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Article

Hydrogen Sulfide Measurement by Headspace-Gas Chromatography-Mass Spectrometry (HS-GC-MS): Application to Gaseous Samples and Gas Dissolved in Muscle

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The aim of our study was to present a new headspace-gas chromatography-mass spectrometry (HS-GC-MS) method applicable to the routine determination of hydrogen sulfide (H_2S) concentrations in biological and gaseous samples. The primary analytical drawback of the GC/MS methods for H_2S measurement discussed in the literature was the absence of a specific H_2S internal standard required to perform quantification. Although a deuterated hydrogen sulfide (D_2S) standard is currently available, this standard is not often used because this standard is expensive and is only available in the gas phase. As an alternative approach, D_2S can be generated *in situ* by reacting deuterated chloride with sodium sulfide; however, this technique can lead to low recovery yield and potential isotopic fractionation. Therefore, N_2O was chosen for use as an internal standard. This method allows precise measurements of H_2S concentrations in biological and gaseous samples. Therefore, a full validation using accuracy profile based on the β-expectation tolerance interval is presented. Finally, this method was applied to quantify H_2S in an actual case of H_2S fatal intoxication.

Introduction

Hydrogen sulfide (H_2S) is a highly toxic gas that becomes flammable at percentages from 4.3 to 46% in ambient air. H_2S tends to accumulate at the bottom of poorly ventilated spaces because this gas is heavier than air (specific gravity of 1.19 compared with air).

H_2S is considered a broad-spectrum poison, although the nervous system is most affected. Its toxicity is comparable to that of hydrogen cyanide or carbon monoxide. H_2S forms a complex bond with iron in mitochondrial cytochrome *c* oxidases, thus inhibiting cellular respiration (1).

Because H_2S occurs naturally in the body, in the environment and in the gut, enzymes exist in the body that are capable of detoxifying H_2S by oxidation to sulfates such that low levels of H_2S may be tolerated. Indeed, H_2S is produced in small amounts by some cells of the mammalian body and has several biological signaling functions. Produced from cysteine by the enzymes cystathionine beta-synthase and cystathionine gamma-lyase, H_2S acts as a myorelaxant and vasodilator and is also active in the brain as a neurotransmitter, where H_2S increases the response of the *N*-methyl-D-aspartate receptor and facilitates long-term potentiation.

H_2S gas is converted to sulfite in mitochondria by thiosulfate reductase, and sulfite is further oxidized to thiosulfate (TS) by hemoglobin and hepatic enzymes, then to sulfate by sulfite oxidase. Finally, sulfates are excreted in the urine. A portion of H_2S can also be excreted unchanged by the lungs. Medical treatment for hydrogen sulfide exposure involves immediate removal from exposure and supportive care (2). Oxygen should be administered at the scene of the incident and in the emergency department (3). Amyl and sodium nitrite are often recommended, and several rapid recoveries have been reported (4, 5); however, their effectiveness as antidotes has not been sufficiently proved (6).

TS has been already reported as an indicator of antemortem H_2S exposure (7–9) because TS was believed to be weakly related to postmortem changes, such as redistribution or putrefaction. However, recent studies have shown that H_2S was less influenced by putrefaction than TS (7). Direct H_2S monitoring could also provide a direct measurement of the magnitude of the H_2S exposure or intoxication. Indeed, sulfates are only measurable in blood and urine, whereas H_2S is measurable in all biological matrices. Because TS concentrations can result in the misinterpretation of H_2S exposure, the direct measurement of H_2S could be a better indicator of H_2S exposure. However, because H_2S may be oxidized to TS non-enzymatically by hemoglobin or enzymatically by bacteria during the postmortem interval, considering both parameters when possible is important. H_2S and TS are easily detected by gas chromatography coupled to mass spectrometry (GC-MS) after pentafluorobenzyl bromide (PFBBr) derivatization (10). H_2S can also be detected without derivatization (11, 12). Most of the reported works have used 1,3,5-tribromobenzene (TBB) as an internal standard; however, nitrous oxide (N_2O) could be more relevant and has never been reported.

H_2S was used by the British Army as a chemical weapon during World War I. More recently, H_2S has been associated with numerous accidents, most of which were lethal. Most of the reported cases are accidental intoxications due to natural volcanic fume (13–16), waste and sewage work (10, 17–22) and industrial gas (4, 23, 24) exposure; sulfur product ingestion (25); organic material and food decomposition (26–29); and individual enzymatic disorders (30). However, some of the cases are suicides. The combination of household ingredients, such as cleansers, with a strong acid, such as hydrochloric acid, results in H_2S generation (31, 32). This mixture has been reported in numerous suicides in Japan (33, 34) and in the United States (35). Other deliberate instances of suicidal ingestion of sulfur products have also been reported (36).

The aim of this study is to present a new analytical method for H_2S measurement using N_2O as an internal standard and its full validation using an accuracy profile based on the β-expectation tolerance interval and to apply this method to actual forensic cases.

Experimental

Materials and reagents

Sodium sulfide (Na_2S) was purchased from Sigma-Aldrich (Saint Louis, MO, USA). Deuterium chloride (D, 99.5%) diluted at 35%

in D_2O was obtained from Cambridge Isotope Laboratories (CIL) Inc. (Andover, MA, USA). Hydrochloric acid (HCl) diluted at 32% in H_2O was obtained from Merck (Darmstadt, Germany). Nitrous oxide was purchased from Carbagas (Lausanne, Switzerland). All headspace extractions were performed in 20 mL headspace vials. A certified H_2S cylinder from Multigas (Domdidier, Switzerland) was used as the external control. Airtight gas syringes from VICI (Houston, TX, USA) were used for the gas sampling and analysis.

GC/MS analysis

An Agilent 6890N GC (Agilent Technologies, Palo Alto, CA, USA) equipped with an Agilent Select Permanent Gases column was used. This column is specifically designed for gas analysis and is composed of two capillary columns set in parallel: a molecular sieve 5 Å PLOT capillary column (10 m × 0.32 mm) and a Porabond Q column (50 m × 0.53 mm). H_2S can be directly eluted on a Porabond Q column ; however, this chromatographic arrangement is the best for a complete gas screening not specifically targeted on a particular gas. The temperature program was as follows: 45°C, held for 8 min and then raised at 20°C/min to 180°C; the injector (split mode 3:1) was set to 180°C, and the interface MS temperature was set to 230°C. Helium was employed as the carrier gas. The detection was performed with an Agilent 5,973 mass spectrometer (Agilent Technologies, Palo Alto, CA, USA), operating in the electron ionisation mode (EI) at 70 eV. The selected ion monitoring (SIM) mode was used to acquire the H_2S signal at m/z 34 (retention time: 6.5 min) and at m/z 30 and 44 for N_2O, which was used as the internal standard (retention time: 3.9 min). The ion m/z 32 (retention time: 6.5 min) was also investigated during the simultaneous scan monitoring to guarantee H2S identification (Figure 1).

Calibration standards and controls

H_2S was prepared by reacting Na_2S with HCl in 20 mL headspace vials according to equation (1). Na_2S was stored at +4°C, and HCl was stored at room temperature when not in use.

$$Na_2S + 2HCl \rightarrow H_2S + 2NaCl \quad (1)$$

An excess amount of hydrochloric acid (100 µL) was carefully introduced to two 20 mL headspace vials. Next, an 11 mm (i.d.) aluminium cap without septa or holes containing a weighted amount of Na_2S (7.8 mg) was introduced in each HS vial. Then, the headspace vials were hermetically sealed with 20 mm (i.d.) magnetic PTFE/silicone septum caps, vigorously shaken and vortexed to allow for direct contact between the acid and Na_2S.

The yield from H_2S generation was previously verified by comparison with H_2S dilutions obtained from pure commercial H_2S, and H_2S generation above these conditions was quantitative.

Five working calibration standards at concentrations corresponding to 12.5, 25.0, 37.5, 50.0 and 62.5 nmol of H_2S/mL of vial HS were prepared daily by diluting generated H_2S. From 7.8 mg of Na_2S, a concentration of 5 µmol/mL HS was reached.

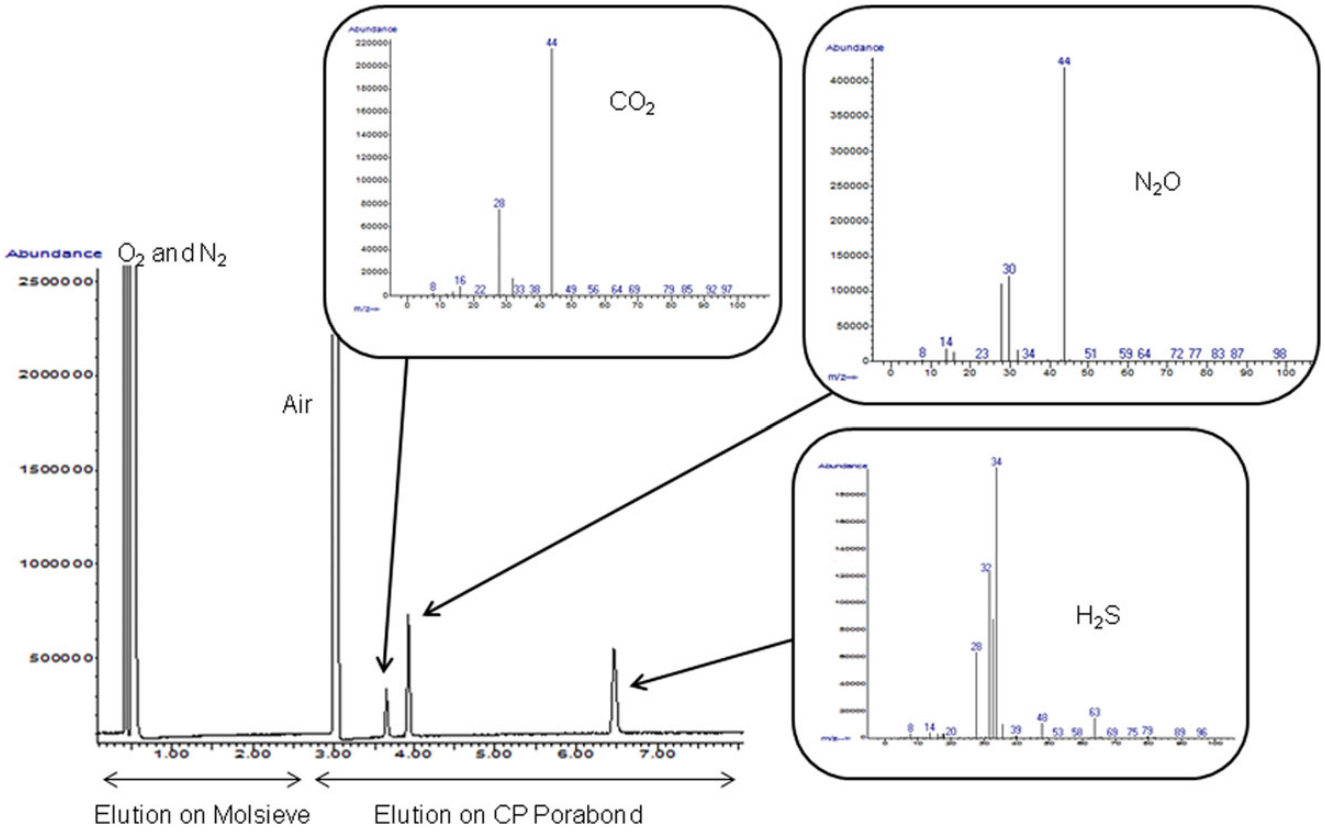

Figure 1. Chromatogram and mass spectra of gases present in the HS vial of H_2S calibrator (62.5 nmol/mL HS).

In total, 500 μL was sampled using a gastight syringe and transferred into another 20 mL vial, reaching a concentration of 125 nmol/mL, which was used to build the calibration standards (100, 200, 300, 400 and 500 μL in individual 20 mL HS vials). The working internal standard was prepared daily at a concentration of 280 nmol N_2O/mL of vial HS by diluting pure N_2O.

Intermediate quality control samples were also prepared daily from the same reactions with the following concentrations: 18.8, 31.3 and 56.3 nmol of H_2S/mL of vial HS. For internal standard sampling, 100 μL of the working internal standard was sampled in a gas syringe, resulting in a final concentration of 28.0 nmol of N_2O/mL of vial HS. For gas sampling, after sampling the internal standard in a gas syringe, sampling of calibrators or actual samples was performed using the same gas syringe. The different gases were mixed in the gas syringe, and the total volume was injected in the GC injector.

Validation procedure

The validation procedure was performed according to the guidelines of the French Society of Pharmaceutical Sciences and Techniques (SFSTP), which are based on the β−expectation tolerance interval (Hubert *et al.*, 2007a, 2007b and 2008) and on the following criteria: selectivity, response function (calibration curve), linearity, trueness, precision (repeatability and intermediate precision), accuracy, limit of detection (LOD) and limit of quantification (LOQ). Linearity was achieved with a minimal coefficient of determination equal to 0.9657. The validation experiments were performed with calibration standards and control samples over three non-consecutive days ($P = 3$) and were not analyzed during the same week. The trueness was assessed using control repetitions and an external control (pure H_2S diluted in air at a final concentration of 31.47 nmol/mL HS).

Postmortem specimens

Gaseous samples collected in altered bodies from our forensic institute were used to investigate the selectivity of the protocol. These gaseous samples were collected according to the CT scan (computed tomography) laser guidance protocol developed in our institute (37). Among alteration gases, H_2S is not the major gas. Therefore, evaluating the eventual coelutions that might interfere with the H_2S signal is important.

Moreover, an actual postmortem sample (skeletal muscle) was used for additional toxicological analyses and was stored at −20°C until GC analysis. The sample was collected from the body of a 37-year-old man who committed suicide with H_2S at home and was found in a specially outfitted tent after 2 months of postmortem delay. The autopsy for this case was conducted at the Lyon Institute of Forensic Medicine. An aliquot of 0.373 g was placed in a HS vial and sealed before heating the sample at 70°C for 10 min (H_2S thermodesorption of 100% from the matrix). Then, a 200 μL volume from the headspace was sampled and injected in the GC injector.

Other skeletal muscle samples ($n = 11$, quantity <1 g for all samples) from other altered cases (with postmortem delays ranging from 1 to 10 weeks) that underwent autopsies in our forensic center were used to study the performance of the developed method. These samples were prepared according to the actual postmortem sample protocol as described above.

Results and discussion

Choice of the internal standard

Initially, *in situ* generated deuterium sulfide (D_2S) was considered for use as an internal standard. However, although D_2S is commercially available, D_2S remains expensive. Therefore, using deuterated hydrochloric acid in deuterium oxide, producing D_2S from equation 2 is chemically correct:

$$Na_2S + 2DCl \rightarrow D_2S + 2NaCl \quad (2)$$

However, the *in-situ* chemical generation of D_2S was impossible because of an isotopic fractionation during D_2S formation. In fact, D_2S appears unstable during this generation, primarily generating H_2S. Another possibility could have been to use labeled Na_2S^* with a sulfur atom isotope S^*; however, this option was not possible due to economic reasons.

Therefore, the best alternative to using a stable labeled isotope as the internal standard was to choose an extremely stable gaseous substance or an ion of an extremely stable gaseous substance whose molecular mass was close to that of H_2S. Therefore, N_2O, with ions monitored at m/z 30 and 44, was a satisfactory choice.

Selectivity

The selectivity of this method was investigated by analyzing gaseous mixtures originating from putrefaction gases sampled from altered bodies from our forensic institute. Several gaseous samples (cardiac, abdominal and thoracic cavities, $n = 11$), as well as various samples from one autopsy, including the kidney, lung, liver, bile, heart, muscle, urine, peripheral blood and cardiac blood, were collected (37) and analyzed. All these analyses were evaluated for co-eluting chromatographic peaks that might interfere with H_2S and N_2O detection. No interference peak was observed at the H_2S retention time or for $m/z = 34$ and for $m/z = 30$ and 44, indicating that this method provides satisfactory selectivity for H_2S determination. The only limit of the method is the CO_2 concentration because CO_2 can coelute with N_2O if present in extremely high concentrations and could influence H_2S quantification. Preliminary dilution of the sample can resolve this inconvenience.

Calibration curve

Each point on the calibration curve was defined as the area ratio of H_2S to N_2O within a concentration range. Three assay calibration curves were performed for H_2S determination, which were prepared on three non-consecutive days ($P = 3$) over 3 weeks. Calibration standards were prepared at 5 ($k = 5$) concentration levels: 12.5, 25.0, 37.5, 50.0 and 62.5 nmol of H_2S/mL of vial HS, each in triplicate ($n = 3$). Calculated concentrations of each calibrator were compared with target values and were found to be within ±20%, except for the first calibrator, which showed a higher variability (±25%). A linear relation was established between the H_2S concentrations diluted from the pure H_2S cylinder and the measured responses in the calibration range. The validation results for the calibration curves are presented in Table I.

Linearity

The linearity of this method was assessed by fitting back-calculated concentrations of the control samples against the

Table I.
Validation parameters of the H_2S measurement method

Calibration curve (12.5–62.5 nmol/mL vial HS) ($k = 5$, $n = 3$, $P = 3$)			
	Day 1	Day 2	Day 3
Slope	0.0114	0.0129	0.0103
Intercept	0.0438	0.0253	0.0279
r^2	0.9677	0.9762	0.9813
Linearity (12.5–62.5 nmol/mL vial HS) ($k = 3$, $n = 3$, $P = 3$)			
Slope		1.0492	
Intercept		−1.8138	
r^2		0.9657	
Trueness (relative bias %) ($k = 3$, $n = 3$, $P = 3$)			
Levels (nmol/mL HS)		Trueness (%)	
18.8		−0.1	
31.3		−1.3	
56.3		+0.2	
External control (31.47 nmol/mL vial HS)		−2.44 (±5.43)	
Precision (RSD %) ($k = 3$, $n = 3$, $P = 3$)			
Levels (μmol/mL HS)	Repeatability	Intermediate precision	
18.8	10.5	10.5	
31.3	3.1	3.9	
56.3	8.5	8.5	

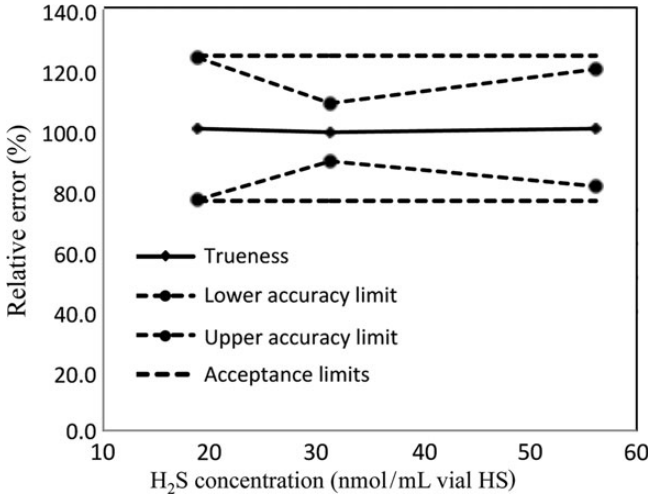

Figure 2. H_2S accuracy profile within a range of 12.5–62.5 nmol/mL vial HS (continuous line: trueness; bold dashed lines: acceptance limits set at ±25%; dashed lines: lower and upper accuracy limits in relative values).

theoretical concentrations. Each non-consecutive day, control samples were measured at three concentration levels ($k = 3$) in triplicate ($n = 3$). The control sample concentrations were calculated using a calibration curve determined for each measurement day. As presented in Table I, satisfactory linearity was obtained, with a coefficient of determination above 0.965 in the range from 12.5 to 62.5 nmol/mL vial HS.

Trueness

The trueness test, which is also called the bias, expresses the closeness between the experimental average value and the accepted reference value. This test, which detects systematic errors, is expressed as a percent deviation from the accepted reference value. Several daily repetitions of control samples were analyzed over several weeks at their respective concentrations, which were used to establish a true value for each concentration. Trueness was measured within ±10% of the accepted reference value in the considered range (12.5–62.5 nmol/mL) and was consequently satisfactory for the H_2S analysis. The evaluation of trueness with the external quality control of certified H_2S was performed at a concentration of 31.47 nmol/mL vial HS. External quality controls were injected on the three different days of the calibration, with a mean trueness measured at −2.44% of the target value.

Precision (repeatability and intermediate precision)

The precision test detects random errors. Precision was assessed by calculating the repeatability (intra-day precision) and intermediate precision (inter-day precision) for each control sample concentration. The repeatability variance was estimated by calculating the intra-days variance (S_r^2), and the intermediate precision variance was estimated by adding the intra- and inter-day variances (S_{IP}^2). As shown in Table I, the relative standard deviation values for repeatability ranged between 3.1 and 10.5%, and the relative standard deviation values for intermediate precision ranged between 3.9 and 10.5%.

Accuracy and LOQ

The accuracy expresses the total error defined by the sum of trueness (systematic error) and precision (random error). The accuracy profile provided in Figure 2 indicates the ability of this method to provide an analytical result in the considered range. The mean bias (%) confidence interval limits for the control samples were within the ±30% acceptability limits typically allowed by Swiss forensic laboratories.

With a threshold of 24% as the acceptability limit, the lower limit of quantification (LLOQ) was set to 18.5 nmol H_2S/mL vial HS.

Limit of detection

The LOD was determined using the headspace dilution of pure H_2S. Several dilutions of the headspace in air were performed, and the LOD was assessed using a signal-to-noise ratio of $S/N > 3$. The noise was estimated by measuring more than 10 samples at the estimated LOD concentration. Therefore, the LOD for H_2S quantification was estimated to be lower than 1.0 nmol/mL vial HS.

Analyses of postmortem specimens

Actual postmortem samples were analyzed to evaluate the performance of H_2S measurement according to the presented protocol. Because the skeletal muscle sample originating from a case of H_2S suicide was collected from a body altered after 2 months of postmortem delay, distinguishing the H_2S coming from the inhaled gas and the H_2S microbially generated after death was not possible. In this case, 22 μg/g concentration was measured in skeletal muscle (Figure 3). This concentration conforms to H_2S concentrations found in the skeletal muscle of people dead after H_2S intoxication with a postmortem delay of a maximum of 72 h (Miyazato *et al.*, 2013).

H_2S has not been detected in skeletal muscle contents in putrefactive control cases selected from our forensic center ($n = 11$, postmortem delay between 1 week and 10 weeks) (Figure 3).

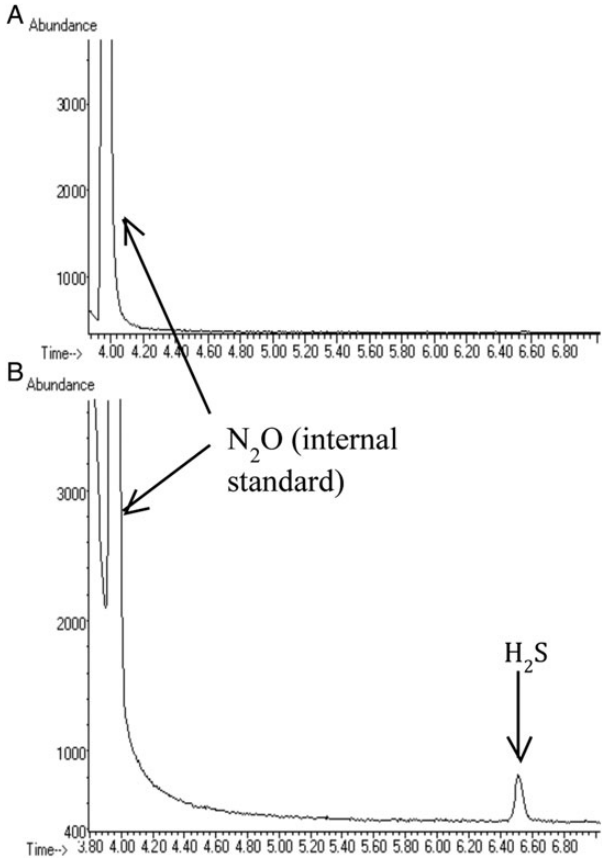

Figure 3. Selected ion monitoring (SIM) chromatograms of time-altered skeletal muscle in our laboratory (A) and of an actual case (H_2S exposure followed by alteration) (B).

Microbial H_2S generation was identified as a crucial parameter of interpretation, which was responsible for extremely variable postmortem H_2S concentrations. Considering recent results (Miyazato *et al.*, 2013) and our observations, H_2S appears to be rapidly produced after death and released from the body with its alteration, which can explain H_2S absence after long postmortem alteration. In our actual case of H_2S intoxication, a non-negligible portion of detected H_2S can be reasonably hypothesized to originate from H_2S intoxication.

The method described in this study was evaluated and determined to be satisfactory for providing reliable, accurate and repeatable H_2S results in a short time from gaseous and biological samples.

Conclusions

A selective and sensitive method for the identification and quantification of H_2S in postmortem muscle samples was presented. This method offers a new opportunity for H_2S measurement in forensic sciences. The technique was validated according to the guidelines of the French Society of Pharmaceutical Sciences and Techniques (SFSTP). This method allows for an accurate and reliable measurement ($\pm 30\%$) of H_2S concentrations in a range from 12.5 to 62.5 nmol/mL vial HS. The method is not time-consuming and is safe because the generation of H_2S occurs in a hermetically closed headspace vial. This method also provides for precise quantification because N_2O is used as the internal standard. The applicability of this method has been tested on actual postmortem cases with a known history of H_2S intoxication and on actual altered bodies and has provided satisfactory results. This method could also be easily extended to other biological matrices, such as blood and other organs.

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