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Analysis of blood for asphyxiant toxicants — Carbon monoxide and hydrogen cyanide

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National foreword

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Analysis of blood for asphyxiant toxicants — Carbon monoxide and hydrogen cyanide

Analyse du sang pour substances toxiques asphyxiantes — Monoxyde de carbone et acide cyanhydrique

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Foreword

ISO (the International Organization for Standardization) is a worldwide federation of national standards bodies (ISO member bodies). The work of preparing International Standards is normally carried out through ISO technical committees. Each member body interested in a subject for which a technical committee has been established has the right to be represented on that committee. International organizations, governmental and non-governmental, in liaison with ISO, also take part in the work. ISO collaborates closely with the International Electrotechnical Commission (IEC) on all matters of electrotechnical standardization.

International Standards are drafted in accordance with the rules given in the ISO/IEC Directives, Part 2.

The main task of technical committees is to prepare International Standards. Draft International Standards adopted by the technical committees are circulated to the member bodies for voting. Publication as an International Standard requires approval by at least 75 % of the member bodies casting a vote.

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights.

ISO 27368 was prepared by Technical Committee ISO/TC 92, *Fire safety*, Subcommittee SC 3, *Fire threat to people and environment*.

Introduction

Carbon monoxide (CO) and hydrogen cyanide (HCN) are two of the primary toxic combustion gases present in fire atmospheres. Upon burning, carbon-containing substances generate CO, whereas nitrogen-containing substances also produce HCN. Since structures surrounding human beings are composed of polymeric materials containing carbon and nitrogen elements as their constituents, these materials generate CO and HCN upon burning and fire victims are exposed to these gases by inhaling smoke. Although ISO 19701 documents methods for the analysis of CO and HCN in fire effluents, the actual toxic insult to exposed persons can be assessed only by the analysis of the fire casualties' blood for CO as carboxyhaemoglobin (COHb) and HCN as cyanide ion (CN^-). These analytical findings are useful for

— estimating life-threatening characteristics of fire atmospheres,

— evaluating the degree of toxicity caused by smoke inhalation in fire victims,

— determining the cause and manner of death of fire victims,

— improving understanding of the direct causes of fire injury and death,

— enhancing understanding of acute and delayed adverse effects of smoke on fire casualties,

— administering immediate treatment for smoke poisoning and monitoring delayed adverse effects of smoke,

— choosing appropriate emergency, long-term and/or follow-up treatments for surviving fire casualties,

— setting priorities for emergency treatment of multiple fire casualties,

— establishing relationships between the concentrations of CO and HCN in a fire atmosphere, blood COHb and CN^- levels, and the degree of toxicity and performance impairment,

— achieving correlations between concentrations of the two gases in fire atmospheres and of COHb and CN^- in blood in order to improve tenability models,

— identifying deficiencies with materials, products, assemblies, structures and escape routes, and

— improving forensic toxicology analytical processes and procedures.

Compliance with this International Standard can help ensure a consistent data set for use in a variety of fields such as

a) fire statistics, which themselves are frequently used to develop regulatory policy,

b) international collaboration on improved design, materials and use of habitable structures, and,

c) ultimately, improvement of international relations and trades.

Such compliance can further assist in developing better and safer fire-safety instruments and structures (residential and commercial buildings; locomotive passenger vans, automobiles, aerospace vehicles and other vehicular structures).

Various different methods are currently used for obtaining blood analysis data for these two fire toxicants and the lack of standardized procedures can result in a wide variation of interpretation. It is, therefore, proposed to set out best-practice, standardized procedures for blood sample collection, sample storage, sample processing/preparation, sample treatment and transfer to analytical instrumentation, analytical instrumentation

and techniques, data presentation and reporting, and guidance for data interpretation. The analytical methods included herein are based upon their suitability for performing an analysis on ante-mortem and post-mortem blood samples from fire victims and are commonly used in forensic toxicological analytical operations.

This International Standard is structured as follows.

— Clause 1 describes the scope of this International Standard.

— Clause 2 cites the normative references.

— Clause 3 provides terms and their definitions.

— Clause 4 lists symbols and abbreviated terms.

— Clause 5 provides a general description of collecting, storing and analysing blood samples.

— Clause 6 covers the quality of materials used during an analysis.

— Clause 7 summarizes common quality analytical elements.

— Clause 8 describes analytical methods for measuring CO as COHb.

— Clause 9 delineates analytical methods for measuring HCN as CN^- in blood.

— Annex A (normative) lists the information crucial for reporting blood analysis results.

— Annex B (informative) outlines additional aspects of analytical methods.

— Annex C (informative) discusses the interpretation of results, including the interactive effects of CO and HCN.

— The bibliography includes references cited in this International Standard.

INTERNATIONAL STANDARD ISO 27368:2008(E)

Analysis of blood for asphyxiant toxicants — Carbon monoxide and hydrogen cyanide

SAFETY PRECAUTIONS — Due consideration shall be given to the fact that both the blood samples for the analyses of asphyxiant toxicants, carbon monoxide (CO) and hydrogen cyanide (HCN), and many of the reagents used for their analyses can be biohazardous and/or toxic and can thereby pose serious health hazards. It is recommended that the collection of blood samples from fire victims be performed by medical practitioners and in accordance with best practices established by the medical authorities in the area. Additionally, it is assumed that the procedures described herein are carried out by suitably qualified professional personnel, adequately trained in the hazards and risks associated with the handling of biological samples and such analyses and aware of any safety regulations that can be in effect. Consideration shall also be given to the safe and ecologically acceptable disposal of all biological samples and chemicals used for analyses. This can require extensive and specific treatment prior to release of the waste into the environment. Again, it is assumed in this International Standard that the personnel responsible for the safe disposal of such bio-samples and reagents are suitably qualified and trained in these procedures and techniques and are aware of the regulations that can be in force.

1 Scope

This International Standard details analytical methods suitable for analysing the two primary toxic combustion gases, carbon monoxide (CO) and hydrogen cyanide (HCN), in blood samples collected from fire casualties. In blood, CO is measured as carboxyhaemoglobin (COHb) and HCN as cyanide ion (CN^-). Although numerous methods are reported in the literature for performing blood COHb and CN^- analyses, the analytical methods included herein are based upon their suitability for performing the analysis on ante-mortem and post-mortem blood samples from fire casualties. The analytical principle, analysis time, repeatability, reproducibility, robustness, effectiveness and instruments used are considered for those methods. Some of the methods described herein might not be suitable for analysing putrid or clotted blood. Burned (solid) blood can be analysed after homogenization.

2 Normative references

The following referenced documents are indispensable for the application of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ISO 3696:1987, *Water for analytical laboratory use — Specification and test methods*

ISO 13344, *Estimation of the lethal toxic potency of fire effluents*

ISO/TS 13571, *Life-threatening components of fire — Guidelines for the estimation of time available for escape using fire data*

ISO 13943, *Fire safety — Vocabulary*

ISO 19701, *Methods for sampling and analysis of fire effluents*

3 Terms and definitions

For the purposes of this document, the terms and definitions given in ISO 19701, ISO 13344, ISO/TS 13571, ISO 13943, ISO 3696, and the following apply.

3.1
analyte
substance that is being identified or determined in a specimen during an analysis

EXAMPLES COHb and CN^-.

3.2
analytical batch
set of aliquots taken out from the specimens associated with various cases (fire casualties) and from negative and positive blind controls for performing a particular type of analysis

3.3
asphyxiant
toxicant causing loss of consciousness and ultimately death resulting from hypoxic (deficiency-of-oxygen) effects, particularly on the central nervous and/or cardiovascular systems

3.4
blind controls
open controls but their identity is unknown to the analysts

See **open controls** (3.20).

3.5
calibrator
material that is based on, or traceable to, a reference preparation or material and whose values are determined by acceptable reference methods

3.6
carboxyhaemoglobin
compound formed when CO combines with haemoglobin

NOTE Haemoglobin has an affinity for binding to CO that is approximately 245 times higher than that for binding to oxygen; thereby the ability of haemoglobin to carry oxygen is seriously compromised during CO poisonings (see C.3.3 and Reference [73]).

3.7
Cheyne-Stokes respiration
breathing pattern characterized by rhythmic waxing and waning of the depth of respiration, with regularly recurring periods of breathing cessation

3.8
cutaneous blood vessels
blood vessels relating to, or affecting, the skin

3.9
cyanogenic glycosides
group of molecules containing a sugar moiety and a cyanide (CN) group

NOTE Cyanogenic glycoside can release the poisonous HCN gas if acted upon by some enzyme.

EXAMPLE Amygadlin from almond.

3.10
cyanomethaemoglobin
compound formed when CN^- combines with methaemoglobin

NOTE During the treatment of CN^- poisonings, haemoglobin is chemically converted to methaemoglobin, which easily binds with CN^-, producing cyanomethaemoglobin. The formation of cyanomethaemoglobin is an essential and critical step in the CN^- detoxification process (see Reference [71]).

3.11
cyanosis
bluish discoloration of the skin caused by the lack of oxygen in the blood

3.12
deoxyhaemoglobin
form of haemoglobin without oxygen, the predominant protein in the red blood cells

NOTE Haemoglobin forms an unstable, reversible bond with oxygen. The oxygen-bonded haemoglobin is known as oxyhaemoglobin. In the oxygen-unloaded form, it is called deoxyhaemoglobin and is purple-blue.

3.13
fire effluent
totality of gases and/or aerosols, including suspended particles, in the atmosphere resulting from combustion or pyrolysis

3.14
fractional toxic concentration
FTC
ratio of the percent of COHb in a blood sample to 70 % COHb (FTC_{COHb}) or of the concentration of CN^-, expressed in micrograms per millilitre, in a blood sample to 3,0 µg/mL CN^- (FTC_{CN^-})

NOTE It is considered that CO at 70 % COHb or HCN at 3,0 µg/mL CN^- individually can cause lethality. For an additive effect of a mixture of the two gases, FTC_{COHb} plus FTC_{CN^-} should be equal to unity. However, the above concept does not rule out other additive effects of these gases (see Clause C.5).

3.15
haemoglobin
biological substance in the red blood cells made up of iron and protein and involved in carrying oxygen to various parts of the body

NOTE Deoxyhaemoglobin or reduced haemoglobin is also referred as to haemoglobin.

3.16
isobestic point
wavelength at which the spectra of various species of a substance have the same absorbance

EXAMPLE The substance haemoglobin and its species oxyhaemoglobin and COHb.

3.17
methaemoglobin
particular type of transformed haemoglobin that is unable to bond with oxygen

NOTE Haemoglobin is converted to methaemoglobin by the oxidation of haemoglobin iron(II) (ferrous iron) into iron(III) (ferric iron). This oxidized form of haemoglobin is in firm union with water and is chemically unable to associate with oxygen; thus, it is ineffective for respiration. Large-scale conversion of haemoglobin to methaemoglobin can cause blueness of skin due to lack of oxygen.

3.18
methanation unit
unit capable of chemically converting CO into methane (CH_4) by using hydrogen in the presence of nickel as a catalyst

3.19
mydriasis
dilatation of the pupil

3.20
open controls
specimens prepared for the purpose of being used as a control and known to the analysts

3.21
oxyhaemoglobin
oxygen-bonded form of haemoglobin, the predominant protein in the red blood cells

NOTE Haemoglobin forms an unstable, reversible bond with oxygen. In its oxygen-loaded form, it is called oxyhaemoglobin and is bright red.

3.22
polymeric materials
materials composed of polymers

NOTE A polymer is a large molecule made up of many smaller repeating chemical units bonded together. These units are known as monomers. Some polymers are naturally occurring, while others are synthetically manufactured.

3.23
post-mortem interval
period after death

EXAMPLE Time between death and blood sample collection from a dead body.

3.24
putrefaction
decomposition of organic matter, especially protein, by microorganisms, resulting in the formation of substances of less complex constitution with the evolution of ammonia, hydrogen sulfide and other substances and, thus, in the production of foul-smelling matter

NOTE This process is usually characterized by the presence of malodorous smell.

3.25
pyocyaneous organisms
group of microorganisms capable of producing CN^-

3.26
reduced haemoglobin
haemoglobin in the red blood cells after the removal of oxygen from oxyhaemoglobin or after the reduction of iron(III) (ferric iron) in methaemoglobin to iron(II) (ferrous iron)

3.27
sulfaemoglobin
product formed by the action of hydrogen sulfide (or sulfides) on iron(III) (ferric iron) in methaemoglobin

NOTE This haemoglobin product is also known as sulfmethaemoglobin.

3.28
tachycardia
excessive rapidity in the action of the heart

3.29
tachypnea
excessive rapidity of respiration

3.30
thermostatization
process of automatic temperature regulation, especially wherein the expansive force of metals or gas acts directly upon the source of heat, ventilation or the like, or controls them indirectly by opening and closing an electric circuit

NOTE Derived from the term “thermostat”.

3.31
toxicants
poisonous substances capable of causing adverse, unwanted or undesired effect(s) on a living system

NOTE For the purpose of this International Standard, these substances are CO and HCN.

3.32
toxic insult
adverse, unwanted or undesired effect(s) on a living system due to, pertaining to, or of the nature of a poison

4 Symbols and abbreviated terms

A	Area
α	Absorbance
C	Concentration
CBI	1-Cyano-2-benzoisoindole or 1-cyano[*f*]benzoisoindole
ClCN	Cyanogen chloride
CN	Cyanide
CN^-	Cyanide ion
CO	Carbon monoxide
COHb	Carboxyhaemoglobin
ECD	Electron capture detector
EDTA	Ethylenediaminetetraacetate
F	Factor
FED(s)	Fractional effective dose(s)
FID	Flame ionization detector
FTC	Fractional toxic concentration
HCN	Hydrogen cyanide
HHb	Deoxyhaemoglobin
HPLC	High-performance liquid chromatograph

IEC	International Electrotechnical Commission
ISO	International Organization for Standardization
MetHb	Methaemoglobin
MSD	Mass spectrometric detector
NDA	2,3-Naphthalenedialdehyde
NPD	Nitrogen phosphorus detector
OxyHb	Oxyhaemoglobin
R	Ratio
TCD	Thermal conductivity detector
tHb	Total haemoglobin
TIC	Total-ion chromatogram
V	Volume
w	Mass fraction

5 Blood samples

5.1 General

For the analyses of COHb and CN^-, blood from fire victims should be properly collected as soon as possible, preserved, stored and analysed as quickly as possible. See also C.3.1 and C.4.1.

5.2 Sample condition

Fresh blood samples can be easily obtained from live fire victims, but collecting quality blood samples from fire fatalities can frequently be challenging. This challenge is linked to the condition of the body, which is affected by the severity of burn, the time between the death and the discovery of the body (post-mortem interval), and the environmental factors, such as temperature and humidity. There are reports of the condition of blood, for example, fresh or putrid blood, having an impact on the outcome of the analyses. Therefore, the documentation of the history, condition and characteristics of the blood samples is crucial, and this information, along with the blood samples, should be submitted to the analytical laboratories performing analyses.

5.3 Sample collection

It is recommended that blood samples from fire casualties be preferably collected in 10 ml (or smaller size) sterile glass tubes containing heparin, or 20 mg of potassium oxalate and 100 mg of sodium fluoride, to prevent blood clotting and/or to preserve the specimens [1]. Some analytical methods use heparinized blood, while other methods can use blood treated with either heparin or potassium oxalate-sodium fluoride. The headspace in the tubes should be kept to a minimum and the tubes containing the blood samples should be airtight sealed to minimize dissociation of CO and HCN and to prevent any escape of these gases from the collected blood. Post-mortem blood samples can be collected from the heart, though no statistically significant difference has been observed between the COHb levels in post-mortem heart blood and peripheral blood specimens [2]. Regardless of the blood collection site, however, it is recommended that the sample collection site be mentioned in the documents submitted with the blood samples for analysis.

5.4 Sample storage

The blood specimens should be stored at 4 °C in the airtight, sealed containers to prevent the loss of CO, denaturation of haemoglobin and release of HCN [3],[4],[5],[6],[7],[8]. If it is necessary to store samples for a long period prior to analysis, then the samples should be frozen [3],[4],[5],[6],[9],[10],[11],[12],[13],[14].

5.5 Sample analysis

Analyses should be performed as quickly as possible after the collection of blood [9],[15],[16]. It is essential for the analysis of COHb to homogenize those blood samples that are not homogeneous. A similar recommendation has also been made for CN^- that autopsy blood should be homogenized before the analysis [17].

6 Materials

All reagents, solvents, gases, and chemicals used in analyses should be of analytical grade quality and of the highest available purity. Water used should be as defined in ISO 3696:1987, quality 3.

7 Common quality analytical elements

7.1 General

Forensic blood samples are precious. Depending upon the nature of the fire accident and condition of the fire victim, a blood sample might, or might not, have been submitted in a large amount for analyses. Once the blood samples are consumed during analyses, it might not be possible to obtain additional samples from the sample submitters. Therefore, it is customary in forensic toxicological operations to use samples submitted for analyses conservatively and cautiously.

Unless stated otherwise, all blind and open controls and calibrators used for analysis shall be prepared in human whole blood. It is important that blood be collected from healthy human subjects who are not smokers and are not exposed to CO. In other words, the collected human blood shall be free from CO and CN^-.

7.2 Qualitative, quantitative and confirmatory analyses

It is recommended that a qualitative analysis (screening) be performed initially on a portion (aliquot) of the blood sample collected from each victim. On the qualitatively positive (presumptive positive) samples, a quantitative analysis should be conducted. Although qualitative and quantitative analyses in some methods can be simultaneously conducted on the same aliquot, it is preferred that the quantitative analysis be performed on a different aliquot of the submitted sample than that which was used during the initial qualitative analysis.

Additionally, quantitative analytical results should be confirmed on a different aliquot of the blood sample by a second method based upon a analytical principle different from the method used during the first quantitative analysis. Such confirmatory analyses can be qualitative or quantitative.

7.3 Replicate analyses

If a sufficient amount of sample is not submitted, then a single analysis is obviously the option. Otherwise, it is recommended that the aliquot of a sample be analysed in duplicate for both qualitative and quantitative analyses. If one or both of the qualitative duplicate results is/are positive, then the sample should be analysed quantitatively.

The mean of the duplicate quantitative values should be reported, provided the duplicate analytical values do not differ by more than 10 % from the mean value. In the event that the duplicate values do not meet this difference criterion, the mean value should be rejected and a new aliquot of the sample should be reanalysed.

As mentioned in 7.2, positive findings should be qualitatively or quantitatively confirmed by a second analytical method using a different aliquot of the sample. For this second analysis, if the sample is not available in sufficient amount, a single analysis can be performed. Otherwise, the analysis should be conducted in duplicate and the mean of the two values should be calculated and evaluated to determine if the value meets the 10 % criterion. If the mean value meets the criterion, the value can be acceptable. Otherwise, the analysis can be accepted as a qualitative analytical finding, provided both duplicate analyses are positive. If the positive findings cannot be confirmed by a second analytical method, then the sample should be considered negative for the analytes.

A laboratory may choose to report the one of the two acceptable quantitative mean values deemed to be obtained from the most reliable analytical method. This decision can also be based upon the laboratory's standard operating procedures.

The total amount of sample required for the analyses is based on the selectivity and sensitivity of the methods adopted by a particular laboratory. It should also be considered that the submitted blood sample will be analysed in duplicate for blood COHb and for blood CN^-. Therefore, these factors should be carefully evaluated and considered by the sample collector, sample submitter and the laboratory receiving the sample and conducting the analyses.

7.4 Analytical batch

In addition to the aliquots of the blood samples from fire victims, each analytical batch shall contain at least two aliquots from blind controls: one from a negative blind control and the other from a positive blind control. In any batch, identity, origin and sequence of the aliquots in relation to the blood samples of the victims or of the blind controls shall not be known to the analysts performing the batch analysis. The analytical result of the negative blind control should be negative and, for the positive blind control, it should be within the limits of the target values established by the respective laboratories. If these two analytical criteria are not met, the batch can be rejected and a new analytical batch can be issued for analysis.

NOTE A negative blind control is a blood specimen free from CO and CN^-. A positive blind control is a blood specimen containing known amounts of COHb and CN^-.

7.5 Open controls

Along with the aliquots of a batch, one negative open control and at least one positive open control shall be processed and analysed by the analysts. Open controls should be known to the analysts. A single analysis is acceptable for open controls. Analytical results for negative open control shall be negative and, for positive open control, it shall be within $\pm$ 20 % of the target value established by the laboratory. If open control results do not meet these criteria, then a new analytical batch should be issued and the samples should be reanalysed.

7.6 Calibrators

The calibrators shall cover the linear range of the calibration curve. The analytical values of the samples shall fall between the lowest and the highest calibrators in the linear range of the curve.

8 Measurement of CO in blood as COHb

8.1 COHb by whole-blood oximeters

8.1.1 Principle

Oximeters are commonly self-contained instruments and include hardware and electronics. By means of these dedicated, special-purpose instruments, the percentage of COHb in suitably diluted whole-blood samples is measured by simultaneous automated differential visible spectrometry at various characteristic wavelengths.

8.1.2 Reagents and materials

The instrument vendors supply necessary reagents/materials, such as blood diluent solution, zeroing solution, cleaning agent solution, calibrators and other necessary reagents and supplies.

8.1.3 Apparatus

Examples of commercially available oximeters[1)] are CO-Oximeter (Instrumentation Laboratory, Inc., Lexington, MA) and AVOXimeter (A-VOX Systems, Inc., San Antonio, TX) [18],[19],[20].

NOTE These devices also measure whole-blood deoxyhaemoglobin (HHb), oxyhaemoglobin (OxyHb), and methaemoglobin (MetHb).

8.1.4 Sample

The amount of blood sample required for the analysis ranges from 100 µl to 400 µl. The recommended amount of the sample is 0,5 ml to 2 ml.

8.1.5 Procedure

Instrument manuals provide details of the analytical procedures. Analysis of the samples shall be performed following the instructions given in the manuals. Oximeters shall be calibrated as instructed by the manufacturers.

8.1.6 Calculation

Digital readout of percentage COHb is usually displayed by the instruments. Percentages of HHb, OxyHb and MetHb are also displayed. The percent mass fraction of COHb, w_{COHb}, is calculated by Equation (1):

$$w_{\mathrm{COHb}} = \left(\frac{C_{\mathrm{COHb}}}{C_{\mathrm{COHb}} + C_{\mathrm{HHb}} + C_{\mathrm{OxyHb}} + C_{\mathrm{MetHb}}} \right) \times 100 \qquad (1)$$

where

C_{COHb} is the concentration of COHb;

C_{HHb} is the concentration of HHb;

C_{OxyHb} is the concentration of OxyHb;

C_{MetHb} is the concentration of MetHb.

NOTE The sum of the concentrations of COHb, HHb, OxyHb, and MetHb, expressed in grams per decilitre, is considered equal to the total haemoglobin (tHb), expressed in grams per decilitre.

8.1.7 Sensitivity

Oximeters are capable of measuring $w_{\mathrm{COHb}} \geqslant 10$ % in fresh blood from live victims with an accuracy of 1 % to 2 %. The main difference between the results obtained from oximeter analyses of 23 blood samples and the analyses by gas chromatography and photometry analyses was 0,35 % [18],[21].

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1) These are examples of suitable products available commercially. This information is given for the convenience of users of ISO 27368 and does not constitute an endorsement by ISO of these products.

8.1.8 Application and limitation

These devices are suitable for determining the mass fraction of COHb in fresh, heparinized blood samples and might not be suitable for the analysis of putrid or clotted blood samples. Ethylenediaminetetraacetate (EDTA) can also be used as an anticoagulant with the CO-Oximeter [19]. With the AVOXimeter, citrate, fluoride, oxalate and EDTA have been reported to cause errors in the measurements [20].

8.2 COHb by palladium chloride reduction

8.2.1 Principle

This method is based upon the release of CO from COHb by sulfuric acid added to the blood sample in the outer rim of a Conway cell [22],[23],[24]. The released CO diffuses in the cell and reduces palladium chloride in the centre well of the cell to palladium, forming a shining black film of the metal on the surface of the palladium chloride solution. The absorbance, α_{278}, of the remaining unreacted palladium chloride solution in the centre well is measured at 278 nm. Additionally, the absorbance of a new aliquot of the palladium chloride solution is measured. These two absorbance values are compared. The difference between the two values can be used as a measure of CO released from the blood sample. By determining the concentration of tHb in the blood, COHb saturation can be calculated.

NOTE tHb can be measured by oximeters [19],[20] or by a colourimetric method [25],[26]. The colourimetric method is based upon the oxidation of HHb and its derivatives to MetHb, its conversion to cyanomethaemoglobin, and measuring absorbance at 540 nm. Reagent kits for determining concentrations of Hb in blood are commercially available (Pointe Scientific, Inc., Canton, MI)[2)].

8.2.2 Reagents and materials

8.2.2.1 Sealant.

8.2.2.2 Hydrochloric acid, 0,1 M.

8.2.2.3 Palladium chloride, 0,002 5 M.

Dissolve 0,440 g of palladium chloride in 500 ml of 0,1 M HCl in a 1 000 ml volumetric flask. After mixing the solution and allowing it to stand overnight, bring the final volume of the solution to 1 000 ml with 0,1 M HCl. One millilitre of this 0,002 5 M palladium chloride solution is equivalent to 0,056 ml of CO [22],[23],[24].

8.2.2.4 Sulfuric acid, 1,8 M; 10 %.

8.2.2.5 Lead acetate-acetic acid solution.

Dilute 10 ml of glacial acetic acid to 100 ml with water and saturate this solution with lead acetate.

8.2.3 Apparatus

8.2.3.1 Spectrophotometer.

8.2.3.2 Conway cells.

8.2.3.3 Cuvettes.

2) This is an example of a suitable product available commercially. This information is given for the convenience of users of ISO 27368 and does not constitute an endorsement by ISO of this product.

8.2.4 Sample

The method requires 0,5 ml of blood per analysis. Considering the determination of tHb also, the preferred amount of sample is 2 ml to 3 ml.

8.2.5 Procedure

a) Spread a thin layer of the sealant on the lid of a Conway cell in a circle comparable to the outer rim of the cell.

b) Pipette 3 ml of the palladium chloride solution into the centre well of the cell.

c) Subsequently, pipette 1 ml of 10 % sulfuric acid into the outer well of the cell and place the lid over the cell, leaving an opening to allow addition of the blood sample.

d) Transfer 0,5 ml of the blood sample, slide the lid over the opening to seal the cell, mix the outer cell contents by gentle rotation, and allow the cell to stand for 2 h at ambient temperature.

e) After the 2 h of diffusion of CO from the blood, remove the lid from the cell and observe the formation of the shining black film of metallic palladium on the surface of the palladium chloride solution in the centre well. The presence of film suggests that the sample is positive for CO; otherwise the sample can be considered negative for CO. The extent of the reduction of palladium chloride to palladium is a function of the CO released from the specimen. Positive samples should be quantitatively analysed as described in the following steps f) to h).

f) For a quantitative analysis, transfer the contents of the centre well to a 50 ml volumetric flask by rinsing three times with 3 ml of 0,1 M HCl and diluting to the final volume of 50 ml with 0,1 M HCl. This solution should be mixed thoroughly.

g) Determine the absorbance of the above solution in a 1 cm silica cuvette at 278 nm, using 0,1 M HCl as a reference.

h) Using the same hydrochloric acid reference solution, determine the absorbance in a 1 cm silica cuvette of the palladium chloride solution obtained by diluting 3 ml of 0,005 M palladium chloride to 50 ml with 0,1 M HCl.

8.2.6 Calibrators and calculation

a) Dilute 0,5 ml; 1,0 ml; 1,5 ml; 2,0 ml; 2,5 ml; and 3,0 ml of 0,002 5 M palladium chloride to 50 ml with 0,1 M HCl.

b) After thoroughly mixing the palladium chloride solutions described in a), determine the absorbance of the solutions in a 1 cm silica cuvette at 278 nm against 0,1 M HCl.

c) Plot the obtained absorbance values on the ordinate against volumes of CO, expressed in millilitres per 100 ml of solution, on the abscissa. The CO values with respect to the palladium chloride solutions are given below.

Table 1 — Correspondence of palladium chloride and CO concentrations

Palladium chloride ml/50 ml of solution	**CO** ml/100 ml of solution
0,5	28,0
1,0	22,4
1,5	16,8
2,0	11,2
2,5	5,6
3,0	0,0

d) From the curve, determine the CO volume for the blood sample. If the absorbance of the palladium chloride solution differs (at zero volume) from that shown on the curve, it is necessary to construct a new reference curve parallel to the old one, passing through the new zero point.

e) After determining the tHb concentration in an aliquot of the original blood specimen [19],[20],[25],[26], calculate the percent mass fraction of COHb, w_{COHb}, by using Equation (2):

$$w_{COHb} = \frac{(V_{CO} \times 100)}{(C_{tHb} \times 1{,}35)} \quad (2)$$

where

V_{CO} is volume of CO, expressed in millilitres per 100 ml of solution (see Table 1);

C_{tHb} is tHb concentration, expressed in grams per 100 ml of the blood sample;

1,35 is a factor [14],[23],[24],[27],[28].

NOTE To calculate the percentage of COHb, it is necessary to know the blood CO capacity, which is calculated by multiplying the tHb concentration by the factor.

8.2.7 Sensitivity

The palladium chloride method does not permit a valid estimation of $w_{COHb} \leqslant 10$ %. The coefficient of variation of the mass fraction of COHb by this method is ± 5,2 %.

8.2.8 Application and limitation

This procedure can be used for the analysis of fresh or uncoagulated ante-mortem or post-mortem blood samples. A visual observation of a shiny black film on the palladium chloride solution suggests the presence of $w_{COHb} > 30$ %. Putrid blood samples might not be suitable for the analysis as sulfides present in putrid blood in large amounts interfere with the analysis. However, such interference can be rectified by using a saturated lead acetate-acetic acid solution in place of sulfuric acid as the CO liberating reagent.

8.3 COHb by visible spectrophotometry (using calibration curve)

8.3.1 Principle

Red cells of the blood specimen are haemolyzed using ammonium hydroxide and the hemolyzate is treated with sodium dithionite to reduce MetHb and OxyHb to HHb. COHb is unaffected by such treatment. The hemolyzate solution is scanned from 450 nm to 650 nm. The absorbance is recorded at 540 nm, a wavelength of maximum absorbance for COHb, and at 579 nm, a wavelength at which the spectra of the various species of HHb have the same absorbance (isobestic point). A ratio of the absorbance values at 540 nm (α_{540}) and 579 nm (α_{579}) is used to determine the percent mass fraction of COHb in the specimen from a calibration curve [14],[29],[30],[31],[32],[33],[34].

8.3.2 Reagents and materials

8.3.2.1 Ammonium hydroxide, 0,4 % aqueous solution.

Dilute approximately 16 ml of concentrated ammonium hydroxide (28 % to 29 %) to 1 000 ml with deionized water.

8.3.2.2 Sodium dithionite (sodium hydrosulfite).

Weigh 10 mg portions of sodium dithionite into individual small test tubes. Stopper the test tubes or cover tubes with Parafilm[3)].

NOTE Sodium dithionite must be freshly obtained and should be stored in a sealed container in a desiccator to prevent its decomposition in contact with moisture.

8.3.2.3 Compressed gases: oxygen, CO, and nitrogen.

8.3.3 Apparatus

8.3.3.1 Spectrophotometer.

8.3.3.2 Separatory funnels.

8.3.3.3 Rotator.

8.3.3.4 Cuvettes.

8.3.4 Sample

The method requires approximately 100 µl of blood per analysis. A sample of approximately 0,5 ml is preferred.

8.3.5 Procedure

a) Pipette 100 µl of whole heparinized blood into 25 ml of the 0,4 % ammonium hydroxide solution. Mix the blood hemolyzate and allow it to stand for 2 min.

b) Transfer 3 ml of the ammonium hydroxide solution (blank) and 3 ml of the hemolyzate (test sample), respectively, into 1 cm cuvettes.

c) Add 10 mg of sodium dithionite to each cuvette, cover the cuvettes with Parafilm, and invert gently 10 times.

d) Exactly 5 min after the addition of sodium dithionite to the sample, scan the sample from 450 nm to 650 nm against the ammonium hydroxide solution blank.

e) Record the absorbance at 540 nm and 579 nm, calculate the ratio of the absorbance at 540 nm to that at 579 nm and determine the percentage mass fraction of COHb in the unknown sample from the calibration curve.

8.3.6 Calibration curve and calculation

a) Collect 20 ml of CO-free blood from healthy human subjects. This blood should be heparinized. The fresh blood collected should be treated immediately.

b) Transfer 4 ml of the fresh, heparinized blood sample into each of two 125 ml separatory funnels. Treat one sample with pure oxygen and the other with pure CO for 15 min while the funnels are gently rotated. After the purging of the gases, close the separatory funnels and rotate them gently for an additional 15 min. Analyse the fully saturated samples and use these results for the establishment of the 0 % and 100 % w_{COHb} calibration points.

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3) Parafilm is an example of a suitable product available commercially. This information is given for the convenience of users of ISO 27368 and does not constitute an endorsement by ISO of this product.

c) Plot the $\alpha_{540}/\alpha_{579}$ ratios for the 0 % w_{COHb} and for the 100 % w_{COHb} and draw a line between the two points.

d) Fill the funnel containing the 100 % w_{COHb} sample with nitrogen and rotate it for 5 min. The treatment with nitrogen removes the dissolved CO from the sample, but a small amount of CO will also dissociate from COHb.

e) Determine the exact percentage mass fraction of COHb of this sample as described in 8.3.5, using the two-point calibration curve as prepared in step c). Prepare intermediate calibration solutions by mixing appropriate proportions of the CO-nitrogen-treated blood sample with the oxygen-treated blood sample.

f) Plot the calculated percentage mass fractions of COHb in the intermediate calibration solutions on the ordinate against the absorbance ratios on the abscissa. These intermediate calibration solutions should fall on the line drawn for the fully oxygen-saturated and fully CO-saturated samples, since the calibration curve is linear over the entire range.

8.3.7 Sensitivity

This method permits an estimation of $w_{COHb} \geqslant 10$ %.

8.3.8 Application and limitation

This procedure can be used for the analysis of fresh or uncoagulated ante-mortem or post-mortem blood samples. Putrid blood might not be suitable for the analysis as pigments resulting from decomposition can distort the combined COHb and HHb spectral scan.

8.4 COHb by visible spectrophotometry (with CO saturation)

8.4.1 Principle

Blood specimens are treated with ammonium hydroxide to haemolyze the red cells. The obtained hemolyzate is split into three parts: part A is saturated with CO and part B with oxygen; part C is not treated with any gas. To each of the three parts, sodium dithionite is added to reduce MetHb and OxyHb to HHb. These three solutions are scanned in the range of 450 nm to 650 nm and absorbance values of each solution are noted at 540 nm and at 579 nm (see also 8.3.1). Ratios of the absorbances of the solutions at 540 nm (α_{540}) and 579 nm (α_{579}) are used to determine the percentage mass fraction of COHb, w_{COHb}, in the specimen using Equation (3) [14],[32],[33],[34],[35],[36],[37].

8.4.2 Reagents and materials

8.4.2.1 **Ammonium hydroxide**, 0,03 % aqueous solution.

8.4.2.2 **Compressed gases**: oxygen and CO.

8.4.2.3 **Sodium dithionite** (sodium hydrosulfite).

8.4.3 Apparatus

8.4.3.1 **Spectrophotometer**.

8.4.3.2 **Vortexer**.

8.4.3.3 **Cuvettes**.

8.4.4 Sample

The method requires 200 µl of blood per analysis. The preferred amount of aliquot needed is 1 ml.

8.4.5 Procedure

a) Dilute 200 µl of the heparinized whole-blood sample with 25 ml of the ammonium hydroxide solution; mix it well and avoid clots.

b) Aliquot 5 ml of this mixture into three tubes, labelled as A, B, and C.

c) Saturate solution A with CO by bubbling the gas very slowly through the mixture for 5 min to 10 min to obtain a 100 % w_{COHb} standard.

d) Saturate solution B with pure oxygen by bubbling the gas very slowly through the mixture for at least 10 min to displace all the bound CO to provide a 0 % w_{COHb} standard.

e) Use solution C without any gas treatment.

f) To each of the three tubes (A, B, and C), add approximately 150 mg of sodium dithionite and 10 ml of the ammonium hydroxide solution.

g) Vortex the mixtures for a few seconds.

h) Scan each mixture from 450 nm to 650 nm against the ammonium hydroxide solution, record the absorbance values of each mixture at 540 nm and 579 nm, and calculate the respective ratios of absorbance of the mixtures at 540 nm to that at 579 nm (see 8.4.6).

8.4.6 Calculation

Calculate the percentage mass fraction of COHb, w_{COHb}, in the blood sample by using Equation (3):

$$w_{\mathrm{COHb}} = \left\{ \left[\left(\frac{\alpha_{540\mathrm{C}}}{\alpha_{579\mathrm{C}}} \right) - \left(\frac{\alpha_{540\mathrm{B}}}{\alpha_{579\mathrm{B}}} \right) \right] \Big/ \left[\left(\frac{\alpha_{540\mathrm{A}}}{\alpha_{579\mathrm{A}}} \right) - \left(\frac{\alpha_{540\mathrm{B}}}{\alpha_{579\mathrm{B}}} \right) \right] \right\} \times 100 \qquad (3)$$

where

$\alpha_{540\mathrm{A}}$, $\alpha_{579\mathrm{A}}$ are the absorbances at 540 nm and 579 nm, respectively, of the CO-saturated sample A;

$\alpha_{540\mathrm{B}}$, $\alpha_{579\mathrm{B}}$ are the absorbances at 540 nm and 579 nm, respectively, of the oxygen-saturated sample B;

$\alpha_{540\mathrm{C}}$, $\alpha_{579\mathrm{C}}$ are the absorbances at 540 nm and 579 nm, respectively, of sample C.

8.4.7 Sensitivity

This method can accurately measure $w_{\mathrm{COHb}} \geqslant 10$ %.

8.4.8 Application and limitation

This method is suitable for the analysis of fresh blood collected from living individuals. The sample should be heparinized. Because of the saturation of samples with CO and oxygen, this method is time-consuming and might not be efficient for analysing several samples on a daily basis, especially for screening purposes in clinical situations or for analysing decomposed blood.

8.5 COHb by visible spectrophotometry (without CO saturation)

8.5.1 Principle

Ammonium hydroxide is used to haemolyze red cells of blood specimens. The hemolyzate is treated with sodium dithionite to reduce MetHb and OxyHb to HHb [14],[32],[33],[34]. The solution thus obtained is scanned from 450 nm to 650 nm, and the absorbance is recorded at 540 nm and at 579 nm (also see 8.3.1). A ratio of the absorbance values of the specimen at 540 nm (α_{540}) and 579 nm (α_{579}) is used to determine the percentage mass fraction of COHb, w_{COHb}, in the specimen by using Equation (4).

NOTE In this method, the samples are not saturated with CO. However, a positive COHb control in human blood is prepared by using CO.

8.5.2 Reagents and materials

8.5.2.1 Sodium dithionite-ammonium hydroxide solution.

Dissolve 5 g of sodium dithionite in 500 ml of water in a 1 000 ml volumetric flask. Bring the volume of the sodium dithionite solution to 1 000 ml with water. Add 4 ml of concentrated ammonium hydroxide into the sodium dithionite solution. After mixing, transfer this sodium dithionite-ammonium hydroxide solution to an amber glass dispensing bottle and let this solution equilibrate for at least 15 min before use.

NOTE This solution is not very stable. It has a short shelf-life of no more than 4 h. Therefore, it is necessary to prepare this solution shortly before analysis.

8.5.2.2 Compressed gases: CO and nitrogen.

8.5.3 Apparatus

8.5.3.1 Spectrophotometer.

8.5.3.2 CO-Oximeter.

8.5.3.3 Tube rocker.

8.5.3.4 Rotator.

8.5.3.5 Cuvettes.

8.5.4 Sample

Per analysis, this method requires 100 µl of blood samples. The preferred amount of sample is 0,5 ml.

8.5.5 Procedure

a) Pipette 100 µl of the specimen into a tube containing 10 ml of the sodium dithionite-ammonium hydroxide solution. Try to avoid clots.

b) Place Parafilm on the tube and invert it several times to mix its contents. Tubes with clots should be shaken vigorously.

c) Five minutes after the addition of the blood specimen, scan the mixture from 450 nm to 650 nm against the sodium dithionite-ammonium hydroxide solution.

d) Record the absorbance readings at 540 nm and 579 nm, calculate the ratio of the absorbance at 540 nm to that at 579 nm and determine the percentage mass fraction of COHb, w_{COHb}, from Equation (4).

8.5.6 Calibration

a) Collect fresh CO-free blood from healthy human subjects into 10 ml sterile glass tubes containing sodium fluoride and potassium oxalate. Rock these tubes for 30 min to ensure that samples are well mixed. The fresh blood collected should be treated immediately after collection.

b) For negative COHb control:

— Transfer the fresh blood sample into a 200 ml volumetric flask, place the flask in a horizontal position, and rotate the flask while nitrogen at a flow rate of $<$ 1,0 ml/min purges the flask for 2 min to 3 min.

— Pipette a portion of this negative control solution into small, plastic, 1,5 ml standard microcentrifuge tubes. Each tube should be filled to its capacity and sealed with the plastic cap attached to the tube. These negative control aliquots should be stored overnight at 4 °C and tested the next day on a CO-Oximeter to obtain the average concentration for the negative COHb control.

c) For positive COHb control:

— Transfer the remaining portion of the negative control solution into a 200 ml volumetric flask, place the flask in a horizontal position, and rotate the flask while CO at a flow rate of $<$ 1,0 ml/min purges the flask for 20 min. This blood sample is considered the positive control for w_{COHb} equal to approximately 100 %.

— Dilute the 100 % w_{COHb} control with the negative COHb control in a proportion of 4,5:5,5 to obtain a control with w_{COHb} equal to approximately 45 %.

— Pipette a portion of this approximately 45 % w_{COHb} positive control into small, plastic, 1,5 ml standard microcentrifuge tubes. Each tube should be filled to its capacity and sealed with the plastic cap attached to the tube. These positive CO control aliquots should be stored overnight at 4 °C and tested the next day on a CO-Oximeter to obtain the average concentration for the positive CO control.

NOTE Allowing all prepared controls to equilibrate overnight is essential in order to reach complete equilibrium between bound and unbound CO. Kinetic experiments suggest that it takes several hours after the preparation of the standard for the CO-saturated blood to reach equilibrium.

d) Run the negative COHb and positive COHb controls on CO-Oximeter 10 times each to obtain an average w_{COHb} level and a standard deviation.

e) Run the COHb negative and COHb positive controls on the spectrophotometer 10 times each to obtain their average intensity ratios for α_{540} to α_{579}.

f) Use the mean CO-Oximeter values of the two controls (negative and positive COHb controls) to calibrate the spectrophotometer.

8.5.7 Calculation

a) If the factors R_{A}, R_{B} and R_{C} are assigned the values of the absorbance ratios for solutions A, B and C (see 8.4.1 and 8.4.6), respectively, the equation [14],[32],[35],[36],[37] for calculating the percentage mass fraction of COHb, w_{COHb}, can be written as Equation (4):

$$w_{\mathrm{COHb}} = \left[\left(R_{\mathrm{C}} - R_{\mathrm{B}}\right) / \left(R_{\mathrm{A}} - R_{\mathrm{B}}\right)\right] \times 100 \qquad (4)$$

where

R_A is the ratio $\frac{\alpha_{540A}}{\alpha_{579A}}$;

R_B is the ratio $\frac{\alpha_{540B}}{\alpha_{579B}}$;

R_C is the ratio $\frac{\alpha_{540C}}{\alpha_{579C}}$.

b) The constant values for R_A and R_B are calculated using Equations (5) and (6), respectively, from the values obtained for the negative and positive COHb controls from the CO-Oximeter and spectrophotometer measurements [33],[34]:

$$R_A = R_B + \left[\frac{\left(\bar{\alpha}_{(540/579),pCOHb} - \bar{\alpha}_{(540/579),nCOHb} \right)}{\left(\bar{C}_{pCOHb} - \bar{C}_{nCOHb} \right)} \right] \quad (5)$$

$$R_B = \bar{\alpha}_{(540/579),pCOHb} - \left\{ \bar{C}_{pCOHb} \times \left[\frac{\left(\bar{\alpha}_{(540/579),pCOHb} - \bar{\alpha}_{(540/579),nCOHb} \right)}{\left(\bar{C}_{pCOHb} - \bar{C}_{nCOHb} \right)} \right] \right\} \quad (6)$$

where

$\bar{\alpha}_{(540/579),pCOHb}$ is the average of the $\frac{\alpha_{540}}{\alpha_{579}}$ ratios for the positive COHb control from spectrophotometer measurements;

$\bar{C}_{pCOHb}$ is the average decimal concentration measured on the CO-Oximeter for the positive COHb control;

$\bar{\alpha}_{(540/579),nCOHb}$ is the average of the $\frac{\alpha_{540}}{\alpha_{579}}$ ratios for the negative COHb control from spectrophotometer measurements;

$\bar{C}_{nCOHb}$ is the average decimal concentration measured on the CO-Oximeter for the negative COHb control.

c) Use the R_A and R_B constants, once calculated, to determine the percentage mass fraction of COHb, w_{COHb}, in a sample from Equation (4). From the procedures in 8.5.6 and Equations (5) and (6), the R_A and R_B constants were determined to be 1,543 and 1,128, respectively, for Equation (4) [33],[34].

8.5.8 Sensitivity

The method is capable of accurately measuring $w_{COHb} \geqslant 10$ %. The values of the percentage mass fraction of COHb obtained by this method are within 3,0 % of the values obtained by CO-Oximeter in the mass fraction range of 3,5 % to 92,4 % COHb for a theoretical range of 0 % to 100 % COHb.

8.5.9 Application and limitation

The method given in 8.5.5 and 8.5.6 is suitable for analysing COHb levels in fresh as well as post-mortem blood samples stored in containers containing sodium fluoride and potassium oxalate. Clotted blood samples may be used after homogenization, but components of old, putrid blood samples can interfere with the analysis.

8.6 COHb by headspace gas chromatography — Nickel-hydrogen reduction and flame ionization detection

8.6.1 Principle

For this gas chromatographic procedure [38],[39], two separate aliquots of blood samples are treated with sodium dithionite to convert MetHb and OxyHb to HHb. One aliquot of the sample is saturated with CO, while the other aliquot is used without any CO treatment. The CO from both blood aliquots is released by using a ferricyanide or phosphoric acid solution. Headspace air samples of the CO-saturated and non-CO-treated sample aliquots are injected on a gas chromatograph equipped with a column, a methanation unit (nickel catalyst and hydrogen unit), and a flame ionization detector (FID). Upon separation on the column, CO is converted by the methanation unit to methane prior to its detection by FID. By comparing the methane peak areas (heights) on the gas chromatograph of the non-CO-treated original blood sample and of the CO-saturated blood sample, the percentage mass fraction of COHb, w_{COHb}, in the blood samples can be calculated.

8.6.2 Reaction

The chemical reaction for the conversion of CO to methane (CH_4) in the presence of hydrogen and a nickel catalyst is given by Equation (7):

$$CO + 3H_2 \rightarrow CH_4 + H_2O \quad (7)$$

8.6.3 Reagents and materials

8.6.3.1 **Sodium dithionite** (sodium hydrosulfite).

8.6.3.2 **Silicone antifoam solution**.

Dilute the product according to the vendor instructions.

8.6.3.3 **CO-liberating solution**:

8.6.3.3.1 **Ferricyanide solution**, 3,2 %.

Dissolve 3,2 g of potassium ferricyanide in water and dilute to a final volume of 100 ml.

8.6.3.3.2 Phosphoric acid solution, 42,5 %.

Dilute 85 % orthophosphoric acid two-fold using water.

8.6.3.4 **Lactic acid solution**.

Dilute 0,8 g of lactic acid with water to a final volume of 100 ml.

8.6.3.5 **Octanol**.

8.6.3.6 **Compressed gases**: CO, hydrogen and a carrier gas (helium or nitrogen).

8.6.4 Apparatus

8.6.4.1 **Gas chromatograph**, with a column, methanation (nickel-hydrogen reduction) unit and flame ionization detector (FID).

8.6.4.2 **Blood collecting tubes**, 16 mm × 100 mm.

8.6.4.3 **Headspace vials**, 6 ml, and **cylindrical tubes**, flat-bottomed, 1 ml.

8.6.4.4 **Crimp caps**, aluminum, with a Teflon seal.

8.6.4.5 **Crimper**.

8.6.4.6 **Tube rocker**.

8.6.4.7 **Syringe** and **hypodermic needle**.

8.6.5 Sample

The method of Griffin [38] requires approximately 1 ml of blood sample per analysis. The preferred amount of sample is approximately 3 ml.

The method of Cardeal *et al*. [39] requires approximately 100 µl of blood sample per analysis. The preferred amount of sample is approximately 300 µl.

8.6.6 Procedure

8.6.6.1 Griffin method

The Griffin method [38] is carried out as follows.

a) Pipette 0,5 ml of blood from each sample into each of two blood collection tubes.

b) To each tube, add 0,5 ml of water, 2 drops of the antifoam solution, and 2 mg to 3 mg of sodium dithionite.

c) Saturate the mixture of one tube with CO by bubbling CO at a low flow rate for 5 min.

d) Flush the tube with helium to remove the excess CO. This sample should be considered as having a 100 % mass fraction COHb.

e) Quickly pipette 0,5 ml of the ferricyanide solution and 0,5 ml of the lactic acid solution into each pair of sample tubes and stopper them tightly.

f) Slowly rotate the tubes for 20 min to ensure complete liberation of CO.

g) Transfer the tubes to a test tube rack and let them stand for an additional period of 10 min to 15 min.

h) Inject 0,5 ml of headspace from each pair of samples onto the gas chromatograph, compare the methane peak areas (heights) of the non-CO-treated sample with that of the CO-saturated sample, and calculate the percentage mass fraction of COHb in the sample by using Equation (8).

i) Gas chromatographic conditions are as follows:

— column: 1,8 m (3 mm OD) packed with 5 Å molecular sieve (100/120 mesh);

— injector port temperature: ≈140 °C to 150 °C;

— oven temperature: 140 °C;

— detector temperature: 150 °C;

— catalyst unit temperature: 275 °C to 300 °C;

— helium and hydrogen flow: 15 ml/min.

8.6.6.2 Cardeal method

The Cardeal *et al*. method [39] is carried out as follows.

a) Transfer 50 µl of blood to a 6 ml headspace vial.

b) Insert into the 6 ml vial a 1 ml cylindrical tube containing 50 µl of octanol (antifoaming agent) and 450 µl of the 42,5 % phosphoric acid solution. The tube is not capped.

c) Cap the vials and then shake them briefly to mix the contents of the vials and the tubes.

d) Repeat steps a) to c) with the blood sample aliquots saturated with CO after adding sodium dithionite as described in 8.6.6.1 b).

e) After a 10 min equilibrium period, inject a suitable volume of the headspace from each vial into the gas chromatograph, compare the methane peak areas (heights) of the non-CO-treated sample with that of the CO-saturated sample, and calculate the percentage mass fraction, w_{COHb}, of COHb in the sample by using Equation (8).

f) Gas chromatographic conditions are as follows:

— column: stainless steel (3 m × 0,9 mm ID) packed with Porapak Q, 80/100 mesh;

— injector port temperature: 80 °C;

— oven temperature: 80 °C;

— detector temperature: 350 °C;

— headspace-injector temperature: 70 °C with a pressurization time of 1,5 min;

— nitrogen flow: 30 ml/min.

8.6.7 Calculation

Calculate the percentage mass fraction of COHb, w_{COHb}, in the sample by using Equation (8).

$$w_{\mathrm{COHb}} = \left(A_{\mathrm{P,O}} / A_{\mathrm{P,S}} \right) \times 100 \tag{8}$$

where

$A_{\mathrm{P,O}}$ is the gas chromatograph peak area (height) of methane from the original blood sample (non-CO-treated sample);

$A_{\mathrm{P,S}}$ is the gas chromatograph peak area (height) of methane from the original sample saturated with CO.

8.6.8 Sensitivity

For the method of Griffin [38], the coefficient of variation is found to be 3,76 % and the sensitivity of the method is more than adequate for measuring COHb in autopsy blood samples. COHb percentage mass fractions as low as 1 % can be detected routinely.

Analytical results of blood samples from victims of CO poisoning obtained by the gas chromatographic method of Cardeal *et al*. [39] are comparable with spectrophotometric results. This method is capable of accurately determining the percentage mass fraction of COHb when $w_{\mathrm{COHb}} \geqslant 10$ %.

8.6.9 Application and limitation

Both heparin- and sodium-fluoride-potassium-oxalate-treated blood samples can be used for analysis. This method is suitable for the analysis of COHb in fresh blood as well as post-mortem blood samples. Clotted blood can be homogenized prior to use. Putrid blood samples, which it might not be possible otherwise to analyse by spectrophotometric methods because of the possible interference with the absorbance of COHb or other species of HHb, can also be analysed by this gas chromatographic method.

8.7 COHb by headspace gas chromatography — Thermal conductivity detection

8.7.1 Principle

The submitted sample is divided in two parts. One part is saturated with CO, while the other part is used without CO treatment. Samples are treated with sodium dithionite to convert MetHb and OxyHb to HHb. CO from the sample is released by sulfuric acid with saponin, which is used to ensure the complete breakdown of the red cells of blood samples. Headspace air samples of the CO-saturated and the non-CO-treated samples are injected into a micro-gas chromatograph for CO analysis [40],[41]. By comparing the areas of the CO peaks in the chromatograms of the original (non-CO-treated) blood sample and of the CO-saturated blood sample, the percentage mass fraction of COHb in a blood sample can be calculated.

8.7.2 Reagents and materials

8.7.2.1 **Reducing reagent**, sodium dithionite (0,287 M) solution in water.

8.7.2.2 **CO-liberating solution**, sulfuric acid (1 M) with saponin, 1,5 g/100 ml.

8.7.2.3 **Ammonium hydroxide**.

8.7.2.4 **Compressed gases**: CO and helium.

8.7.3 Apparatus

8.7.3.1 **Micro-gas chromatograph**, with a capillary column and a thermal conductivity detector (TCD).

8.7.3.2 **Headspace vials**, 10 ml.

8.7.3.3 **Crimp caps**, aluminium, fitted with a silicon septa.

8.7.3.4 **Crimper**.

8.7.3.5 **Tube rocker**.

8.7.3.6 **Syringe** and **hypodermic needle**.

8.7.3.7 **Gas bottles**, 100 ml.

8.7.4 Sample

Approximately 1,5 ml of blood sample is needed for each analysis. The preferred amount of sample is 3 ml to 4 ml.

8.7.5 Procedure

a) Use two aliquots, 0,5 ml and 1 ml, of the same blood specimen.

b) To a 10 ml headspace vial, transfer 0,5 ml of the blood sample followed by 0,5 mL of the reducing agent, and seal the vial. This blood aliquot is not treated with CO.

c) Using a second aliquot of the same blood sample, prepare a CO-saturated blood aliquot by placing 1 ml of the blood sample and 1 ml of the reducing agent in a 10 mm × 75 mm test tube, and by purging the headspace with CO for 30 s. Cap the tube and place it on a rocker for 30 min. After this time, purge the tube again with CO for 30 s and rock it for an additional 30 min. Then, purge headspace with helium for 10 s, and transfer 1 ml of this CO-saturated blood mixture to a 10 ml headspace vial. Seal the vial.

d) Liberate the CO from both the original (non-CO-treated) and the CO-saturated blood sample by adding 1 ml of liberating agent into the vials using a syringe fitted with a hypodermic needle, followed by the agitation of the samples at room temperature on a shaker for 40 min.

e) Inject the headspace of the vials into the gas chromatograph under the conditions given below:

- inlet temperature: 110 °C;
- column module: 20 m 0,32 mm ID column packed with 5 Å molecular sieve;
- column temperature: 120 °C;
- carrier gas: helium;
- column pressure: 241 kPa (35 psi);
- sampling time: 40 s;
- injection time: 100 ms;
- TCD sensitivity: high;
- total chromatographic time: 195 s;
- CO retention time: approximately 2,5 min.

8.7.6 Calculation

Calculate the percentage of COHb in the sample by using Equation (8).

8.7.7 Sensitivity

The results of the gas chromatographic method are comparable with the spectrophotometric results. The coefficient of variation for each of the COHb controls is found to be less than 10 %. This method is capable of accurately measuring $w_{COHb} \geqslant 10$ %.

8.7.8 Application and limitation

See 8.6.9.

9 Measurement of HCN in blood as CN^-

9.1 CN^- by colourimetric method (*p*-nitrobenzaldehyde and *o*-dinitrobenzene)

9.1.1 Principle

CN^- present in blood samples reacts with *p*-nitrobenzaldehyde and *o*-dinitrobenzene under an alkaline condition to produce a violet colour, suggesting the presence of CN^- [42],[43],[44].

9.1.2 Reagents and materials

9.1.2.1 2-Methoxyethanol.

9.1.2.2 Potassium cyanide.

9.1.2.3 *p*-Nitrobenzaldehyde, 0,05 M.

Dissolve 0,755 g of *p*-nitrobenzaldehyde in a final volume of 100 ml of 2-methoxyethanol. Store the solution in an amber-colour glass bottle. A fresh solution should be prepared every six months.

9.1.2.4 *o*-Dinitrobenzene, 0,05 M.

Dissolve 0,840 g of *o*-dinitrobenzene in a final volume of 100 ml of 2-methoxyethanol. Store the solution in an amber-coloured glass bottle. The solution is stable for up to six months.

9.1.2.5 Sodium hydroxide, 0,5 M.

Dissolve 2 g of sodium hydroxide pallets in a final volume of 100 ml of water. Keep exposure to room air at a minimum.

9.1.2.6 CN^- stock solution, aqueous, 100 mg CN^-/100 ml.

Dissolve 0,250 g of potassium cyanide in approximately 50 ml of water. To this solution, add 20 ml of 0,5 M NaOH and bring the volume of the mixture to 100 ml with water. Prepare this solution fresh at least every three months.

9.1.2.7 CN^- reference solution, aqueous, 0,05 mg CN^-/100 ml.

Dilute 0,5 ml of the 100 mg CN^-/100 ml solution to 1 l with water. Prepare this reference solution just prior to use. This reference solution could also be prepared in CN^--free human blood.

9.1.3 Apparatus

9.1.3.1 Test tube vibrator.

9.1.3.2 Clinical centrifuge.

9.1.3.3 Conway dish, three-compartment, with cover.

9.1.4 Sample

This colour-specific method requires 50 µl or 100 µl, respectively, depending on whether the blood sample is used directly [42],[43] or whether the HCN is diffused from the blood sample [43],[44]. The preferred amounts of the samples are 150 µl and 300 µl, respectively.

9.1.5 Procedure

9.1.5.1 Direct use of the blood sample

The procedure for the direct use of the blood sample [42],[43] is as follows:

a) To a clean dry 10 mm × 75 mm glass test tube, add 0,5 ml of the *p*-nitrobenzaldehyde solution, 0,1 ml of the blood specimen, and 0,5 ml of the *o*-dinitrobenzene solution.

b) Add 0,5 ml of the *p*-nitrobenzaldehyde solution and 0,5 ml of the *o*-dinitrobenzene solution to two additional tubes: one containing 0,1 ml of the reference CN^- solution and another 0,1 ml of water.

c) Agitate each mixture for 30 s by flicking the tubes.

d) Centrifuge these tubes at approximately 770 g for 3 min, and transfer 0,5 ml of the supernatant from each tube to a clean dry 10 mm × 75 mm glass test tube.

e) Add 50 µl of the sodium hydroxide solution to each tube and mix by shaking.

f) Observe the samples for 1 min for the appearance of a violet colour and observe the colour again after keeping the tubes in the dark for 15 min.

9.1.5.2 Diffusion of HCN from the blood sample

The procedure for the diffusion of HCN from the blood sample [43],[44] is as follows:

a) Place 50 µl of 0,1 M NaOH in the centre well of a Conway diffusion dish.

b) Add 200 µl each of the *p*-nitrobenzaldehyde and *o*-dinitrobenzene solutions to the well.

c) To the middle and outer rings, add 1 ml and 2 ml of 0,5 M H_2SO_4, respectively.

d) Add 200 µl of the blood specimen into the middle ring, mix the contents by carefully rotating the dish and immediately cover the reaction dish. Be sure not to mix the contents of the outer and centre cells.

e) Allow the diffusion dish to stand for 30 min and observe a violet colour in the centre well.

9.1.6 Sensitivity and selectivity

The intensity of the resulting violet colour is proportional to CN^- concentration. Although the minimum CN^- concentration that can be detected by directly using the specimen in the colour-producing reaction mixture in the test tube is 0,3 µg/ml [42],[43], the diffusion technique increases the sensitivity and specificity of the method to detect CN^- as low as 0,05 µg/ml [44].

Up to 0,5 M fluoride, bromide, chloride, thiocyanate, cyanate, carbonate, sulfate, sulfite, phosphate, nitrate, citrate, tartrate and acetate ions do not interfere with the analysis. However, nitrite and sulfide ions at 0,5 M concentrations result in rapid browning of the solution. Such interference is not observed at concentrations of these ions below 0,1 M.

9.1.7 Application and limitation

This procedure is a quick, qualitative analytical method. The development of a violet colour suggests the presence of a potentially toxic concentration of CN^- in the blood sample. Other biological samples — plasma, serum, gastric contents, cerebrospinal fluid, urine and tissue homogenates — can be analysed to screen for the presence of CN^-. In addition to fresh blood, clotted blood may suitably be used for analysis after homogenization. Suitability of this method for the analysis of putrid blood has not been established.

9.2 CN^- by visible spectrophotometry

9.2.1 Principle

In this method, HCN is liberated from the blood sample by acidification and microdiffusion, trapped in a dilute alkaline solution, and converted to cyanogen chloride after reacting with chloramine-T. Subsequently, cyanogen chloride reacts with pyridine to form *N*-cyanopyridinium chloride, followed by a reaction wherein *N*-cyanopyridinium chloride is cleaved to form an anil of glutaconic aldehyde. This aldehyde then couples with barbituric acid to form a red-pinkish, highly resonant product [27],[45],[46] (see also Clause B.4). The appearance of a red-pinkish product suggests the presence of CN^-. The level of CN^- can be quantitatively determined by measuring the absorbance of the product.

9.2.2 Reaction

The chemical reaction of liberated HCN producing a resonant product is shown in Figure 1.

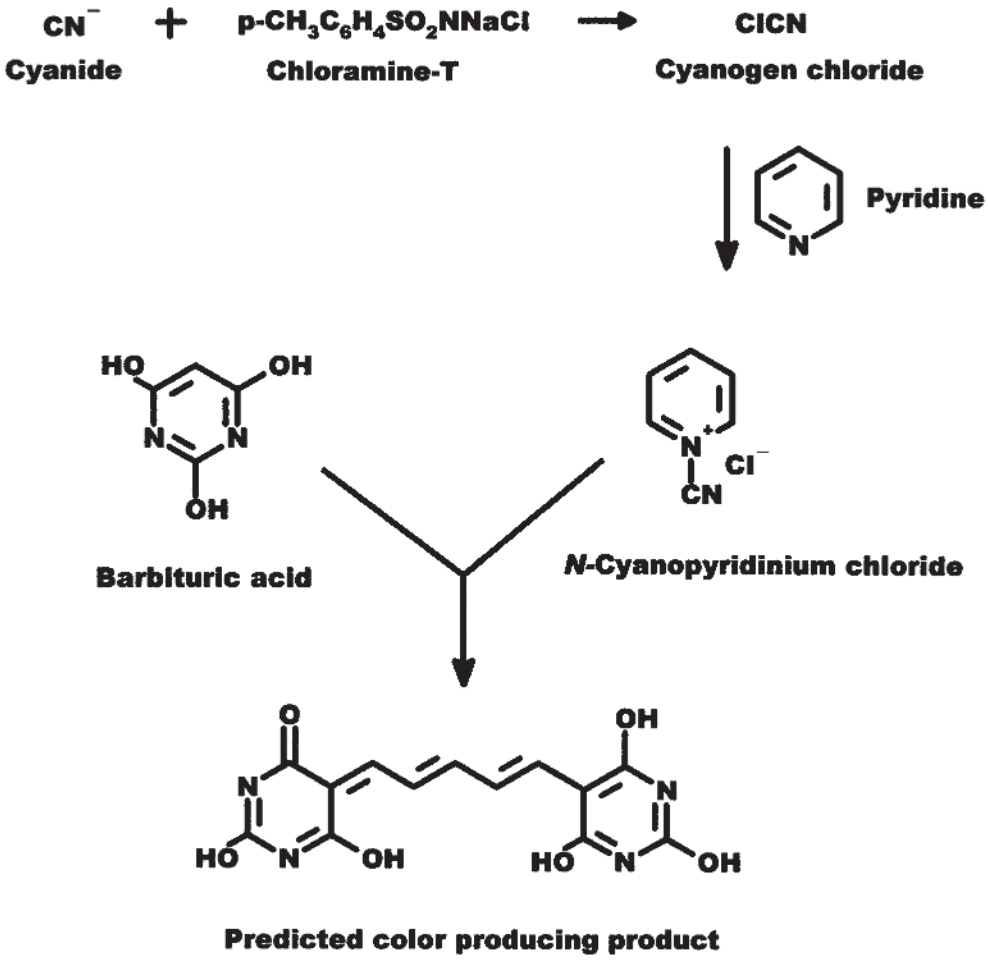

Figure 1 — Chemical reaction of the formation of a coloured, highly resonant product for the determination of CN^-

9.2.3 Reagents and materials

9.2.3.1 **Absorbing solution**, 0,1 M NaOH.

9.2.3.2 **Liberating solution**, 1,8 M H_2SO_4.

9.2.3.3 **Potassium cyanide**.

9.2.3.4 **Silicone lubricant**.

9.2.3.5 **Sodium phosphate**, 1 M.

9.2.3.6 **CN^- stock solution**, aqueous, 100 mg CN^-/100 ml.

Dissolve 0,250 g of KCN in approximately 50 ml of water. To this solution, add 20 ml of 0,5 M NaOH and bring the volume of the mixture to 100 ml with water. Prepare this solution fresh at least every three months.

9.2.3.7 High-CN^- internal control solution, 10 µg CN^-/ml.

Dilute 1 ml of the 100 mg CN^-/100 ml solution to 100 ml with CN^--free human blood. Prepare this solution fresh just prior to use.

9.2.3.8 Low-CN^- internal control solution, 2 µg CN^-/ml.

Dilute 2 ml of the 100 mg CN^-/100 ml solution to 100 ml with water and dilute 10 ml of this 20 µg CN^-/ml to 100 ml with CN^--free human blood. Prepare this solution fresh just prior to use.

9.2.3.9 Chloramine-T, 0,25 g/100 ml.

Dissolve 0,250 g of chloramine-T in water and dilute to 100 ml with water. Store the solution at 4 °C to ensure its stability.

9.2.3.10 Pyridine-barbituric acid colour reagent.

Pipette 15 ml of pyridine into a 50 ml volumetric flask containing 3 g of barbituric acid. Mix this mixture and add 3 ml of concentrated hydrochloric acid. Mix the solution and dilute to 50 ml with water. Mix well as the constituents dissolve slowly. Let it stand for 30 min. Filter if necessary. It is necessary to prepare this solution fresh each day of analysis.

9.2.4 Apparatus

9.2.4.1 Spectrophotometer.

9.2.4.2 Conway microdiffusion dish, with cover; two-compartment porcelain dish with glass cover or three-compartment polypropylene dish with lid.

9.2.4.3 Cuvettes.

9.2.5 Sample

This colour-specific method utilizes 0,5 ml of blood sample. The preferred amount of sample is approximately 1,5 ml.

9.2.6 Procedure

a) The procedure using the two-compartment porcelain Conway dish [27],[45],[46] is as follows.

- Lightly coat the rim of each Conway dish with silicon and add 0,5 ml of 0,1 M NaOH to the inner compartment of each dish.
- Into the outer compartment add 0,5 ml of 1,8 M H_2SO_4.
- Place a glass cover on each dish in such a way that there is an opening to the outer compartment through which sample can be added to the outer compartment.
- Through the opening, add 0,5 ml of the blood sample, immediately slide the cover over the exposed outer compartment to seal the dish, gently tip and rotate the dish to mix the sample and the liberating solution. Precautions should be taken to prevent the contents of the outer and inner compartments from mixing.

b) The procedure using the three-compartment polypropylene Conway dish [27],[44],[46] is as follows.

- To the centre well (inner compartment) add 0,5 ml of 0,1 M NaOH.
- To the middle and outer rings, add 1 ml and 2 ml of 1,8 M H_2SO_4, respectively.

— To the middle ring, add 0,5 ml of the blood sample while making sure that blood does not touch the liberating solution at this time.

— Immediately place the lid on the dish, rotating the lid to ensure that it seals the headspace properly. The liberating solution in the outer compartment serves as a sealant. Gently try to lift the lid by the knob of the lid to check the seal; the lid should not lift.

— Gently tip and rotate the dish to mix the sample and the liberating solution. Precautions should be taken to prevent the contents of the outer and inner compartments from mixing.

c) Allow the samples to diffuse for 2 h at room temperature.

d) After 2 h, transfer 100 µl of the contents of the inner compartment of each dish to a 5 ml test tube.

e) To each tube, add 1 ml of the sodium phosphate solution and 0,5 ml of the chloramine-T solution.

f) Mix the contents of the tubes and wait for 2 min to 3 min.

g) Then, add 1,5 ml of the colour reagent, mix, and let it stand for 10 min for the development of the colour. The appearance of a red-pinkish colour suggests that CN^- is present in the sample.

h) Along with blood samples, process a CN^--free human blood sample, and the high and low internal CN^- controls.

i) As soon as possible, determine the absorbance of each solution at 580 nm against the solution obtained by processing water as described above, since the colour product produced is not very stable.

9.2.7 Calculation

The concentration, $C_{CN^-,S}$, expressed in micrograms per millilitre, of CN^- in the blood sample can be calculated by using Equation (9):

$$C_{CN^-,S} = \alpha_S \times \left(\frac{10}{\alpha_{HIC}} \right) \tag{9}$$

where

α_S is the absorbance of the sample at 580 nm;

α_{HIC} is the absorbance of the high internal control solution (10 µg CN^-/ml) at 580 nm.

The absorbance of the low internal control solution (2 µg CN^-/ml) can also be used for the calculation, but a factor of “2” should be used in place of “10” in Equation (9).

Alternatively, a series of calibration solutions can be prepared and processed throughout the entire process, including the diffusion step. The calibration solutions can be prepared in a manner similar to that for the controls in CN^--free human blood. A calibration curve can be constructed by plotting 580 nm absorbance readings against CN^- concentrations in the respective calibration solutions. The concentration of CN^- in the sample can be determined from its absorbance using the curve. The curve has been reported to be linear up to 2 µg/ml CN^-, depending upon the amount of the sample used during the analysis. However, Equation (9) [45] implies that the curve is also a straight line between 2 µg/ml and 10 µg/ml, which is considered as a toxic-to-lethal blood concentration range of CN^- (see also C.4.3).

9.2.8 Sensitivity

A CN^- concentration of as low as 0,25 µg/ml can be easily detected by processing 0,5 ml of blood specimen. Using larger amounts of blood sample, lower concentrations of CN^- can be measured.

9.2.9 Application and limitation

This method is suitable for analysing fresh and post-mortem blood samples. Other biological samples — plasma, serum, gastric content, cerebrospinal fluid, urine and tissue homogenates — can also be analysed. Clotted blood sample can be used after homogenization. The suitability of this method for analysing putrid blood is not known.

CAUTION — It is necessary to take precautions to avoid physical contamination of the absorbing solution in the centre well by even trace amounts of specimen, because chloramine-T has been reported to produce CN^- after oxidizing certain substances such as glycine.

The controls and calibration solutions can be prepared in water, but it is analytically prudent to prepare these CN^- solutions in CN^--free human blood to be consistent with the biometrics of the sample. A negative blood (CN^--free human blood) control should also be analysed in parallel.

The colorimetric reaction cannot be carried out on blood or tissues that contain formalin, since formaldehyde reacts with CN^- to form cyanohydrin that is quickly hydrolysed into glycolic acid and ammonia.

9.3 CN^- as HCN by headspace gas chromatography — Nitrogen phosphorous detection

9.3.1 Principle

The blood sample is equilibrated at room temperature for 30 min in the presence of acetonitrile as an internal standard in a vial [47],[48]. The headspace of the vial is injected into a gas chromatograph equipped with a nitrogen phosphorus detector (NPD) to detect HCN. CN^- in blood samples is determined from a calibration curve.

9.3.2 Reagents and materials

9.3.2.1 **Acetonitrile**.

9.3.2.2 **Internal standard stock solution**, acetonitrile.

Pipette 25 µl of acetonitrile into 100 ml of water.

9.3.2.3 **Sodium cyanide stock solution**, aqueous, 1 mg/ml.

9.3.2.4 **Glacial acetic acid**.

9.3.2.5 **1-Octanol**.

9.3.2.6 **Compressed gases**: hydrogen, air, and helium.

9.3.3 Apparatus

9.3.3.1 **Gas chromatograph**, with a silanized glass column and an NPD.

9.3.3.2 **Porapak Q**.

9.3.3.3 **Vials**, disposable, airtight, 1 ml.

9.3.3.4 **Caps**, aluminium, Teflon-lined, with a rubber septum.

9.3.3.5 **Crimper**.

9.3.3.6 **Syringe**, airtight, 100 µl, with a Teflon-tipped plunger.

9.3.3.7 **Vortexer**.

9.3.3.8 **Clinical centrifuge**.

9.3.4 Sample

This method is easily capable of measuring 0,25 µg/ml CN^- using 0,5 ml of blood samples. The preferred amount of sample for the assay is approximately 1,5 ml.

9.3.5 Procedure

a) Aliquot 0,5 ml of blood sample into a 1 ml disposable vial containing 100 µl of the internal standard solution and 5 µl of 1-octanol.

b) Seal the vials with the aluminium caps.

c) Inject 50 µl of glacial acetic acid into all vials and vortex the contents of vials.

d) Centrifuge these vials at approximately 770 g for 5 min, and let the vials equilibrate for 30 min at room temperature.

e) After 30 min, inject 50 µl of the headspace of each vial onto the gas chromatograph under the operating conditions given below:

— column:	silanized glass column (180 cm × 2 mm ID) packed with 100/120 mesh Porapak Q;
— injector temperature:	200 °C;
— column temperature:	120 °C;
— detector temperature:	250 °C;
— carrier gas:	helium (20 ml/min);
— detector gases:	8,5 % of hydrogen in helium (28 ml/min) and air (50 ml/min);
— retention time for HCN:	0,6 min;
— retention time for acetonitrile (internal standard):	2,5 min.

f) After each vial headspace injection, flush the syringe many times with room air to ensure that there is no carry-over of residual HCN.

g) Run blank air injections routinely between samples, controls or calibration solutions to ensure that there is no carry-over from injection to injection.

9.3.6 Calibrators and calculation

a) Prepare CN^- calibration solutions (0,25 µg/ml; 0,5 µg/ml; 1 µg/ml; 5 µg/ml; and 15 µg/ml) in negative (CN^--free) human blood using the 1 mg/ml sodium cyanide stock solution.

b) Process and analyse these calibrators along with samples.

c) Obtain ratios of peak areas of the calibration solutions and the internal standard, and generate the calibration curve against the CN^- concentration in the calibration solutions.

d) Knowing the ratio of the areas of the HCN peak and the internal standard in the unknown sample, calculate the CN^- concentration in the sample from the curve.

9.3.7 Sensitivity

The assay is linear over the range of 0,25 µg/ml to 15 µg/ml CN^-. The sensitivity of the method is 0,05 µg/ml CN^-, and intra- and inter-assay coefficients of variation were 1,31 % and 9,16 %, respectively.

9.3.8 Application and limitation

Fresh and post-mortem blood samples can be analysed by this method. No interfering peaks have been noted with post-mortem whole-blood samples. Clotted blood samples can be analysed after homogenization.

9.4 CN^- by headspace gas chromatography — Electron capture detection

9.4.1 Principle

Blood CN^- is quantitatively determined by headspace gas chromatography using an electron capture detector (ECD) [49]. During the analysis, CN^- is detected after conversion of HCN to cyanogen chloride by reaction with chloramine-T. The liberation of HCN and its chlorination are carried out in a single preparatory step and in the same reaction medium.

9.4.2 Reaction

The associated chemical reaction along with the sample processing is shown in Figure 2.

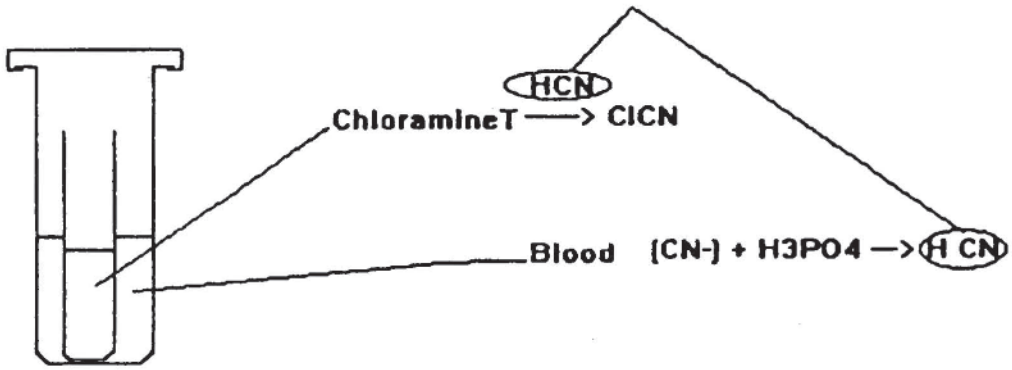

Figure 2 — Schematic representation of CN^- determination by conversion of HCN into cyanogen chloride (CICN)[4)]

9.4.3 Reagents and material

9.4.3.1 **CN^- solution**, aqueous 100 µg/ml in 4 % sodium hydroxide.

Store this solution at 4 °C.

9.4.3.2 **Orthophosphoric acid**.

9.4.3.3 **Chloramine-T**, 0,5 % in water.

Prepare fresh daily.

9.4.3.4 **Compressed gases**: argon, methane, and helium.

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4) Reproduced from Odoul *et al*. (1994) [49], by permission of Preston Publications, a Division of Preston Industries, Inc., 6600 West Touhy Avenue, Niles, IL 60714.

9.4.4 Apparatus

9.4.4.1 **Gas chromatograph**, equipped with a fused-silica capillary column, a headspace autosampler, and an ECD.

9.4.4.2 **Tubes**, special rounded, 3 ml, 45 mm long, 10 mm in diameter.

9.4.4.3 **Headspace vials**, 20 ml.

9.4.4.4 **Crimp caps**, aluminium, with a Teflon-faced butyl-rubber septum.

9.4.4.5 **Crimper**.

9.4.4.6 **Vortexer**.

9.4.5 Sample

The required amount of blood sample per analysis is 250 µl, but the desired amount of blood sample is approximately 750 µl.

9.4.6 Procedure

a) Dilute blood 1:20 in water (250 µl of blood in 5 ml of water) to minimize matrix effects.

b) Transfer 5 ml of the diluted blood sample into a 20 ml headspace vial.

c) To the vial, add 100 µl of phosphoric acid, and vortex the mixture in the vial for 5 s.

d) Introduce immediately into the vials the 3 ml special tubes containing 1 ml of the chloramine-T solution, and seal the vials with the aluminium caps.

e) Incubate the sealed vials at 65 °C for 90 min and analyse headspace by gas chromatography under the operating conditions given below:

   — vial thermostatization temperature in the headspace analyser: 55 °C;

   — transfer line temperature: 60 °C;

   — thermostatization time: 60 min;

   — pressurization time: 2 min;

   — injection time: 0,08 min;

   — volume of headspace injection into the gas chromatograph: 200 µl;

   — column: fused-silica capillary column (CP Sil 8B methylsilicone; 50 m × 0,23 mm ID; 1,2 µm film thickness);

   — column temperature: 60 °C for 10 min;

   — detector temperature: 300 °C;

   — carrier gas: helium (2 ml/min);

   — detector gases: a makeup argon-methane gas (60 ml/min);

   — gas chromatograph running mode: split (split ratio of 1:20);

   — retention time for cyanogen chloride: 2,7 min.

9.4.7 Calibrators and calculation

a) Spike CN^--free human whole blood (1:20 dilution; 250 µl of whole blood in 5 ml of water for one assay) with the aqueous CN^- solution to obtain reference calibration solutions ranging from 5 ng/ml to 1 000 ng/ml CN^-.

b) Process and analyse these calibration solutions along with samples.

c) Obtain the heights (areas) of the cyanogen chloride peaks corresponding to the respective CN^- calibration solutions and plot the heights against the CN^- concentrations of calibration solutions to obtain a calibration curve.

d) Knowing the height of the cyanogen chloride peak of the unknown sample, calculate the CN^- concentration in the sample from the curve.

9.4.8 Sensitivity

This method is easily capable of quantitating 100 ng/ml CN^- in blood samples. The assay is linear over the range of 5 ng/ml to 1 000 ng/ml CN^- and its sensitivity is 5 ng/ml. The intra-assay coefficient of variation was 1 % to 8 %. Within-run studies were also conducted by replicate analysis, but the coefficient of variation was not reported. Cyanate and thiocyanate do not interfere with the CN^- quantitation.

9.4.9 Application and limitation

This technique is easy to perform and is specific. It requires a minimum of handling of the samples and a small sample size, and can accurately measure CN^- in the whole blood of healthy persons. This method should be suitable for clinical and toxicological purposes. Fresh and post-mortem blood samples can be analysed by this method. Homogenized clotted blood samples can be used for analysis by this method.

9.5 CN^- by spectrophotofluorimetry or high-performance liquid chromatography using a fluorescence detector

9.5.1 Principle

This technique is based upon the transformation of CN^- by acidification from blood to HCN and the subsequent reaction of CN^- in HCN with 2,3-naphthalenedialdehyde (NDA) and taurine in a self-contained system [50]. The reaction product, 1-cyano-2-benzoisoindole [1-cyano[*f*]benzoisoindole (CBI)] derivative (see also Clause B.5) thus formed, is a suitable candidate for fluorimetric measurement ($\lambda_{ex} = 418$ nm; $\lambda_{em} = 460$ nm).

9.5.2 Reaction

The chemical reaction along with the associated sample preparation/processing is shown in Figure 3.

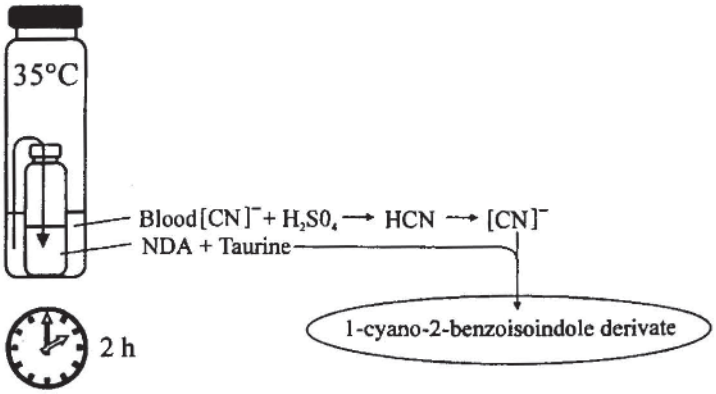

Figure 3 — Sample preparation for the detection of CN^- by fluorimetry[5)]

9.5.3 Reagents and material

9.5.3.1 Acetonitrile.

9.5.3.2 Sulfuric acid, concentrated.

9.5.3.3 Methanol.

9.5.3.4 2,3-Naphthalenedialdehyde (NDA).

9.5.3.5 Sodium hydroxide.

9.5.3.6 Potassium cyanide.

9.5.3.7 Potassium dihydrogen phosphate.

9.5.3.8 Sodium hydrogen phosphate.

9.5.3.9 Sodium hydrogen sulfate.

9.5.3.10 Taurine.

9.5.3.11 CN^- stock standard solution, aqueous, 10 mM.

Prepare this solution by weighing the appropriate amount of potassium cyanide and dissolving in 0,1 M NaOH.

9.5.3.12 CN^- working standard solutions, aqueous.

Prepare various working standard solutions of CN^- by diluting the CN^- sock solution with water.

9.5.3.13 Phosphate buffer, pH 8,0.

Mix 969 ml of a sodium hydrogen phosphate solution (11,878 g/l) and 31 ml of a potassium dihydrogen phosphate solution (9,073 g/l).

9.5.3.14 Methanolic 2,3-naphthalenedialdehyde (**NDA**) **solution**, 4 mM.

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5) Reproduced from Felscher and Wulfmeyer (1998) [50], by permission of Preston Publications, a Division of Preston Industries, Inc., 6600 West Touhy Avenue, Niles, IL 60714.

9.5.3.15 **NDA working solution**, 1 mM.

Dilute the 4 mM NDA solution with the phosphate buffer to obtain 1 mM NDA solution. Store this solution in an amber glass bottle at 4 °C. This dilute NDA solution may be used for up to seven days.

9.5.3.16 **Taurine solution**, 5 mM in phosphate buffer, pH 8,0.

This solution may be used for up to four weeks when stored at 4 °C.

9.5.3.17 **Sulfuric acid**, 10 % mass fraction containing 200 mg $NaHSO_4$.

9.5.4 Apparatus

9.5.4.1 Fluorescence spectrophotometer, with a 0,3 ml precision quartz glass cuvette (path length: 0,5 cm).

9.5.4.2 **High-performance liquid chromatograph** (**HPLC**), equipped with a fluorescence detector and a 5 µm Hypersil ODS RP18 column (100 mm × 2,1 mm ID).

9.5.4.3 **Tube**, 2 ml.

9.5.4.4 **Headspace vials**, 20 ml.

9.5.4.5 **Crimp caps**, aluminium, with a Teflon-faced butyl-rubber septum.

9.5.4.6 **Crimper**.

9.5.4.7 **Vortexer**.

9.5.5 Sample

This method utilizes 2 ml of blood sample per analysis. The preferred amount of sample is approximately 5 ml.

9.5.6 Procedure

9.5.6.1 Pipette 2 ml of blood into a 20 ml vial and, to this vial, add 0,5 ml of the sulfuric acid solution containing $NaHSO_4$.

9.5.6.2 Into the 20 ml vials, immediately place the 2 ml special tubes containing 200 µl each of the diluted NDA solution and the taurine solution.

9.5.6.3 Stopper the 20 ml vials using the aluminium crimp-top caps with the Teflon-faced butyl-rubber septum.

9.5.6.4 Carefully vortex the contents of the vials for 5 s.

9.5.6.5 Incubate the sealed vials for 120 min at 35 °C to allow the diffusion of HCN from the sample to the mixture of NDA and taurine solutions in the 2 ml tubes.

9.5.6.6 After the incubation, measure the fluorescence intensity of an aliquot of contents from the 2 ml tubes using the 0,3 ml cuvette at 418 nm excitation and 460 nm emission.

9.5.6.7 Alternatively, inject a fixed portion of the contents of the 2 ml vial into the HPLC equipped with a fluorescence detector (λ_{ex} = 418 nm; λ_{em} = 460 nm) under the following instrumental conditions:

— column temperature: ambient;

— HPLC elution solvent: water (92 %) acetonitrile (8 %) mixture;

— solvent flow rate: 0,4 ml/min;

— 1-cyano-2-benzoisoindole (CBI) derivative retention time: 1,7 min.

Injection volume and filter factors vary depending upon CN^- concentration range.

9.5.7 Calibration curves and calculation

9.5.7.1 Fluorescence spectrophotometric determination

a) Prepare two calibration curves representing two different concentration ranges of CN^-. The calibration solutions should be prepared in CN^--free human whole blood at the following concentrations:

   — curve 1: 0 µg/ml to 0,5 µg/ml;

   — curve 2: 0 µg/ml to 1,0 µg/ml.

b) Plot the fluorescence intensity values of different calibrators against the respective CN^- concentrations in calibration solutions.

c) From the curves, obtain the corresponding CN^- level in the unknown blood samples from their fluorescence values.

9.5.7.2 HPLC determination

a) Prepare three calibration curves representing three concentration ranges of CN^-. These calibrators should be prepared in CN^--free human whole blood at the following concentrations:

   — physiological range curve: 0 µg/ml to 0,5 µg/ml;

   — toxic range curve: 0 µg/ml to 1,0 µg/ml;

   — lethal range curve: 0 µg/ml to 5,0 µg/ml.

b) Obtain the areas of CBI derivative peaks corresponding to the respective CN^- calibration solutions and plot the peak areas against the CN^- concentrations of the calibration solutions to generate the calibration curves.

c) Knowing the areas of the CBI derivative peak of the unknown sample, calculate the CN^- concentration in the sample from the curves.

9.5.8 Sensitivity

The method is easy to perform and is specific for CN^-. The detection limit of this method is 0,002 µg/ml CN^-. The linearity has been found to be excellent (correlation coefficient $\geqslant$ 0,980) from 0,002 µg/ml to 1 µg/ml CN^- for spectrophotometric determination and from 0,002 µg/ml to 5 µg/ml CN^- for HPLC determination. The coefficient of repeatability is $\leqslant$ 8 %. Thiocyanate and sulfide ions do not interfere with the method, even at high concentrations (200 µg/ml).

9.5.9 Application and limitation

This method can be used for analysing CN^- in fresh and post-mortem blood samples. It requires a minimum handling of the samples and can accurately measure CN^- in the whole blood of healthy persons and of individuals exposed to CN^-. This method is expected to be suitable for clinical and toxicological purposes. This method might not be suitable for the analysis of coagulated blood samples, unless the samples are homogenized.

9.6 CN^- by high-performance liquid chromatography-mass spectrometry

9.6.1 Principle

This technique is based upon the microdiffusion of CN^- from blood as HCN and the subsequent reaction of CN^- in HCN with NDA and taurine in a self-contained system [51]. The reaction produces a CBI derivative (see 9.6.2). The diffusion of CN^- from blood is carried out after the addition of isotopic potassium cyanide ($K^{13}C^{15}N$) as an internal standard. Thus, the CN^- and $^{13}C^{14}N^-$ species, respectively, produce non-isotopically tagged and isotopically tagged analogs of CBI. Both CBI analogs thus formed during the reaction are qualitatively and quantitatively determined by means of a high-performance liquid chromatography-mass spectrometry.

NOTE The relative molecular mass of the isotopically tagged CBI-$^{13}C^{14}N$ derivative is two atomic mass units greater than that of non-isotopically tagged CBI-CN derivative. Examples of mass spectra and chromatograms are depicted in Figures B.2 and B3, respectively.

9.6.2 Reaction

The chemical reaction between CN^- (isotopically or non-isotopically tagged), NDA and taurine is shown in Figure 4.

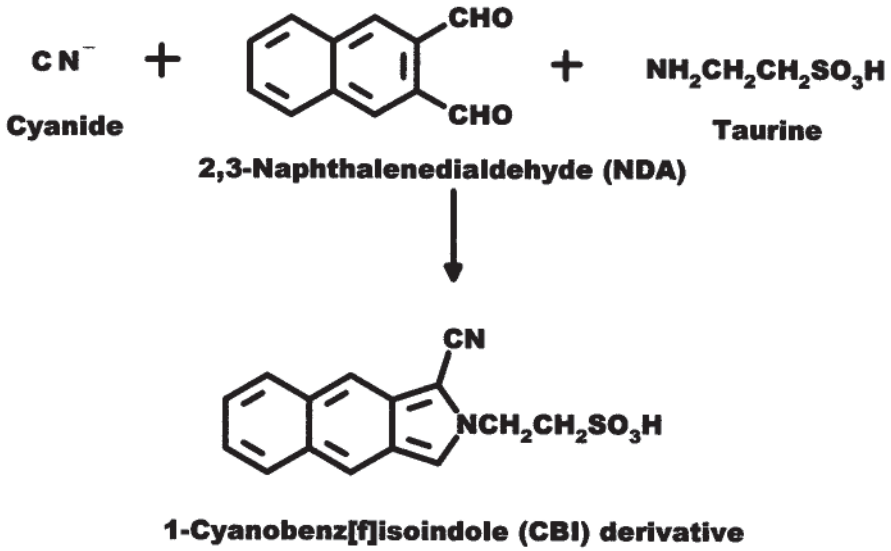


Figure 4 — Reaction of cyanide ion (CN^-) with 2,3-naphthalenediadehyde (NDA) and taurine, producing 1-cyanobenz[*f*]isoindole [1-cyano-2-benzoisoindole (CBI)] derivative

9.6.3 Reagents and materials

9.6.3.1 **Acetonitrile**.

9.6.3.2 **Ammonium formate** ($HCOONH_4$).

9.6.3.3 **Ammonium hydroxide**.

9.6.3.4 **Sulfuric acid**, concentrated.

9.6.3.5 **Formic acid**, 99 % to 100 %.

9.6.3.6 **Methanol**.

9.6.3.7 **2,3-Naphthalenedialdehyde** (NDA).

9.6.3.8 **Sodium hydroxide**.

9.6.3.9 **Potassium cyanide**.

9.6.3.10 **$K^{13}C^{15}N$**.

9.6.3.11 **Taurine**.

9.6.3.12 **CN^- stock standard solution**, aqueous, 10 mM.

Prepare this solution by weighing the appropriate amount of potassium cyanide in 0,1 M NaOH.

9.6.3.13 **$^{13}C^{15}N^-$ stock standard solution**, aqueous, 10 mM.

Prepare this solution by weighing the appropriate amount of $K^{13}C^{15}N$ in 0,1 M NaOH.

9.6.3.14 **CN^- working standard solutions**, aqueous.

Prepare, just before use, various working standard solutions of CN^- by diluting the CN^- stock solution with water. It is preferable that the solutions be prepared in CN^--free human blood.

9.6.3.15 **$^{13}C^{15}N^-$ working standard solution**, aqueous, 1,43 mM.

Prepare, just before use, the working standard solution of $^{13}C^{15}N^-$ by diluting the $^{13}C^{15}N^-$ stock solution with water. This solution is equivalent to a $^{13}C^{15}N^-$ concentration of 40 µg/ml.

9.6.3.16 **Methanolic 2,3-naphthalenedialdehyde (NDA) stock solution**, 10 mM.

Prepare this solution by weighing the appropriate amount of NDA and dissolving it in methanol. Store at 4 °C in the dark. The solution is stable for four weeks.

9.6.3.17 **Taurine stock solution**, aqueous, 50 mM.

Prepare this solution by weighing a suitable amount of taurine and dissolving it in water. This solution can be used for up to four weeks when stored at 4 °C.

9.6.3.18 **Derivatizing reagent**.

Prepare just before use by mixing appropriate amounts of NDA and taurine solutions with methanol and ammonium hydroxide (NDA/taurine/methanol/ammonium hydroxide, 25:25:45:5 volume percent).

9.6.3.19 **$HCOONH_4$-HCOOH buffer**, pH 3,0.

Prepare a 2 mM $HCOONH_4$ solution (126,2 µg/ml) in water and adjust the pH of this solution to 3,0 by using the concentrated formic acid.

9.6.3.20 **Compressed gas**: nitrogen, 99,95 %.

9.6.4 Apparatus

9.6.4.1 **High-performance liquid chromatograph** (HPLC), consisting of

— NovaPack C18 column, 4 µm, (150 mm × 2,0 mm ID), protected by a 5 µm Opti-Guard C18 guard cartridge (15 mm × 1,0 mm ID);

— 20 ml dual-syringe pump and manual injection valve;

— pneumatically assisted electrospray interface;

— single-quadrupole mass analyser [mass spectrometric detector (MSD)].

9.6.4.2 **Headspace vials**, 20 ml, for use as a microdiffusion chamber.

9.6.4.3 **Microtubes**, plastic, 1,5 ml, for use as the inner chamber of the microdiffusion chamber.

9.6.4.4 **Caps**, Teflon-lined, aluminium.

9.6.4.5 **Crimper**.

9.6.5 Sample

A volume of 2 ml of blood sample is required for each analysis. The preferred amount of sample is approximately 5 ml.

9.6.6 Procedure

a) To a 20 ml headspace vial used as a microdiffusion chamber, pipette 2 ml of blood and, then, add 50 µl of the 1,43 mM (40 µg/ml of $^{13}C^{15}N^{-}$) solution of $K^{13}C^{15}N$.

b) Insert into this vial a 1,5 ml plastic microtube containing 40 µl of the derivatization reagent.

c) To the blood, carefully add 2 ml of concentrated sulfuric acid by dripping the acid along the inner wall of the microdiffusion chamber.

d) Then, seal the headspace vial with a Teflon-lined aluminium cap.

e) After 30 min of gentle, periodic agitation at ambient temperature, remove the caps of the vials.

f) Inject 2 µl of the contents of the inner plastic microtube directly into the HPLC equipped with an MSD under the following instrumental conditions:

   — column temperature: ambient;

   — mobile phase: acetonitrile-$HCOONH_4$ (2 mM; pH 3,0 buffer), following a gradient of 35 % to 80 % acetonitrile in 10 min;

   — mobile phase flow rate: 200 µl/min with a post-column split of 1:3;

   — equilibrium time: 5 min at 35 % acetonitrile between two successive runs;

   — nebulizing gas: nitrogen (1,16 l/min) at 40 psi;

   — curtain gas: nitrogen (1,08 l/min) to flush the ion sampling orifice of the vacuum chamber;

   — detector: negative ionization mode with − 4,5 kV applied to the sprayer and − 50 V applied to the sampling orifice;

   — electron multiplier setting: + 2,2 kV;

   — 1-cyano[*f*]benzoisoindole (CBI) derivative retention time: 4,69 min;

   — data collection: total-ion chromatogram (TIC) mass range m/z 70 to 320; selected ion monitoring at m/z 299 and 191 for the CBI derivative containing –CN functional group and at m/z 301 and 193 for the CBI derivative containing $-^{13}C^{15}N$ functional group.

9.6.7 Calibrators and calculation

a) Calibration solutions of appropriate concentrations of CN^- can be prepared in CN^--free human whole blood using the aqueous CN^- stock standard solution. The CN^- concentration range can be 0,015 µg/ml to 3 µg/ml.

b) CN^- can be quantified by computing the peak-height ratios $\frac{m/z191}{m/z193}$ or $\frac{m/z299}{m/z301}$ from the CBI-CN and CBI-$^{13}C^{15}N$ derivatives.

c) The calibration curve can be constructed by plotting the peak-height ion ratios as a function of the CN^- concentrations of the calibrators. From the curve, knowing the ion ratios of the unknown sample, the CN^- concentration in the sample can be obtained.

d) In practice, because the blood samples are spiked with $^{13}C^{15}N^-$ at a resulting concentration in 1 µg/ml, the CN^- concentration, C_{CN^-}, expressed in micrograms per millilitre, can be directly approximated by the ratio of the peak heights (areas) of the CBI-CN to CBI-$^{13}C^{15}N$ derivatives as given in Equation (10):

$$C_{CN^-} = \frac{A_{P,CBI\text{-}CN}}{A_{P,CBI\text{-}^{13}C^{15}N}} \times F \quad (10)$$

where

$A_{P,\,CBI–CN}$ is the peak height (area) of the non-isotopically tagged CBI derivative;

$A_{P,\,CBI–^{13}C^{15}N}$ is the peak height (area) of the isotopically tagged CBI derivative;

F is a factor equal to 1 µg/ml.

9.6.8 Sensitivity

This method is simple, rapid and extremely specific. The detection limit of this method is 0,005 µg/ml CN^-. The linearity has been found to be excellent in the range of 0,015 µg/ml to 3 µg/ml CN^-. At 0,5 µg/ml CN^-, accuracy and precision values were 2,3 % and 7,4 % ($n = 6$), respectively, for within-run conditions, and 2,7 % and 8,9 %, respectively, for day-to-day runs over a 10-day period. No interferences are observed from the NDA and taurine that is not consumed during the derivatization. The complete analytical process can be carried out within 45 min.

9.6.9 Application and limitation

This technique can be used for accurately analysing fresh and post-mortem blood samples, including putrid blood samples and tissue homogenates. This method can be suitable for the analysis of coagulated blood samples after preparing their homogenates. It requires minimum handling of the samples and can accurately measure CN^- in the whole blood of healthy persons and of individuals exposed to CN^-. This method should be suitable for clinical and toxicological purposes.

Annex A
(normative)

Analytical report pro forma

A.1 General

Blood samples collected from fire victims shall be analysed for COHb and CN^-. The analytical report for COHb and CN^- shall provide a best blood concentration of each of these chemical species from the results of all analyses conducted for each victim. The reported concentrations shall be based upon the quantitative analytical results, instead of the qualitative-analysis findings, and shall be in a numerical format.

A.2 Analytical report

Each analytical report shall include at least the following information pertaining to the analyses of COHb and CN^- in blood samples for each fire victim:

a) analytical laboratory:

- name, address, and contact number of the laboratory analysing the sample;
- name(s) of responsible person(s) at the analytical laboratory;
- date of the report;
- laboratory report identification number;
- laboratory case reference number;

b) sample submitter:

- name and address of agency (or individual) submitting the sample;
- submitting agency's identification number;

c) accident/incident:

- place of accident/incident;
- date of accident/incident;
- time of fire;
- type of fire (description);

d) victim:

- name;
- age and sex;
- physical condition;
- any previously known medical condition(s);

- additional known information, such as any oxygen treatments, taking any medications, smoker (or non-smoker), worker in an atmosphere rich in gasoline exhaust fumes, or operator (traveller) of a vehicle with a faulty exhaust system;
- degree of burn;
- state of victim (alive or dead);
- date and time of death, if applicable;

e) analytical blood sample:

- date and time of blood collection;
- time between the removal of the victim from the fire atmosphere and the blood collection from live victim;
- post-mortem interval, that is time between death and blood collection for a dead victim;
- site of blood collection, for example central (heart) or peripheral;
- date and time sample received at the laboratory;
- type of blood collection container (for example test tubes with preservatives and/or anticoagulants);
- characteristics of blood sample (for example cherry-red, green, putrefied, coagulated and/or burned);
- storage condition of blood sample (ambient temperature, 4 °C, or − 20 °C);

f) sample analysis:

- date and time of analysis;
- analysis interval, that is the time between blood collection and analysis;
- method of analysis:
  - i) brief description of the method by which the results included in the report were obtained;
  - ii) sensitivity of the method (detection limit);
- units of results:
  - iii) COHb values should be reported as percentage of COHb;
  - iv) CN^- values should be reported in micrograms per millilitre of blood;
  - v) laboratory cutoff for reporting COHb and CN^- values:
    - for COHb, it should be 10 %;
    - for CN^-, it should be 0,25 µg/ml;

g) analytical report approval:

- name and signature of approving official;
- date of approval.

Annex B
(informative)

Additional aspects of analytical methods

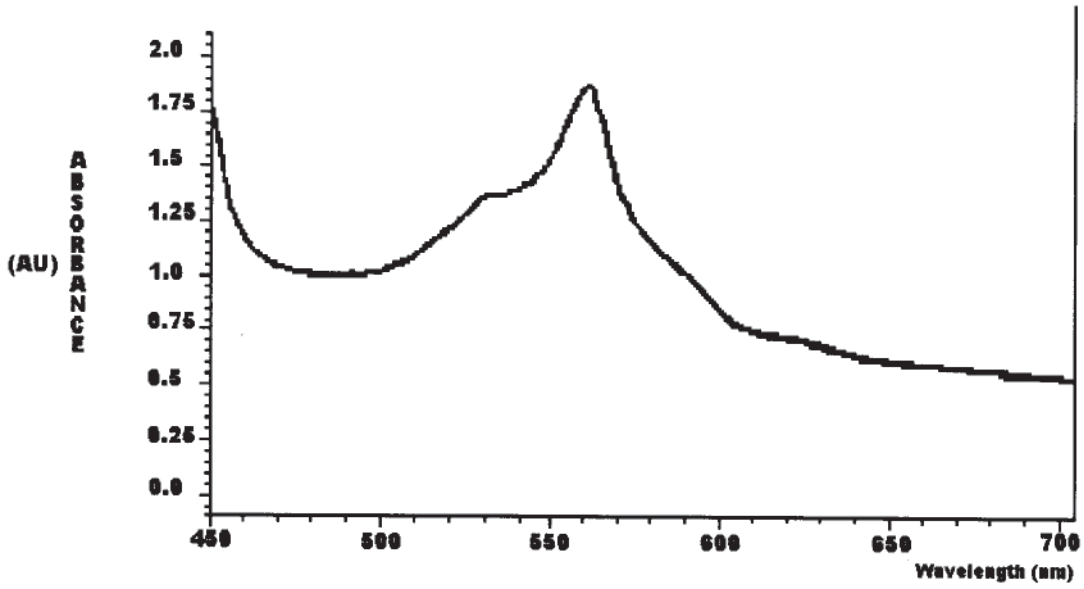
B.1 COHb by visible spectrophotometry (with CO saturation)

In this method (also refer to 8.4), sodium dithionite is used to convert MetHb and OxyHb to HHb [14],[32],[33],[34],[35],[36],[37]. COHb is unaffected by the treatment of sodium dithionite. A mixture of sodium fluoride and potassium oxalate is commonly used in collecting blood samples for post-mortem forensic toxicology in order to minimize blood clotting and to preserve the specimen. Lack of preservation of samples, including exposure to heat [52] and long-term storage at and above temperatures of −30 °C [53],[54], could cause increase in MetHb concentrations in blood samples because of post-mortem oxidation of HHb to MetHb [55],[56],[57]. If sodium dithionite in a method is used after saturating the sample with CO or oxygen, elevated levels of MetHb in a sample will result in a loss of CO binding capacity of the blood. Therefore, the elevated MetHb levels will produce an erroneously high % COHb value as MetHb does not bind to CO [58]. In view of this, sodium dithionite should be used prior to the saturation of sample with CO or oxygen when determining % COHb in post-mortem blood samples particularly when they are decomposed [21],[41],[59].

B.2 COHb by visible spectrophotometry (without CO saturation)

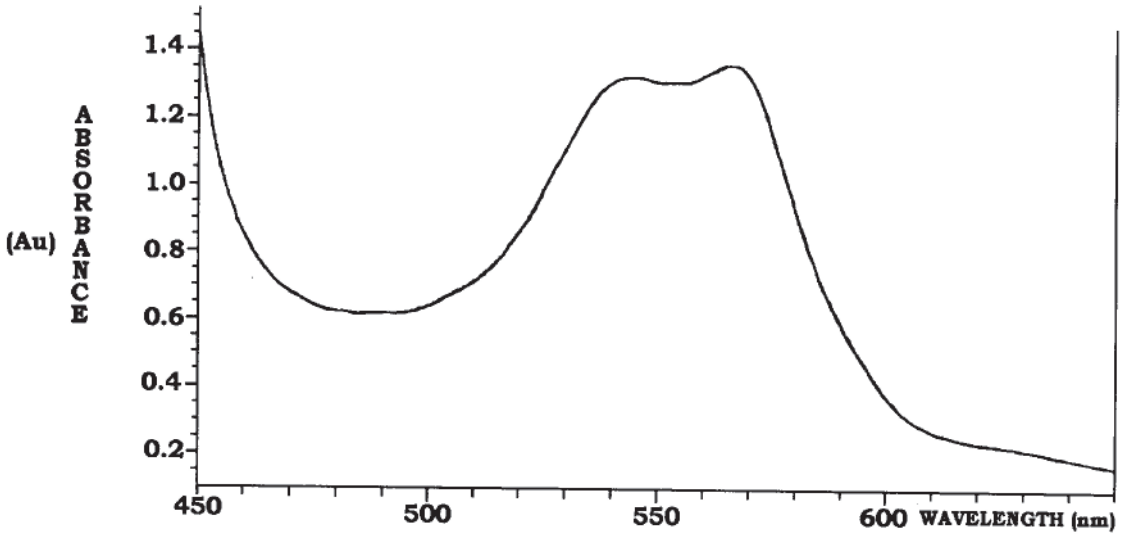
For this procedure (also see 8.5), a CO-Oximeter can be used for determining the $\bar{C}_{\text{pCOHb}}$ and $\bar{C}_{\text{nCOHb}}$ constant values for Equations (5) and (6) after analysing negative and positive COHb controls [14],[32],[33],[34], but an AVOXimeter cannot be used as citrate, fluoride, oxalate and EDTA have been reported to interfere with the analysis using this device. However, heparin-treated blood can be used for establishing the constant values by CO-Oximeter [19] or by AVOXimeter [20].

Putrid blood might not be suitable for this analysis as pigments resulting from decomposition can distort the combined COHb and HHb spectral scan. Figure B.1 shows examples of visible spectra of an unsuitable blood sample [a)], a blood sample containing 46,6 % COHb [b)], and a blood sample containing 2,9 % COHb [c)].

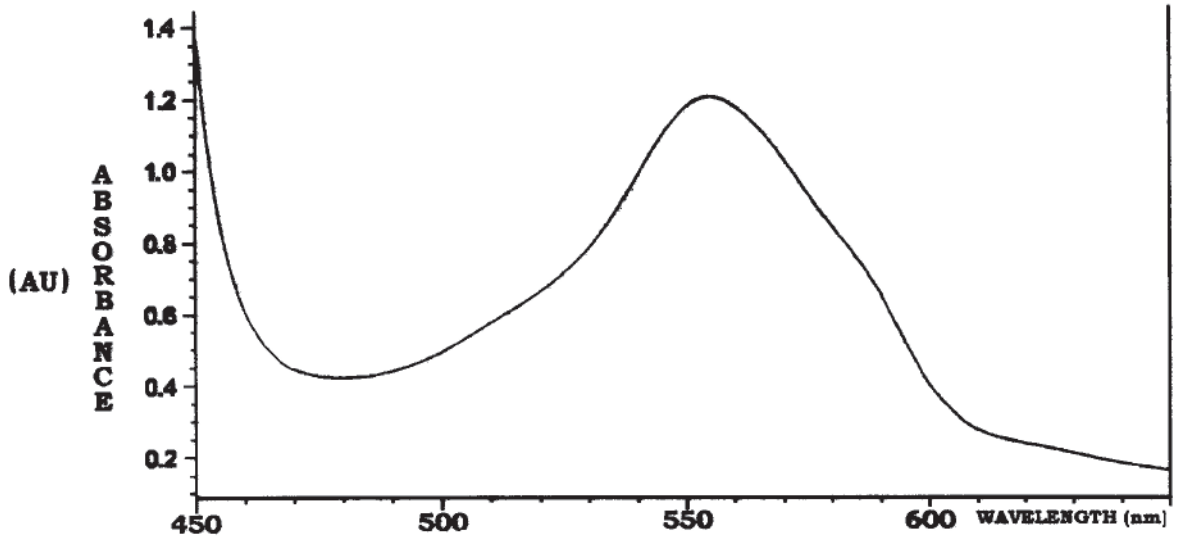


a) Unsuitable sample

Figure B.1 (*continued*)



b) Sample containing 46,6 % COHb



c) Sample containing 2,9 % COHb

Figure B.1 — Visible spectra of different types of blood samples[6)]

6) Reprinted, with permission, from Canfield *et al*.(1999) [34], copyright ASTM International, 100 Barr Harbor Drive, West Conshohocken, PA 19428. Since these figures are also published in Canfield *et al*.(1998) [33], a public-domain technical report of the U.S. Department of Transportation, Washington, DC, is also being acknowledged.

B.3 COHb by headspace gas chromatography — Thermal conductivity detection

In this method, sodium dithionate is used. Refer to 8.7 and Clause B.1.

B.4 CN^- by visible spectrophotometry

In the colorimetric method (see 9.2), CN^- from blood is converted to a red-pinkish, highly resonant product after reacting with chloramine-T, pyridine and barbituric acid [27],[45],[46]. For the chemical reaction, see Figure 1.

B.5 CN^- by spectrophotofluorimeter or high-performance liquid chromatograph equipped with a fluorescence detector

The chemical reaction between CN^-, NDA, and taurine upon which this method [50] is based (also see 9.5) is outlined in 9.5.2. The reaction product (a CBI derivative) thus formed is a suitable candidate for fluorimetric measurement.

B.6 CN^- by high-performance liquid chromatography-mass spectrometric detection

As shown in Figure 4, CN^- from blood reacts with NDA and taurine and produces the CBI–CN derivative [51]. (See also 9.6.) Since isotopically tagged potassium cyanide ($K^{13}C^{15}N$) is used as an internal standard, $^{13}C^{15}N^-$ from blood also simultaneously reacts with the two reagents, producing the CBI–$^{13}C^{15}N$ derivative. Examples of the mass spectra (mass range *m/z* 70 to 320) and chromatograms of CBI–CN and CBI–$^{13}C^{15}N$ are shown in Figures B.2 and B.3, respectively.

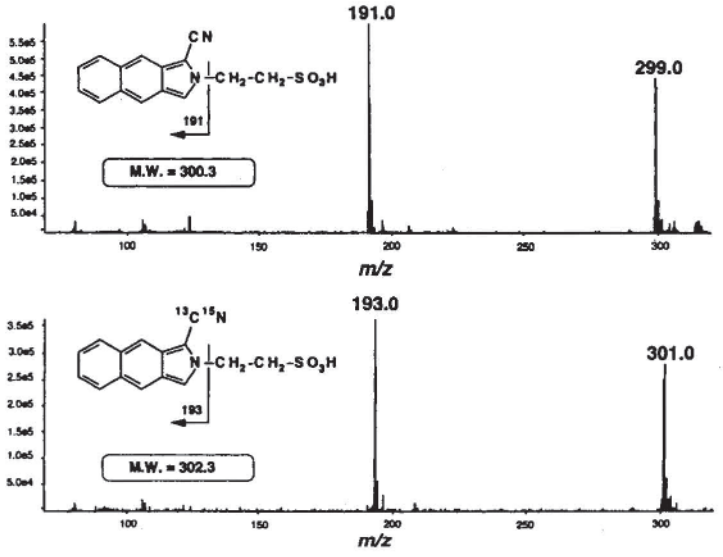
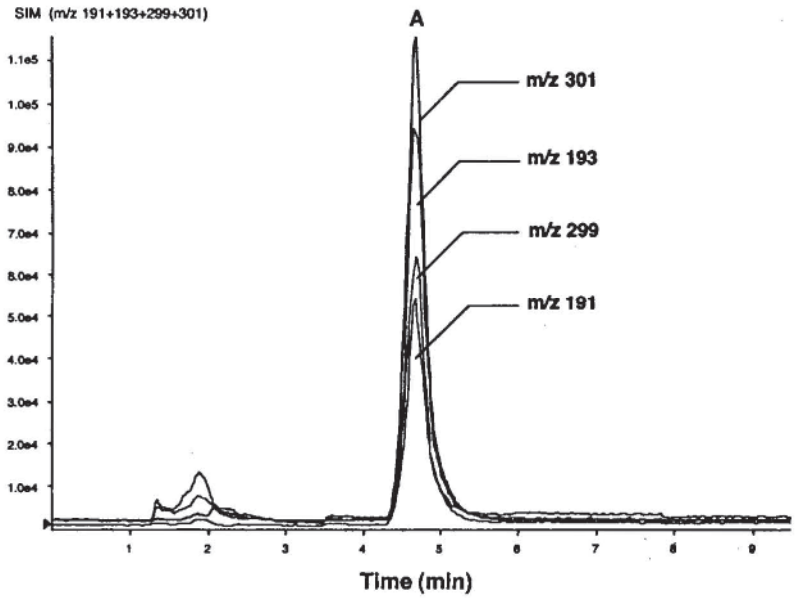


Figure B.2 — High-performance liquid chromatography — Negative-ion ion spray mass spectra of the CBI derivatives (*m/z* 70 to 320)[7)]

7) Reproduced from Tracqui *et al.* (2002) [51], by permission of Preston Publications, a Division of Preston Industries, Inc., 6600 West Touhy Avenue, Niles, IL 60714.



Peak A at 4,66 min is a summation of signals from the CBI derivatives of CN^- (*m/z* 191 and 299) and $^{13}C^{15}N^-$ (*m/z* 193 and 301). CN^- concentration: 0,56 μg/ml.

Figure B.3 — High-performance liquid chromatography — Mass spectrometric chromatograms, using selected-ion monitoring at *m/z* 191, 193, 299 and 301 of the post-mortem blood extract from a fire victim[7)]

Annex C
(informative)

Interpretation of results

C.1 General

During fire, carbon- and nitrogen-containing materials produce CO and HCN [40],[60],[61],[62],[63],[64]. Since these chemical species are the two primary toxic combustion gases and both gases are present in the blood of fire victims [12],[65],[66],[67],[68],[69],[70], blood is the specimen of choice for the analysis of these gases. Based upon the COHb and CN^- concentrations in blood, the degree of toxicity produced by smoke can be established for the victims of fire. Since both CO and HCN have adverse effects on the central nervous system [71],[72],[73], these toxic gases have the potential for producing interactive effects on the victims. However, the contribution of CO and HCN to the toxicity on, or the death of, a fire victim should be determined while taking into account the degree of burns, blood levels of COHb and CN^-, the MetHb content, the temperature at which the body has been left, the post-mortem interval and the age and physical condition of the victim [74],[75]. The presence or absence of these species in blood suggests whether the individual died during a fire or was dead prior to the fire. This type of information is useful in the investigation of fires involving casualties.

C.2 Blood samples

Old or putrid blood samples can interfere with the analysis leading to erroneous results. Clotted blood samples can be homogenized prior to their use for analysis. Solid, burned blood samples might not be suitable, though aqueous homogenates of such blood samples can be analysed; however, interpretation of such analytical results can be difficult. The quality of the blood

— can be related to the time between death and blood collection (post-mortem interval),

— has been a toxicological and analytical concern,

— can impact on the accuracy of the COHb and CN^- concentrations.

The blood collection technique and container, the types of anticoagulants and/or preservatives used, the sample storage conditions and the analysis time after sample collection can also adversely affect the outcome of the COHb and CN^- analyses. Therefore, such analytical values might not reflect the true concentrations of these species at the time of death.

C.3 COHb concentrations

C.3.1 Factors affecting COHb concentrations

Factors that have been found to influence the COHb concentrations include the type of container and preservative/coagulant used for blood collection, the ratio of surface area to volume of blood exposed to the atmosphere, storage condition and temperature and the initial percentage COHb saturation [76]. A decrease of approximately 20 % in COHb levels as a function of time has been reported in post-mortem blood samples collected from fire victims [12].

C.3.2 Normal COHb levels

Although analytical methods are capable of measuring COHb concentrations below 10 %, this value is forensically considered a cut-off for COHb concentrations. Blood COHb concentrations below 5 % are considered normal. Normal, healthy individuals can reach a COHb concentration of up to 10 % from inhaling air contaminated with CO [7],[24], such as smoking, working in an atmosphere rich in gasoline exhaust fumes or operating (travelling in) a vehicle with a faulty exhaust system. In heavy smokers, COHb levels have been observed to be as high as 15 % to 17 % [77].

C.3.3 COHb-associated signs and symptoms

Toxic manifestations of CO poisoning are associated with the central nervous and cardiovascular systems wherein the need of oxygen is high [71],[72],[73]. COHb concentrations and related signs and symptoms are summarized in Table C.1. The signs and symptoms in Table C.1 relate primarily to the effects of short exposures (of the order of minutes) in otherwise healthy subjects at rest. For a given percentage COHb concentration, effects can be more severe in physically active subjects or following prolonged exposures of several hours [78],[79]. There is, therefore, often a poor relationship between the severity of signs and symptoms and percentage COHb concentrations measured in CO victims on presentation at hospital [80]. There is also a considerable range of sensitivity to CO poisoning in the population, so that fatalities from CO exposures or from fire-effluent exposures show a wide range of COHb concentrations from approximately < 30 % to 95 % COHb [81].

Table C.1 — COHb concentrations and related sign and symptoms [71]

COHb %	**Typical signs and symptoms**
0 to 10	No symptoms
10 to 20	Tightness in the forehead, possible slight headache and dilation of cutaneous blood vessels
20 to 30	Headache and throbbing in temples, easily fatigued and possible dizziness
30 to 40	Severe headache, weakness, dizziness, confusion, vision dimness, nausea, vomiting and collapse
40 to 50	Same as above, but severity is higher; increased pulse and respiratory rate
50 to 60	Increased respiratory and pulse rate, coma, intermittent convulsions and Cheyne-Stokes respiration
60 to 70	Coma, intermittent convulsions, depressed heart action and respiratory rate and possible death
70 to 80	Weak pulse, slow respiration, respiratory failure and death within a few hours
80 to 90	Death in less than an hour
> 90	Death in a few minutes

C.4 Blood CN^- concentrations

C.4.1 Factors affecting CN^- concentrations

Blood CN^- concentrations are strongly affected by the time between death and sample collection, decreasing by approximately 50 % per day in a cadaver [16],[82]. Once a sample has been taken, it is also affected by the type of container and preservative/coagulant used for blood collection, the ratio of surface area to volume of blood exposed to the atmosphere, the sample storage condition and temperature and the initial blood CN^- concentration. Chemically, CN^- in blood (pH 7.4) can be converted into its protonated form (HCN; pK_a 9.2); thereby HCN can easily be diffused through the body and also released into the surrounding atmosphere.

C.4.2 Normal CN^- levels

Normal human blood concentrations range from 0,0 µg/ml to 0,30 µg/ml for non-smokers and from 0,02 µg/ml to 0,50 µg/ml for smokers [5],[83],[84]. Levels up to 0,15 µg/ml CN^- originating from cyanogenic glycosides or pyocyaneous organisms can be found in adults without symptoms [8],[10],[46],[71],[85],[86],[87]. An analytical method with a detectability of less than 0,25 µg/ml might not have toxicological relevance as clinical manifestations are observed at CN^- concentrations of $\geqslant$ 0,5 µg/ml [71].

Only one-third of the unmeasurable CN^- can be attributed to the conversion from CN^- to thiocyanate. The antidote, sodium thiosulfate, for the treatment of CN^- poisoning has been reported to interfere with colourimetric, fluorimetric and potentiometric methods for the measurement of CN^- concentrations [88],[89],[90]. The majority of the analytical methods measure total CN^- originating from both critical (cyanide-cytochrome oxidase complex) and noncritical (cyanomethaemoglobin; erythrocytes) sites [71],[72],[91],[92],[93]. However, release of HCN from cyanomethaemoglobin has been reported by heat denaturation [94].

C.4.3 CN^--related sign and symptoms

CN^- is a rapidly acting, toxic species and is capable of causing incapacitation and death quickly [71],[72],[73]. As CN^- concentrations increase, unavailability of oxygen to the central nervous system can induce hypoxic convulsions followed by death due to respiratory arrest. In fires, inhalation of HCN induces hyperventilation, thereby increasing the rate of uptake until the subject collapses and becomes comatose, whereupon the rate of uptake decreases and the subject can recover somewhat before going into a gradual and eventually fatal decline [79],[95] unless rescued. Blood has high CN^- concentrations probably due to the sequestration of CN^- in the red blood cells [4],[6],[91],[96],[97],[98] and blood concentrations of CN^- can be correlated with its toxicity [71],[92],[99]; however, signs can be more closely related to plasma CN^- concentrations than whole-blood CN^-, so that an unconscious subject can recover within a few minutes of the cessation of exposure without any decline in whole-blood CN^- [79],[95]. Blood CN^- concentrations and related toxic manifestations for blood samples taken soon after a non-fatal or fatal exposure are tabulated below (see Table C.2), though toxic effects of CN^- on fire victims should not be based solely on its concentration in the blood [74],[75]. Due to the rapid decline in blood CN^- in cadavers, post-mortem blood CN^- concentrations can be considerably lower than those present at the time of death, so that it can be necessary to adjust the measured values for such decreases for comparison with the values in Table C.2.

Table C.2 — Blood CN^- concentrations and associated toxic manifestations [71]

Degree of toxicity	Blood CN^- µg/ml	Typical signs and symptoms
Mild	0,5 to 1,0	Flushed, rapid pulse, conscious and headache
Moderate	1,0 to 2,5	Stuporous but responsive to stimuli, tachycardia and tachypnea
Severe	$\geqslant$ 2,5	Comatose, unresponsive, hypotension, slow respiration, gasping, mydriasis, cyanosis at high concentration and death

C.5 Interactive effects of CO and HCN

When both COHb and CN^- concentrations are elevated, the interpretation of such levels as to the degree of toxicity caused by CO and HCN becomes challenging because both asphyxiants have interactive effects on the central nervous system [64],[71],[72],[73]. The "fractional effective dose" (FED) concept [93],[100],[101],[102] can be applied wherein FED_{CO} plus FED_{HCN} should equal unity for an additive effect of CO and HCN. By employing the FED model using blood percentage of COHb, w_{COHb}, expressed as a mass fraction, and the concentration of CN^-, C_{CN^-}, expressed in micrograms per millilitre, in place of the effective exposure doses (FEDs) of CO and HCN, an attempt has been made to account for the additive effects caused by these gases on the basis of the fractional toxic concentration, X_{FTC}, in the blood as given in Equations (C.1) and (C.2) [103].

$$X_{FTC} = X_{FTC,COHb} + X_{FTC,CN^-} \tag{C.1}$$

where

$X_{FTC,COHb}$ is the fractional toxic concentration of COHb;

X_{FTC,CN^-} is the fractional toxic concentration of CN^-.

$$X_{FTC} = \left(\frac{w_{COHb}}{0,7 w_{COHb}}\right) + \left(\frac{C_{CN^-}}{3}\right) \tag{C.2}$$

$$= 1$$

where

w_{COHb} is the concentration of COHb in the sample, expressed as a percentage mass fraction;

$0,7 w_{COHb}$ is the concentration of COHb that can alone cause lethality [104],[105], expressed as a percentage mass fraction;

C_{CN^-} is the concentration of CN^-, expressed in micrograms per millilitre;

3 is the concentration of CN^- that can alone cause lethality [104],[105], expressed in micrograms per millilitre.

However, the above concept does not rule out other additive effects of these gases. It is suggested that the concentrations of both COHb and CN^- in blood and interactive potentials of both species should be carefully considered during interpreting and correlating their blood concentrations with the adverse effects.

C.6 Sulfmethaemoglobin and cyanomethaemoglobin

In addition to CO and HCN, numerous other species can be produced during the combustion of substances [40],[60],[63],[64],[106],[107] (see also ISO 19701). Depending upon the chemical nature of the substances involved in the fire, these species can be very reactive. Exposure to these combustion products can convert HHb to MetHb. Exposure to heat can also produce MetHb [52], and post-mortem oxidation of HHb to MetHb has been reported [55],[56],[57]. If smoke rich in HCN, hydrogen sulfide or sulfur-containing substances is inhaled by fire victims, then these products can react with MetHb to form sulfmethaemoglobin (also known as sulfaemoglobin) and cyanomethaemoglobin. Sulfmethaemoglobin can also be formed post-mortem as hydrogen sulfide and other sulfur species are generated during putrefaction. Thus, there is a strong potential for the presence of sulfmethaemoglobin and cyanomethaemoglobin in blood samples collected from fire casualties.

Sulfmethaemoglobin has been reported to interfere with spectrophotometrically based analytical methods [19],[73], particularly if this HHb species is present in high concentrations, as the spectral absorbance of sulfmethaemoglobin is similar to that of MetHb. Additionally, some methods do not account for

sulfmethaemoglobin and cyanomethaemoglobin in tHb (see 8.1 and 8.2), thus the percentage COHb results would be erroneously high by those methods.

In several methods, sodium dithionite is used as a reducing agent to convert OxyHb and MetHb to HHb to maximize the CO binding capacity of the blood sample (see 8.3 to 8.7). It is not certain whether such dithionite treatment would also reduce sulfmethaemoglobin and cyanomethaemoglobin to HHb, though polysulfides, thiosufate and sulfate have been reported to reduce sulfmethaemoglobin to HHb [71]. If sulfmethaemoglobin and cyanomethaemoglobin (or even OxyHb and MetHb) are not totally reduced to HHb by sodium dithionite, then the binding capacity of the blood sample would not be maximum; and the observed percentage COHb values would be erroneously high in comparison with the situation when these HHb species were completely reduced to HHb.

Therefore, the two species of HHb, sulfmethaemoglobin and cyanomethaemoglobin, at high concentrations have the potential for interfering with the analyses of COHb and causing inaccurate analytical results.

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