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Short communication

Occupational asphyxiation by unknown compound(s): Environmental and toxicological approach

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ABSTRACT

During a routine truck-tank washing operation, five healthy workers were found motionless inside an empty tanker. Four of them died inside the tanker while the fifth died the following day in hospital. Since the true nature of the fatal compound(s) were not known, a rigorous environmental and toxicological approach supported by autopsy findings was essential to clarify the cause of death.

Environmental results indicated that H_2S fumes arising from the liquid sulfur previously shipped were responsible for the serial deaths, also confirmed by a simulation performed on two similar truck-tanks.

These environmental findings were supported by toxicological analyses through the measurement of thiosulfate, one of the main H_2S metabolites. Abnormal thiosulfate concentrations from 1.1 to 186.2 mg/kg were revealed in all *post-mortem* biological samples (blood, lung, liver, kidney, brain and fat). Finally, the cluster analysis performed on thiosulfate body distribution contributed to establishing the time of death according to the accident scene reconstruction.

This report presents valuable findings in correctly identifying the cause of death in gas asphyxiation cases by unknown compound(s).

1. Introduction

Asphyxiation in the workplace is mainly accidental and frequently due to a slow, insidious accumulation of toxic gases in low-lying areas such as sewers, mines, wells, silos and storage tanks [1,2].

The determination of the cause of death in chemical asphyxiation cases is very difficult because of varying circumstances surrounding any accident [3,4]. To clarify the cause of death and identify the factors involved, a rigorous environmental and toxicological approach supported by autopsy findings is essential mainly when the true nature and toxicity of the compound(s) involved is not clear.

In literature different kinds of asphyxiation are described depending on the concentration of O_2 together with the presence of fatal substances [4]. In particular, asphyxiation can happen in a matter of minutes (rapid asphyxia) when O_2 concentration is completely lacking, or it can be prolonged (20–25 min) in the case of gradual O_2 depletion and its replacement with gaseous substance(s).

However, asphyxiation can also be caused by inhalation of toxic gases without depletion in O_2 air levels (20%, v/v) and therefore, the time of death is strictly related to their toxicity.

To accurately classify both the cause and manner of death, it is essential to perform accurate scene investigations supported by environmental–toxicological monitoring and compare the results with those described in previous studies [5]. Variation in tissue gas concentrations or its metabolites has been observed according to the kind of asphyxiation, the physical–chemical properties of the gas and the time of exposure [4].

2. Case history

Five healthy Caucasian workers attending to a routine tank-truck washing operation, were found motionless inside an empty truck-tank (length 5.0 m, width and height 2.0 m) by a co-worker who called for help. Approximately 40–45 min later 4 of them, already dead, were removed from the tanker by rescuers with self-contained air supply. The youngest worker survived and was transported to the hospital, but died later due to complications of a massive pulmonary edema.

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Accident scene reconstruction revealed that a worker, the first victim (a 46-year-old man), entered the empty tanker through the upper manhole and in a few minutes fell unconscious. Thinking there had been an accident, a second worker (a 20-year-old man), the only one extracted alive, entered the tanker where he too fell unconscious. The last three men (a 37-year-old man, a 23-year-old man and a 64-year-old man) died trying to take their fellow workers out of the tanker.

Further investigation revealed that the tanker had previously contained sulfur liquid and before the accident the empty tanker had been stored unused for several months. No safety sheet was supplied to the Company and no additional information on conditions and storage time was provided.

Additionally, personal protective devices were not available because the main job of the Company is to wash the exterior parts of tank-trucks. In fact, the inspection of the interior had been an extraordinary event performed on the assumption that the tanker was empty and unused.

Sulfur liquid not being toxic by itself, required a rigorous investigative approach in order to detect the fatal compound(s) and define the cause and manner of death.

The aim of this study was to identify the cause of death by relying on environmental findings through analysis of the residue in the tanker and by performing a simulated experiment. Additionally, toxicological analyses are also illustrated.

3. Materials and methods

3.1. Chemicals

Hydrogen sulfide, sodium thiosulfate pentahydrate, pentafluorobenzyl bromide (PFBBr), L-ascorbic acid, 1,3,5-tribromobenzene (TBB, used as internal standard), sodium chloride, iodine, acetone and ethyl acetate were purchased from Sigma–Aldrich (Milan, Italy). Before use, the NaCl was oven dried at 80 °C in order to avoid contamination with laboratory air.

3.2. Subjects

The five workers were aged between 20 and 64 years (average: 37.6 years) and were all in good health.

Their working aim was to clean the exterior and to inspect the interior of the tanker. None of them wore personal protective devices.

The characteristics of all workers together with the main pathological findings are reported in Table 1.

Immediately after death, all the bodies were kept at a temperature below 15 °C. The autopsies were carried out 36 h after the accident on bodies which were well preserved.

During autopsies, femoral blood and tissues (lung, liver, kidney, brain and fat) were collected, weighed, and transferred to 10-ml airtight vials. The samples were stored at −20 °C until analysis.

3.3. Environmental analyses

Environmental analyses were performed on air samples withdrawn from the upper porthole, and on yellow solid samples and dregs of blackish liquid collected from the bottom of the tanker.

Air monitoring was performed 1 week and 1 month after the accident by using Gastec® Color Dosimeter Tubes connected to a pump device. Each monitoring was carried out in triplicate ($n = 3$).

Elemental analysis (Leco Analyzer, Leco Corporation, Michigan, USA) was performed on the yellow solid sample, while gas chromatography/mass spectrometry (GC/MS) analysis was performed on the liquid sample. Air monitoring revealed the presence of H_2S; its presence was also verified in the blackish liquid by potentiometric titration with standard $Fe(NO_3)_2$ solution after stripping with N_2 (flow rate: 150 ml/min).

Additionally, a simulation on 2 similar truck-tanks used for liquid sulfur transport was carried out.

H_2S air concentration was measured as previously described before loading (into empty tankers), during loading, when the tankers were fully loaded and after sulfur discharge. Air samples were withdrawn from the upper manhole. The sulfur liquid was also tested by potentiometric titration to identify and quantify the presence of H_2S as the contaminant.

3.4. Toxicological analyses

Toxicological analyses were performed about 2 months after the autopsy on femoral blood, lung, liver, kidney, brain, and fat taken from the workers. Further experiments confirmed that thiosulfate, one of the main H_2S metabolites [6,7], is stable even if the analysis is performed more than 6 months later (data not shown).

For method validation and for purposes of comparison, we also analyzed *post-mortem* biological samples taken from subjects not exposed to H_2S. Femoral blood and tissues were taken from well-refrigerated cadavers 32–36 h after death. All samples were collected in 10-ml airtight vials and stored at −20° C. Analysis was carried out within a range of 1 week to 2 months after the autopsy. Further experiments performed on the same samples excluded thiosulfate production even 6 months after autopsy (data not shown).

3.4.1. Sample preparation

Thiosulfate in blood samples was detected as bis(pentafluorobenzyl)disulfide ($C_6F_5CH_2SSCH_2C_6F_5$) after derivatization with pentafluorobenzyl bromide following the procedure described by Kage et al. [8]. Briefly, 0.2 ml were added to 0.05 ml aqueous solution of L-ascorbic acid 0.2 M, 0.05 ml NaCl aqueous solution (5%) and 0.5 ml solution of PFBBr (alkylating agent) 0.02 M in acetone and thus vortexed for 1 min. Then, 2 ml of 0.025 M iodine solution (oxidating agent) and 0.5 ml of TBB (I.S.) 0.04 mM in ethyl acetate were added (final blood concentration: 0.01 mM–3.15 mg/kg). The phases were then separated by means of centrifugation at 2500 rpm for 20 min and 1 μl of the organic phase was injected into GC/MS system.

A similar procedure was applied to tissue analyses (about 200 mg) after homogenization in a 10% KCl aqueous solution (10 μl of aqueous solution/100 mg of tissue), following the same sample manipulation described in one of our recent works [9].

TBB 0.04 M in ethyl acetate was added to each sample (10 μl of solution/100 mg of tissue) as an internal standard (I.S.).

3.4.2. Assay validation

Assay validation was performed in matrix (blood and tissue *post-mortem* samples) obtained from unexposed subjects, by studying linearity, the limit of detection (LOD), accuracy, and intra- and inter-day analytical precision (calculated as % RSD) for all determinations.

In particular, thiosulfate linearity in blood samples and in *post-mortem* tissues (blood, lung, liver, kidney, brain and fat tissues) was calculated by adding 0, 5, 10, 25, 50, 100 and 200 mg/kg.

LOD was calculated on 3 spiked samples as the standard concentration at which signal to noise ratio was equal to 3.

Accuracy was performed by means of a recovery study on the basis of the found and added amount of thiosulfate [10].

Table 1
Pathological findings. The number of the subjects indicates the order in which they entered the tanker.

Victims (males)	Incidental findings	Pulmonary Findings	Other Findings
1 (44 years old)	Blunt force injuries on the occipital areas with subgaleal contusions	Edema, aspiration of gastric content	Cerebral edema
2 (20 years old)[a]	Froth from the mouth on arrival at the Hospital	Massive hemorrhagic edema,	Cerebral edema
3 (36 years old)	Blunt force injuries on the occipital areas with subgaleal contusions	High-grade edema and congestion	Massive cerebral edema
4 (23 years old)	Greenish discoloration face and trunk	High-grade edema and congestion	Cerebral edema, greenish discoloration torax muscles and stomach surface
5 (64 years old)	Aortocoronary bypass scars	High-grade edema, emphysema	Cerebral edema, old myocardial infarct

[a] The worker was extracted alive from the tanker and died at hospital 12 h later

Table 2
Assay validation of the GC/MS method for the determination of thiosulfate levels in blood and tissue samples.

	Blood	Liver	Kidney	Lung	Brain	Fat Tissue
[a]Linear range (mg/kg)	0–200	0–100	0–100	0–100	0–100	0–100
[a]r^2	0.99	0.98	0.98	0.97	0.98	0.96
[b]LOD (mg/kg)	0.5	0.7	0.4	0.9	0.7	1.0
[c]Accuracy (%)	99.1 ± 1.5	96.7 ± 4.8	97.0 ± 3.4	95.8 ± 5.5	96.2 ± 5.9	91.5 ± 10.1
[d]Precision (% RSD)						
Intra-day	1.1–3.2	4.1–7.7	5.6–8.5	6.2–9.0	5.2–8.0	11.0–15.1
Inter-day	2.5–5.0	5.3–10.1	6.1–9.0	7.2–12.8	7.0–10.7	12.0–17.8

[a] Calibration fitting: $y = bx + a$ ($n = 6$ analyzed in duplicate) ± 95% CI; linear regression analysis performed using the last-square method.
[b] Limit of detection (LOD) calculated as Signal/Noise = 3.
[c] Accuracy ($n = 3$) calculated on samples by means of a recovery study on the basis of the found and added amount of thiosulfate [8].
[d] Intra- and inter-day precision ($n = 3$) calculated on samples by spiking each matrix with 10 mg/kg of H_2S.

Intra- and inter-day analytical precision ($n = 3$) was assessed spiking each matrix with 10 mg/kg of H_2S.

Results are displayed in Table 2.

3.4.3. Gas chromatography/mass spectrometry (GC/MS) analysis

The analyses were made out using a gas chromatograph Hewlett Packard HP 6890 coupled with a HP 5973 mass selective detector (Agilent Technologies, Palo Alto, CA, USA). The compounds were separated on a HP-5MS column (Agilent Technologies, 30 m × 0.25 mm I.D., 0.50 μm film) using H_2 as the carrier gas (flow rate 1.3 ml/min). The GC conditions were: 30 °C/min from 100 °C to 220 °C hold for 3 min. The injection and auxiliary temperature were 250 °C and 280 °C, respectively.

Analyses were made in selected ion monitoring SIM mode by acquiring the signal of the following ions (dwell time in parenthesis): m/z 314 (200 ms) for I.S. while 426 (200 ms) for bis(pentafluorobenzyl)disulfide.

A solvent delay of 2.70 min was set to protect the filament.

3.5. Statistical analysis

To test the differences among thiosulfate concentration in different tissues, Friedman test followed by Dunn's post hoc test was performed (Table 3) [11].

To assess whether thiosulfate distribution in tissues could be related to time of exposure and death, hierarchical cluster analysis was performed using standardized z scores, squared Euclidean distance and the Ward's method to perform the dendrogram (Fig. 3). In brief, Cluster Analysis is a multivariate statistical approach to group the cases based on the similarity of responses and it is highly used in social and economical sciences, but also in psychology and in medicine [12,13]

In other words, variables are transformed in z-scores and the difference among cases and thus the grouping process are assessed on the basis of a calculated multivariate distance (e.g. a measure of similarity). The Ward's method joins cases into clusters in order to minimize the variance [13].

A p value <0.05 was always considered significant.

The statistical analyses were made using SPSS 13.0 (SPSS inc. Chicago, IL, USA) and Graphpad Prism 3.0 (Graphpad Software, La Jolla, CA).

4. Results

4.1. Autopsy findings

The autopsies were carried out 36 h after the accident on bodies well preserved.

External examination of the bodies revealed congestion of the head, neck and shoulders with cyanosis of lips and fingernails in all cases. The ocular conjunctiva showed marked hyperemia and few petechiae. Two workers (the first and the fourth who entered the tanker) displayed traces of yellow solid sulfur on their faces and under the sole of their shoes. Two of the men (the first and the third who entered the tanker) presented with blunt force injuries on the occipital area with subgaleal contusion which resulted from the falling.

Table 3
Summary of the main physical–chemical properties of H_2S [22].

H_2S physical–chemical properties	
Boiling point	−60 °C
Vapor pressure	19.6 atm
Relatively vapor density to air (p°)	1.19
Henry's constant ($conc_{liquid}/p_{gas}$)	8.7×10^{-2} M/atm [38]
Auto-ignition temperature	250 °C
Conversion factor for H_2S in air	1 mg/m^3 = 0.717 ppm
Odor threshold	0.011 mg/m^3 [43]

One of the workers (the fourth who entered the tanker), displayed a very characteristic greenish discoloration on his eyes, anterior cervical region and precordia.

Upon internal examination, it was noted that the lungs of all the workers were heavy with edema and congestion that was present also in kidneys and spleen. The 23-year-old worker (the fourth who entered the tanker) revealed a greenish discoloration both in thorax muscles, and on the surface of the stomach. There were no remarkable findings related to the other organs except for slight cerebral edema which was presented in all five victims. In addition, an aortocoronary bypass graft was present in the oldest victim.

Microscopic examination revealed passive congestion that was evident in lungs, spleen, kidneys and adrenal glands.

Massive hemorrhagic edemas were found in all the workers, most notably in the youngest victim who died 12 h after the tragic event.

A summary of the main pathological finding together with the characteristic of the subjects is reported in Table 1.

Blood, lung, liver, kidney, brain and fat samples collected during necropsy had been submitted to toxicological evaluation.

4.2. Environmental findings

Analyses performed on several specimens taken from the tanker confirmed that the yellow granular solid samples were composed of sulfur. The dregs of blackish liquid were a mixture mainly composed of sulfur and H_2S as the major contaminant (2.5 mg/l), and with an almost neutral pH (7.6). GC/MS analyses of these dregs established the absence of toxic volatile organic substances such as hydrocarbon-aliphatic compounds.

Air monitoring inside the tanker revealed H_2S concentrations higher than the permissible exposure limit in an occupational setting [14], while sulfur oxides were negligible. In particular, one week after the accident (corresponding to the third inspection inside the tanker) H_2S concentration was equal to 95 ± 5 ppm and became 0.25 ± 0.1 ppm one month after the accident (corresponding to the fourth inspection).

To better understand these results a simulation on two similar truck-tanks used for liquid sulfur transport was performed. H_2S air concentration was measured before loading (into empty tankers), during loading, when the tankers were fully loaded and after sulfur discharge. Sulfur liquid shipped was also tested to identify and quantify the presence of H_2S as contaminant.

In detail, although before loading H_2S was undetectable, during the loading phase H_2S air concentration in the two tankers was equal to 45 and 80.9 ppm, respectively and became 480 and 600 ppm when the tankers were fully loaded. After sulfur

discharge, H_2S air levels were 750 and 910 ppm, respectively. Finally, analyses on the liquid sulfur withdrawn from the tankers revealed high H_2S contamination (85 and 108 mg/kg, respectively).

4.3. Toxicological findings

Blood and tissue *post-mortem* specimens obtained from the workers were all positive to thiosulfate, which was negligible in samples from unexposed subjects. The relative chromatogram profiles are displayed in Fig. 1.

Further experiments performed 6 months after the autopsy showed that thiosulfate levels in workers' blood and tissues were stable, while they remained undetectable in non-exposed subjects (data not shown).

Thiosulfate blood concentrations for 4 workers who died inside the tanker (median: 8, range 2.6–183 mg/kg), were similar to that measured in the rescued worker who died the following day (10.5 mg/kg). These values were also similar or higher with respect to the levels reported in other fatal cases [6,8,15–17] and from 8 to more than 500 times higher then those measured in healthy exposed subjects [16]. A similar trend was observed in thiosulfate brain levels (median 14.8, range 6.5–87.5 mg/kg, *vs* 6.0 mg/kg) and data were consistent with literature [6,8, 15,17,18].

Fig. 2 displays thiosulfate levels in lung, kidney, liver and fat tissue samples reporting the values (median and range) distinguishing the workers who died inside the tanker and the fifth one who died in hospital the following day.

No significant differences were found among tissues in the workers who died inside the tanker, with the exception of fat tissue, where the median thiosulfate concentration was the lowest.

On the other hand, the rescued worker who died the following day, had lower thiosulfate levels in lung and kidney (2 orders of magnitude) and higher concentrations in liver and fat tissues (more than 1 order of magnitude).

For kidney, liver and fat tissues no data were reported in literature, while for lung they were consistent with literature [6,8,15,17,18].

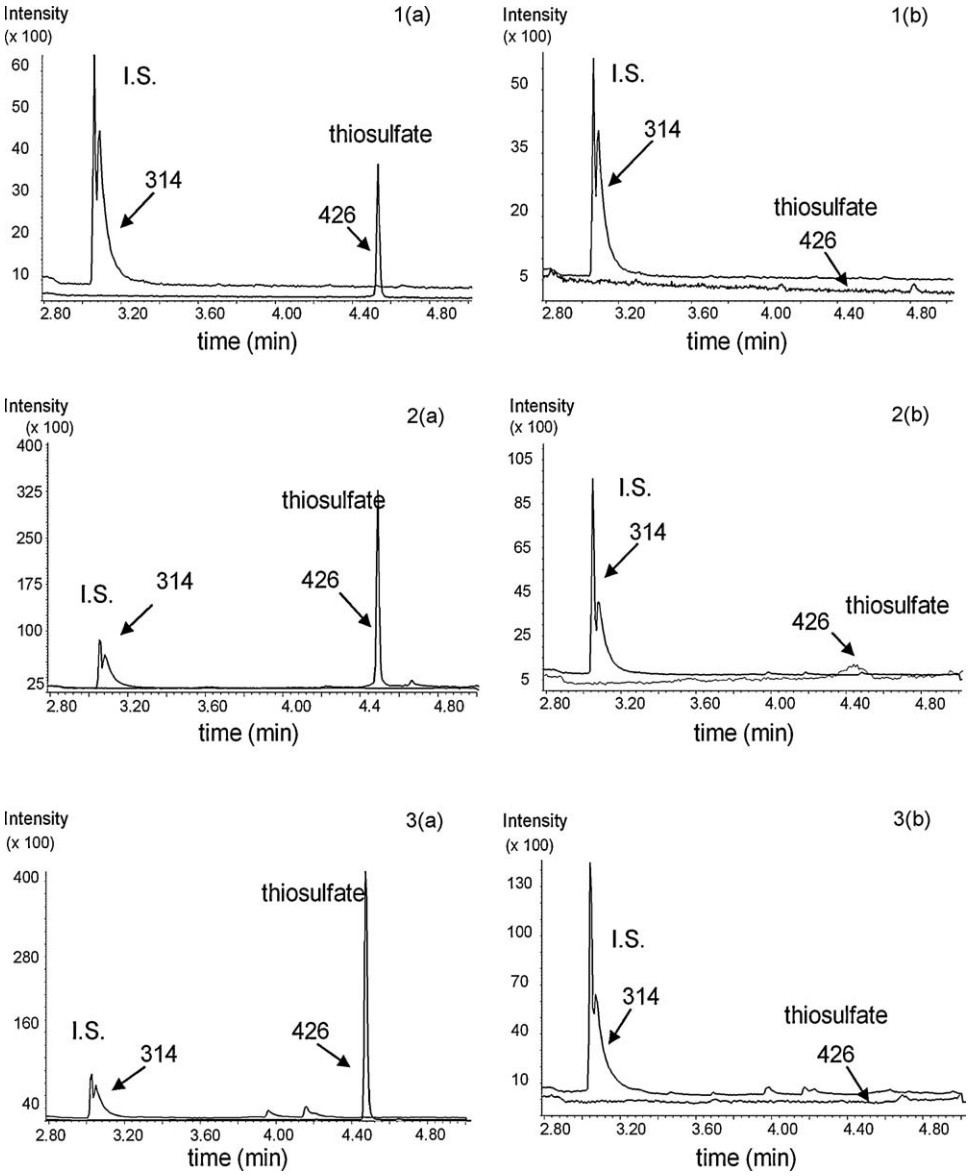

Fig. 1. Chromatogram profiles of representative *post-mortem* samples from the workers and from unexposed subjects (controls). (1) Blood samples: (a) subject 5—10.2 mg/kg, (b) unexposed subject. (2) Lung tissues: (a) subject 1—115.7 mg/kg, (b) unexposed subject. (3) Liver samples: (a) subject 2—149.3 mg/kg, (b) unexposed subject. (4) Kidney samples: (a) subject 3—7.0 mg/kg, (b) unexposed subject. (5) Brain samples: (a) subject 4—87.6 mg/kg, (b) unexposed subject. (6) Fat samples: (a) subject 2—7.4 mg/kg, (b) unexposed subject. Ions used for quantitation: I.S. *m*/*z* 314 and thiosulfate *m*/*z* 426.

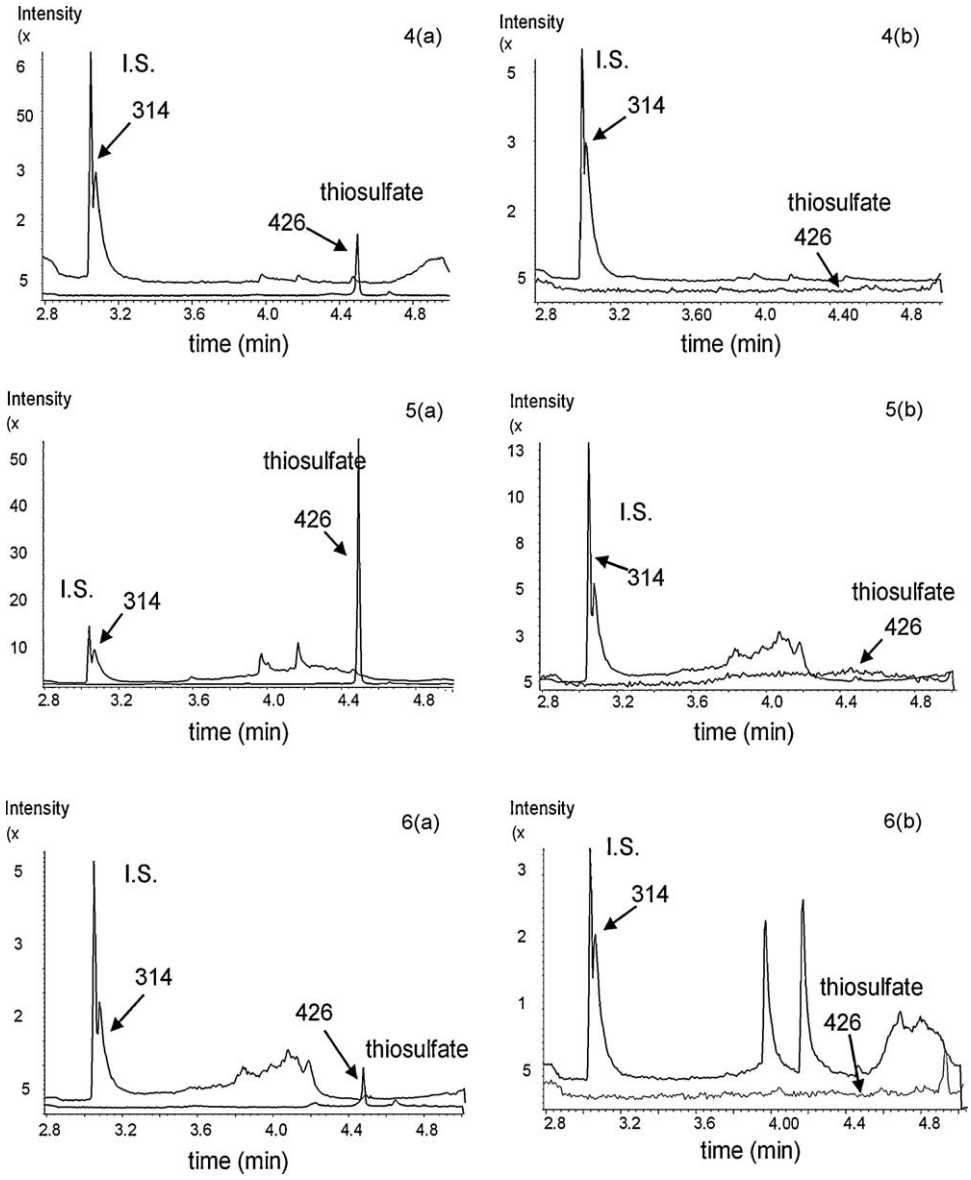

Fig. 1. (*Continued*).

Finally, cluster analysis (Fig. 3) showed a first cluster of two subjects (the third and the fifth who entered the tanker) with the lowest rescaled distance, whereas the other three subjects could be considered as single cases.

5. Discussion

Environmental findings demonstrated that deaths were caused by lethal H_2S levels inside the tanker.

It is naturally generated during putrefaction of organic substances while in industry it is produced and used as an important reagent or intermediate in the manufacturing of chemicals [19–22].

With regard to basal environmental exposure to H_2S, WHO (World Health Organization) has reported H_2S air concentrations generally below 0.0015 mg/m^3 (0.001 ppm) even if they may occasionally be as high as 0.050 mg/m^3 (0.034 ppm). Peak concentrations of 0.20 mg/m^3 (0.14 ppm) have been described near point sources while in a geothermal area mean concentrations of 2 mg/m^3 (1.43 ppm) have been observed [22].

Taking into account H_2S toxicity and its presence in several industrial settings and in rural areas [6,19,23–25] the federal OSHA permissible exposure limit in occupational settings (8-h work shift) is 20 ppm [24]. TWA (8-h time weighted average) and STEL (short-term exposure limit) occupational limits of exposure recommended by ACGIH are 10 ppm (14 mg/m^3) and 15 ppm (21 mg/m^3), respectively [14].

Accidental hazardous exposure to H_2S can occur in several occupational fields as a result either of leaks in industrial gas streams or H_2S accumulation in relatively confined spaces, due to its specific gravity (1.19) which is higher than air [26].

Most human data on H_2S toxicity are derived from acute poisoning case reports in occupational exposures [7,25,27,28], and from published community studies [20–22,29].

Human health effects have been reported following exposure to H_2S starting from 2.8 mg/m^3 (2.00 ppm) where bronchial constriction in asthmatic individuals is observed [30], to 140 mg/m^3 (100.38 ppm) with olfactory paralysis [31] and 560 mg/m^3 (401.52 ppm) with respiratory insufficiency [32]. Deaths are reported at environmental concentrations higher than 700 mg/m^3 (501.90 ppm) [33].

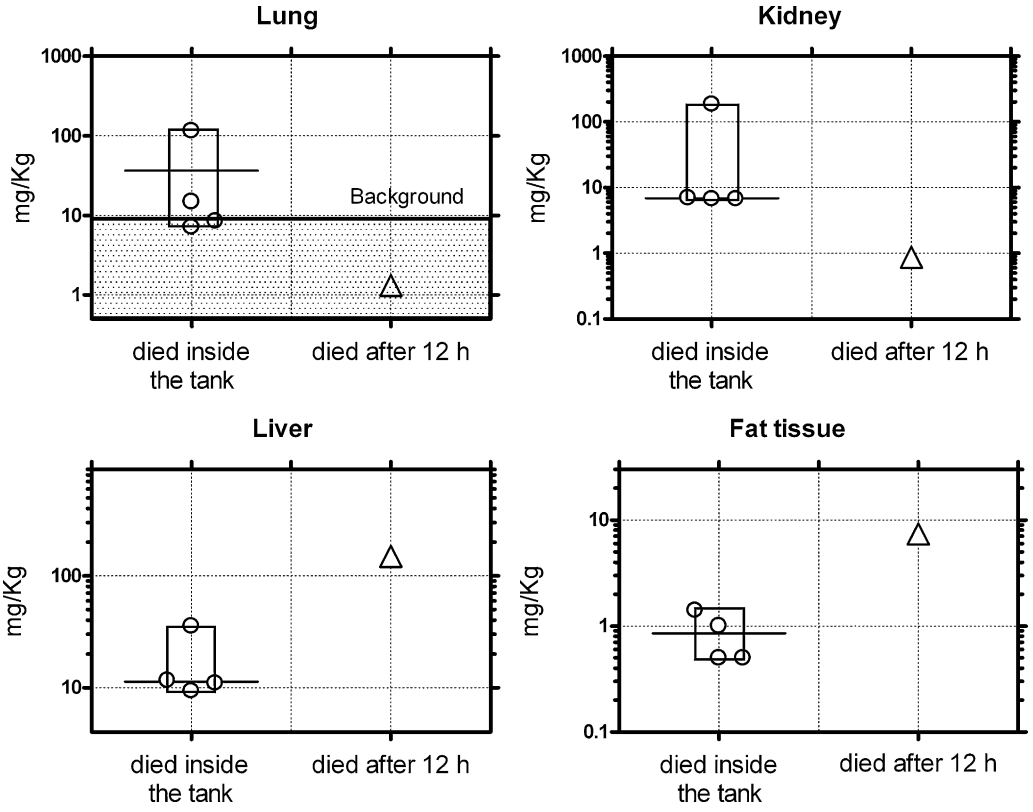

Fig. 2. Thiosulfate distribution and median lung, liver, kidney and fat tissues considering the time of death. Thiosulfate levels measured in other fatal deaths due to H_2S poisoning are indicated as background [6,9–12].

In particular, in the range of 750–1000 ppm, prolonged inhalation of H_2S causes sudden loss of consciousness ("knock-down" effect) that can lead to fatal respiratory paralysis if rescue operations are not rapid [27]. In many cases this level of exposure was not fatal if promptly terminated [23,25], even if additional trauma due to the "knockdown" effects may complicate the clinical condition associated with the exposure.

Studies on occupational H_2S exposure performed in the United States from 1984 to 1994 reported that more than 80% of incidents happened in confined spaces and 23% of the deaths were due to co-workers' rescue attempts [16,29,34,35].

In the present fatal case, at the beginning H_2S was not suspected as being responsible for the worker's asphyxiation because at the moment of the accident the tanker was empty and the nature of the substance previously shipped was unknown.

In fact, even if the presence of the gas could have been identified by the characteristic odor of rotten eggs, detection by odor is not always possible because at high concentrations H_2S paralyzes the olfactory nerves [7,19,35]. Owing to this unique property, it is difficult to detect its presence at high concentrations and therefore the level danger is under-estimated.

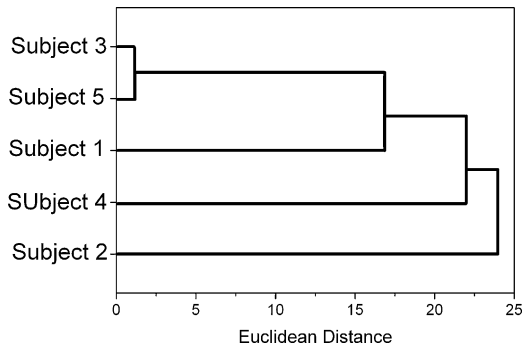

Fig. 3. Cluster analysis. Subjects are classified in the dendrogram on the basis of their reciprocal distance related to the thiosulfate tissue concentrations.

Further investigation revealed that the tanker had previously shipped sulfur liquid. However, the analyses performed on liquid dregs taken from the tanker revealed the presence of H_2S. The absence of hydrocarbon-aliphatic compounds and solvents together with its almost neutral pH demonstrated that the workers had not used detergents or basic compounds to dissolve and clean the dregs.

Analyses performed on air confirmed that sulfur oxides were negligible, which excluded a sulfur combustion induced by the workers cleaning the dregs. This aspect is particularly relevant because it demonstrates that the workers were merely performing an inspection.

Air monitoring inside the tanker performed one week after the accident, revealed H_2S concentrations higher than the permitted occupational exposure values [14]. Even one month after the accident, the H2S levels were comparable to those reported by the WHO near the point sources [22].

This depletion was probably due to the continuous opening of the manhole during the rescue operations and the following inspections, as demonstrated by the strong characteristic odor of rotten eggs that could be smelled in the area outside the tanker in the following days.

However, despite the possible loss of H_2S from the tanker during rescue operations even one week after the accident, H_2S air concentration was 8–9 times higher than TWA and STEL occupational exposure values (10 and 15 ppm, respectively) and OSHA permissible exposure limit in occupational settings (20 ppm).

These results were difficult to understand, especially considering that the tanker had previously contained sulfur liquid and that air H_2S levels were a long way from those reported for human death (>501.90 ppm) [33].

On the other hand, only in a few fatal cases has it been possible to evaluate real H_2S air concentration immediately after an accident [7,16,36]. In particular, when air H_2S determination was carried out immediately, the levels were higher than 1,000 ppm (1394.7 mg/m^3) [23,24], and became lower (from a few ppm to about 100 ppm) when monitored after area ventilation (forced or passive) [6,36]. In fact, Kage et al. described how a simulation performed on the scene of the accident revealed H_2S air concentration of 850 ppm (1185.5 mg/m^3) even if the concentration after rescue operation was equal to 1 ppm (1.4 mg/m^3) [16].

The high levels measured inside the tanker were thought to be related to contamination by the original liquid sulfur shipped. In fact, H_2S can exist as a residual compound from sulfur synthesis produced by Claus's process, the most common industrial method used to recover elemental sulfur from gaseous H_2S [37]. This process involves several steps and the overall main equation in its simplest form is:

$$8H_2S + 4O_2 \rightarrow S_8 + 8H_2O$$

Therefore, we could suppose that, since the sulfur was shipped in liquid form (sulfur melting point 113 °C), H_2S easily passes from the liquid to gas phase in the tanker due to its high vapor pressure (252 psi at 21 °C) and low boiling point (−60 °C) (see Table 3). Additionally, its low Henry's constant (8.7×10^{-2} M/atm) confirmed its low solubility [38,39] which is similar to those reported for other gases such as O_2 ($K_H = 1.3 \times 10^{-3}$ M/atm) and CO_2 ($K_H = 3.4 \times 10^{-2}$ M/atm) [40].

H_2S evaporation was certainly enhanced by the movement of the liquid during shipping which increased the evaporation kinetics. When liquid sulfur was emptied from the lower outlet of the tanker, it is probable that H_2S remained in the gas phase at high concentration, especially since, after sulfur discharge, the manhole and bottom exit of the tanker had remained closed until the accident.

To support the hypothesis that the abnormal concentrations of H_2S arising from liquid sulfur was responsible for the deaths, two similar tank-trucks used for liquid sulfur transport were tested.

Results revealed that before loading liquid sulfur, air inside the tankers contained no H_2S and the O_2 levels were similar to environmental percentage of O_2 (about 20%, v/v), whereas H_2S concentrations gradually increased during the loading phase, with the tankers fully loaded and even after sulfur discharge.

Lastly, the high sulfur liquid contamination by H_2S confirmed that the H_2S itself was indeed a residual compound arising from sulfur synthesis.

This simulation demonstrated that after liquid sulfur had been removed, H_2S remained in the gas phase at concentrations consistent with both those described by Beauchamp et al. for human death after single exposure [33] and those reported in other fatal cases [7,36].

These environmental findings were confirmed by toxicological analyses through the measurement of thiosulfate, one of the main H_2S metabolites [6,7].

In the case of H_2S poisoning, thiosulfate has been proposed as a more reliable indicator with respect to H_2S because sulfide is rapidly metabolized [6,15,41], and H_2S production can also occur *post-mortem* due to the putrefaction of blood and tissues [8]. Additionally, while sulfide can spread from the airway and gastric fluid, thiosulfate can diffuse only from the airway because the acid condition of the gastric fluids can easily decompose it [16].

In fatal cases, the presence of thiosulfate can confirm the intake and bio-transformation of the parent compound, thereby excluding potential H_2S exogenous contamination of the samples.

Analysis performed on biological samples from all workers revealed high thiosulfate concentrations. To exclude its *post-mortem* production, tissue samples from unexposed subjects were also tested (Fig. 1)

With regard to the 4 workers who died inside the tanker, tissues had thiosulfate concentrations similar to those measured in blood. On the other hand, fat tissues had the lowest levels ($p < 005$) in respect to all other tissues due to its lipophilic property which requires higher distribution time. This confirms that death happened in a short time. On the other hand, the fact that it had spread throughout the body excludes immediate death.

Results showed that blood and brain thiosulfate values were similar in all of the dead workers, whereas in the survived one, who died the following day at the hospital, lung and kidney concentrations were lower. This is probably due to the fact that he was catheterized and under assisted ventilation through an endotracheal tube, which promoted the clearance of the toxicant. On the other hand, this worker had the highest thiosulfate levels in liver, the tissue with the highest metabolic rate, and in fat tissue. This latter result indicates that time of death had allowed accumulation of the substance, since fat-soluble gas concentrations in the adipose tissue are strictly dependent on the time of exposure [4].

The difference in the results obtained in the four workers who died inside the tanker and in the surviving worker, are in accordance with the time of death as shown by cluster analysis. Cluster analysis indicated a similar time of exposure and death in the third and fifth workers, while the first and the fourth had higher thiosulfate tissue concentration. A more evident difference was also observed for the second worker.

The variability in data distribution could also be related both to the inter-individual differences in metabolic rates, and the impossibility of knowing the exact time of death.

Only in a few cases has thiosulfate been measured in biological tissues (e.g. brain, lung and muscle) [18], and to the best of our knowledge, such distribution after fatal exposure has never been described. Several authors have reported sulfide concentrations measured in other tissues besides blood and urine [18,27,42], but this information is not totally reliable due to H_2S *post-mortem* production.

The measurement of thiosulfate in several tissues is an important but relatively unusual step in toxicological investigation of H_2S poisoning, which adds important information about time of death and toxicokinetics.

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