which can be performed using an automated instrument, is the most popular and common method used for measuring COHb (Luchini *et al.*, 2009). In this study, a CO-oximeter (which is an automated spectrophotometer) will be used as method of choice because it is able to analyse blood samples swiftly and with ease, while measuring multiple haemoglobin derivatives.

1.4.1 Principle of CO-oximetric analysis

Haemoglobin derivatives obey Beer-Lambert's law, and this enables the use of the CO-oximeter, which measures the absorbance of blood at specific wavelengths (Kinoshita *et al.*, 2017). CO-oximeters utilise four, or more wavelengths of monochromatic light to quantify the spectral signature of blood to distinguish haemoglobins (Haymond *et al.*, 2005). The composition of the derivatives (oxyhaemoglobin (O_2Hb), methaemoglobin (MetHb), carboxyhaemoglobin (COHb) and deoxyhaemoglobin (HHb)) in blood is important for forensic diagnosis because their presence may indicate exposure to CO or nitrite/nitrate substances. These haemoglobin derivatives are measured simultaneously as fractions of total haemoglobin (tHb) (Kinoshita *et al.*, 2017).

1.4.2 Benefits and limitations of CO-oximetry

CO-oximetry is the technique of preference for measuring haemoglobin derivatives in the forensic setting because: it is rapid, requires little pre-treatment of samples and is automated/semi-automated. It requires a small sample volume, making it a reasonable choice in cases of problematic blood sampling due to charring or blood loss (Kinoshita *et al.*, 2017). However, this technique is not without limitations, which include interferences such as sulfhaemoglobin, lipids, and putrefaction (Kinoshita *et al.*, 2017).

1.5 The relevance of investigating MetHb in cases of CO exposure

Reports have revealed that elevated levels of MetHb are associated with elevated levels of COHb in suspected CO poisoning cases (specifically from fire and exhaust fumes). Reporting the MetHb value may assist the forensic practitioners in determining the cause of death especially in cases where normal COHb levels have been observed.

Ferrari and Giannuzzi (2015) found a linear relationship between COHb and MetHb levels in the blood of fire victims. Elevated levels of MetHb (4.9-31.6%) were reported in fire cases and exhaust fumes poisonings in addition to high COHb levels

(Katsumata *et al*., 1980). In fire-related fatalities, MetHb is formed as a result of heat denaturation, and inhalation of nitric oxides which, in addition to CO, may also contribute to death (Saukko and Knight, 2016). In cases of exhaust fume poisonings, inhalation of nitric oxide may solely be responsible for MetHb production (Katsumata *et al*., 1980). When elevated amounts of nitrogen oxides are inhaled leading to MetHb formation, methaemoglobinemia may result. According to Kumagai *et al.* (2005), methemoglobinemia and CO-haemoglobinemia could coexist in situations of cyanosis caused by vehicle exhaust fumes. Schwerd and Schulz (1978) observed similar results (MetHb 5-37%) in blood collected from casualties of a fire and agreed with Katsumata *et al*. (1980) in claiming that inhalation of nitrogen oxides from burning plastic was responsible for the elevated levels. Inhalation of nitrogen oxides should be taken into consideration when determining the cause of death, especially in cases of poisonings by exhaust fumes and fires (Ferrari and Giannuzzi, 2015; Vevelstad and Morild, 2009, Katsumata *et al*., 1980; Schwerd and Schulz, 1978).

1.6 Formation of methaemoglobin and methaemoglobinemia

Methaemoglobin results when Hb is involved in redox reactions. Redox reactions are so-called because they always occur in pairs, i.e., for one molecule to be reduced, another must be oxidised. Oxidation is the process of extracting electrons from a substrate; if a substance loses one electron, it has been oxidised. The transfer of electrons to a substrate is known as reduction; the substrate has been reduced if it gains an electron (Wright *et al*., 1999). Free radicals, which are highly reactive and unstable molecules (e.g., superoxide), oxidise certain proteins (such as haemoglobin), thereby hindering their function. These reactions lead to the production of more free radicals (Wright *et al*., 1999), resulting in progressive oxidation. The iron moiety in Hb is usually in the ferrous (Fe^{2+}) form and can be oxidised by these free radicals to the ferric (Fe^{3+}) state (Wright *et al*., 1999), resulting in MetHb formation.

Methaemoglobin (MetHb) is a form of haemoglobin that carries a ferric ion in its haem group instead of the normal haemoglobin ferrous ion (Varlet *et al*., 2018). Haemoglobin oxidation occurs both endogenously and exogenously. Diet, genetics, presence of nitrates, chlorates and automobile exhaust gases (which contain nitrogen dioxide and

nitric oxides) are some factors that contribute to MetHb formation (Saukko and Knight, 2015). Oxidizers can be divided into two classes: direct and indirect oxidizers. Direct oxidizers react directly with Hb *in vivo* or *in vitro* to form MetHb. Indirect oxidizers undergo metabolic modifications before they induce the formation of MetHb (Kosaka and Tyuma, 1987). Indirect oxidizers are potent reducing agents that reduce oxygen to superoxide, which, in turn, oxidizes Hb (Wright *et al.*, 1999).

Methaemoglobinemia is a well-known but rare and life-threatening (Velvestad and Morild, 2009) blood disorder that is characterized by abnormal levels of MetHb in blood (Varlet *et al.*, 2018). It can be congenital or acquired (Velvestad and Morild, 2009), and results from exposure to oxidizing agents, inadequate reductase activity or considerable production of MetHb (Varlet *et al.*, 2018). In acquired methaemoglobinemia, the rate of Hb oxidation is accelerated, surpassing the reducing capacity of red blood cells (Lukens, 1999) resulting in elevated MetHb levels. Methaemoglobinemia is also found in individuals with hereditary diseases wherein the MetHb reductase enzyme is structurally abnormal or absent (Young *et al.*, 1994).

1.6.1 Endogenous methaemoglobin production

Within the human body, MetHb is produced under normal physiological conditions because red blood cells are constantly bathed in oxygen, which results in a small quantity of haemoglobin undergoing oxidation (Gehle, 2007). Endogenous oxidation of haemoglobin by molecular oxygen will eventually convert sufficient haemoglobin to MetHb to impair cellular respiration (Wright *et al.*, 1999). Normal oxygenation of Hb triggers a partial transfer of an electron from ferrous iron to oxygen, forming a loose bond. The loose bond results in a ferric state iron and superoxide free radical (Figure 1A). Under normal physiological conditions, the electron is reverted to the iron when oxygen is transferred to the tissues (Figure 1B). Under oxidative conditions, the electron will not be reverted to iron, resulting in MetHb formation (Figure 1C)

(Dean, Looman and Topmiller, 2021).

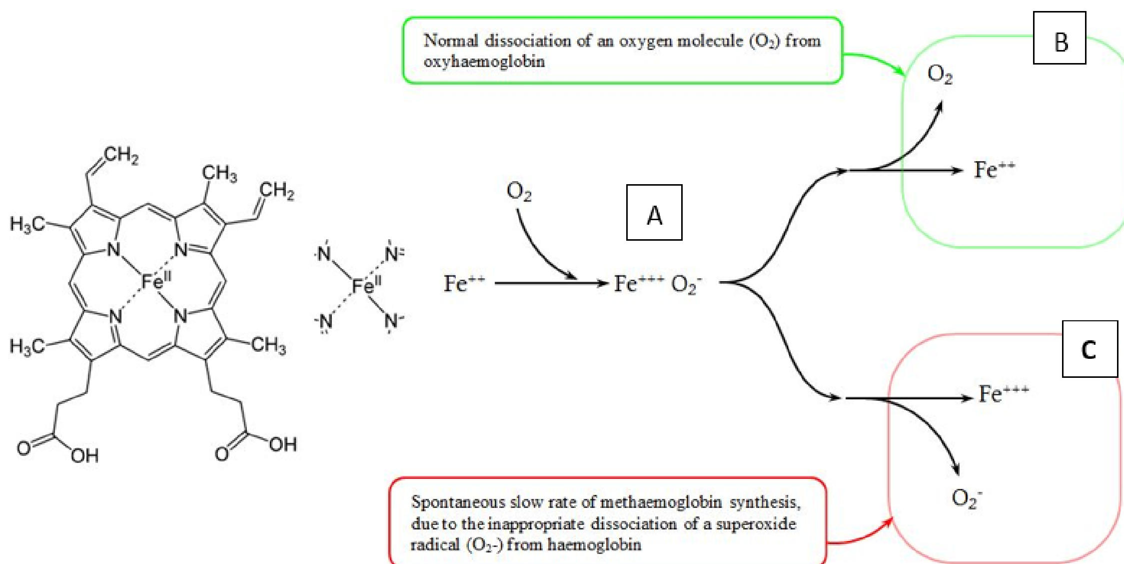

Figure 1.1: Depiction of the formation of methaemoglobin. A). Haem (a prosthetic group of haemoglobin) reacts with oxygen, resulting in the formation of ferric iron and superoxide. B) Under normal conditions, the bond is broken and the oxygen from haemoglobin is released to tissues. C) Under oxidative conditions, the electron will be donated to oxygen, resulting in the spontaneous production of MetHb and superoxide. From Yartsev, 2021. *home | Deranged Physiology*. [online] Derangedphysiology.com.

The cycle of converting MetHb back to Hb occurs spontaneously and regularly within the body under normal conditions to maintain MetHb levels at less than 1% of total haemoglobin in blood (Dean, Looman and Topmiller, 2021). To maintain low MetHb levels in the body, there are numerous systems that hinder oxidation or reduce MetHb back to Hb. The nicotinamide adenine dinucleotide (reduced form) $NADH_2$- and nicotinamide adenine dinucleotide phosphate (reduced form) $NADPH_2$-MetHb reductase systems are the main enzyme systems which account for roughly 95% of everyday MetHb reduction *in vivo* (Wright *et al.*, 1999; Young *et al.*, 1994; Dixon, Reisch and Santinga, 1981). Ascorbic acid and glutathione account for little quantities of reduction (Wright *et al.*, 1999; Young *et al.*, 1994; Dixon, Reisch and Santinga, 1981). Each of these reactions may be compromised by the hereditary inability to synthesize glutathione and by congenital deficiencies of MetHb reductase (Dixon, Reisch and Santinga, 1981).

1.6.2 Toxicity of oxidised haemoglobin

The MetHb reductase systems responsible for reducing MetHb back to Hb may be overpowered in the presence of substantial quantities of oxidants, resulting in elevated MetHb levels (Dean, Looman and Topmiller, 2021). Haemoglobin is a tetrameric molecule, and a four-electron loss will result in complete oxidation (Wright *et al.*, 1999). Oxidation of one of the four haem subunits of the Hb molecule represents a partial oxidation (Wright *et al.*, 1999). The oxidised haem subunit of Hb has a high affinity for negative anions as opposed to uncharged molecules such as CO_2 and O_2 (Wright *et al.*, 1999). The non-oxidized subunits of a partially oxidised Hb will bind more tightly to oxygen than usual, resulting in poor oxygen delivery to tissues and lethal anoxia (Dean, Looman and Topmiller, 2021; Varlet *et al.*, 2018; Young *et al.*, 1994;). The build-up of MetHb and carbon dioxide (due to the incapability of Hb to transport CO_2) causes metabolic acidosis, cyanosis, poor aerobic respiration, and in serious cases, death (Wright *et al.*, 1999).

1.6.3 MetHb relevance in cases of exposure to nitrite and nitrate

There are several ways in which exogenous oxidants enter the human body and cause the oxidation of Hb. Skin exposure and consumption of oxidizing agents are the most common causes of toxin-induced MetHb (Wright *et al.*, 1999). Burning plastic releases nitrogen oxides into the atmosphere, which can cause high MetHb concentrations, if inhaled (Schwerd and Schulz, 1978). Nitrites and nitrates are found in fruits and vegetables, used in pharmaceutical products (to treat cyanide poisoning), available as salts (such as potassium, sodium and ammonium) and also used as anti-microbial agents (Dean, Looman and Topmiller, 2021).

The administration of a class of drugs collectively known as alkyl nitrites also results in the production of MetHb. When the haemoglobin molecule reacts with nitrates or nitrites, it donates an electron, leading to methaemoglobin formation (Umbreit, 2007). The major physiological danger from the alkyl nitrites is methaemoglobinemia secondary to the oxidation of Hb by the nitrite ion (Dixon, Reisch and Santinga, 1981). Amyl nitrite and isobutyl nitrite are members of this class. These recreational drugs

have a high potential for abuse and are popular due to their euphoric effects (Chandrasekaran and Erickson, 2021). Oral ingestion of amyl nitrites produces more rapid and sudden onset of methaemoglobinemia but inhalation is the most common route of administration (Taylor *et al.*, 2021).

Isobutyl nitrites are substances with widespread recreational use during sexual activity (Dixon, Reisch and Santinga, 1981), to enhance sexual pleasure and prolong orgasm (Saito *et al.*, 2000). Isobutyl nitrites are marketed as nail polish in glass-vials (Taylor *et al.*, 2021), or as “room oxidizers” with the instruction that they are not to be inhaled (Saito *et al.*, 2000). Products with isobutyl nitrite colloquially referred to as poppers (Taylor *et al.*, 2021), are sold under various names including “gold rush”, “climax”, “bullet”, “rush”, “bolt” or “thrust”. These drugs contain up to 96% isobutyl nitrite with small amounts of vegetable oil and isobutyl alcohol, rendering the formulation less volatile (Bradberry *et al.*, 1994). Isobutyl nitrite is a volatile gas that is inhaled more often than ingested (Saito *et al.*, 2000; Dixon, Reisch and Santinga, 1981) and is readily absorbed on any body surface (Taylor *et al.*, 2021).

Sodium nitrite is one of the oxidants known to induce methaemoglobinemia when ingested (Mudan *et al.*, 2020). Sodium nitrite is an odorless white crystalline material that resembles and tastes like sodium chloride (table salt) and easily dissolves in water. Sodium nitrite is easily accessable both online and retail because it is a household chemical that is inexpensive and not restricted (Dean, Looman and Topmiller, 2021). It is used as a food preservative and as a curing salt, but when taken alone in large quantities can be lethal (Dean, Looman and Topmiller, 2021). Additionally, it is used as a food coloring agent and antimicrobial in fish, meats and cheeses (Mudan *et al.*, 2020). Sodium nitrite use in suicide cases has been reported, where the substance is bought online in suicide kits that contain 35 grams of sodium nitrite in a packet (Dean, Looman and Topmiller, 2021). Some of the sites even contain explicit step-by-step instructions for committing suicide (Mudan *et al.*, 2020). In cases where sodium nitrite is used to commit suicide, the mechanism of death is severe hypoxia due to increased MetHb (Dean, Looman and Topmiller, 2021).

1.6.4 Physiological effects of MetHb blood levels

Clinical symptoms that are associated with MetHb levels are well recorded by Wright *et al*. (1999) and Olson *et al*. (2007). According to Wright and colleagues (1999), methaemoglobin saturation less than 10% are associated with no symptoms. It is possible to be asymptomatic with MetHb concentrations up to 20%, particularly in individuals with congenital methaemoglobinemia, where 10-20% MetHb is endured with no ill effects (Dixon *et al*., 1981; Young *et al*.,1994). The first symptoms of high methaemoglobin levels include cyanotic skin discoloration (Wright *et al*., 1999). Levels between 20-50% are associated with fatigue, weakness, dyspnoea, tachycardia, weakness, dizziness, cyanosis, vomiting, nausea (Olson *et al*., 2007; Dixon, Reisch and Santinga, 1981). When MetHb levels are greater than 55%, coma can result (Olson *et al*., 2007) and levels greater than 70% are fatal (Olson *et al*., 2007; Dixon, Reisch and Santinga, 1981).

In cases of fatal methaemoglobinemia, blue-grey discoloration of the face and lips of the decedent is often noted at autopsy (Dean, Looman and Topmiller, 2021). Blood that is thin (Dean, Looman and Topmiller, 2021) and chocolate-brown in colour (Dean, Looman and Topmiller, 2021; Mudan *et al*., 2020; Guay, 2009; Saito *et al*., 2000; Dixon, Reisch and Santinga, 1981) may also suggest methaemoglobinemia (usually at 15-20% of MetHb saturation levels).

1.6.5 Stability of MetHb in blood

Although it is known that various factors may contribute to post-mortem formation of MetHb, there is a paucity of literature on recommended storage conditions of blood before spectrophotometric analysis of this analyte. Two main factors that may affect the MetHb saturation include storage temperature and the preservative used for collection of the blood sample. Storage of blood samples at room temperature results in highly unstable MetHb values, with fluctuating and irreproducible results and blood samples getting putrefied after 12 weeks of storage (Domingo *et al*., 2017). The refrigeration of blood (at 4˚C) stored with sodium fluoride, EDTA or without preservative is one of the best ways to prevent increases in MetHb levels for short-term storage (2 weeks). Varlet *et al*. (2018) found that blood samples stored at these conditions resulted in MetHb increases less than 10%. This was supported by

Domingo *et al.* (2017) who found more stable results, where MetHb saturation demonstrated a less than 4% variation of the initial value over a 3-week period.

The study conducted by Lim and Tan (1999) found controversial results when they investigated the stability of MetHb saturation using samples prepared *in vitro*. The samples were aliquoted into vacutainer tubes containing either lithium heparin, dipotassium EDTA or fluoride/heparin and refrigerated (4˚C) for 18 hours. The results demonstrated that MetHb content of blood in lithium heparin, dipotassium EDTA and potassium oxalate tubes decreased gradually with time. The MetHb content dropped to 88-93% after 2 hours, and to 63-69% after 18 hours, compared to the initial value.

Varlet and colleagues (2018) recommend freezing for samples that are stored for extended periods (more than two weeks) because intra-erythrocyte MetHb reductase rapidly degrades MetHb to Hb during prolonged refrigeration, leading to underestimation of MetHb levels. In blood samples that are frozen at –20˚C, autoxidation occurs (Varlet *et al.*, 2018). This was supported by a study conducted by Domingo and colleagues (2017) which revealed a rapid increase of MetHb in blood samples stored at –20˚C, which may be attributed to high autoxidation. When the superoxide is confined within the binding pocket of Hb during freezing, it spontaneously oxidizes Hb, yielding these results. The increase may alternatively be due to the altered state of the tertiary structure of the Hb which cannot protect the haem group against autoxidation (Domingo *et al.*, 2016). However, freezing samples at –80˚C is recommended for long-term storage to promote MetHb stability (Varlet *et al.*, 2018).

Lim and Tan (1999) concluded that MetHb is not stable since it can be converted enzymatically to Hb, and becomes oxyhaemoglobin, if measurement is delayed. MetHb levels have been found to be stable for approximately two hours in lithium heparin, dipotassium EDTA and potassium oxalate (Lim and Tan, 1999). Varlet *et al.* (2018) stated that MetHb has not been studied as a valid post-mortem biomarker because of the random MetHb production. Measured COHb saturation levels

represent ante-mortem COHb levels; however, with MetHb there are doubts related to its value as a suitable biomarker of ante-mortem MetHb due to secondary modifications that occur in bodies following death and during sample processing and storage (Vevelstad and Morild, 2009). Thus, to obtain a clear indication of MetHb levels at the time of death, post-mortem MetHb saturation should be measured in blood where the sampling and storage conditions promote its stability as far as possible (Varlet *et al.*, 2018).

In addition to storage conditions, there are various other factors that may affect the MetHb levels in blood such as haemolysis, freezing, putrefaction, bacterial action, heating, thawing, pH, and temperature (Vevelstad and Morild, 2009). Post-mortem decreases occur because of intracellular enzymatic reduction of MetHb to Hb, which continues after death. However, the enzyme responsible for this can be deactivated by heat (Vevelstad and Morild, 2009). Methaemoglobin is highly unstable due to the post-mortem oxidation of the haem group, and spontaneous reduction of MetHb (Fukui, Yamamoto and Matsubara, 1980). Haemolyzed post-mortem blood samples that are highly contaminated with bacteria as seen during putrefaction show reduction in MetHb levels (Dean, Looman and Topmiller, 2021; Rodney and O'neal, 1974).

For the abovementioned reasons, MetHb levels should be measured immediately after collection, and it is not recommended for samples that are suspected to be contaminated by bacteria. However, if the analysis is delayed, MetHb reductase enzymes should be inhibited (Varlet *et al.*, 2018). Measurement of MetHb in nitrite related cases may be challenging if the involvement of the substance is not known at the time of analysis and requires re-analysis after long-term storage (Domingo *et al.*, 2017).

1.7 Thoracic cavity fluid as an alternative specimen for CO-oximetry analysis

CO-oximetry is a gold-standard method for the determination of COHb in blood (Oliverio and Varlet, 2020) and there is a variety of literature on COHb in blood. In post-mortem forensic cases, blood availability is sometimes limited or not available because the bodies are either decomposed, severely charred, and/or have traumatic injuries that result in major blood loss (Tanaka *et al*, 2015). In such cases, alternative

specimens such as thoracic cavity fluid (bloody fluid collected from the decedent following the removal of the thoracic block), may prove useful. The author investigated COHb levels between blood and body cavity fluid in a putrefied drowned body and found that COHb levels in body cavity fluid were elevated compared to those in blood, which may be due to high CO levels before death and the decomposition of myoglobin and Hb during putrefaction (Kojima *et al*., 1982). Kojima and colleagues concluded that the significance of COHb in thoracic cavity fluid is challenging to interpret without the value of COHb in blood (Kojima *et al*., 1982). Kinoshita *et al*. (2020) advised against the use of cavity fluids for measurements of CO in cases of severe putrefaction. In contrast, Lee *et al*. (2003) found that thoracic cavity fluid is a viable alternative specimen for the determination of COHb. For this reason, the question of the suitability of thoracic cavity as an alternative specimen for COHb determination remains relevant for forensic laboratories and is open for further investigation.

1.8 Knowledge gap and motivation of study

The Forensic Toxicology Unit (FTU) – a toxicology laboratory within the Forensic Pathology Services (FPS) in the Western Cape, South Africa, receives requests from mortuaries in the Western Cape to determine COHb concentration in cases of suspected CO exposure. The FTU uses a Radiometer ABL825 FLEX analyser (a CO-oximeter) for measuring haemoglobin derivatives (oxyhaemoglobin, methaemoglobin, carboxyhaemoglobin, and deoxyhaemoglobin) as fractions of total haemoglobin in blood samples. The technique is currently only validated for measuring COHb saturation in the aforementioned laboratory. One of the haemoglobin derivatives that is measured by the instrument is methaemoglobin (MetHb), an oxidised form of Hb that can cause or contribute to fatality at high concentrations. Elevated levels of MetHb are toxic to humans because they cause tissue hypoxia, which may be fatal. High concentrations of both COHb and MetHb have been observed in cases wherein the victims were exposed to fire or car-exhaust fumes (Kumagai *et al*., 2005). However, MetHb is highly unstable in post-mortem blood (Domingo *et al*., 2017). The FTU laboratory currently does not have a validated procedure in place that specifies optimal conditions for handling post-mortem specimens on which the measurement of MetHb is intended. Considering that the Radiometer ABL825 FLEX analyser measures COHb and MetHb simultaneously, it would be beneficial to determine the conditions that

maintain the stability of both analytes. The establishment of such conditions would allow the laboratory to advise mortuaries in the Western Cape on the correct specimen collection and handling requirements for the determination of potential exposure to substances that raise COHb and MetHb to toxic levels. The development of such a method would benefit the Western Cape province by expanding the CO-oximetry analysis currently on offer to include the measurement of MetHb in cases where the defective haemoglobin derivative may have contributed to death.

Several studies that investigated MetHb (Domingo *et al*., 2017; Ferrari and Giannuzzi, 2015; Rodkey and O'Neal, 1974; Saito *et al*., 2000; Varlet *et al*., 2018; Wallace and Curry, 2002) and COHb (Kojima *et al*., 1982; Oliverio and Varlet., 2019; Sato *et al*., 1990; Tanaka *et al*, 2015; Vevelstad and Morild, 2009) collected samples from either cardiac or peripheral or from both sites. This suggests that the site of collection is not a major factor for COHb/MetHb analysis. This is substantiated by a study conducted by Varlet and colleagues (2018), who found that MetHb measurement is not influenced by the site of collection. It is for this reason that the current study made use of both peripheral and cardiac blood.

The blood samples were stored in three blood-collection tubes, namely red (no additive)-, purple (containing ethylenediaminetetraacetic acid (EDTA))- and green-top (containing lithium heparin) tubes. The green-top tube was selected because it is the tube that is currently being used at the Forensic Toxicology Unit and is also recommended by Radiometer (the manufacturer of the instrument used for COHb analysis). The purple-top tube was investigated as an alternative to the lithium heparin tube. The blood collection tube commonly used for toxicological analyses is the grey-top tube, which contains potassium oxalate (anticoagulant) and sodium fluoride (drug preservative). The study had no specific requirement for drug preservation in COHb/MetHb analysis. Moreover, interference studies performed during the validation of the CO-oximetry method used by the FTU, the grey-top tube was demonstrated to cause COHb underestimation.

In summary, the FTU seeks to determine handling conditions best suited for the stability of MetHb in post-mortem blood such that the ABL825 FLEX analyser may be used for the simultaneous measurement of COHb and MetHb.

Moreover, the nature of post-mortem forensic casework is such that suitable blood specimens may not always be available for toxicological analyses. In such cases, it is ideal to have alternative specimens on which the required analyses may still be performed. There are contradicting reports on the use of thoracic cavity fluid as a viable alternative specimen for the determination of COHb. It is, therefore, imperative to investigate the suitability of thoracic cavity fluid as a potential alternative specimen for the measurement of COHb using the ABL825 FLEX analyser. Thus, in addition to determining the storage conditions best suited for maintaining the MetHb and COHb saturation levels, the study will further investigate the suitability of thoracic cavity fluid as an alternative specimen. This affords the FTU laboratory the possibility of being able to measure COHb (in thoracic cavity fluid) in instances where sufficient femoral or cardiac blood cannot be obtained from a deceased individual.

1.9 Aims and objectives

1.9.1 Aim 1

To investigate the specimen handling conditions that are suitable for the simultaneous measurement of carboxyhaemoglobin and methaemoglobin by CO-oximetry.

1.9.1.1 Primary objectives

1. Collection of blood specimens from suitable cases (with suspected carbon monoxide exposure) at Salt River Mortuary.
2. Aliquoting the collected blood specimens into different types of blood collection tubes:
   a. Green-top tube (containing lithium heparin)
   b. Purple-top tube (containing ethylenediaminetetraacetic acid – EDTA)
   c. Red-top tube (no additive).
3. Storing the samples at different temperatures:

    a. Refrigerated (4˚C)
    b. Frozen (–80˚C).
4. Analysing the samples using the Radiometer ABL825 FLEX analyser on days 0, 1, 4, 7, 14 and 30.
5. Performing data analysis to determine the combination of handling parameters that results in the least concentration variation for COHb and MetHb.

1.9.2 Aim 2

To investigate the suitability of thoracic cavity fluid as an alternative specimen for the measurement of carboxyhaemoglobin.

1.9.2.1 Secondary objectives

1. Collection of blood and thoracic cavity fluid into green-top tubes from cases of suspected CO exposure at Salt River Mortuary.
2. Sample preparation and analysis of the different specimen types on the day of collection, using the Radiometer ABL825 FLEX analyser.
3. Comparison of the COHb results obtained for the two specimen types.

2. CHAPTER TWO: METHOD AND MATERIALS

2.1 Study population

A prospective toxicological analysis study was conducted between February and December 2021 on specimens collected from deceased individuals admitted to Salt River Mortuary (SRM) with suspected carbon monoxide exposure. The SRM is one of 16 mortuaries in the Western Cape, South Africa, and receives cases from the West Metropole of Cape Town. This was a pilot study with a total of 15 cases included to confer statistical significance to the outcome of the project. This study was approved, prior to commencement, by the Human Research Ethics Committee (HREC) at the University of Cape Town, HREC REF:291/2021.

2.2 Inclusion criteria

The post-mortem cases that were considered for inclusion in the study are those for which the nature of the death was suspected to involve exposure to CO. Only cases admitted to SRM were included.

2.3 Exclusion criteria

Minors (persons younger than 18 years old) were not included in the study. Individuals classified by the mortuary as decomposed were excluded from the study. Cases for which there was inadequate blood for the study samples to be collected were also excluded.

2.4 Study site

Specimen collection took place at SRM and analysis of the specimens was performed at the Forensic Toxicology Unit, located in the Department of Pathology, at the University of Cape Town (UCT), Western Cape, South Africa.

2.5 Sample collection

2.5.1 For aim 1:

Femoral or cardiac blood was drawn from the deceased using a sterile syringe (Becton, Dickson and company, New Jersey, USA) and transferred into a no-additive 15 mL blood-collection tube (Wuxi Nest Biotechnology Co., Ltd, Wuxi, Jiangsu, China).

2.5.2 For aim 2:

For each of the selected 15 cases, a sterile syringe was used to aspirate thoracic cavity fluid from the decedent and transfer it into a 4 mL green-top tube containing lithium heparin.

The method of collection that was utilised is one that is already in practice at SRM. The study samples were only collected after the assigned forensic medical practitioner had collected all the specimens required for the case's routine ancillary investigations (as per the Inquest Act 58 of 1959). To track collected specimens and the data subsequently generated, unique identification codes were assigned to each sample after collection. This also served to anonymise and maintain the confidentiality of the study participants. Immediately after collection, all the specimens were placed in a polystyrene box containing ice packs and transported from SRM to the FTU laboratory at UCT, where post-collection processing was performed.

2.6 Post collection processing

2.6.1 For aim 1:

The blood sample collected in a 15 mL tube was aliquoted equally into red-, green-, and purple-top tubes (Zans African Medical, Pretoria, South Africa). The tubes with the colour-coded caps were then vortex-mixed (Gemmy industrial corporation, Taipei, Taiwan) for 40 seconds. From each tube, an air-displacement pipette (Gilson, Inc., Wisconsin, USA) was used to aliquot a volume of 80 µL into a microtube (Inqaba Biotechnical industries (Pty) Ltd, Pretoria, South Africa) containing 240 µL of ultrapure water obtained from the Direct-Q3 UV Water Purification System (Merck Millipore, Massachusetts, USA) for the time (or day) zero (D_0) analysis. Half of the volume of the

sample that remained in each tube was transferred into a 2 mL cryotube (Greiner Bio One Ltd, Kremsmuster, Austria) such that there was a total of six containers with equal volumes of blood: the red-, green-, and purple-top tubes and the three corresponding cryotubes. The cryotubes were immediately stored in an ultra-low temperature freezer (Haier Biomedical Bulle, Switzerland) set to –80˚C and the blood collection tubes were stored in a refrigerator (Leibhrr, Qingdao, Shandong, China) set at 4˚C as depicted in Figure 2. The samples were retrieved from the fridge and freezer for analysis after the following days of storage: 1, 4, 7, 14 and 30.

2.6.2 For aim 2:

The thoracic cavity fluid samples were transported to the FTU laboratory as described above. The samples were analysed immediately upon arrival at the FTU laboratory. This is the only specimen analysis that was performed for aim two. The obtained COHb results were compared to those obtained from the analysis of the blood transferred into green-top tubes and analysed on day zero (D_0).

2.7 Sample analysis

Samples were analysed according to the validated protocol currently being used by the FTU laboratory for routine analysis. Specimens were retrieved from the refrigerator and the freezer and allowed to stand at controlled room temperature (at approximately 22°C maintained by an air-conditioner) for 30 and 60 minutes, respectively, so that they reach ambient temperature. Upon thawing and stabilisation at room temperature, all the specimens were subjected to the same procedure for sample preparation and analysis.

Each specimen tube was vortex-mixed for 20 seconds before 80 µL of its contents was transferred into a microtube containing 240 µL of ultrapure water, producing a sample with a dilution ratio of 1:3 (specimen to water). The diluted sample was vortex-mixed for 10 seconds and analysed on the ABL825 FLEX analyser (Radiometer, Copenhagen, Denmark), using the 95 µL syringe mode. One Radiometer AutoCheck5+ Level 3 quality control sample was analysed before the study samples

were analysed, and one Radiometer AutoCheck5+ Level 4 quality control sample was analysed after the study samples were analysed.

2.8 Flow chart

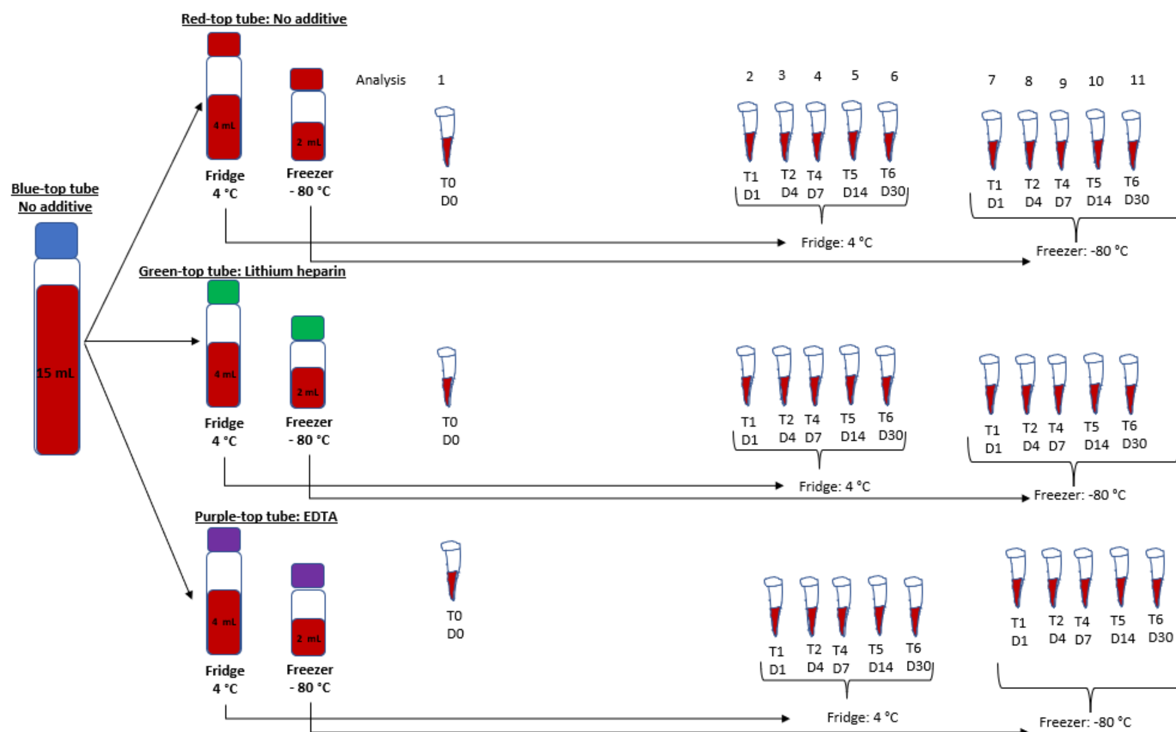

Figure 2.1: Flow diagram illustrating the assignment of sample blood into tubes, storage temperature and days of analysis. A blood specimen collected in a 15 mL blue-top tube (with no-additive) was equally aliquoted into three 4 mL blood collection tubes: red-top tube (with no additive), green-top tube (containing lithium heparin) and purple-top tube (containing EDTA). Following vortex-mixing of the blood sample, 80 µl was aliquoted from each of the 4 ml blood collection tube into a microtube for analysis, and half of the remaining sample was aliquoted into a 2 ml cryo-tube for storage in the freezer. The remaining half of the sample in the blood collection tube was refrigerated at 4 ºC. The stored samples were retrieved from storage on days 1, 4, 7, 14 and 30 for analysis by CO-oximetry.

2.9 Data management

The data collected from the analyses performed was stored on the local hard drive of the main investigator's computer and on Google Drive cloud. Raw data from the ABL825 FLEX analyser were kept as hard copies. To maintain anonymity, the WC number (a case identification number used by SRM) and the decedent's personal information were not retained.

2.10 Data analysis

The results obtained from the ABL825 FLEX analyser were entered onto Microsoft Excel 2016 (Microsoft Corporation, New Mexico, USA) for storage and data analysis. Statistical Package of Social Sciences (SPSS) version 16 (SPSS Inc., Chicago, Illinois, USA) was used for statistical analysis of the results.

2.11 Statistical analysis

For the primary aim, percentage difference scatter plot was used to represent the stability of carboxyhaemoglobin and methaemoglobin during the period of analysis. The scatter plots constructed show the percentage change in carboxyhaemoglobin and methaemoglobin over time, for each type of collection tube used and storage temperature.

For the secondary aim, results were analysed using simple Pearson's correlation to determine association between the two variables. Bland-Altman analysis was used to determine the level of agreement between blood and thoracic cavity fluid samples.

3. CHAPTER THREE: RESULTS

This chapter presents the results of the current study, the aim of which was to investigate the specimen handling conditions that are suitable for the simultaneous measurement of COHb and MetHb using the Radiometer ABL825 FLEX analyser. To this end, post-mortem blood specimens collected in 15 mL centrifuge tubes were aliquoted into 4 mL red-, purple- or green-top blood collection tubes containing no additive, EDTA and lithium heparin, respectively. The blood samples in the 4 mL blood collection tubes were equally aliquoted into 2 mL cryotubes for storage in the freezer (at –80˚C). The remainder of the blood samples in the blood collection tubes were stored in a refrigerator (at 4˚C). On days 0, 1, 4, 7, 14, and 30, the samples were removed from storage and analysed. The study assessed whether the COHb and MetHb concentrations were maintained throughout the 30-day period under the abovementioned conditions. The chapter is, therefore, divided into parts presenting data on the stability of (1) COHb at 4˚C, (2) COHb at –80˚C, (3) MetHb at 4˚C, (4) and MetHb at –80˚C. The author also presents the results of an experiment conducted to assess the suitability of thoracic cavity fluid as a potential alternative to post-mortem blood for the measurement of COHb.

3.1 Stability of COHb in green-, purple-, and red-top tubes at 4˚C

The COHb concentrations (presented as percentage fractions of tHb) obtained during analysis, their respective means, and calculated percentage coefficient of variation (%CV) within each tube type are presented in Table 3.1. Percentage CV was used to assess the variation of COHb concentrations obtained for each case from a single tube type over the course of thirty days. This was done for all cases and the three tube types that were under assessment. A mean %CV for each tube type was calculated from the individual %CV values obtained for each of the 15 cases included in the study.

Table 3.1: COHb concentrations obtained from blood samples stored in red-, purple-, and green-top tubes at 4˚C over a period of 30 days.

		COHb concentration (%)																		%CV		
	Day	0			1			4			7			14			30					
	Tube cap	Red	Purple	Green	Red	Purple	Green	Red	Purple	Green	Red	Purple	Green	Red	Purple	Green	Red	Purple	Green	Red	Purple	Green
Study cases	1	-0.4	-1.1	-1.0	-1.1	-1.1	-1.2	-1.2	-1.4	-1.3	-1.0	-0.7	-0.7	-1.4	0.4	-1.0	-0.8	-0.6	-1.1	-35.5	-84.6	-19.7
	2	42.3	40.8	42.0	41.2	40.8	41.1	41.5	41.0	41.6	42.4	41.8	42.2	42.2	40.3	41.9	41.9	40.6	41.4	1.1	1.2	1.0
	3	30.1	29.3	29.9	29.3	28.9	29.2	29.5	29.2	29.5	30.2	29.9	30.0	29.2	29.1	29.6	30.2	29.2	29.9	1.6	1.2	1.0
	4	33.1	33.0	33.0	32.1	32.0	30.8	32.7	32.3	32.7	32.8	31.6	32.7	32.9	31.9	32.5	32.8	32.3	32.9	1.0	1.5	2.5
	5	39.5	39.3	40.4	38.7	38.1	39.3	38.6	37.4	38.7	38.1	36.7	38.3	37.5	35.8	38.5	38.6	36.8	38	1.7	3.3	2.2
	6	54.1	56.7	57.0	58.2	57.7	58.1	58.2	57.3	58.0	56.2	56.2	56.8	57.7	55.5	57.4	58.1	56.7	57.4	2.9	1.4	0.9
	7	65.1	64.4	64.4	64.9	63.8	64.7	65.1	60.4	61.4	63.0	61.7	61.3	64.9	63.6	63.8	63.3	60.3	63.5	1.5	2.9	2.3
	8	67.7	67.6	67.7	65.9	65.2	65.6	64.8	63.7	63.2	63.7	64.8	65.5	64.7	64.4	64.2	63.2	63.4	64.6	2.5	2.3	2.4
	9	23.4	22.2	22.8	23.8	22.1	22.9	22.5	22.1	22.4	21.7	21.7	22.2	23.3	22.3	21.7	22.8	21.5	21.8	3.3	1.4	2.2
	10	30.1	30.3	30.1	31.2	30.3	30.4	31.3	30.4	30.5	30.4	30.2	29.8	29.7	29.4	29.9	31.3	30.7	31.4	2.3	1.4	1.9
	11	75.5	74.8	73.7	73.9	73.2	71.9	72.1	71.9	66.4	71.4	70.6	66.0	70.0	68.2	66.5	73.5	72.2	68.5	2.7	3.1	4.7
	12	0.3	0.1	0.1	0.5	0.2	0.4	0.5	0.2	0.4	0.7	0.3	0.6	0.8	0.2	0.7	0.8	0.6	0.7	33.3	65.7	47.9
	13	62.7	61.6	62.0	64.1	61.8	61.9	61.6	60.9	60.1	60.9	60.8	60.5	63.7	61.4	63.5	63.8	61.8	63.9	2.1	0.7	2.5
	14	2.0	2.0	2.1	2.0	1.9	2.0	2.4	2.0	2.2	2.5	1.9	2.6	2.8	2.2	2.8	3.0	2.5	3.0	16.7	11.1	16.7
	15	40.7	39.5	40.6	39.6	39.8	40.1	38.8	38.3	38.1	38.7	38.2	39.1	38.9	39.2	39.4	40.6	40.7	37.0	2.3	2.4	3.4
	Mean	**37.7**	**37.4**	**37.7**	**37.6**	**37.0**	**37.1**	**37.2**	**36.4**	**36.3**	**36.8**	**36.4**	**36.5**	**37.1**	**36.3**	**36.8**	**37.5**	**36.6**	**36.9**	**2.6**	**1.0**	**4.8**

The mean %CV values for all tube types were below 5%, suggesting a generally low variation between the COHb concentrations measured for every case over 30 days (Table 3.1). Out of the 15 cases studied, only three (case 1, 12, and 14 in red font) presented %CV values that were greater than 5%. The researchers note that the baseline COHb (obtained on day 0) was considerably low for the three cases compared to the rest of the cases (maximum COHb: –0.4% for case 1, 0.3% for case 12, and 2.1% for case 14). The internal validation studies conducted for the ABL825 FLEX analyser used in the current investigation found that when COHb saturation is below 2.7%, it cannot be quantified with acceptable precision (signified by a %CV above 20%) and accuracy, as noted in this study.

The results suggest that storing blood samples with anticoagulants (EDTA and lithium heparin) at refrigerated temperature (4˚C) or refrigerating without anticoagulants were both effective ways of maintaining COHb concentrations. The three cases with notably high %CV values were, thus, deemed to be outliers. For this reason, data from these cases was omitted when plotting the % difference scatter graph presented in Figure 3.1. The plot wherein the outliers were included is presented in Figure S3.1 (Appendix A: Supplementary data).

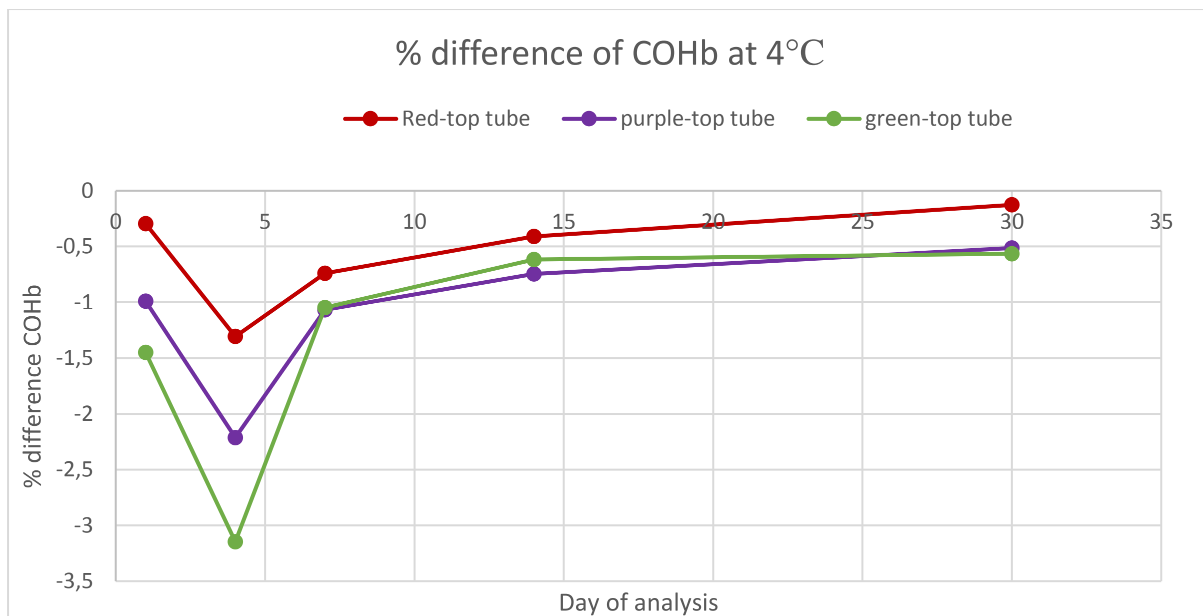

Figure 3.1: Mean % difference of COHb in blood stored at 4°C over a period of 30 days. Post-mortem blood specimens collected and stored at 4°C in three different tube types (red-top, purple-top, and green-top) were analysed on days 0, 1, 4, 7, 14, and 30 of collection. Individual % differences were calculated between the COHb concentrations obtained on day 0 and that of each of the subsequent time intervals. The mean % difference for each tube type (calculated from the individual % difference values obtained for each of the 12 cases included in the analysis) was below 3.5% throughout the study period.

For all tube types, the % differences of COHb saturation remained below 3.5% throughout the study duration. The tube with no additive suggests that refrigeration alone is sufficient for maintaining COHb saturation over a 30-day period. The same was observed when refrigeration was investigated in combination with anticoagulants EDTA and lithium heparin. A repeated measures ANOVA for the red- ($p = 0.117$) and green-top tube types ($p = 0.117$) revealed that COHb saturation did not significantly change between days 0 and 30. The level of significance (α) used for the statistical analysis is 0.05. The purple-top tube returned a p-value of 0.043 suggesting a significant difference of COHb saturations between the different time intervals but the results obtained from the post-hoc tests were contradictory showing no significant difference (p-values less than 0.05).

3.2 Stability of COHb in green-, purple-, and red-top tubes at –80˚C

Table 3.2 presents the COHb saturations, the respective means for each case and the calculated %CV for the red-, green-, and purple-top tubes at –80˚C.

Table 3.2: COHb concentrations obtained from blood samples stored in red-, purple-, and green-top tubes at –80˚C over a period of 30 days.

		COHb concentration (%)																		%CV		
	Day	0			1			4			7			14			30					
	Tube cap	Red	Purple	Green	Red	Purple	Green	Red	Purple	Green	Red	Purple	Green	Red	Purple	Green	Red	Purple	Green	Red	Purple	Green
Study cases	1	-0.4	-1.1	-1.0	-0.7	-1,3	-1,1	-0,7	-1,1	-1,1	-0,8	-0.9	-0.9	-0.7	-0.8	-0.8	-0.7	-0.6	-0.6	-20.5	-25.9	-21.2
	2	42.3	40.8	42.0	38.5	40.0	41.3	41.8	41.0	41.4	42.9	42.0	42.7	42.4	41.4	41.6	41.0	40.4	40.9	3.8	1.7	1.5
	3	30.1	29.3	29.9	29.5	28.9	29.5	29.8	28.8	29.6	30.4	29.9	28.9	30.0	28.2	29.7	29.4	28.9	27.0	1.3	2.0	3.7
	4	33.1	33.0	33.0	31.9	30.4	32.4	32.4	32.4	32.8	32.9	32.7	32.5	32.8	32.4	33.0	32.3	32.2	32.5	1.4	2.9	0.8
	5	39.5	39.3	40.4	38.9	38.3	38.1	38.7	39.4	39.9	39.2	39.2	37.7	35.6	37.8	39.2	40.0	39.1	39.1	4.0	1.7	2.6
	6	54.1	56.7	57.0	56.2	57.7	58.0	58.4	58.4	58.5	58	57.9	57.4	58.3	58.2	57.4	53.8	58.4	57.7	3.7	1.1	0.9
	7	65.1	64.4	64.4	65.4	64.5	63.4	63.8	62.8	63.6	61.6	62.0	61.7	65.1	64.2	64.6	64.5	63.6	64.2	2.2	1.6	1.7
	8	67.7	67.6	67.7	64.6	65.2	65.4	63.7	64.3	63.5	64.5	63.4	63.7	64.1	63.2	62.0	66.0	65.2	64.5	2.3	2.5	3.0
	9	23.4	22.2	22.8	22.9	22.1	22.9	22.7	22.1	22.6	22.6	21.1	22.2	22.9	22.2	22.8	22.6	21.7	22.5	1.3	2.0	1.1
	10	30.1	30.3	30.1	30.9	30.5	30.7	30.4	30.4	30.4	30.5	29.4	30.1	29.8	29.7	29.8	31.0	30.7	30.4	1.5	1.7	1.0
	11	75.5	74.8	73.7	69.7	71.7	74.3	73.2	70.3	71.1	72.1	70.6	71.7	68.6	69.1	68.9	72.0	69.7	70.8	3.4	2.9	2.8
	12	0.3	0.1	0.1	0.6	0.3	0.6	0.7	0.4	0.8	1.0	0.6	1.0	1.2	0.6	1.1	1.3	0.8	1.4	45.1	53.6	54.0
	13	62.7	61.6	62	62.9	62.8	62.6	61.6	60.8	62.3	61.2	61.5	62.0	63.0	62.8	62.4	60.1	61.1	61.4	1.9	1.4	0.7
	14	2.0	2.0	2.1	2.1	2.1	2.2	2.4	2.3	2.4	2.4	2.1	2.5	2.7	2.3	2.6	2.9	2.6	2.9	14.2	9.7	11.8
	15	40.7	39.5	40.6	40.0	39.9	40.2	38.4	39.1	39.8	39.4	39.3	39.7	39.6	39.5	40.2	39.3	39.5	40.3	1.9	0.7	0.8
	Mean	**37.7**	**37.4**	**37.7**	**36.9**	**36.9**	**37.4**	**37.2**	**36.8**	**37.2**	**37.2**	**36.7**	**36.9**	**37.0**	**36.7**	**37.0**	**37.0**	**36.9**	**37.0**	**4.5**	**4.0**	**4.4**

The mean %CV value for the green-top tube for blood samples stored at –80˚C was similar to the one obtained for samples stored at 4˚C. For red- and purple-top tubes at −80˚C the %CV values were slightly above the ones obtained in refrigeration, but still below 5%. Both temperatures with and without the addition of anticoagulants indicated a generally low variation for the COHb concentrations measured for every case between the different time intervals. The same cases noted in section 3.1 to present very low COHb were also outliers in this section, with %CV values greater than 5%. The three tube types under assessment produced similar mean %CV values of 4.5, 4.0, and 4.4 for the red-top, purple-top, and green-top tube, respectively. This suggests that EDTA and lithium heparin in combination with freezing at –80˚C, as well as freezing unaided were effective in maintaining the COHb concentration. For the same reason stated in 3.1, the % differences obtained from the three cases with considerably higher %CV values (case 1, 12 and 14) were excluded in the construction of the scatter plot below (Figure 3.2). The original plot indicating the % differences for all 15 samples is presented in Figure S3.2 (Appendix A: Supplementary data).

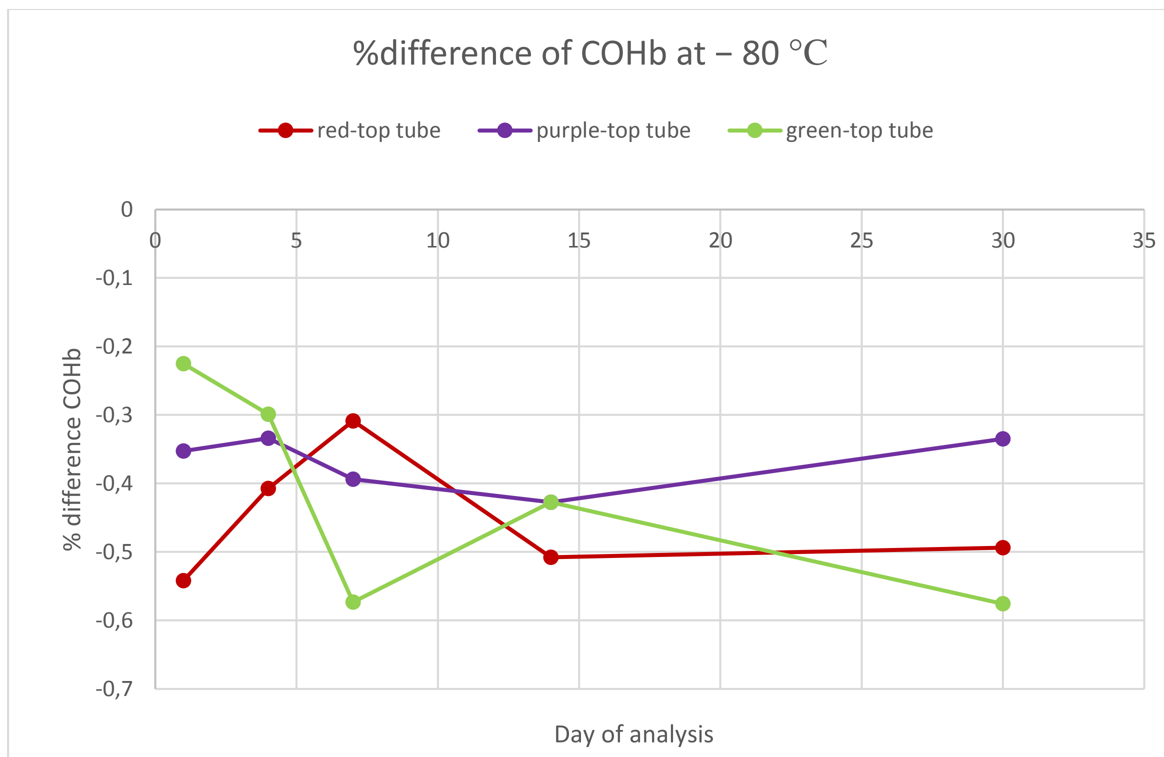

Figure 3.2: Mean % difference of COHb in blood stored at −80°C over a period of 30 days. Post-mortem blood specimens collected and stored at −80°C in three different tube types (red-top, purple-top, and green-top) were analysed on days 0, 1, 4, 7, 14, and 30 of collection. Individual % differences were calculated between the COHb concentration obtained on day 0 and that of each of the subsequent time intervals. The mean % difference for each tube type (was calculated from the individual % difference values obtained for each of the 12 cases included in the analysis), was below 0.6%.

The mean variations for COHb (as indicated by % difference) between the initial day of analysis and other time intervals for all frozen blood samples were below 0.6% throughout the 30-day period (Figure 3.2). No obvious trend was observed in the trajectory of the curves representing the three tube types of interest. A repeated measures ANOVA revealed that COHb levels did not significantly change from day 0 to day 30 for all frozen samples. The red-, purple- and green-top tubes returned p-values 0.453, 0.326 and 0.205, respectively.

3.3 Stability of MetHb in green-, purple-, and red-top tubes at 4˚C

The stability of MetHb saturation was investigated in three different blood tube types (red-, green-, and purple-top) stored at 4˚C. The MetHb concentrations (presented as fractions of tHb – in %) obtained during analysis at different time intervals, their

respective means, and the %CV calculated for all tube types are presented in Table 3.3.

Table 3.3: MetHb concentrations obtained from blood samples stored in red-, purple-, and green-top tubes at 4˚C over a period of 30 days.

		MetHb concentration (%)																		%CV		
	Day	0			1			4			7			14			30					
	Tube cap	Red	Purple	Green	Red	Purple	Green	Red	Purple	Green	Red	Purple	Green	Red	Purple	Green	Red	Purple	Green	Red	Purple	Green
Study cases	1	0,8	2.6	2.7	2.3	7	3,5	3,9	8	5,6	9,6	18,2	15,6	8,8	36,3	13,8	2,3	10,8	4,1	80,0	88,0	74,8
	2	2,4	4,7	3,1	2,6	3,9	2,9	3,9	5,1	4,1	1,7	4	2.3	0.5	3	1.5	0.4	3.1	2.1	70.0	21.1	34.0
	3	3.4	6.6	3.7	4	6.4	4.3	5.8	7	5.9	1.7	3.4	1.9	1.8	3.5	1.9	1.7	4.2	2.0	54.2	32.0	50.1
	4	3.3	4.1	3.4	3.3	4.8	3.9	4.8	5.6	5.1	7.3	6.7	7.9	5.7	5.9	6.9	3.7	4.1	3.6	34.0	20.1	36.6
	5	3.6	4.8	3.6	4.1	5.5	4.2	4.3	5.0	4.1	3.5	5.7	3.8	4.7	7.7	4.6	3.4	7.9	3.7	13.1	22.3	9.4
	6	3.1	3.3	3.2	3.3	3.8	3.4	3.8	4.3	4.2	4.6	4.5	4.6	3.6	5.3	4.6	2.3	3.2	3.6	22.2	19.6	15.6
	7	3.2	3.5	3.1	2.7	3.3	3.2	2.4	3.1	3.1	3.2	3.6	3.4	3.1	3.5	4.1	2.0	3.3	3.6	17.8	5.4	11.3
	8	3.2	3.5	3.5	3.6	3.7	3.6	3.4	3.6	3.1	3.6	3.7	3.8	5.9	4.4	5.3	4.1	5.1	3.9	25.0	15.7	19.5
	9	1.9	3.5	2.5	1.5	5.4	2.9	3.4	7.6	4.4	6.7	10.1	7.2	10.6	13.0	13.0	4.8	14.5	8.0	71.0	47.7	62.4
	10	4.4	5.1	4.4	3.9	5.9	4.4	6.6	8.9	6.9	4.8	12.3	4.9	3.6	7.6	3.8	3.0	3.8	3.5	28.6	42.1	26.0
	11	3.8	5.1	5.0	4.4	5.2	4.6	5.5	5.8	5.8	5.8	7.0	4.6	5.4	6.3	3.9	5.1	6.6	6.0	15.1	12.8	16.0
	12	2.2	2.5	2.2	2.5	3.3	2.7	4.3	5.2	4.4	3.9	6.1	3.6	1.9	5.3	1.9	1.9	2.5	2.3	37.8	37.9	33.7
	13	1.8	2.5	2.8	1.1	2.8	2.9	3.2	3.4	3.4	3.1	3.6	3.6	0.9	3.0	1.7	1.9	2.6	1.9	48.6	14.8	28.5
	14	2.5	3.0	2.6	3.7	4.7	4.0	2.1	4.7	2.3	1.6	2.8	1.4	1.4	2.0	1.3	1.1	1.3	1.0	45.7	45.1	53.2
	15	1.2	2.3	1.8	2.5	5.2	1.6	4.3	9.4	5.2	2.9	5.1	0.3	0.7	0.7	0.3	-0.6	-0.4	1.5	95.3	96.6	100.9
	Mean	**2.7**	**3.8**	**3.2**	**3.0**	**4.7**	**3.5**	**4.1**	**5.8**	**4.5**	**4.3**	**6.5**	**4.6**	**3.9**	**7.2**	**4.6**	**2.5**	**4.8**	**3.4**	**43.9**	**34.7**	**38.1**

Unlike the mean %CVs obtained for COHb saturation which were below 5%, the mean %CV for MetHb were all above 30%, suggesting a generally high variation for MetHb concentrations between the various time intervals over the course of 30 days (Table 3.3). The range for %CV from the individual cases was between 5.4% and 100.9%. The MetHb concentrations were relatively low (<10%) for majority of the cases, which could be partly responsible for the large variation, as noted with COHb. This suggests that the anticoagulants: EDTA and lithium heparin in combination with refrigeration were not effective in maintaining the MetHb concentration. The same is true for samples that were stored without additives. A scatter plot (presented in Figure 3.3) was drafted to assess the trend of MetHb deviation throughout the 30-day period in all tube types.

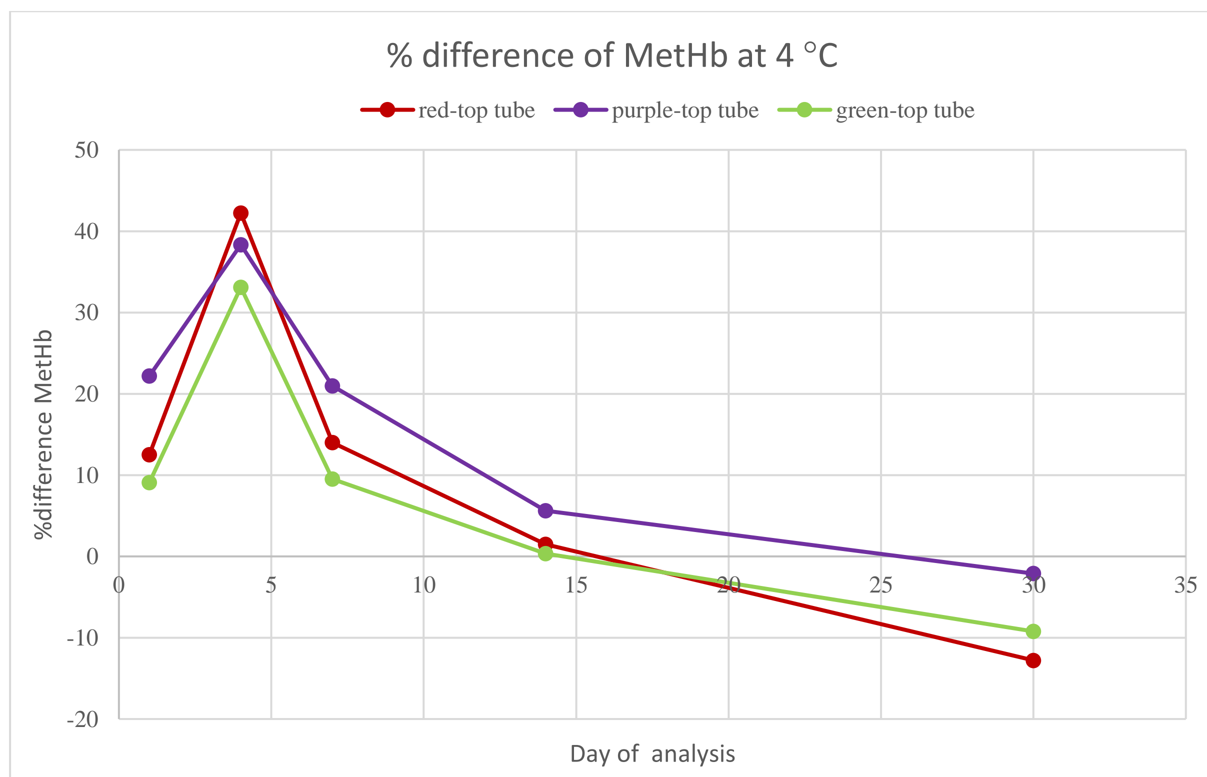

Figure 3.3: Mean % difference of MetHb in blood stored at 4°C over a period of 30 days. Post-mortem blood specimens collected and stored at 4°C in three different tube types (red-top, purple-top, and green-top) were analysed on days 0, 1, 4, 7, 14, and 30 of collection. Individual % differences were calculated between the MetHb concentration obtained on day 0 and that of each of the subsequent time intervals. The mean % difference for each tube type was calculated from the individual % difference values obtained for each of the 15 cases included in the analysis. At first, there was a rise in MetHb, which was followed by a steady decline back to the initial values, which continued until the values were below the initial values.

All the tube types followed the same trend, beginning with an increase of more than 8%, followed by a sharp increase to the highest % difference, and then a continuous decrease to negative values below the X-axis (Figure 3.3). The mean % difference for all tube types was above 8% following 24 hours of the initial analysis. The highest % difference for all tube types was recorded on the 4th day of storage. From this point, a continuous decline in concentration was observed. Following two weeks of storage, the % differences for all tube types was below 6%, the lowest observed in the entire period of assessment. For blood samples stored in green- and purple-top tubes, the % difference was less than 10% between days 14 to 30. For the red-top tube this was the case only on day 14. The increase during the first week of analysis suggests that Hb was oxidized to MetHb. Following the increase, a continuous decline in MetHb saturation was noted until the last day of the study.

A repeated measures ANOVA for the green- ($p = 0.178$) and purple-top tube ($p = 0.195$), indicated that overall, there was no significant difference for MetHb concentrations obtained between the different time intervals. This contrasts with the graph, which shows up to 30% in % differences for both tubes. The stated statistical results could be attributable to the fact that after the initial spike, the percent differences return to values closer to zero, possibly offsetting the initial effect. A repeated measures ANOVA for red-top tube produced a p-value of 0.047, indicating that overall, there is a significant difference for MetHb saturation between the various time intervals. The post-hoc test revealed that MetHb concentrations differed significantly only on the 4th day ($p = 0.005$) but was not on other days of analysis. On this day, the red-top tube showed a % difference greater than all the other datapoints on the graph.

3.4 Stability of MetHb in green-, purple-, and red-top tubes at –80˚C

The stability of MetHb saturation was further evaluated using the same blood collection tubes (red-, purple- and green-top tube) frozen at –80°C. Table 3.4 presents the MetHb concentrations (presented as fractions of tHb – in %) obtained during examination, their respective means, and the calculated coefficient of variation within each tube type.

Table 3.4: MetHb concentrations obtained from blood samples stored in red-, purple-, and green-top tubes at –80˚C over a period of 30 days.

	MetHb concentration (%)																		%CV		
Day	**0**			**1**			**4**			**7**			**14**			**30**					
Tube cap	**Red**	**Purple**	**Green**	**Red**	**Purple**	**Green**	**Red**	**Purple**	**Green**	**Red**	**Purple**	**Green**	**Red**	**Purple**	**Green**	**Red**	**Purple**	**Green**	**Red**	**Purple**	**Green**
Study cases																					
1	0.8	2.6	2.7	4.9	6.5	6.6	6.6	12.4	8.5	11.1	18.0	12.1	12.1	20.8	13.5	13.6	21.7	14.3	60.1	57.6	46.8
2	2.4	4.7	3.1	3.9	5.5	3.9	5.3	6.7	5.1	5.7	8.0	5.3	5.7	8.5	5.5	6.9	9.9	6.5	31.9	27.0	24.8
3	3.4	6.6	3.7	6.1	9.0	5.7	7.2	10.5	7.1	7.8	10.9	7.6	8.3	11.8	8	9.4	12.8	8.3	29.8	21.5	25.9
4	3.3	4.1	3.4	5.9	6.8	5.3	7.4	8.0	7.0	8.3	9.7	8.1	9.3	10.7	8.1	9.9	10.8	8.3	33.2	31.2	29.4
5	3.6	4.8	3.6	4.0	5.4	2.2	4.9	5.1	4.6	4.5	5.5	4.5	5.1	6.0	4.6	4.8	6.0	5.1	12.9	8.8	25.6
6	3.1	3.3	3.2	3.3	3.5	3.3	4.2	5.0	4.3	4.4	5.4	4.5	4.6	5.8	4.8	4.9	6.4	5.1	17.8	25.6	18.7
7	3.2	3.5	3.1	3.4	3.5	3.4	3.4	3.7	3.7	4.5	4.6	4.4	5.0	4.9	4.8	5.2	5.3	5.2	21.6	18.5	20.2
8	3.2	3.5	3.5	3.6	3.9	3.5	4.2	4.6	4.2	4.7	5.1	4.4	5.2	5.6	5.0	5.4	5.8	5.1	20.0	19.4	16.3
9	1.9	3.5	2.5	4.1	5.6	4.0	5.0	6.2	5.0	3.9	6.9	5.3	5.2	7.0	5.2	4.9	6.9	4.9	29.4	22.4	24.0
10	4.4	5.1	4.4	5.8	6.4	5.8	7.3	8.4	7.4	8.9	10.6	8.8	9.3	11.7	9.8	9.4	11.6	10.0	27.5	31.1	29.3
11	3.8	5.1	5	4.9	5.1	3.6	4.6	5.5	4.8	5.4	5.8	5.0	5.2	6.6	5.4	5.1	6.1	4.9	11.9	10.3	12.8
12	2.2	2.5	2.2	5.5	6.5	5.4	10.5	11.6	10.5	15.7	16.4	15.6	19.5	19.7	19.3	21.8	21.4	21.5	62.3	57.6	62.1
13	1.8	2.5	2.8	3.0	3.2	3.2	3.4	3.6	3.6	3.6	4.4	3.7	4.0	5.0	4.3	4.3	4.8	4.3	26.4	25.0	16.3
14	2.5	3.0	2.6	6.6	7.1	6.3	10.1	10.5	9.3	12.4	12.5	11.2	14.6	14.7	12.9	16.7	15.8	14.4	50.2	45.7	46.4
15	1.2	2.3	1.8	2.2	3.8	1.6	3.4	5.2	2.2	3.0	4.7	2.1	2.5	5.0	2.0	3.8	5.5	1.5	34.7	26.9	15.0
Mean	**2.7**	**3.8**	**3.2**	**4.5**	**5.5**	**4.3**	**5.8**	**7.1**	**5.8**	**6.9**	**8.6**	**6.8**	**7.7**	**9.6**	**7.5**	**8.4**	**10.1**	**8.0**	**31.3**	**28.6**	**27.6**

The mean %CV obtained indicates a high variation with all above 20% (Table 3.4). The calculated %CV values for individual cases ranged between 8.8% and 62.3%. Similar to the observation made in section 3.3, the mean %CV values in 3.4 also imply that the anticoagulants EDTA and lithium heparin, as well as freezing alone, were ineffective in sustaining MetHb concentrations over the 30-day period. Unique to this dataset, however, is the observed constant increase of the mean values of MetHb with time for all the tube types, which was also reflected in the representative graph shown in Figure 3.4.

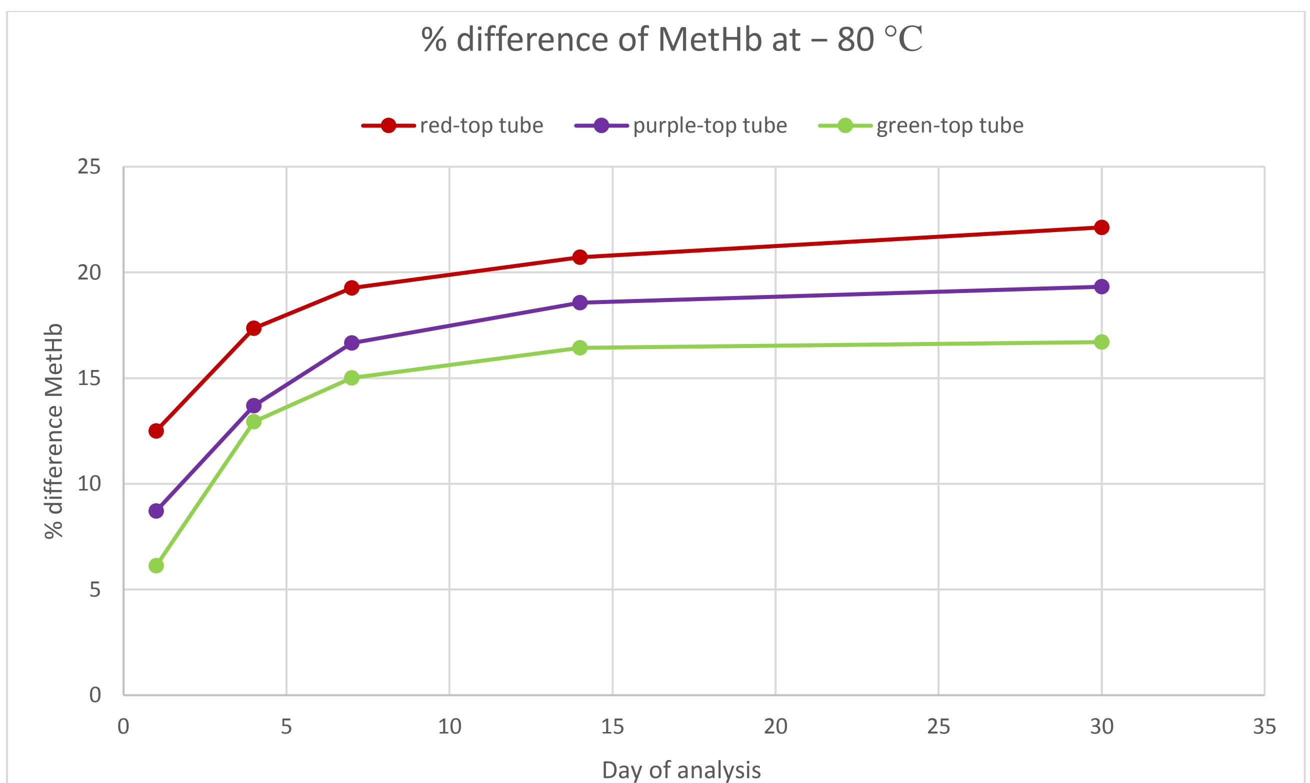

Figure 3.4: Mean % difference of MetHb in blood stored at −80°C over a period of 30 days. Post-mortem blood specimens collected and stored at –80°C in three different tube types (red-top, purple-top, and green-top) were analysed on days 0, 1, 4, 7, 14, and 30 of collection. Individual % differences were calculated between the MetHb concentration obtained on day 0 and that of each of the subsequent time intervals. The mean % difference for each tube type was calculated from the individual % difference values obtained for each of the 15 cases included in the analysis. A continuous increase was noted from the first day of storage until the last day of storage.

The curves for all three tube types followed a similar trend, showing a continuous increase throughout the study period (Figure 3.4). All the percentage differences were greater than 5%, with the green and purple-top tubes showing % differences that were

less than 10% only on the first day upon storage. The sharpest increase in % difference occurred in the early days of storage. With an increase in storage time, the elevated % difference was seen to approach a plateau for all tube types. The increases are suggestive of oxidation and that the MetHb reductase enzyme is either inactive or inhibited by the freezing.

A repeated measures ANOVA for the green-top tube produced a p-value of 0.003 indicating that overall, there was a significant difference for MetHb concentration obtained at subsequent time intervals. The post-hoc test revealed that MetHb concentrations obtained on days 1, 14 and 30 with p-values 0.362, 0.05 and 0.062, respectively, were not significantly different from the values obtained on the day 0 of analysis. Methaemoglobin concentration for days 4 and 7 (p-values 0.018 and 0.042) were significantly different from the values obtained on day 0 (reference values). A repeated measures ANOVA for the purple-top and red-top tube produced a p-value of 0.001 and 0.002, respectively, indicating that overall, there is a significant difference for MetHb concentrations obtained on day 0 as compared to that obtained on subsequent time intervals. The post-hoc test revealed that MetHb concentrations changed significantly over the 30-day period producing p-values less than 0.05 for all time intervals.

3.5 Assessment of thoracic cavity fluid as an alternative specimen to post-mortem blood for the determination of COHb

All blood and thoracic cavity fluid samples aliquoted/collected into green-top tubes were analysed on the day of collection (day 0) to determine COHb concentrations and the total haemoglobin in each sample. The tHb values were reported to indicate the blood viscosity, which affects COHb assessment during CO-oximetry analysis. The results for this analysis are presented in Table 3.5. The aim of the analysis was to investigate the suitability of thoracic cavity fluid as an alternative specimen for the measurement of COHb, in cases where a blood specimen is not available. This was achieved by analysing the obtained COHb concentrations using Pearson's correlation to assess if there was a linear relationship between the values and determine how the COHb levels change in relation to one another. The Bland-Altman plot was used to

determine the level of agreement between the two specimen types and the results of this analysis are presented Figure 3.5 below.

Table 3.5: COHb concentration and total haemoglobin values for blood and thoracic cavity fluid.

Sample cases	COHb concentration (%)		total haemoglobin (g/dL)	
	Thoracic cavity fluid	Blood	Thoracic cavity fluid	Blood
1	-1.1	-1.0	3.7	4.2
2	46.0	42.0	2.4	3.7
3	29.9	30.0	4.5	4.3
4	41.5	33.0	2.8	4.8
5	38.4	40.0	3.0	2.4
6	54.5	57.0	3.0	5.4
7	**NR**	64.0	0.14	4.7
8	**NR**	68.0	0.1	6.3
9	21.8	23.0	3.1	2.4
10	38.1	30.0	4.8	5.3
11	**NR**	74.0	**NR**	3.9
12	**NR**	0	0.15	7.5
13	63.4	62.0	3.9	5.9
14	**NR**	2.0	0.54	6.4
15	39.7	41.0	2.9	2.5

NR – no result

The reported COHb saturations for both blood and thoracic-cavity fluid ranged between –1.1% and 74%. The range for total haemoglobin was between 0.1 and 7.5%. The instrument did not report COHb values for five thoracic cavity fluid samples with very low tHb levels (between 0.1 to 0.54 g/dL), with one sample failing to report both the COHb and tHb values (case 11 had tHb below reportable range). The ABL825 FLEX analyser has a tHb threshold of 1.61 g/dL, below which it is unable to report COHb values. For this reason, only the remaining ten specimen pairs were used to conduct statistical analysis. The Pearson's correlation coefficient measured the strength of the relationship and evaluated whether a linear relationship exists between the two linear variables. One of the assumptions that must be met before performing a Pearson's correlation is that both variables must be normally distributed. The Shapiro-Wilk test was used to evaluate data distribution and the test produced p =

0.531 for thoracic cavity fluid and p = 0.602 for blood, demonstrating that both data sets were normally distributed. The calculated Pearson's correlation coefficient (r) was 0.975, with a p-value less than 0.001. These results suggests that there is a strong positive relationship that exists between thoracic cavity fluid and blood.

The Bland-Altman plot, also known as the Tukey Mean-Difference plot, was used to investigate agreement between the two specimens, as well as identify bias and potential outliers. This was accomplished by investigating the mean difference and the limits of agreement for the ten pairs of specimens.

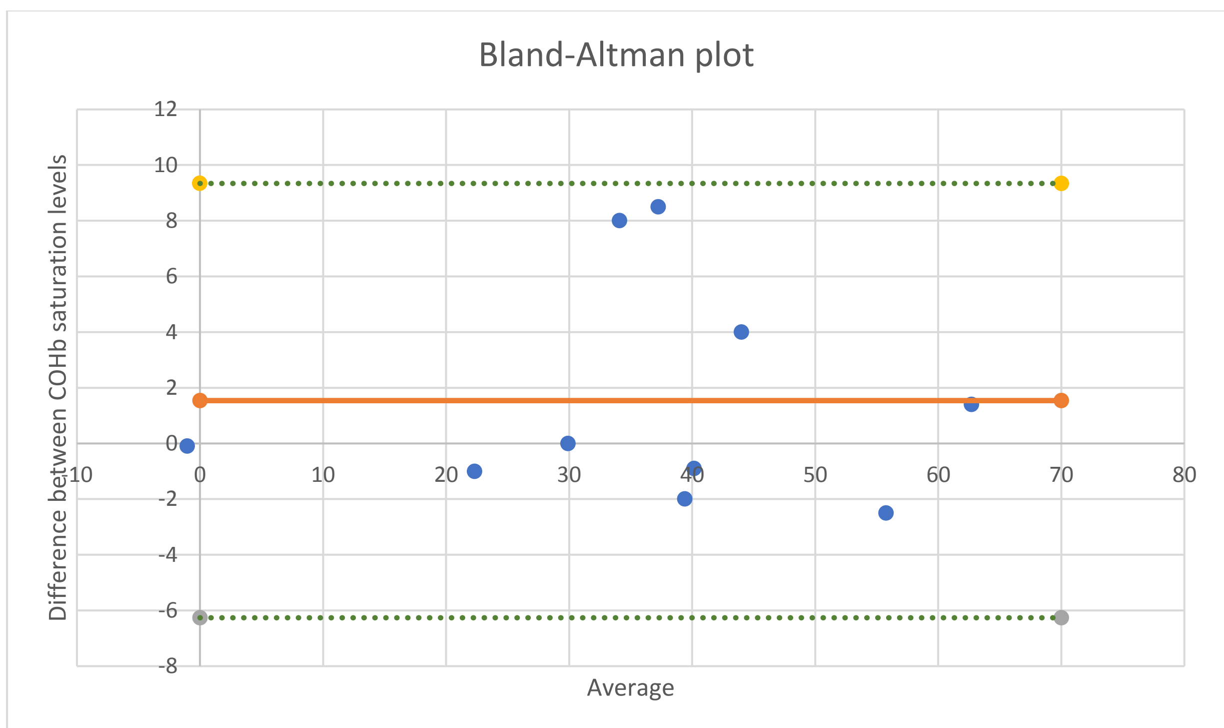

Figure 3.5: Bland-Altman for COHb saturation in blood and thoracic cavity fluid. Post-mortem blood and thoracic cavity fluid specimens were collected in green-top tubes and analysed on the day of collection. The calculated average difference (solid orange line) together with the upper and lower limits of agreement or confidence intervals (green broken lines) indicated that COHb saturation from both specimens agreed with each other.

The mean difference (the gap between the X-axis and the orange line) represents the estimated bias of 1.54% between the two specimens, while the data points measure random fluctuations around the mean difference (Figure 3.5). Blood was taken as the standard sample, and it illustrated that thoracic cavity fluid had the ability to

underestimate COHb. The plot's green-dotted lines represent the upper and lower limits of agreement and there were no outliers falling outside these limits of agreement. Since the bias is insignificant (does not change the interpretation of the results) and there are no outliers observed, this implies that the COHb levels from the two specimens agree with each other. Thus, this finding supports the correlation observed above and suggests that thoracic cavity fluid may be used as an alternative specimen for COHb analysis. However, this will need to be investigated further using a larger sample size.

4. CHAPTER FOUR: DISCUSSION AND CONCLUSION

Carbon monoxide is considered the most hazardous gas due to its high affinity for haemoglobin. As a result of the great affinity, COHb has emerged as the most useful biomarker for CO poisoning (Oliverio and Varlet., 2019). The ABL825 FLEX analyser used in this study for measurement of COHb also measures other haemoglobin derivatives such as MetHb, HHb, and O_2Hb. One of the derivatives, MetHb, is used in the determination of exposure to nitrite/nitrates. Exposure to exogenous oxidative compounds such as nitrites, nitrates and chlorate containing substances are some of the factors that lead to elevated levels of MetHb (Saukko and Knight, 2015). Numerous studies have also found elevated levels of MetHb in addition to COHb in cases of CO poisoning caused by fires and automobile exhaust fumes (Ferrari and Giannuzzi, 2015; Kumagai *et al.,* 2005; Katsumata *et al*., 1980; Schwerd and Schulz, 1978). The formation and reduction of MetHb after sample collection, make it challenging to find sample storage conditions that maintain the levels of MetHb, thus making it difficult to establish it as a suitable biomarker for estimating ante-mortem MetHb levels (Ferrari and Giannuzzi, 2015; Vevelstad and Morild, 2009; Katsumata *et al*., 1980; Schwerd and Schulz, 1978). The MetHb instability is a result of the MetHb reductase activity and the spontaneous oxidation that occurs following death and during storage of blood samples after collection. In order to maintain MetHb saturation these two processes i.e., MetHb reductase activity and spontaneous autoxidation, should be inhibited throughout the storage period. The method used to preserve the blood specimen after collection, such as storage temperature, may thus have a significant impact on the results obtained during measurement (F).

In the attempt to find storage conditions that maintain both COHb and MetHb saturations, the primary goal of this study was to investigate the specimen handling and storage conditions that are appropriate for subsequent simultaneous measurement of COHb and MetHb using the Radiometer ABL825 FLEX analyser. This was undertaken by evaluating the effects of the choice of preservative (EDTA, lithium heparin or none) and storage temperature (4˚C and –80˚C) on MetHb and COHb saturation levels over a period of 30 days. For each of the 15 post-mortem cases

included in the study, blood specimens were drawn from the decedent's body into a 15 mL centrifuge tube and equally aliquoted into red-, green-, and purple-top blood collection tubes. The samples were analysed on days 0, 1, 4, 7, 14 and 30, and the results are presented in Tables 3.1 – 3.4. The COHb and MetHb saturation were used to calculate the percentage differences which were, in turn, used to construct the scatter plots presented in Figures 3.1 – 3.4.

Furthermore, the study investigated the suitability of the thoracic cavity fluid as an alternative specimen for COHb measurement. The thoracic cavity fluid collected in green-top blood collection tubes was analysed on the day of collection along with the blood aliquoted into the green-top blood collection tubes. The COHb saturation and total haemoglobin levels are presented in Table 3.5. The association and agreement between the two specimens were evaluated using Pearson correlation coefficient and Bland-Altman plot (presented in Figure 3.5), respectively.

4.1 Assessment of the stability of COHb in red-, purple-, and green-top tubes at 4˚C

Carboxyhaemoglobin saturation in blood samples ranged from -1.4 to 75.5% (Table 3.1). Preservative type and temperature are the two factors that were controlled in this study. The %CV was calculated to investigate the COHb variation during the study period. No significant differences ($p > 0.05$) were found over the 30-day period for COHb in all blood collection tubes, with % difference remaining below 3.5%. According to the findings of this study, COHb was stable in post-mortem samples stored under the conditions described.

The COHb stability findings in this study are in agreement with the following publications, which also found that COHb remained stable under different storage conditions. Ocak and colleagues (1985) observed a change that was below 8% of the original value when they investigated COHb saturation levels in sealed samples (capped vials) over a four-month period. An extended investigation found that a

second analysis two years later yielded no significant differences in COHb saturation interpretation from the initial values for blood samples stored without preservative or with EDTA at 3˚C (Kunsman, Presses and Rodriguez, 2000). Ghanem and colleagues (2012) demonstrated that there is an insignificant change in COHb levels after refrigeration (4˚C) of samples for up to 3 years in heparinized blood tubes. Oliverio and Varlet (2020) suggested storing samples in EDTA tubes for short-term storage (up to 1 month) at 4˚C and –80˚C. They further indicated that when analysing COHb, it is critical to fill the collection tube to more than 50% of its capacity and analyse it as soon as possible. Preservative type, volume of headspace, and initial COHb did not significantly affect the COHb level in post-mortem blood samples in a study conducted by Kunsman *et al*. (2000).

Even though the current study did not run for longer than a month; it should be noted that COHb remained stable even when the blood volume was below 50% of the blood collection tube. The researchers in the current study assume that the type of preservative, headspace volume, and initial COHb had no effect on COHb saturation. This may have also been the case in the current study, where these factors did not seem to influence the COHb saturation in post-mortem blood specimens refrigerated for 30 days after specimen collection at autopsy.

4.2 Assessment of the stability of COHb in red-, purple-, and green-top tube at –80˚C

Apart from three outliers, all the cases included for this assessment had %CVs below 5% with COHb saturation ranging between −1.3 to 75.5% (Table 3.2). The COHb saturation for blood samples stored in red-, purple-, and green-top tubes in the freezer (–80˚C) remained stable and there was no significant difference observed over the 30-day period. For all the assessed tube types the mean % difference was below 0.6% throughout the 30-day period, which may be a result of the freezing. There were no significant changes observed irrespective of the number of re-opening or freeze-and-thaw cycles and the volume of the headspace. This was supported by Oliverio and Varlet (2020) who stated that during storage, a negligible amount of CO is released

into the headspace of the blood tube and measurement values were unaffected by re-opening or freeze-and-thaw cycles. Carboxyhaemoglobin measurement by spectrophotometer is influenced by changes in optical blood quality, which are primarily caused by catabolism of blood constituents as time and temperature change. This might account for the observed variations during storage. Since COHb is stable in both fridge and freezer, samples may be stored for a period of 30 days, and re-analysed, if required.

4.3 Assessment of the stability of MetHb in red-, purple-, and green-top tube at 4˚C

Spontaneous autoxidation and rapid reduction by MetHb reductase enzymes make it difficult for MetHb to remain stable in the blood before and after sample collection. This is a major problem for analysts, and thus, it is necessary to find suitable conditions that preserve MetHb concentration. There is limited data available on the recommended sample collection and the storage conditions that can preserve MetHb levels in blood. The current study investigated the effects of various storage conditions on MetHb analysis.

The MetHb saturation from blood samples stored in red-, purple-, and green-top tubes at 4˚C ranged from −0.6 to 36.3% (Table 3.3). The results suggest that the preservatives (EDTA and lithium heparin) at 4˚C had little to no effect on MetHb stability, since they were not able to prevent either the formation or the breakdown of MetHb. The same observation was made in tubes without any additive kept at 4˚C. These preservatives/temperature combinations were not able to prevent MetHb modifications. For MetHb levels to be stable, there should be a balance between activity of MetHb-reductase and the process of autoxidation i.e., the storage temperature and choice of preservative used should be able to inhibit the activity of the MetHb-reductase enzyme and prevent autoxidation.

For all tube types, in the early days of storage a considerable increase in MetHb saturation was observed which is suggestive of MetHb oxidation far exceeding the

ability of the reductase enzyme to break down the MetHb back to Hb. The highest MetHb increase was reached on the fourth day and for this reason it is not recommended to analyse blood samples for MetHb saturation four days after storage. Following the fourth day of storage, a decrease in the MetHb saturation continued until day 30. A decrease below the X-axis (i.e., a decrease below the initial values obtained on the day of collection) was seen in MetHb saturations from day 14 onwards under all the three collection tube types at 4˚C. The lowest % difference values in all tube types were observed following two weeks of storage at 4˚C. The obtained curves suggest this to be the mid-point at which the amount of MetHb oxidation is matched by the reduction that has taken place, creating an effect in which the measured MetHb approximates that which was determined before storage. Thus, based on the reported findings, it may be suggested, pending further investigation with a larger sample size, that MetHb saturations be measured after 2 weeks of storage at 4˚C if not analysed immediately after collection. This continuous decrease after day 4 may indicate that the MetHb-reductase activity is still active and not inhibited by either the temperature or the preservatives used.

The following studies reported varying findings regarding the behaviour of MetHb during the early stages of storage. Sato *et al*. (1987) found that when whole blood was stored at 3˚C, there was a rapid reduction of MetHb in nitrite-treated blood, but neither formation nor reduction of MetHb was observed in untreated or heated blood within seven days. Slight autoxidation was observed in the untreated blood seven days after storage, but not in the nitrite-treated blood (Sato *et al.*, 1987). In a different study, MetHb content decreased by 7 to 12% after 2 hours and by 32 to 37% after 18 hours for samples stored in EDTA, lithium heparin and fluoride/heparin at 4˚C (Lim and Tan, 1999). Sato and colleagues also observed comparable results to Lim and Tan's study with MetHb reduction in whole blood samples collected from nitrite-administered rats and post-mortem blood stored at 3˚C (Sato *et al.*, 1990).

Other studies found that when blood samples were stored at 4˚C, they exhibited more stable MetHb saturations, which appear to slowly increase over time by up to 4% of the initial values only after 3 weeks of storage (Domingo *et al.*, 2017). However, after

3 weeks of storage, these increases occurred more rapidly (Domingo *et al.*, 2017). Sato *et al.* (1988) concluded that storing whole blood at 4-5˚C resulted in significant underestimation of MetHb, due to the breakdown of MetHb by the reductase enzyme. Varlet and colleagues (2018) recommend storing blood without preservative under refrigeration (4 − 6˚C) for short-term storage, as they found that after 2 weeks MetHb % change was still below 5%. Samples with EDTA or without preservative stored at refrigerated temperatures can be considered to have prevented MetHb increases over time because MetHb % change was below 10% after four weeks (Varlet *et al.*, 2018).

Similar observations were made in the current study for the red- (no additive) and green-top (lithium heparin) tube which have a percentage difference below 5% after 2 weeks whereas the underestimation of MetHb was only observed following two weeks of storage. Contrary to the study's (Varlet *et al.*, 2018; Domingo *et al.*, 2017) observations, the MetHb saturation increased by more than 8% on the first day in the current study with a rapid increase until day 4. The reported inconsistency may be due to the studies' use of different methods of treating and storing blood samples.

4.4 Assessment of the stability of MetHb in red-, purple-, and green-top tubes at −80°C

The stability of MetHb was further investigated in the same blood tubes at −80˚C. The obtained MetHb saturation from blood samples at −80˚C ranged between 0.8 and 21.8% with %CV values (> 20%) indicating a high variation for MetHb saturation (Table 3.4). The curves (in Figure 3.4) show a continuous increase throughout the study period, suggesting that freezing supports oxidation of Hb to MetHb. The rapid increase suggests that freezing deactivates the MetHb reductase enzyme and promotes oxidation. Following two weeks of freezing, MetHb saturation approached a plateau which persisted up to 30 days of storage and suggesting that a balance was reached between MetHb formation and break-down. Although all tube types have identical curve shapes, the mean % differences were lower for the green-top tube, followed by the purple and then red-top tubes. This correlated with the results for % CVs, which followed the same trend.

Wallace and Curry (2002) conducted a study over a period of six days which investigated the rise of MetHb saturation in post-mortem frozen blood and found that MetHb saturation in frozen-thawed EDTA-containing specimens increased in a quadratic fashion over time. They also observed that there was no discernible effect of the anticoagulant in the blood collection tube on MetHb levels in frozen specimens. The increase in MetHb levels observed in frozen storage of blood specimens could be attributed to a temperature-dependent imbalance between enzymatic reduction and non-enzymatic oxidation (Wallace and Curry, 2002).

On the other hand, there were studies that made contradicting observations. Sato and colleagues (1987) discovered that MetHb concentrations were stable for at least 30 days when stored at –80˚C, irrespective of the initial values. Sato *et al*. (1987) further suggested that due to the mechanism of Hb autoxidation in the frozen state, the freezing-induced modification of the Hb structure may allow oxygen access to Hb iron, but this modification can be inhibited by a cryoprotectant. Varlet *et al*. (2018) advised that opening/closing cycles should be avoided as they may result in increased autoxidation and further recommended the addition of a cryoprotectant for blood samples frozen at –85˚C or –196˚C for longer periods (more than 2 weeks).

The literature does also not provide any conclusive findings with regards to post-mortem stability of MetHb. Sato *et al*. (1987) concluded that MetHb is not stable in a fridge (at 3-4 ˚C) due to reduction or in a freezer because of considerable autoxidation. Varlet *et al.* (2018) recommended that blood samples should be analysed as soon as they arrive at the laboratory or frozen with the addition of a cryoprotectant for long-term storage. As such, the consensus seems to be that to the random MetHb increases, post-mortem MetHb concentrations are not reliable indicators of ante-mortem methemoglobinemia (Reay, Insalaco, and Eisele, 1984; Varlet *et al*., 2018).

A continuous increase was observed in the current study for frozen samples at –80˚C for all blood collection tube types over the 30-day period. The non-enzymatic oxidation might have outweighed the enzymatic reduction of Hb resulting in an increase of

MetHb. The results of the current study were similar to those observed by Wallace and Curry (2002) and Sato *et al.* (1987). The absence of a cryoprotectant and the number of opening/closing cycles may explain the elevated levels of MetHb observed in the current study. Thus, freezing blood samples is not recommended for 30-days.

4.5 Assessment of the association and agreement between COHb saturation from blood and thoracic cavity fluid

Total haemoglobin and COHb levels for thoracic cavity fluid and blood samples stored in green-top tubes and analysed on the day of collection are presented in Table 3.5. Carboxyhaemoglobin saturation in the abovementioned tubes obtained in both specimens ranged from −1.1 to 63.4%. A relationship between the two specimens was evaluated using the Pearson's correlation coefficient (Table 3.5). A strong relationship ($p = 0.001$) was demonstrated between COHb values within the range of −1.1 to 63.4% from thoracic cavity fluid and blood ($n = 10$; $r = 0.975$). The Pearson's correlation coefficient indicates a strong linear relationship for COHb levels between thoracic cavity fluids and blood specimens. No significant difference ($p = 0.252$) was apparent in COHb saturation levels between the two specimen types.

Data with a good linear relationship or a strong association may not agree with each other. A Bland-Altman plot was generated to determine the level of agreement between COHb levels from blood and thoracic cavity fluid (Figure 3.5). When the blood specimen was used as a standard, thoracic cavity fluid underestimated the COHb saturation by 1.54%, with none of the values falling outside the 95% limits of agreement. Agreement between the specimens was judged acceptable because the underestimation will not change the interpretation of the COHb saturation. Therefore, we conclude that based on these results thoracic cavity fluids can be used as an alternative specimen. Although blood is essential for the determination of COHb levels, sometimes it is not readily obtainable, in such instances thoracic cavity fluid may present a useful alternative. However, it should be noted that thoracic cavity fluid is prone to having low tHb, which may inhibit COHb determination. The thoracic cavity fluid used in the current study was mixed with blood, which in turn, increased the tHb.

4.6 Strengths and limitations of the study

The study aimed to determine the best specimen handling and storage conditions for COHb and MetHb in post-mortem blood samples. Three blood collection tube types (no additive, lithium heparin and EDTA) under two storage conditions (freezer and refrigeration) were investigated in this study. All the storage conditions investigated in this study were able to effectively maintain COHb saturation throughout the 30-day period, with % differences less than 10%. However, the same could not be said for MetHb under the same conditions. The least variation from the initial values was observed after approximately two weeks of storage for the green- and red-top tubes and after 4 weeks for the purple-top tube under refrigeration. This study demonstrated that for the simultaneous analysis of COHb and MetHb to occur, the blood samples should be refrigerated in any of the blood collection tubes and analysed on the day of collection or after two weeks of storage to obtain the least variation.

Nonetheless, despite the potentially significant information that can be drawn from this study, some limitations exist, as noted in the following text. All blood samples collected in this study produced a total haemoglobin above the required threshold (> 1.61 g/dL) and reported haemoglobin derivatives values during analysis. This, however, is not guaranteed from putrefied samples and samples with low total haemoglobin such as the five thoracic cavity fluid samples observed in the current study. The inclusion criteria used excluded individuals under the age of eighteen. Thus, the results do not give a clear indication of the behaviour of COHb measured in thoracic cavity in the young. All the cases were accidental burnings as opposed to other forms of CO poisonings and all the samples were collected at SRM mortuary. So, the findings do not give a representation of how COHb behaves in other forms of CO poisoning cases received at SRM or in the Western Cape as a whole. The study used a sample size of 15 cases to give the research's findings statistical significance. A larger sample size, on the other hand, would provide greater statistical strength to the data analysis performed and, by extension, greater confidence in the findings made.

One other limitation noted by the author is the inclusion of samples that produced COHb concentrations below 2.7%, the value under which the instrument is known to lose acceptable accuracy and precision, as was partially reflected by the anomalously high %CV. This necessitated the exclusion of these samples from data analysis, further reducing the sample size and statistical strength of the study. Ideally, a day zero COHb concentration threshold should have been implemented by the investigators to exclude non-suitable cases to be replaced with ones viable for the intended data analysis. All the blood samples had an initial MetHb saturation that was below 7%, thus the results do not give an indication of how samples with moderate to high MetHb saturation behaves in the same conditions.

4.7 Conclusion

The study aimed to investigate suitable conditions best suited for the simultaneous determination of COHb and MetHb. To achieve this, COHb and MetHb levels were measured in post-mortem blood samples stored in different blood collection tubes (red, purple, and green-top tubes) at two temperatures of 4°C and –80°C. Based on the findings, COHb concentrations in stored blood samples are stable in both the refrigerator and freezer for up to a month in EDTA, lithium heparin, or no-additive containing tubes. Methaemoglobin formation occurred by oxidation when blood samples were stored at −80˚C, but MetHb was reduced after four days of storage when blood samples were refrigerated at 4˚C for a month. Refrigerating blood samples in either red-, purple-, or green-top tube were suitable for the accurate determination of both COHb and MetHb, if the analytes are measured immediately or after two weeks of storage.

Regarding the second aim, the COHb saturation from thoracic cavity fluid and blood showed a strong positive association and agreement with each other, with a bias of 1.54% when blood was used as a standard. This suggests that thoracic cavity fluid is useful as an alternative specimen for COHb measurement.

4.8 Recommendations for future studies

The addition of a cryoprotectant solution for the prevention of oxidation in frozen blood samples should be investigated for the stability of both MetHb and COHb. The preservative effect of other anticoagulants not utilised in the current study should be evaluated for the stability of MetHb. The author would also suggest a larger sample size in general and extended storage period.

5. CHAPTER FIVE: REFERENCES

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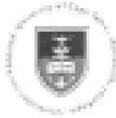
6. CHAPTER SIX: APPENDICES

APPENDIX A: UCT HREC ETHICS APPROVAL LETTER



UNIVERSITY OF CAPE TOWN
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Human Research Ethics Committee

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Website: www.health.uct.ac.za/fhs/research/humanethics/forms

05 May 2021

HREC REF: 291/2021

Mr L Vuko
Division of Forensic Medicine & Toxicology
FHS
Email: Loyiso.vuko@uct.ac.za
Student: mlyjan001@myuct.ac.za

Dear Mr Vuko

PROJECT TITLE: INVESTIGATING THE USE OF CO-OXIMETRY FOR SIMULTANEOUS MEASUREMENT OF CARBOXYHAEMOGLOBIN AND MATHAEMOGLOBIN IN POST-MORTEM BLOOD-MASTERS CANDIDATE-MISS JANE MULEYA-SUB-STUDY LINKED TO R029/2018

Thank you for submitting your study to the Faculty of Health Sciences Human Research Ethics Committee (HREC) for review.

It is a pleasure to inform you that the HREC has **formally approved** the above-mentioned study.

This approval is subject to strict adherence to the HREC recommendations regarding research involving human participants during COVID -19, dated 17 March 2020 & 06 July 2020.

Approval is granted for one year until the 30 May 2022.

Please submit a progress form, using the standardised Annual Report Form if the study continues beyond the approval period. Please submit a Standard Closure form if the study is completed within the approval period.
(Forms can be found on our website: www.health.uct.ac.za/fhs/research/humanethics/forms)

The HREC acknowledge that the student: - Miss Jane Muleya will also be involved in this study.

Please quote the HREC REF 291/2021 in all your correspondence.

Please note that the ongoing ethical conduct of the study remains the responsibility of the principal investigator.

Please note that for all studies approved by the HREC, the principal investigator **must** obtain appropriate institutional approval, where necessary, before the research may occur.

HREC/REF 291/2021sa

APPENDIX B: SRM APPROVAL LETTER

Acting Head of Division: Division of Forensic Medicine and Toxicology

Dr Gavin Kirk

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07 May 2021

To whom it may concern,

As the Acting Clinical Head of the Division of Forensic Medicine and Toxicology, I grant permission for the following researchers to have access to Salt River Mortuary records and/or samples (as specified below) for the research project as stipulated:

Principal Investigator: *Mr Loyiso Vuko (Staff number: 01472127)*
Co-investigators: *Mrs Jade Mader (Staff number: 01472135), Ms Bronwen Davies (Staff number: 01458527)*
Student: *Jane Muleya (Student number: MLYJAN001)*
Project Title: Investigating the use of co-oximetry for simultaneous measurement of carboxyhaemoglobin and methaemoglobin in post-mortem blood

Access to:

Please tick all that apply	
	The autopsy allocations
✓	The Office Autopsy Database and related records
	Forensic Pathology Services Laboratory, Salt River for observation and collection of data
✓	Forensic Pathology Services Laboratory, Salt River for the collection of tissue samples
	Forensic Pathology Services Laboratory, Salt River for conducting Interviews
	Forensic Pathology Services Laboratory, Salt River for obtaining informed consent

For the data collection period of 05/05/2021 to 30/05/2022

Dr Gavin Kirk *(Signature)*

07/05/2021

Date *(dd/mm/yyyy)*

APPENDIX C: SUPPLEMENTARY DATA

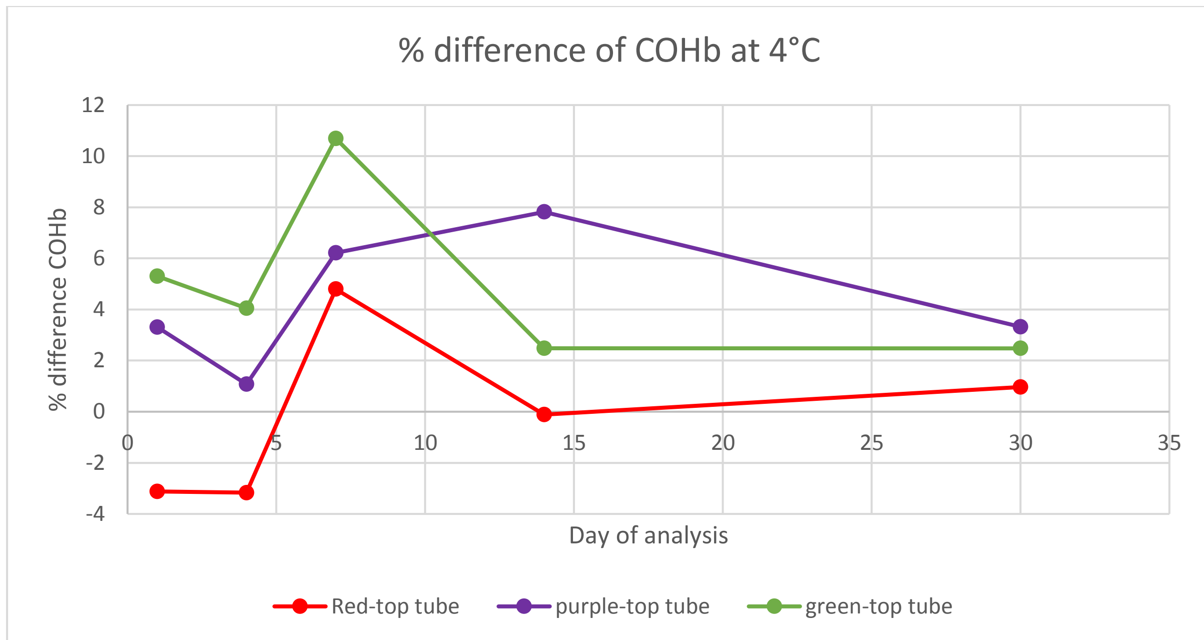

Figure S3.1: Mean % difference of COHb in blood stored at 4°C over a period of 30 days. Post-mortem blood specimens collected and stored at 4°C in three different tube types (red-top, purple-top, and green-top) were analysed on days 0, 1, 4, 7, 14, and 30 of collection. Individual % differences were calculated between the COHb concentrations obtained on day 0 and that of each of the subsequent time intervals. The mean % difference for each tube type was calculated from the individual % difference values obtained for each of the 15 cases included in the analysis.

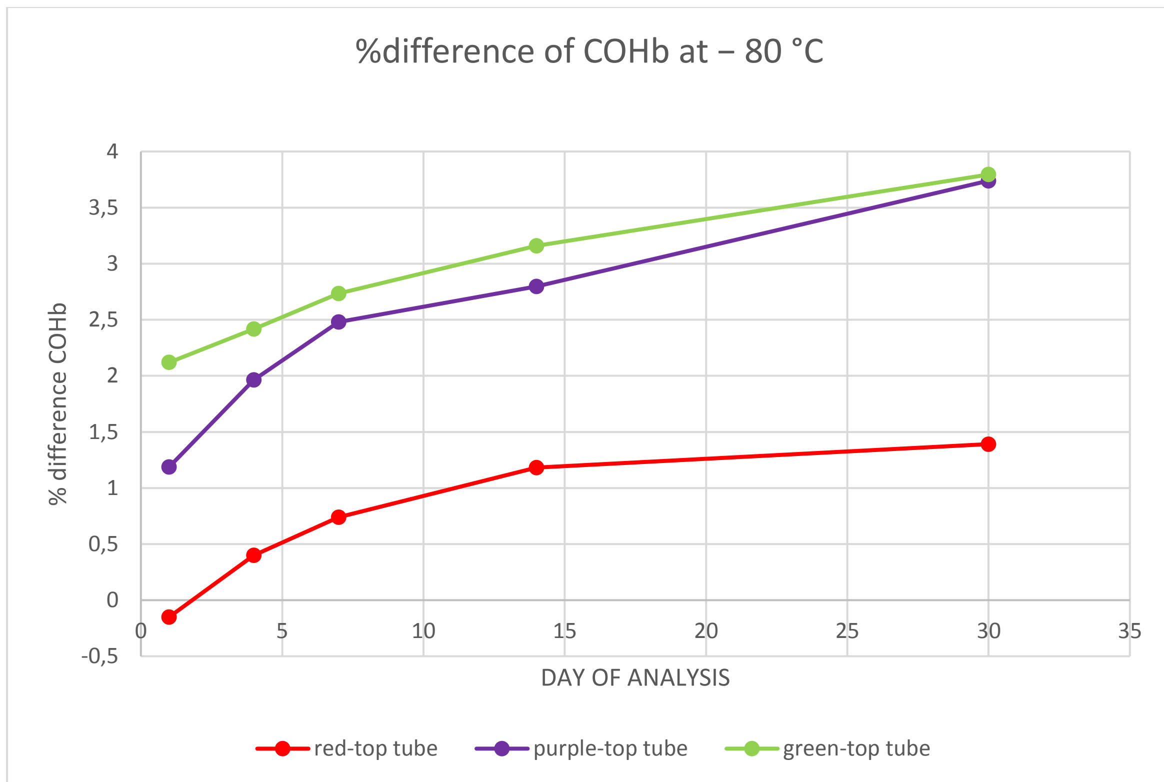

Figure S3.2: Mean % difference of COHb in blood stored at −80°C over a period of 30 days. Post-mortem blood specimens collected and stored at −80°C in three different tube types (red-top, purple-top, and green-top) were analysed on days 0, 1, 4, 7, 14, and 30 of collection. Individual % differences were calculated between the COHb concentration obtained on day 0 and that of each of the subsequent time intervals. The mean % difference for each tube type was calculated from the individual % difference values obtained for each of the 15 cases included in the analysis.