Modified congener analysis: Quantification of cyanide in whole blood, other body fluids, and diverse beverages

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Abstract
The congener analysis is routinely used for the determination of volatile compounds in body fluids and beverages for forensic investigations. Although intoxications with cyanide via smoke inhalation or ingestion of cyanide salts are frequently encountered in forensic medicine, the inclusion of hydrogen cyanide in this analysis was never studied in detail. In this work, a very simple, fast, and sensitive quantification method with headspace gas chromatography and flame ionization detection for the analysis of cyanide in whole blood—was developed and validated. In contrast to the standard sample preparation of the congener analysis, an acidification step with tartaric acid was added. A limit of detection of 50 ng/ml, good linearity (coefficient of correlation > 0.9997), high accuracy (101.5%–106.4%), and precision (relative standard deviation 1.8%–3.7%) were achieved. Authentic blood samples of 10 forensic cases were investigated with the new method. Furthermore, the method was used for the quantification of cyanide in other body fluids (serum and urine) and diverse beverages. Interferences were investigated, and the addition of aldehydes produced a clear concentration-dependent decrease of the cyanide signal. Besides, the method offers an economical use of limited sample material by the simultaneous determination of cyanide, ethanol, and congener alcohols.

Keywords
beverages, blood, congener analysis, cyanide, gas chromatography

1 | INTRODUCTION

The congener analysis was originally introduced for the determination and quantification of congener alcohols (i.e., methanol, 1-propanol, and isobutanol) in whole blood and in diverse beverages. The composition of such volatile compounds varies significantly between particular alcoholic beverages. Therefore, ingestion of a specific beverage can be verified by comparing the congeners measured in blood and in the previously consumed liquids. Cyanide (CN), a rapidly acting and effective poison, is commonly analyzed in poisoning and smoke inhalation cases in forensic toxicology. Intoxications can be a result of inhalation of hydrogen cyanide (HCN) vapors or ingestion of cyanide salts. Deaths by accidental or intentional cyanide intoxication are frequently encountered in legal medicine (i.e., forensic pathology, and toxicology). Smoke inhalation is the most common accidental cyanide exposure and contributes to 80% of the deaths related to residential fires. Investigations of suicides and homicides involving cyanide salts are historically well-known and still ongoing topic in the field of forensic toxicology.
oral doses of sodium and potassium cyanide for adults are 0.15–0.30 g. Although endogenous or dietary blood levels of cyanide can vary significantly between 0.0005 and 0.27 μg/ml, the average endogenous level of a nonsmoker is considered to be approximately 0.06 μg/ml and of a smoker approximately 0.12 μg/ml.8,9 Whereas blood cyanide concentrations less than 0.25 μg/ml are considered nontoxic, cyanide concentrations of more than 1 μg/ml are generally considered toxic.10 Lethal concentrations found in suicide cases range from 1 to 53 μg/ml (average: 12.4 μg/ml).8,11,12 In fire cases with fatalities, reported cyanide blood concentrations range from 0 to 3.5 μg/ml (average: 0.68 μg/ml).5

In a routine toxicological analysis of cases with potential cyanide intoxication, the determination of CN is preferably performed in whole blood but, if required, also in serum, other body fluids (urine, gastric content, and liquor) and confiscated liquids from the crime scene. In blood, most of the CN is presumably bound to methemoglobin and accumulated in the erythrocytes.13 Consequently, CN concentrations in nonhemolytic serum tend to be lower in comparison with the concentrations in whole blood. Although the free CN can be converted to the volatile HCN by pure acidification, the bound form must be treated with heat under acidic conditions for complete conversion.14 Cyanide is a typical metabolite in humans and can be both produced and degraded in gastric content and blood samples.15 Postmortem transformation of cyanide includes the conversion to thiocyanate, hydrolysis to ammonium formate, and reaction with aldehydes and polysulphides in postmortem tissues.13,16 The quantification of cyanide in confiscated food or beverages from the crime scene can be challenging due to matrix effects like extraneous cyanide production or included interfering components.17 Cyanide naturally appears in bacteria, algae, fungi, and numerous plants. Cyanogenic glycosides can be found in the whole plants (cabbage, clover, and bamboo) as well as in specific plant parts such as roots (potato, radish, and turnip), grains (sorghum), and fruits (coffee, almond, cashew, seeds, and pits of apple, apricot, peach, and plum).17,18 Beverages made with these organisms or plant parts can contain cyanide or cyanogenic glycosides and measureable CN concentrations are expectable.19,20 A significant interference can be caused by aldehydes like sugars, alphaketoglutarate, acetaldehyde, or benzaldehyde, which decrease the cyanide concentration via cyanohydrin formation.21,22

Among the wide range of methodologies proposed for the determination of CN in biological specimens, methods using headspace-gas chromatography (HS-GC) coupled with different detection systems such as flame ionization detection (FID),23,24 electron capture detection,25 mass spectrometry,26 and nitrogen-phosphorus detection27,28 are predominant in forensic laboratories. So far, the congener analysis, which is routinely performed by HS-GC-FID, was never evaluated for a specific detection and quantification of CN.

In this article, the authors developed a method based on the congener analysis for the quantification of cyanide in whole blood. Following the forensic guidelines, the method was validated, and blood samples of 10 authentic forensic cases were investigated. In addition, the method was evaluated for the quantification of cyanide in other body fluids such as serum, gastric content, and urine and in diverse beverages. Moreover, the influence of congeners like methanol, ethanol, and aldehydes (acetaldehyde and benzaldehyde) on the determination of cyanide was investigated systematically.

2 | EXPERIMENTAL

2.1 | Reagents

Potassium cyanide (KCN) and tartaric acid were purchased from Merck KGaA (Darmstadt, Germany). Sodium hydroxide was obtained from Carl Roth GmbH CoKG (Karlsruhe, Germany) and Ampuwa (dest. water) from Fresenius Kabi (Bad Homburg, Germany). A certified cyanide solution (1,000 mg/L) in 0.1 M NaOH was purchased from LGC Promochem GmbH (Wesel, Germany). The gases for GC analysis were obtained from Grandpair (Heidelberg, Germany). All chemicals and solvents were used without further purification.

2.2 | Sample collection and storage

Blood specimens for method development and validation were obtained from whole blood donations of the blood bank of the University Hospital Heidelberg. The blood was drawn from a vein, stabilized with citrate-phosphate-dextrose (CPD) solution, and frozen immediately. Prior to its use, the frozen blood was thawed, aliquoted, and kept at 4°C. All postmortem blood samples used for method verification were collected in tubes during forensic autopsy and were kept at 4°C until analysis.

2.3 | Sample preparation

Whole blood (0.5 ml) was dispensed into a 10-ml headspace vial, and 0.5 ml of a saturated aqueous tartaric acid solution was added. The vial was immediately crimp sealed, vortexed, and placed on the sample tray of the autosampler from the HS-GC-FID system. For external calibration, two stock solutions were prepared from potassium cyanide with cyanide concentrations of 250 and 25 μg/ml in 0.1 M NaOH. The calibration standards were prepared following the procedure outlined in the above paragraph using the stock solutions and whole blood for a total volume of 0.5 ml followed by the addition of the saturated tartaric acid solution. For the quality controls (QC), cyanide stock solutions with concentrations of 250 and 25 μg/ml in 0.1 M NaOH were prepared from a certified cyanide solution from LGC to afford three QC samples in whole blood at concentrations of 0.6, 1.8, and 4.0 μg/ml.

If the concentration or content of cyanide was determined in other matrices (e.g., serum, urine, gastric content, and other beverages), the calibration and QCs were prepared by using the respective matrix.
2.4 | HS-GC analysis

The automated HS-GC-FID analyses were carried out on a Clarus 500 gas chromatograph (PerkinElmer, Waltham, MA, USA) equipped with FID and interfaced to an automated TurboMatrix 40 GC sampler (PerkinElmer, Waltham, MA, USA). During the injection period, carrier gas flowing to the GC system is replaced by pressurized sample gas (pneumatic, pressure-balanced sampling). The headspace time parameters were set as follows: thermostating time 30 min, injection time 0.08 min, withdrawal time 0.2 min, and GC cycle time 35 min. The headspace temperatures were set as follows: oven thermostating temperature 66°C, needle temperature 100°C, and transfer line temperature 100°C. The gas chromatograph was equipped with a DB-1701 (Agilent Technologies, Wilmington, DE, USA) capillary column (60 m × 0.32 mm I.D., 1.0-μm film thickness). The nitrogen flow rate was maintained at 3 ml/min. Column temperature was programmed at 30°C for 7 min, increased by 10°C/min to a temperature of 155°C, held for 1 min, ramped at 30°C/min to 230°C, and held at final temperature for 10 min (33 min total run time). The FID detector temperature was held at 250°C.

2.5 | Data analysis

All samples, blanks, standards, and QC materials were processed identically, and the data were evaluated for accuracy of integration. The retention time of CN was used for identification and the integrated peak area for quantification. Five standard CN concentrations (0.25, 0.5, 1.0, 2.5, and 5.0 μg/ml) encompassing the entire linear range of the method were used for recording a calibration curve of peak area versus standard concentration, which were used for the quantification of CN concentrations in unknown samples. Data were automatically processed using the quantitation software TotalChrom Workstation. Statistical analysis of the data was carried out using the software Microsoft Excel. The validation of the method and calculation of validation parameters like linearity, accuracy, precision, limit of detection (LOD) and limit of quantification (LOQ) (calibration approach following DIN 32645) stability, recovery, and matrix effects was performed with the software Valistat®.

3 | RESULTS AND DISCUSSION

3.1 | Method development

The method was primarily intended for the determination of cyanide in whole blood and other biological samples collected during an autopsy in cases of suspected intoxication with cyanide, for example, smoke inhalation, homicide, or suicide. Because the method should be suitable for the determination of cyanide and other volatile compounds in alcoholic and nonalcoholic beverages, we started with the original method of the congener analysis. Although we wanted to maintain the HS-GC-FID parameters of our method, the sample preparation was modified to achieve a better release of CN from the sample.

In other methods, the blood is acidified with diluted aqueous solutions of phosphoric, sulfuric, or hydrochloric acid. To maintain a salinity effect ("salting out effect") and achieve simultaneous acidification of the sample, we replaced the originally added sodium sulfate with solid tartaric acid or a saturated aqueous tartaric acid solution. Based on the easy sample preparation and a lower LOD, the addition of the saturated aqueous tartaric acid solution was primarily used for further experiments.

The use of an internal standard might improve the results of analysis by compensating matrix effects and was therefore thoroughly investigated. Because neither the addition of tert-butyl alcohol nor acetonitrile improved the analytical performance of the method (i.e., linearity, accuracy, and recovery), the application of an internal standard in the method was not pursued further. Whereas other published HS-GC-FID methods require sample volumes of 1-to 5-ml blood, our method was performed with only 0.5-ml blood.

With the original HS-GC-FID parameters, the HCN signal at a retention time of 3.75 min is accompanied by acetaldehyde at 3.58 min and methanol at 3.98 min (Figure 1). A baseline separation of these signals was always observed at endogenous levels of acetaldehyde (0–30 mg/L) and methanol (0–3 mg/L). A chromatographic peak resolution of 1.54 for the acetaldehyde and HCN peak and of 1.53 for the HCN and methanol peaks indicates a good separation (overlap ≤ 0.15%), respectively. Other volatile compounds of the congener analysis like ethanol at 5.39 min and n-propanol at 9.69 min can be analyzed in parallel without signal interferences. A blood blank was processed in every batch along with the rest of the samples to monitor contaminations of reagents or blood.

3.2 | Method validation

The method was validated for the determination of cyanide in whole blood samples according to the guidelines of the "Society of Toxicological and Forensic Chemistry" (GTFCh). In order to assess the method linearity, a series of spiked whole blood samples (0.25, 0.5, 0.75, 1, 1.5, 2.5, and 5 μg/ml of cyanide) were prepared and analyzed. Standard response curves were generated using a least-square linear regression and showed excellent linearity over the investigated range (i.e., linear correlation coefficient R² = 0.999). A percent residual accuracy (%RA) of 97.39 also demonstrates good and reliable calibration data. Moreover, the interday variability of daily calibration curves was negligible, and the relative standard deviation given as a percentage (%RSD) of the slope of 10 calibration curves over 5 months was 1.7%. The average calibration curve of CN in whole blood is shown in Figure 2A.

Precision and accuracy were assessed by the analysis of blank blood samples spiked with cyanide (0.6, 1.8, and 4 μg/ml). For the determination of the intraassay precision, eight prepared samples of each concentration were analyzed within 1 day. For the determination of the interassay precision, a series of each concentration was measured on eight different days in total. The analytical results of the
intraassay (%RSD 2.2%–3%) and interassay (%RSD 1.8%–3.7%) precision and accuracy (101.5%–106.4%) were in acceptable ranges. The average mean recovery was 103.3%. The slope of a linear regression analysis of the calculated versus the expected concentration was 0.999, which confirmed the excellent accuracy of the method.

The replicate analysis of 15 blood samples spiked with low-level standards (i.e., 0.1, 0.2, 0.3, 0.4, and 0.5 μg/ml) was used to calculate the lower LOD and LOQ according to DIN 32645.29,32 The calculated LOD of our method was 0.05 μg/ml, and the LOQ 0.16 μg/ml, which is comparable with previously reported values from other HS-GC-FID

**FIGURE 1** Gas chromatography (GC) chromatograms of blood samples from a (A) nonsmoker, specimens spiked with (B) 0.25 and (C) 5.0 μg/ml of CN, and a fire victim (Case 6) who died as a result of (D) smoke inhalation [Colour figure can be viewed at wileyonlinelibrary.com]

**FIGURE 2** Calibration curves of CN in (A) whole blood, (B) other body fluids and (C) diverse beverages across the linear range ±SD with linear correlation coefficients of >0.99. Detected CN in aqueous samples with (D) 5.0 μg/ml of cyanide spiked with different volumes of potentially interfering substances.
methods (LOD: 0.02 and 1 μg/ml).\textsuperscript{23,24} Physiological levels of CN can range from 0.005 to 0.08 μg/ml and can be determined with MS and NPD detectors, which provide the best sensitivity for cyanide detection (LOD: 0.005–0.05 μg/ml).\textsuperscript{28,34–36} Although toxic and lethal CN blood levels start at 0.2 and 1.0 μg/ml, the level of sensitivity of our technique appears quite sufficient to permit reliable measurements of cases with accidental or self-induced CN poisoning in clinical and forensic situations.

Recovery was calculated from the measurement of standard aqueous solutions and spiked blood samples with standard cyanide concentrations of 0.60 and 4.0 μg/ml. The recovery was found to be excellent with 98.5% and 96.3%.

In addition, the stability of cyanide was investigated with spiked samples of cyanide (1.0 μg/ml) in blood. After preparation in crimp-sealed headspace vials, the samples were stored at different temperatures (−20°C, 4°C, and room temperature). At each day for 1 week, three vials of the respective storage temperatures were opened, acidified with tartaric acid solution, and crimp sealed, and the CN concentrations were determined with the presented method. In the spiked samples, a decrease of the CN concentration by 1%–2% after 1 day and by 13% after 7 days was observed independently of storage temperatures, respectively.

3.3 | Method verification with post-mortem specimens

Fatal cases of fire victims with smoke inhalation are routinely examined for the presence of carbon monoxide and cyanide as part of a forensic investigation. The CN concentrations in blood samples of authentic cases (Table 1), which were sent to the Institute of Forensic and Traffic Medicine in Heidelberg for a forensic autopsy, were investigated with the presented method. The blood samples were collected during the autopsy and cyanide-antidote medication was not used. Cyanide was not detected in cases with suicidal or accidental CO intoxication without open fire (Cases 1–3). In Case 4, the victim presumably died before the fire started, and without extensive smoke inhalation, only low carboxyhemoglobin (COHb) and CN concentration were observed. Amounts of hydrogen cyanide in the smoke of residential fires depend on the burning goods (e.g., cotton and plastic) and can vary significantly. Therefore, victims of deadly smoke inhalations (Cases 5–9) can show similar COHb values but different CN blood concentrations. The victims of Cases 8 and 9 died in the same incident and showed nearly identical COHb and CN concentrations. The highest determined CN concentration of 2.72 μg/ml was found in the blood of a person who died as a result of a vehicle fire with extensive burns (Case 10). These results indicate that the new method is useful for the detection of cyanide in blood at levels above and below toxic levels, thus confirming the applicability of the method in forensic toxicology.

### TABLE 1  
Autopsy cases from the Institute of Forensic and Traffic Medicine in Heidelberg with assumed smoke inhalation (CO and CN)

| Case | Casuistic | Burns | COHb (%) | CN (μg/ml) |
|------|-----------|-------|----------|------------|
| 1    | Generator gas | None  | 45       | n.d.       |
| 2    | Charcoal grill  | None  | 87       | n.d.       |
| 3    | Smoldering fire | Minor | 73       | n.d.       |
| 4    | Residential fire | Extensive | 14      | 0.07       |
| 5    | Trailer fire | Extensive | 47       | 0.53       |
| 6    | Residential fire | Extensive | 44      | 1.06       |
| 7    | Residential fire | Extensive | 67       | 1.32       |
| 8    | Residential fire | Minor  | 52       | 2.00       |
| 9    | Residential fire | Minor  | 50       | 2.02       |
| 10   | Vehicle fire | Extensive | 50       | 2.72       |

Abbreviations: COHb, carboxyhemoglobin measured with photometric detection; n.d. not detected (≤LOD).

3.4 | Measurement of CN in other matrices

Although blood is the preferred matrix for the determination of cyanide, other body fluids like gastric content and urine or confiscated liquids from the crime scene might be investigated as part of a forensic investigation. Moreover, known CN concentration in gastric content and urine is useful for an estimation of the total ingested or administrated amount of cyanide salts or cyanogenic glycosides. Therefore, the presented method was adapted for the determination of cyanide in diverse body fluids and beverages (Figure 2B,C).

All calibration curves of cyanide in body fluids (blood, serum, and urine) showed excellent linearity ($R^2 > 0.99$; %RA > 90). Whereas the slope of the curves of blood, urine, and water was nearly identical, the slope of the calibration curves of serum was significantly decreased by 23%–33% (Figure 2B). In addition, the calibration curves measured in spiked samples of various beverages, like coffee, milk, tonic water, juice, and brandy, were also compared (Figure 2C). A calibration curve for brandy could not be obtained in the investigated range of the method because only the highest spiked amount of 5.0 μg/ml of CN generated a detectable signal. All other calibration curves showed excellent linearity ($R^2 > 0.997$; %RA > 90) with a similar slope from curves of water, tonic water, milk, and juice. For coffee, the slope of the calibration curve was decreased by 38%.

A decrease of the cyanide signal might be obtained by other volatile or interfering compounds, which were further investigated (Figure 2D). Aqueous samples with 5.0 μg/ml of CN were spiked with increasing amounts of methanol, ethanol, gamma-butyrolactone, acetaldehyde, benzaldehyde, and acetone were measured with the described method, respectively. High amounts (up to 5% [v/v]) of ethanol and gamma-butyrolactone had no significant effect on the cyanide detection and even 5% (v/v) of methanol and acetone only slightly decreased the detected amount of CN. In comparison, even small amounts of acetaldehyde (0.05% [v/v]) and benzaldehyde (2% [v/v]) totally negated the cyanide signal in the chromatograms. Aldehydes like acetaldehyde or benzaldehyde are well-known to react with cyanide via cyanohydrin formation.\textsuperscript{21,22} Therefore, the presence...
of aldehydes in coffee and brandy might be a reason for the observed reduction of the detected CN in such beverages.

3.5 | Limitations

Extraneous production of HCN is described for heating of blood at high temperatures and from thiocyanate (SCN) upon denaturation of the hemoglobin under acidic conditions. In the presented method, the samples were incubated at 66°C, and tartaric acid was used for acidification. Under these conditions in more than 50 blank blood samples, the CN signals were below the LOD (50 ng/ml of cyanide). Therefore, an extraneous production of HCN was not observed or could not be differentiated from endogenous CN levels.

4 | CONCLUSION

In this work, a simple, sensitive, and reliable HS-GC-FID method for the quantification of cyanide in whole blood was developed and validated according to forensic guidelines. In comparison with other HS-GC-FID methods, only a low sample volume of 0.5 ml is required, and tartaric acid as a gentle acidic compound was used for acidification. With the time-efficient manual sample preparation (<2 min/sample) and measurement (35 min/sample), the method is suitable for the diagnosis of cyanide intoxications in clinical and forensic cases. A single method for the determination of cyanide in different body fluids from forensic autopsies (blood, serum, urine, and gastric content) and confiscated liquids from crime scene can simplify the forensic investigation and provide more insight in diagnosing cyanide intoxication in forensic practice. In forensic cases with limited sample material, the determination of cyanide can be performed simultaneously with ethanol and congener alcohols. Interfering compounds like acetaldehyde and benzaldehyde were investigated, and their presence can lead to a significant reduction of the measured CN concentrations in beverages or other liquids.

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