

# 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<sub>2</sub>S) concentrations in biological and gaseous samples. The primary analytical drawback of the GC/MS methods for H<sub>2</sub>S measurement discussed in the literature was the absence of a specific H<sub>2</sub>S internal standard required to perform quantification. Although a deuterated hydrogen sulfide (D<sub>2</sub>S) 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<sub>2</sub>S 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<sub>2</sub>O was chosen for use as an internal standard. This method allows precise measurements of H<sub>2</sub>S concentrations in biological and gaseous samples. Therefore, a full validation using accuracy profile based on the  $\beta$ -expectation tolerance interval is presented. Finally, this method was applied to quantify H<sub>2</sub>S in an actual case of H<sub>2</sub>S fatal intoxication.**

## Introduction

Hydrogen sulfide (H<sub>2</sub>S) is a highly toxic gas that becomes flammable at percentages from 4.3 to 46% in ambient air. H<sub>2</sub>S 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<sub>2</sub>S 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<sub>2</sub>S forms a complex bond with iron in mitochondrial cytochrome *c* oxidases, thus inhibiting cellular respiration (1).

Because H<sub>2</sub>S occurs naturally in the body, in the environment and in the gut, enzymes exist in the body that are capable of detoxifying H<sub>2</sub>S by oxidation to sulfates such that low levels of H<sub>2</sub>S may be tolerated. Indeed, H<sub>2</sub>S 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<sub>2</sub>S acts as a myorelaxant and vasodilator and is also active in the brain as a neurotransmitter, where H<sub>2</sub>S increases the response of the *N*-methyl-D-aspartate receptor and facilitates long-term potentiation.

H<sub>2</sub>S 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<sub>2</sub>S 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<sub>2</sub>S 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<sub>2</sub>S was less influenced by putrefaction than TS (7). Direct H<sub>2</sub>S monitoring could also provide a direct measurement of the magnitude of the H<sub>2</sub>S exposure or intoxication. Indeed, sulfates are only measurable in blood and urine, whereas H<sub>2</sub>S is measurable in all biological matrices. Because TS concentrations can result in the misinterpretation of H<sub>2</sub>S exposure, the direct measurement of H<sub>2</sub>S could be a better indicator of H<sub>2</sub>S exposure. However, because H<sub>2</sub>S 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<sub>2</sub>S and TS are easily detected by gas chromatography coupled to mass spectrometry (GC-MS) after pentafluorobenzyl bromide (PFBBR) derivatization (10). H<sub>2</sub>S 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<sub>2</sub>O) could be more relevant and has never been reported.

H<sub>2</sub>S was used by the British Army as a chemical weapon during World War I. More recently, H<sub>2</sub>S 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<sub>2</sub>S 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<sub>2</sub>S measurement using N<sub>2</sub>O as an internal standard and its full validation using an accuracy profile based on the  $\beta$ -expectation tolerance interval and to apply this method to actual forensic cases.

## Experimental

### Materials and reagents

Sodium sulfide (Na<sub>2</sub>S) was purchased from Sigma-Aldrich (Saint Louis, MO, USA). Deuterium chloride (D, 99.5%) diluted at 35%

in D<sub>2</sub>O was obtained from Cambridge Isotope Laboratories (CIL) Inc. (Andover, MA, USA). Hydrochloric acid (HCl) diluted at 32% in H<sub>2</sub>O 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<sub>2</sub>S 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.

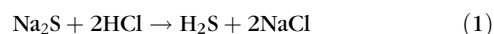
### GC/MS analysis

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<sub>2</sub>S 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<sub>2</sub>S signal at *m/z* 34 (retention

time: 6.5 min) and at *m/z* 30 and 44 for N<sub>2</sub>O, 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 H<sub>2</sub>S identification (Figure 1).

### Calibration standards and controls

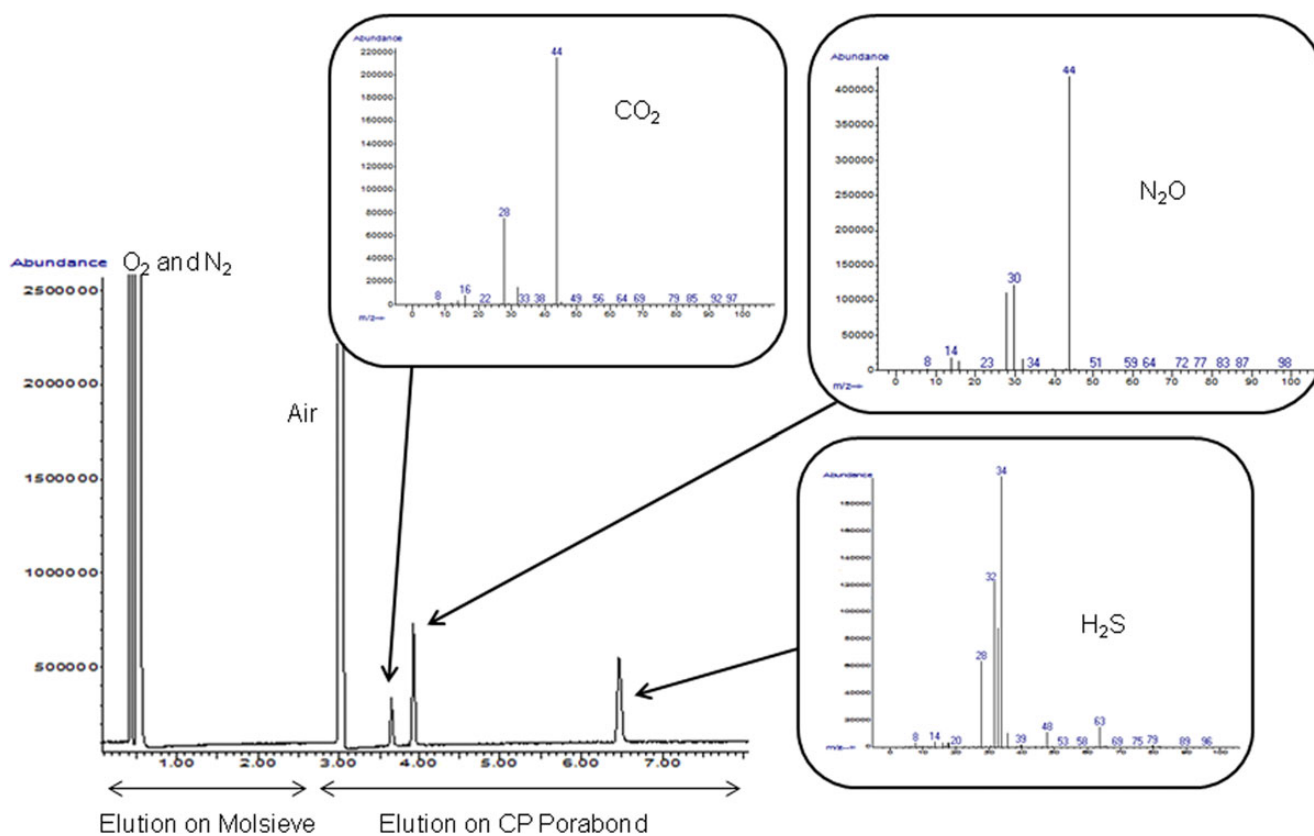
H<sub>2</sub>S was prepared by reacting Na<sub>2</sub>S with HCl in 20 mL headspace vials according to equation (1). Na<sub>2</sub>S was stored at +4°C, and HCl was stored at room temperature when not in use.



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<sub>2</sub>S (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<sub>2</sub>S.

The yield from H<sub>2</sub>S generation was previously verified by comparison with H<sub>2</sub>S dilutions obtained from pure commercial H<sub>2</sub>S, and H<sub>2</sub>S 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<sub>2</sub>S/mL of vial HS were prepared daily by diluting generated H<sub>2</sub>S. From 7.8 mg of Na<sub>2</sub>S, a concentration of 5 µmol/mL HS was reached.



**Figure 1.** Chromatogram and mass spectra of gases present in the HS vial of H<sub>2</sub>S calibrator (62.5 nmol/mL HS).

In total, 500  $\mu\text{L}$  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  $\mu\text{L}$  in individual 20 mL HS vials). The working internal standard was prepared daily at a concentration of 280 nmol  $\text{N}_2\text{O}$ /mL of vial HS by diluting pure  $\text{N}_2\text{O}$ .

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  $\text{H}_2\text{S}$ /mL of vial HS. For internal standard sampling, 100  $\mu\text{L}$  of the working internal standard was sampled in a gas syringe, resulting in a final concentration of 28.0 nmol of  $\text{N}_2\text{O}$ /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  $\beta$ -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  $\text{H}_2\text{S}$  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,  $\text{H}_2\text{S}$  is not the major gas. Therefore, evaluating the eventual coelutions that might interfere with the  $\text{H}_2\text{S}$  signal is important.

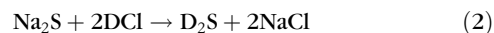
Moreover, an actual postmortem sample (skeletal muscle) was used for additional toxicological analyses and was stored at  $-20^\circ\text{C}$  until GC analysis. The sample was collected from the body of a 37-year-old man who committed suicide with  $\text{H}_2\text{S}$  at home and was found in a specially 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  $70^\circ\text{C}$  for 10 min ( $\text{H}_2\text{S}$  thermodesorption of 100% from the matrix). Then, a 200  $\mu\text{L}$  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 ( $\text{D}_2\text{S}$ ) was considered for use as an internal standard. However, although  $\text{D}_2\text{S}$  is commercially available,  $\text{D}_2\text{S}$  remains expensive. Therefore, using deuterated hydrochloric acid in deuterium oxide, producing  $\text{D}_2\text{S}$  from equation 2 is chemically correct:



However, the *in-situ* chemical generation of  $\text{D}_2\text{S}$  was impossible because of an isotopic fractionation during  $\text{D}_2\text{S}$  formation. In fact,  $\text{D}_2\text{S}$  appears unstable during this generation, primarily generating  $\text{H}_2\text{S}$ . Another possibility could have been to use labeled  $\text{Na}_2\text{S}^*$  with a sulfur atom isotope  $\text{S}^*$ ; 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  $\text{H}_2\text{S}$ . Therefore,  $\text{N}_2\text{O}$ , 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  $\text{H}_2\text{S}$  and  $\text{N}_2\text{O}$  detection. No interference peak was observed at the  $\text{H}_2\text{S}$  retention time or for  $m/z=34$  and for  $m/z=30$  and 44, indicating that this method provides satisfactory selectivity for  $\text{H}_2\text{S}$  determination. The only limit of the method is the  $\text{CO}_2$  concentration because  $\text{CO}_2$  can coelute with  $\text{N}_2\text{O}$  if present in extremely high concentrations and could influence  $\text{H}_2\text{S}$  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  $\text{H}_2\text{S}$  to  $\text{N}_2\text{O}$  within a concentration range. Three assay calibration curves were performed for  $\text{H}_2\text{S}$  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  $\text{H}_2\text{S}$ /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  $\pm 20\%$ , except for the first calibrator, which showed a higher variability ( $\pm 25\%$ ). A linear relation was established between the  $\text{H}_2\text{S}$  concentrations diluted from the pure  $\text{H}_2\text{S}$  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<sub>2</sub>S measurement methodCalibration 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 ( $\pm 5.43$ )

Precision (RSD %) ( $k = 3, n = 3, P = 3$ )

Levels ( $\mu\text{mol/mL HS}$ )	Repeatability	Intermediate precision
18.8	10.5	10.5
31.3	3.1	3.9
56.3	8.5	8.5

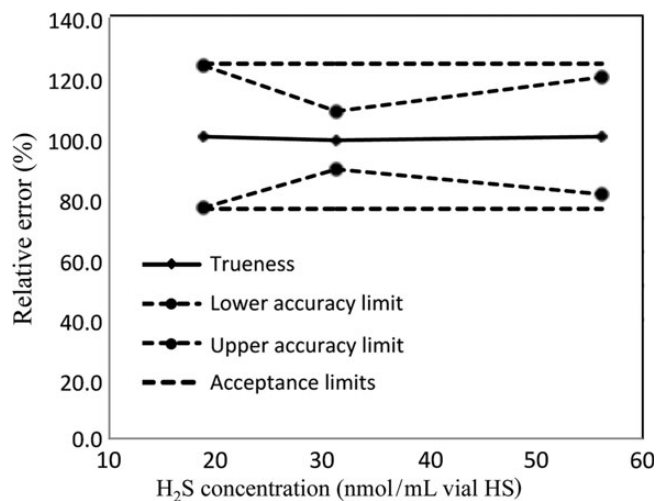
theoretical concentrations. Each non-consecutive day, control samples were measured at three concentration levels ( $k = 3$ ) in triplicate ( $n = 3$ ). The control sample concentrations were calculated using a calibration curve determined for each measurement day. As presented in Table I, satisfactory 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  $\pm 10\%$  of the accepted reference value in the considered range (12.5–62.5 nmol/mL) and was consequently satisfactory for the H<sub>2</sub>S analysis. The evaluation of trueness with the external quality control of certified H<sub>2</sub>S 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%.



**Figure 2.** H<sub>2</sub>S accuracy profile within a range of 12.5–62.5 nmol/mL vial HS (continuous line: trueness; bold dashed lines: acceptance limits set at  $\pm 25\%$ ; dashed lines: lower and upper accuracy limits in relative values).

### 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  $\pm 30\%$  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<sub>2</sub>S/mL vial HS.

### Limit of detection

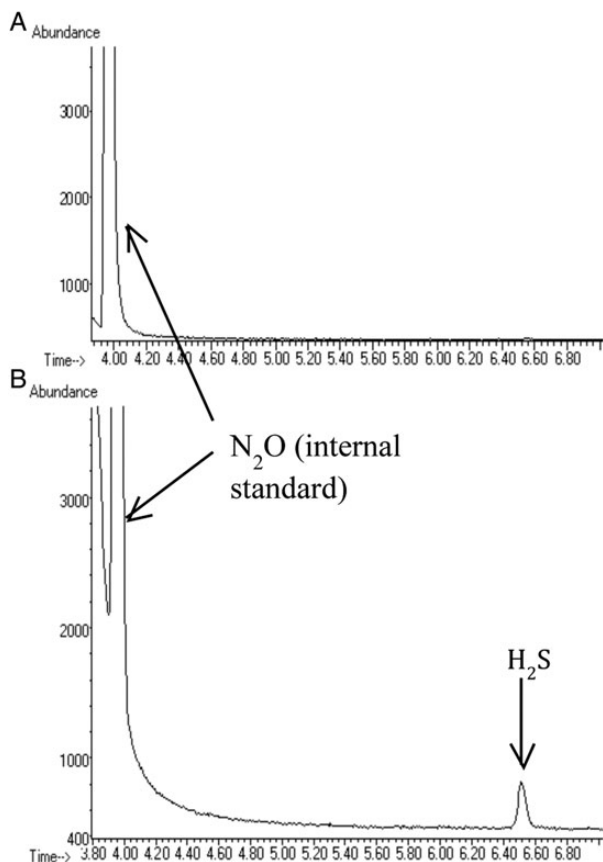
The LOD was determined using the headspace dilution of pure H<sub>2</sub>S. Several dilutions of the headspace in air were performed, and the LOD was assessed using a signal-to-noise ratio of  $S/N > 3$ . The noise was estimated by measuring more than 10 samples at the estimated LOD concentration. Therefore, the LOD for H<sub>2</sub>S 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<sub>2</sub>S measurement according to the presented protocol. Because the skeletal muscle sample originating from a case of H<sub>2</sub>S suicide was collected from a body altered after 2 months of postmortem delay, distinguishing the H<sub>2</sub>S coming from the inhaled gas and the H<sub>2</sub>S microbially generated after death was not possible. In this case, 22  $\mu\text{g/g}$  concentration was measured in skeletal muscle (Figure 3). This concentration conforms to H<sub>2</sub>S concentrations found in the skeletal muscle of people dead after H<sub>2</sub>S intoxication with a postmortem delay of a maximum of 72 h (Miyazato *et al.*, 2013).

H<sub>2</sub>S 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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