Review

Measurement and biological significance of the volatile sulfur compounds hydrogen sulfide, methanethiol and dimethyl sulfide in various biological matrices☆

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ABSTRACT

This review deals with the measurement of the volatile sulfur compounds hydrogen sulfide, methanethiol and dimethyl sulfide in various biological matrices of rats and humans (blood, serum, tissues, urine, breath, feces and flatus). Hydrogen sulfide and methanethiol both contain the active thiol (–SH) group and appear in the free gaseous form, in the acid-labile form and in the dithiothreitol-labile form. Dimethyl sulfide is a neutral molecule and exists only in the free form. The foul odor of these sulfur volatiles is a striking characteristic and plays a major role in bad breath, feces and flatus. Because sulfur is a biologically active element, the biological significance of the sulfur volatiles are also highlighted. Despite its highly toxic properties, hydrogen sulfide has been lately recommended to become the third gasotransmitter, next to nitric oxide and carbon monoxide, based on high concentration found in healthy tissues, such as blood and brain. However, there is much doubt about the reliability of the assay methods used. Many artifacts in the sulfide assays exist. The methods to detect the various forms of hydrogen sulfide are critically reviewed and compared with findings of our group. Recent findings that free gaseous hydrogen sulfide is absent in whole blood urged the need to revisit its role as a blood-borne signaling molecule.

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Contents

1. Introduction

This review deals with the measurement of the volatile sulfur compounds (VSCs) hydrogen sulfide (H$_2$S), methanethiol (methyl mercaptan, CH$_3$SH, MT) and dimethyl sulfide (CH$_3$SCH$_3$, DMS) in various biological mammalian matrices, *viz.* blood, serum, tissues, urine, breath, feces and flatus. The VSCs have a bad reputation, mainly because of their toxicity and of their bad odor. The three VSCs are metabolites of the sulfur-containing amino acids cysteine and methionine. The various pathways are summarized in Fig. 1.

H$_2$S is synthesized endogenously in various mammalian tissues by desulfhydration of cysteine, catalyzed by at least two enzymes responsible for metabolizing L-cysteine: cystathionine β-synthase (EC 4.2.1.22) and cystathionine γ-lyase (EC 4.4.1.1). It can also be synthesized endogenously from L-methionine through the transsulfuration pathway, with cysteine being an intermediate in the process [1,2]. Considerable amounts of H$_2$S are formed by anaerobic bacteria in the intestinal tract [3,4,5]. MT is formed from methionine by the enzyme L-methionine γ-lyase (EC 4.4.1.11) [6,7], in mammalian tissues as well as by bacteria in the intestinal tract. MT may also be formed via transamination of methionine out of 3-methylthiopropionate [8,9]. DMS is also a product of methionine transamination [9,10]. DMS can be formed via methylation of MT by the enzyme thiol S-methyltransferase (EC 2.1.1.9) [11]. The same enzyme can convert H$_2$S into MT. The importance of these methylations in the gut were questioned by Levitt's group [12,13]. They found that in cecal mucosa MT was first demethylated to H$_2$S and H$_2$S was then oxidized to thiosulfate and sulfate. Conversion of MT into H$_2$S and sulfate was also observed by Benevenga [14] and by us in whole blood [15]. Conversion of MT into H$_2$S is catalyzed by methanethiol oxidase (EC 1.8.3.4) [16]. DMS is the dominant biogenic sulfur compound in the marine atmosphere and essential to the global sulfur cycle. It is formed out of dimethylsulfoniopropionate, a sulfonium ion abundantly present in algae [17].

H$_2$S is extremely toxic to humans [18]. Its toxicity resembles that of hydrogen cyanide. The main metabolism of H$_2$S is: (1) oxidation to sulfate; (2) methylation to MT and DMS; and (3) reaction with metallo- or disulfide-containing proteins. The first two metabolic pathways can be regarded as detoxification routes. The methylation reactions [11] were regarded as a detoxification mechanism since the mono- (MT) and dimethylated (DMS) products have been described as progressively less toxic than H$_2$S [19]. Especially the toxicity of DMS was substantially lower than that of H$_2$S and MT [20]. The reaction of H$_2$S with essential proteins is believed to be largely responsible for the toxic action of H$_2$S. Similar to cyanide, H$_2$S is a potent inhibitor of cytochrome oxidase and has also been shown to inhibit certain metalloproteins [18].

In the past few years, H$_2$S has also been postulated to be a biologically gaseous mediator with potential roles in several physiological processes and disease states [21–24]. Most results were based on the finding of high concentrations of H$_2$S (10–300 μM) in healthy tissues and blood [21–25]. Two reports recently found that tissue H$_2$S concentrations were orders of magnitude lower than presently accepted values [26] and that H$_2$S was undetectable in blood [27], casting doubt about H$_2$S serving as a blood-borne signaling molecule. It is therefore highly important to critically review the analytical methods to measure H$_2$S. Results on the presence of MT and DMS in biological matrices come mainly from our group. Other VSCs, such as dimethyl disulfide (CH$_3$SSCH$_3$, DMDS, oxidation product of MT), dimethyl sulfoxide and dimethyl sulfone (CH$_3$S(O)CH$_3$ and CH$_3$S(O)$_2$CH$_3$, oxidation products of DMS), carbonyl sulfide (COS), carbonyl disulfide (CS$_2$) and sulfur dioxide (SO$_2$) are less abundant in mammals and less frequently cited and stay outside the scope of this review.

1.1. Definition of the various forms of the VSCs

1.1.1. Forms of H$_2$S

There is some lack of clarity about the various forms of H$_2$S. In this review analysis of the following forms will be discussed: free H$_2$S, acid-labile sulfide (ALS) and dithiothreitol-labile sulfide (DLS). Each form probably has completely different physiological aspects. It is therefore highly important to differentiate between the various forms which was not always done in literature. In most reports where analysis of sulfide has been described, the exact form of H$_2$S has not been clarified.

1.1.1.1. Free H$_2$S. This is the form also known as inorganic sulfide. Inorganic sulfide can be present as H$_2$S, HS$^-$ or S^{2-} in the aqueous state and as H$_2$S in the gas phase. The pK$_a$ values for the first and second dissociation steps of H$_2$S are 7.04 and 11.96, respectively. Therefore, in the aqueous state, at physiologic pH 7.4 approximately one third exists in the undissociated volatile form (H$_2$S) and the remainder largely as the hydrosulfide anion (HS$^-$). Very small amounts of sulfide anion (S^{2-}) are present [18]. There is considerable ambiguity in literature regarding the terms describing free H$_2$S. Besides inorganic sulfide, hydrogen sulfide, sulfide and total sulfide are used. Whitfield et al. [27] denotes the sum of H$_2$S and HS$^-$ as total sulfide. However, this is confusing because other reports also use this term for ALS or DLS. We prefer to use the term "free H$_2$S" for denoting the sum of H$_2$S and HS$^-$. It is not known whether H$_2$S, HS$^-$, or both are biologically active. Even the other forms of H$_2$S, ALS and DLS, might contribute to the biological activity. Precautions must be taken to measure free H$_2$S in aqueous samples (blood, urine) to prevent volatilization of H$_2$S in the open air, thereby escaping detection. After withdrawal from the patient, the sample (blood, urine) was immediately injected in evacuated vials after which the H$_2$S-containing gas phase was sampled quantitatively and measured by
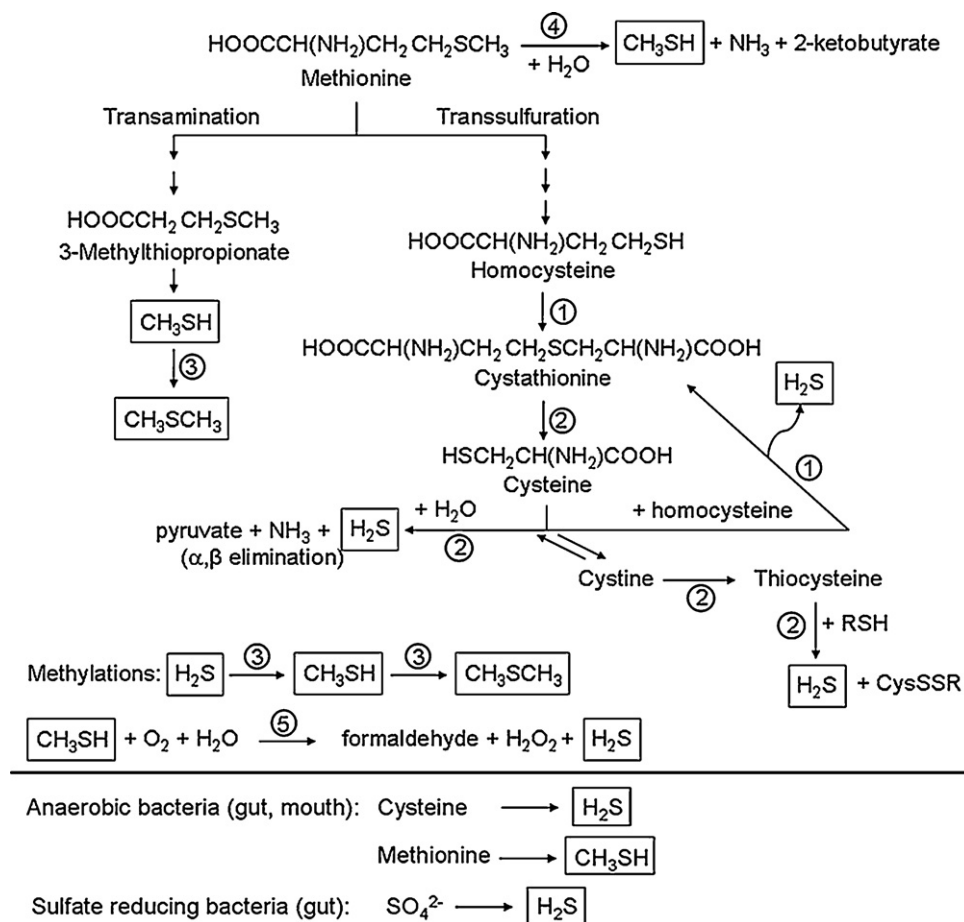
Fig. 1. Pathways of production of VSCs from methionine and cysteine in mammalian tissues (above) and by bacteria (below). Reactions 1 are catalyzed by cystathionine β-synthase, reactions 2 by cystathionine γ-lyase, reactions 3 by thiol S-methyltransferase, reaction 4 by L-methionine γ-lyase, and reaction 5 by methanethiol oxidase (methyl mercaptan oxidase). CysSH, cysteine; RSH, thiol; RSSR, disulfide.

gas chromatography (GC) [28]. It is difficult to quantitatively measure free H_2S in serum or plasma because appreciable amounts will escape during the process of centrifugation. Free H_2S is the only form of H_2S present in gaseous samples (breath, flatus).

1.1.1.2. Acid-labile sulfide (ALS). ALS is the form from which H_2S is liberated when a biological matrix is treated under acidic conditions. Two terms, namely acid-labile sulfide and acid-labile sulfur, are used [25]. The latter term is misleading because the acid-labile MT would also fall under the acid-labile sulfur. It has been established that ALS is contained in iron–sulfur clusters of non-heme iron–sulfur proteins (e.g. ferredoxins) [25,29]. However, not all ALS is contained in iron–sulfur clusters. When free H_2S, if present, was not completely removed before ALS determination, ALS might be contaminated with free H_2S. Removal of free H_2S from aqueous samples can be done by purging the solution with nitrogen, forcing the free H_2S to escape in the open air.

1.1.1.3. Dithiothreitol-labile sulfide (DLS). DLS is the form from which H_2S is liberated when a biological matrix is treated with dithiothreitol (DTT). This form belongs to the class of biological sulfane sulfur. Sulfane sulfur atoms are defined as divalent sulfur atoms bonded only to other sulfur, except that they may also bear ionizable hydrogen at some pH values [30–33]. The following labile sulfane compounds have been shown to be involved in biochemical systems [25,29,32]: elemental sulfur (S_8) associated with protein, hydrodisulfides, frequently called persulfides (R–SSH), polysul-

fides (R–S_n–R) where $n \geq 3$, polythionates (^-O_3S–S_n–SO_3^-) where $n \geq 1$, thiosulfate (SSO_3^{2-}), and thiosulfonates (R–$S(O_2)S^-$). Various physiological functions of sulfane sulfur have been reported, in particular of persulfides.

Not all sulfane compounds do release H_2S on treatment with DTT. Sulfide liberated from proteins by DTT (DLS) appears to include persulfides (protein–S–SH) and elemental sulfur. Instead of DLS, many other terms, such as protein-associated sulfur, protein-bound sulfur, tissue-bound sulfide, bound sulfur, sulfide liberated by DTT (or reduction), and non-acid-labile sulfide, have been used in the literature [25]. Again, just as described for ALS, DLS might be contaminated with free H_2S, if present, when free H_2S was not completely removed before DLS determination.

1.1.2. Forms of MT (CH_3SH)

In contrast to H_2S, much less is known about the physiological significance of MT and of the various forms of MT. Most of it comes from our group [9,15,28,34–38]. Besides free MT, an acid-labile MT (ALM) and a dithiothreitol-labile MT (DLM) were found. The exact structures of ALM and DLM are not yet known. MT may also exist in its oxidized form DMDS (CH_3SSCH_3). No special attention in this review will be given to the latter one.

1.1.2.1. Free MT. MT is a very weak acid with a pK_a of 10.4. At physiologic pH 7.4, nearly all MT exists in the undissociated volatile form. Very minor amounts (one thousandth) are present as the anion (CH_3S^-). Therefore, just as described above for free H_2S, precau-

tions must be taken to measure free MT in aqueous samples (blood, urine) to prevent volatilization of MT in the open air, thereby escaping detection. Free MT is the only form of MT present in gaseous samples (breath, flatus).

1.1.2.2. Acid-labile MT (ALM). ALM is the form from which MT is liberated when a biological matrix is treated under acidic conditions. ALM was first detected in serum and urine [28]. Diaflo ultrafiltration experiments showed that its molecular weight was below 500. At that time, methyl-β-D-thioglucuronide was proposed to be a possible candidate. The exact nature is still unknown but experiments with DEAE-Sephadex column chromatography have shown that ALM consists of at least two compounds. When free MT, if present, was not completely removed before ALM determination, ALM might be contaminated with free MT. Removal of free MT from aqueous samples can be done by purging the solution with nitrogen, forcing the free MT to escape in the open air.

1.1.2.3. Dithiothreitol-labile MT (DLM). DLM is the form from which MT is liberated when a biological matrix is treated with DTT. DLM was detected in serum [9,28] and urine [9,10]. In serum, part of MT is bound to proteins in a disulfide linkage: protein–S–SCH$_3$, and part as a disulfide linkage to a compound X: X–S–SCH$_3$ (X yet unknown). The first form reacts with DTT at neutral pH and the second form at pH 10. In urine, MT is bound via a disulfide linkage to the compound X with a molecular weight <500: X–S–SCH$_3$. Just as described for ALM, DLM might be contaminated with free MT, if present, when free MT was not completely removed before DLM determination.

1.1.3. Forms of DMS (CH$_3$SCH$_3$)
1.1.3.1. Free DMS. In contrast to the thiols H$_2$S and MT, containing a reactive –SH group, DMS is a neutral molecule which does not react with proteins and which is quite stable in various biological matrices. Free DMS is the only form known which is present in mammals. In plants, two precursors of DMS have been found, *viz.* S-methylmethionine [39], also known as vitamin U, and dimethylsulfoniopropionate [17,39]. Both belong to the class of sulfonium ions and can be catabolyzed to DMS after thermal or enzymatic degradation. DMS has an important role in flavoring many cooked vegetables and beer [39].

2. Experimental (authors newly presented experiments)

Our procedures for measuring the various forms of VSCs are summarized in Table 1. A detailed description is given in the text.

2.1. Free concentrations of the VSCs in blood and urine

Free concentrations of VSCs in blood and urine were measured by GC in essentially the same way as described for DMS in blood [28]. Complete release of DMS from blood required 8 consecutive samplings of 60 ml of headspace gas. For the more volatile H$_2$S and MT, 3 headspace samplings were already sufficient for a recovery of more than 90%. For routine measurements one might also apply one headspace gas sampling of 60 ml. In that case the GC outcome has to be corrected for the recovery in that first sampling [28].

2.2. Bound forms of H$_2$S and MT in serum and urine

DLS and ALS in serum and urine were measured in essentially the same way as described for DLM and ALM, respectively, in serum and urine [9,28]. DLS: To 50–250 μl serum or urine in a stoppered 15 ml vial were added 250 μl of DTT (10 mg/ml = 65 mM) in 0.1 M Tris (pH 8). The mixture was vortexed for 10 s and left at room temperature for 1 min. The H$_2$S was then released into the headspace by addition of 50–100 μl of 3 times diluted acetic acid, sampled quantitatively as shown above (Section 2.1) and measured by GC. Addition of diluted acetic acid facilitates the release of H$_2$S into the headspace. Without acid addition the H$_2$S can also be sampled quantitatively by consecutive sampling. The concentration of DTT (65 mM) may vary (2–65 mM) without a different outcome in DLS. ALS: To 50–250 μl serum or urine in a stoppered 15 ml vial were added 250 μl of 5 M HCl. The mixture was vortexed for 10 s and left at room temperature for 1 min. The released H$_2$S was then sampled quantitatively (see Section 2.1) and measured by GC.

2.3. Bound forms of H$_2$S and MT in tissues

Tissues of male Wistar rats (1 g) were homogenized with 2 ml of 0.25 M sucrose in 20 mM Tris (pH 7.4) with an all glass tissue grinder (Potter-Elvehjem). DLS, DLM, ALS and ALM in tissue homogenates were measured in essentially the same way as described for DLM and ALM in serum and urine [9,28]. DLS and DLM: To 100 μl tissue homogenate in a stoppered 15 ml vial were added 250 μl of DTT (10 mg/ml = 65 mM) in 0.1 M Tris (pH 8). The mixture was vortexed

Table 1
A broad scheme of the procedures for measuring the various forms of the VSCs.

Free concentrations of VSCs in blood, tissues, urine and feces

 - No preceding chemical modification of the sample.
 - To prevent losses of the free gaseous VSCs, immediate transfer of the sample into a stoppered 15 ml vial, after sample collection from the patient.
 - Exhaustive sampling of the free VSCs by consecutive headspace samplings.
 - Preconcentration of the headspace samplings onto Tenax trap tubes, followed by GC analysis.
 - Breath and flatus only contain free VSCs.
 - DMS only exists in its free form in all biological matrices.

Bound concentrations of the VSCs H$_2$S and MT in blood, plasma, urine and feces

Acid-labile forms
 - First removal of free VSCs by purging the sample with nitrogen.
 - Collection of the sample in a stoppered 15 ml vial.
 - Release of the VSCs by pretreatment of the sample with HCl.
 - Preconcentration of the released VSCs by headspace sampling onto Tenax trap tubes, followed by GC analysis, or, in case of feces, direct injection of a headspace sample for GC.

Dithiothreitol-labile forms
 - First removal of free VSCs by purging the sample with nitrogen.
 - Collection of the sample in a stoppered 15 ml vial.
 - Release of the VSCs by pretreatment of the sample with DTT and then with three times diluted HAc to facilitate this release.
 - Preconcentration of the released VSCs by headspace sampling onto Tenax trap tubes, followed by GC analysis, or direct injection of a headspace sample for GC.

for 10 s and left at room temperature for 2 min. The H_2S and MT were then released into the headspace by addition of 100 µl of 3 times diluted acetic acid, sampled quantitatively as shown above (Section 2.1) and measured by GC. Experiments were also performed with 1.5 mM DTT. Because tissues normally also contain ALS, acidification with diluted acetic acid might have released some ALS. Tissues were therefore also treated with only diluted acetic acid, without prior DTT treatment. The outcome of the latter reaction must be subtracted from that of the first reaction to obtain the DLS value. ALS and ALM: To 100 µl tissue homogenate in a stoppered 15 ml vial were added 100 µl of 5 M HCl. The mixture was vortexed for 10 s and left at room temperature for 1 min. The released H_2S was then sampled quantitatively (see Section 2.1) and measured by GC.

2.4. Fecal H_2S

2.4.1. Total H_2S (sum of free and bound H_2S)
Fecal material was collected in a plastic bag during defecation. The bag was immediately closed, thereby expelling any air. The feces was analyzed as quickly as possible. Feces (1 g) was transferred into a 15 ml glass vial, containing 5 ml of 0.5 M zinc acetate to trap all the sulfide including the free H_2S. The vial was closed with a rubber septum and capped with an aluminum cap. After vigorously vortexing, 0.2 ml of the homogeneous suspension was transferred to another capped empty 15 ml vial by means of a 1 ml syringe with a blunt needle (i.d. 1 mm). The H_2S was then completely released into the headspace by addition of 0.3 ml of 6 M HCl. After vortexing for 10 s, 1 ml of headspace was withdrawn from the vial and directly injected in the GC for H_2S analysis.

2.4.2. Free H_2S
Fecal material was collected as described above. Feces (1 g) was transferred into a 15 ml glass vial, containing 5 ml of 0.1 M phosphate buffer (pH 7.0). The vial was closed immediately and the mixture was homogenized by vortexing, after which the headspace was sampled quantitatively by consecutive samplings of 60 ml of headspace gas [28]. The headspace samplings were concentrated onto Tenax trap tubes and measured by GC. For fecal suspensions with high concentrations of H_2S, smaller amounts can be injected in the GC.

2.4.3. Bound H_2S
Feces (1 g) was transferred into a 15 ml glass vial, containing 5 ml of 0.1 M phosphate buffer (pH 7.0). The vial was closed and the mixture was homogenized by vortexing, after which 0.2 ml of the homogeneous suspension was transferred to another empty 15 ml vial by means of a 1 ml syringe. This 0.2 ml sample was flushed with nitrogen for 5 min, to remove free H_2S. The vial was then capped and the bound H_2S was released into the headspace by addition of 0.3 ml of 6 M HCl and measured by GC as described for total H_2S.

2.5. Flatus

2.5.1. Flatus perception thresholds
Perception threshold values are normally assessed by injection of an odorant into the nostril of panel members [40]. However, in the odor recognition of a flatus, the nose of the observer of a flatus is in most instances at least at 1 m from the bottom of the offender. A dilution of a flatus of 100 ml into 4–8 m³ has been assumed before the smell reaches the nose, resulting in a dilution factor of 40000–80000. The theoretical flatus perception threshold values have now been defined as the perception threshold values [40] times the dilution factor of $4–8 \times 10^4$. The experimental flatus perception threshold value has been defined as the lowest concentration of the odorant where all panel members detected any odor, when 100 ml of an odorant
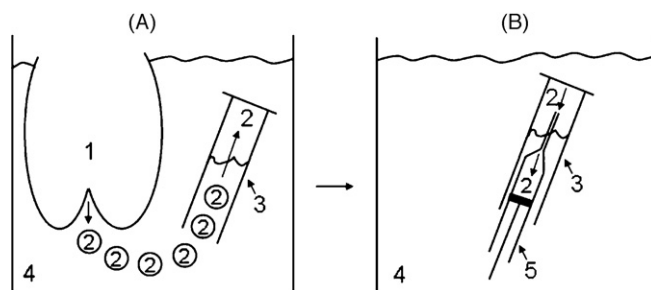

Fig. 2. Bathtub method for quantitative flatus sampling. (A) Collection of one flatus in a measuring beaker. (B) Transfer of a known volume of pure flatus from the measuring beaker into a 60 ml sampling syringe. 1, The bottom of the flatus supplier; 2, flatus; 3, measuring beaker (500 ml); 4, bathtub, filled with water; 5, 60 ml sampling syringe.

mixture was delivered from a large 250 ml syringe in about 1 s, 1 meter beneath the nose of the panel members, mimicking a flatus emission. The theoretical and experimental flatus perception thresholds of the VSCs lie in the same range, indicating that the assumed dilution factor has the right order of magnitude.

2.5.2. Bathtub flatus sampling
We developed a bathtub method for quantitative flatus sampling (Fig. 2).

The volunteers submerged the lower part of their body in a warm bath each time they felt an urge to deflate. The flatus emission was completely sampled by catching all the flatus bubbles in a measuring beaker, which was submerged, fully filled with water and turned upside down before the emission. After some practice, it is very easy to quantitatively sample all the bubbles. The volume of the emission was the same as the volume of the displaced water in the measuring beaker. Part of the pure flatus was then sampled by means of a large 60 ml sampling syringe. For storage, 10 ml of flatus was then collected in an evacuated 15 ml glass vial sealed with an aluminum cap and containing 1 g of the drying agent calcium chloride. Without this drying agent, a sharp fall in H_2S and to a minor extent in MT was observed, due to reaction of the thiols with the water [41] in the humid flatus emission. The concentrations of all VSCs remained stable for at least one week during storage above calcium chloride. The contents of the glass vial were sampled quantitatively by means of a 60 ml syringe, preconcentrated onto Tenax trap tubes and analyzed by GC [28,34]. Gaseous H_2S and MT, especially at low concentrations, may adhere to several materials, e.g. glass [42,43]. Breath samples are therefore stored in polyethylene foil (mylar) balloons [43] and not in glass vials. The use of glass vials in flatus research satisfied the need for quantitative recoveries of the VSCs, probably because of the much higher concentrations of VSCs in flatus compared with breath. Nevertheless, it is advisable to use polypropylene or polyethylene materials in all handlings with VSCs.

3. Analysis of the VSCs

3.1. General remarks

The results from literature will be compared with the results from our group, some results already published and some presented here for the first time. In the latter case, the assay methods used have been described in Section 2. The assay methods from literature for sulfide will not been described here in detail, because this was already done in an excellent review in 2002 by Ubuka [25] and supplemented in 2008 by Whitfield et al. [27]. In short, the four most used methods to detect sulfide are colorimetric assays

(mainly the methylene blue method), ion selective electrode assays, high-performance liquid chromatography (HPLC) and GC. The first two methods always use acidic conditions with the consequence that they also include ALS in their measurements. These assays can therefore not distinguish between free H_2S and ALS and cannot measure free H_2S. The methylene blue method is also subjected to interference with colored substances, lowering its sensitivity. The HPLC methods are neither direct methods and also require a chemical modification, such as the conversion of sulfide to, e.g. methylene blue or thionine, before measurement and are therefore also not suited to measure free H_2S. GC is a direct method and can measure free H_2S without a chemical modification. GC might be the method of choice to detect the various forms of H_2S. Recently [27,44], a polarographic H_2S sensor was used to directly measure free H_2S without a chemical modification, also with promising results.

Analysis of VSCs, especially that of H_2S, can be very tricky and various artifacts must be avoided. Many studies have not been aware of these artifacts, resulting in falsely elevated or reduced levels of VSCs. H_2S and MT, containing a reactive thiol (–SH) group, are very reactive compounds and adhere to several materials, e.g. glass [42]. Therefore, liquid samples (e.g. blood, urine) should be preferably stored in inert polypropylene or polyethylene vials and gaseous samples (e.g. breath and flatus) in polyethylene foil (mylar) balloons [43]. The latter are aluminum foil balloons, coated inside with the inert polyethylene. Care must also be taken to avoid rubber (in septa or stoppers of reaction vials or blood vials and in the barrel seal of disposable syringes) which might adsorb or release VSCs [43,45]. When using syringes during analyses, all-plastic syringes are the preferred ones.

Many studies apply a harsh chemical treatment (strong acid or base, respectively) before analysis of H_2S [27]. Some of these treatments might give rise to artificially elevated H_2S levels. For example, it is known that mixing concentrated sulfuric acid with water (2:1) is a highly exothermic process. We found that this process in a blank experiment sometimes gave rise to artificially, highly elevated H_2S levels up to 80 μM, excluding the use of sulfuric acid in H_2S analyses. Probably, concentrated sulfuric acid contains trace amounts of H_2S. The widespread use of DTT to release H_2S from DLS also sometimes resulted in high blanks for H_2S, especially in older DTT solutions and in DTT solutions with a higher pH (≥ 9). Therefore, freshly prepared DTT solutions should always be used. These experiments stress the importance of using appropriate blank experiments in all analyses. In such a blank experiment, we found that relevant low concentrations of H_2S in the headspace above water in a closed vial rapidly decreased within minutes. The lost H_2S could not be recovered by addition of acid (ALS) or DTT (DLS). It is known that in aqueous solutions H_2S may react with oxygen dissolved in the water resulting in elemental sulfur and/or sulfuric acid [41]. It is therefore highly important to quickly measure H_2S once it has been released from aqueous matrices (e.g. blood, urine). MT is much less subjected to these artifacts than H_2S whereas analysis of DMS is relatively easy because the neutral DMS is quite stable in biological matrices.

In all our analyses of VSCs in biological matrices we used the technique of GC, applying dynamic headspace sampling onto replaceable Tenax trap tubes [28,34,37,38]. These are glass trap tubes (length 8 cm), containing 200 mg Tenax GC (80–100 mesh). Tenax is a perfect material to adsorb the VSCs. For blood, serum, tissues, urine and feces, reactions were performed in stoppered 15 ml glass vials. After release of the VSCs, quantitative sampling was performed by consecutive samplings of 60 ml of headspace gas [28]. All these samplings were concentrated onto one Tenax trap tube maintained in liquid nitrogen (cryogenic preconcentration). All the VSCs were completely adsorbed onto the Tenax [34]. The trap tube was then transferred into the injection port of the GC where the adsorbed VSCs were thermally liberated (200 °C) directly into the carrier gas stream and transferred to the GC column (20% SE-

30 on Chromosorb P 60–80 mesh). After GC separation, the VSCs were detected by means of a flame photometric detector (FPD). For routine measurements one might also apply one headspace gas sampling of 60 ml instead of quantitative sampling by exhaustive consecutive samplings of the headspace gas. In that case the outcome should be corrected for the recovery in that first sampling [28]. This recovery depends on the biological matrix used and the VSC of interest and amounted between 40 and 80%. Instead of headspace gas sampling by hand, one might also apply the often used technique of flushing the headspace gas with a stream of nitrogen directly onto adsorbent traps. For gaseous biological matrices (breath, flatus) a known volume is simply concentrated onto the Tenax trap tube for GC. For GC analyses of VSCs in biological matrices, dynamic headspace sampling onto sorbent trap tubes is the preferred technique, giving very low detection limits. For example, for 1 ml of an aqueous sample (blood, urine) one calculates a detection limit for H_2S of 5 nM, when using the absolute GC detection limit of 5 pmol for H_2S [34]. Static (equilibrium) headspace injection is commonly used for GC determination of volatiles in solid and liquid samples. Since this technique relies on the analyte partitioning between the sample and headspace, and uses a fixed small injection volume, it does not provide adequate detection limits in this field. The GC technique with FPD detection, applying dynamic headspace sampling onto replaceable Tenax trap tubes, is one of the most reliable and suited techniques for detection of VSCs in biological material. Once released, the gaseous VSCs are immediately transferred into the trap tubes. There is no interference with other compounds or with the biological material. Representative chromatograms have been presented in several publications [28,34,38,85,87]. The same GC column is now in use for more than 20 years in our laboratory, without any deterioration.

3.2. VSCs in blood, serum and tissues

VSCs, especially the thiols H_2S and MT, behave differently in a biological matrix containing proteins than in a matrix without proteins. Therefore, blood, serum and tissues will be discussed as a single group, apart from urine, breath, feces and flatus.

3.2.1. H_2S in blood, serum and tissues
3.2.1.1. Free H_2S. Most reports, while claiming to measure sulfide levels in blood or tissue, do measure the other forms of H_2S, *viz*. ALS and DLS, whereas free H_2S might be the biologically most active form of H_2S, when serving as a blood-borne signaling molecule [21–24,26,27]. Free H_2S was not found in human serum [46,47], and rat tissues [29,48]. Nagata et al. [49] did not find free H_2S in blood of control rats and of humans but did find markedly increased values in blood and some tissues in autopsied rats and humans with elapsed time after death. Furne et al. [26], using GC with FPD detection, found very low levels of free H_2S in brain (14 nM) and liver tissue (17 nM) of mice and in human blood of an estimated 100 pM. Whitfield et al. [27] used a polarographic H_2S sensor [44] to directly measure H_2S gas in blood and plasma without any chemical modification other than adding an anticoagulant. They did not detect any free H_2S in blood and plasma of various animals and found that exogenously applied sulfide was rapidly removed. Using the described GC technique, we also directly measured H_2S gas in blood and plasma without any chemical modification and neither detected any free H_2S in blood and serum of men and rats nor in tissues of rats.

In vitro experiments by us have shown that, just as was described for free MT [15,28], addition of free gaseous H_2S to human or rat serum or plasma gave a complete binding of H_2S to proteins, probably as persulfides (protein–S–SH). A quantitative recovery of H_2S was obtained from DLS by addition of DTT. Similar results were obtained by Ogasawara et al. [46] by spiking human serum with

Na_2S or elemental sulfur. However, addition of free gaseous H_2S to whole blood did not result in the formation of any DLS in plasma. The H_2S was completely oxidized by the red blood cells. Heme catalyzed sulfide oxidation to elemental sulfur, thiosulfate or sulfate [50–52] is probably a much faster process than the formation of persulfides. In in vitro experiments, we did not find any exchange between DLS in plasma and red blood cells. Incubation of the DLS-containing plasma (DLS: 41.5 μM, artificially elevated by addition of free gaseous H_2S to the plasma) during 2 h with the original red cell fraction did not change the concentration of the DLS in plasma (DLS: 40.1 μM). These results do not support the theory of Evans [51] that free H_2S, when injected into the circulation, is first carried for a time in plasma, after which it slowly penetrates into the red blood cells, where it is destroyed in reactions with oxyhaemoglobin. Our results suggest that free H_2S immediately enters the red cell where it becomes oxidized. This fast process is the main reason why free H_2S is normally absent in whole blood or serum. The same holds for free MT [15]. Evans [51] showed that a very short transient existence of free H_2S in blood may appear after in vivo injection of large doses of H_2S into the bloodstream of cats and rabbits, as was concluded from the appearance of H_2S in the expired air in the first 15 s after injection. Slow injection of the same doses did not show any trace of H_2S in the expired air, indicating that metabolism of free H_2S by blood is a fast process, normally preventing the escape of H_2S from blood into expired air. Curtis et al. [53] stated that blood is not the principle site of H_2S oxidation in vivo. This probably also depends on the dose of H_2S injected.

3.2.1.2. ALS and DLS. The described methods to detect bound sulfide do not always distinguish between ALS and DLS. This is highly important because ALS and DLS probably have complete different physiological functions. As a component of non-heme iron–sulfur proteins, ALS functions in electron transfer in the mitochondrial electron-transfer system and in enzyme activity (for references see [25]). Probably, not all ALS is contained in iron–sulfur clusters. More biological importance has been ascribed to DLS (sulfane sulfur, persulfides, [30–33]).

Many reports claim to measure "sulfide" in tissues [54–57] and serum or blood [47,58–60] without mentioning that in fact they measured ALS. For example, Goodwin et al. [55] determined sulfide in brain tissue by first binding the sulfide as zinc sulfide by addition of zinc acetate to the sample. It is unlikely that zinc acetate traps DLS and it has not been ascertained if zinc acetate traps ALS. They then added strong acid (6 M HCl) to release the H_2S out of the zinc sulfide after which a continuous flow gas dialysis system was used to separate the sulfide from the biological matrix and to measure the sulfide by ion chromatography. Addition of strong acid not only releases H_2S out of the zinc sulfide but also H_2S out of ALS. It is possible and even plausible that the measured sulfide completely existed as ALS. This holds for all the methods performed under acidic conditions, such as the often used methylene blue method to measure sulfide [25,27]. This method is performed at a pH < 1 and therefore also measures ALS. Only a limited amount of reports [29,46,48,61–63] clearly distinguish between the analysis of ALS and DLS.

Ogasawara et al. [46] did not find ALS in human serum and very small amounts (0.16 μM) in red blood cells [61]. Others also reported low values of ALS in human serum (1.3 μM, [47]) and whole blood (<1.5 μM [59]). Lindell et al. [58] reported ALS values of 1.3–18.8 μM in blood of non-fatal cases of H_2S poisoning and McAnalley et al. [59] of 50–117 μM in the blood of fatal cases. Whitfield et al. [27] stated that there is a very clear demarcation in blood sulfide levels reported in studies published before or after 2000. The earlier studies all show low plasma or blood values of 2 μM or less. Studies after 2000 reported plasma or blood sulfide levels between 10 and 300 μM. All these latter studies use "acidic" methods and

therefore also include ALS in their measurements. These high values are highly unlikely. They are based on colorimetric assays or ion selective electrode assays, each of which use a harsh chemical treatment (strong acid and sometimes also strong base) before analysis. The majority of the latter studies used the methylene blue method, a method subjected to interfering colored substances from blood [25] and therefore less suited for biological samples.

The ALS values in tissues (brain, liver, kidney, lung, heart, spleen [29,48,54–57,62]) varied between 6.8 and 274.1 nmol/g (0.23–9.32 μg/g). A large variation was seen between the outcome in the various reports (for a compilation see also Table 1 in [25]). For example, Ogasawara et al. [29] reported a value of 26 nmol/g for ALS in liver whereas Ubuka et al. [48] gave a value of 112.2 nmol/g. The reports of Ogasawara et al. [29] and Warenycia et al. [62] were the only ones found which clearly differentiated between ALS and DLS in rat tissues. The ALS values were generally lower than the DLS values, except for heart tissue [29], where high ALS values (129 nmol/g) were found and no "bound sulfur" or DLS. Although we did not study the ALS form in detail, we found that in tissues addition of three times diluted acetic acid (pH 3) did release smaller amounts of H_2S (7–15 nmol/g) from liver, kidney and heart. Addition of a stronger acid (6 M HCl, pH < 1) released higher amounts of H_2S. Release of H_2S out of the iron–sulfur clusters proceeds better at lower pH.

Ogasawara et al. [46] found that DLS was the only form of H_2S present in human serum and in various animal sera in concentrations usually below 2 μM. They showed that this DLS existed exclusively in the high-molecular-weight fraction of serum and that some part of DLS existed in the persulfide form (protein–S–S$^-$). Using the triphenylphosphine method, no protein-associated sulfur or sulfane sulfur was detected in human or rat plasma and erythrocytes [63] (below or near the detection limit of 0.3 μM). Both reports [46,63] reported fairly high values of DLS or sulfane sulfur in rat tissues (4.2–324.1 nmol/g).

We measured low concentrations of DLS in serum of normal men (0.97 ± 0.22 μM, mean ± SD, n = 9) and rats 0.8 ± 0.6 μM (n = 10), comparable with literature values. However, elevated concentrations were found in men with hepatic encephalopathy (3.38 ± 3.38 μM, n = 11) and highly elevated concentrations of serum DLS were found [64] in rats with acute fulminant hepatic failure due to thioacetamide administration [65] (22.3 ± 10.6 μM, n = 17) and in rats with hepatic encephalopathy due to 80% or total liver ischemia. The liver remained in the body during liver ischemia [66]. In these latter rats, the DLS gradually increased until death to values of about 100 μM, 14–20 h after the start of ischemia [64]. In one experiment it was shown that ALS was also elevated in the serum of these rats (22.8 μM). In rats with 100% hepatectomy, no elevation at all was seen during the 6 h until death. These results indicate that encephalopathy is not the cause of DLS elevation because no DLS elevation was seen in the encephalopathic rats after 100% hepatectomy. Deterioration of liver cells is most likely the cause of DLS elevation. The H_2S formed during deterioration will become bound to proteins as DLS and will leak out of the deteriorating cell into the bloodstream. Indeed, deterioration might lead to elevated DLS levels. Highly elevated DLS levels (10–140 μM) were also found by us in serum which was kept at room temperature for several days. DLS levels remained constant for at least one year during preservation of serum at −20 or −80 °C.

We measured DLS concentrations in rat tissues, varying from 11 nmol/g for spleen to 189 nmol/g for kidney. It was found that the levels were highly dependent on the concentration of DTT used in the reaction, on the reaction temperature and on the duration of the reaction. This was not the case for the DLS levels in serum which were fairly constant. Firstly, deterioration of tissue might lead to artificially elevated DLS levels. Secondly, leakage of enzymes out of the tissues might react with the reactant DTT, giving artificially elevated H_2S levels out of DTT. DTT is also a thiol, containing two

thiol groups. When lowering the DTT concentration to 1.5 mM, no DLS was measured in fresh tissues. These results cast doubts about the reliability of the high DLS values found in tissues. Such a phenomenon of dependency of DLS levels on the DTT concentration used was found earlier [67].

The absence or very low concentrations of free H_2S found in blood and tissues prompted Furne et al. [26], Whitfield et al. [27] and from the same group Olsen in a recent review [68] to cast doubts about H_2S serving as a blood-borne signaling molecule. They compared their values of free H_2S with the much higher values found in literature. However, these latter values do not contain free H_2S but ALS and/or DLS. The relatively high values of ALS and DLS might be artificially elevated. Many methods do use a harsh chemical treatment with strong acid or base. The influence of such a treatment on the values of ALS and DLS is not known. However, it was shown that the use of alkaline conditions might result in desulfuration of proteins via alkaline hydrolysis, resulting in artificially elevated whole blood sulfide levels of $100 \mu M$, instead of the $2 \mu M$ when applying a method without alkalization of the blood [27,69]. We strongly believe that the methodology to measure ALS and DLS has to be refined, in order to determine their possible physiological role. Free H_2S in blood and tissues is very low or absent. Given that virtually all in vitro studies have required a concentration of $100 \mu M$ H_2S to alter cell function and to induce a biological effect, the very low blood and tissue concentrations of free H_2S are insufficient for H_2S to serve as a blood-borne signaling molecule. Its role should be reconsidered, using validated methods to measure the various forms of H_2S. Dynamic GC might be a powerful tool to explore this field in more detail.

3.2.2. MT in blood, serum and tissues

Numerous methods, including GC, have been described for detection of H_2S. For the detection of MT and also of DMS in biological material, GC is the only appropriate method to detect and identify these VSCs. Most research comes from our group.

3.2.2.1. Free MT.
Free MT was absent in blood [10,15,28] and tissues of healthy men and rats. MT, when added to blood, was almost completely oxidized to thiosulfate and sulfate [15]. H_2S was an intermediate in this process. Only very minor amounts of DMS were formed, the latter through thiolmethylation of MT [11]. Same oxidation processes of MT were seen in cecal mucosa [12,13]. Addition of free gaseous MT to human or rat serum or plasma gave a complete binding of MT to proteins (high-molecular-weight fraction) in the form of DLM (protein–S–SCH$_3$) [15,28]. A nearly quantitative recovery of MT was obtained from DLM by addition of DTT. However, addition of free gaseous MT to whole blood did result in the formation of only 1–2% of DLM in plasma. The MT was almost completely oxidized by the red blood cells. Heme catalyzed oxidation is probably a much faster process than the formation of DLM. Same oxidation processes were found for free H_2S (see above). In in vitro experiments, we did not find any exchange between DLM in plasma and red blood cells. Incubation of the DLM-containing plasma during 2 h with the original red cell fraction did not change the concentration of the DLM in plasma.

Only a short transient existence of free MT in blood may appear when blood is subjected to large doses of MT. A very short transient existence of free MT in blood was observed after in vivo injection of large doses of MT into rats, as was concluded from the appearance of MT in the expired air in the first 2 min after injection [35]. Very small amounts of free MT (5–9 nM, just above the detection limit of 3 nM) were detected in blood of cystinotic patients, 2–3 h after oral cysteamine intake [70]. The formation of MT is an unwanted side product of cysteamine.

3.2.2.2. ALM.
This form was first detected [28] in the serum of normal persons ($0.22 \pm 0.04 \mu M$). Slightly elevated concentrations were found in serum of liver cirrhotics ($0.41 \pm 0.19 \mu M$). Preliminary experiments have revealed that ALM was always elevated ($0.4–1.9 \mu M$) in the plasma of patients with cystathionine β-synthase (CBS) deficiency [71] and in patients with isolated persistent hypermethioninemia due to glycine N-methyltransferase deficiency ($3.5 \mu M$) [72], together with elevations of S-adenosylmethionine, the most important methyl donor. Formation of ALM may well depend upon some methyl transfer reaction. No ALM was detected in rat tissues.

3.2.2.3. DLM.
This form was first detected [28] in the serum of normal persons ($0.16 \pm 0.03 \mu M$). Minor elevations of DLM were found in serum of liver cirrhotics without and with hepatic encephalopathy [73]. No differences were observed between the different grades of encephalopathy. No elevations were observed in the serum of rats and dogs with hepatic encephalopathy [35]. Because DLM is a relative measure of exposure to MT, it was concluded that the often suggested role of MT in the pathogenesis of hepatic encephalopathy is probably minor or insignificant. Highly elevated concentrations in serum, up to 41 μM, were found in patients with isolated persistent hypermethioninemia due to hepatic methionine adenosyltransferase (MAT) deficiency [9,10,36]. This was ascribed to an elevated transamination of methionine, at methionine concentrations exceeding 300–350 μM, leading to elevated MT and DLM, the latter also known as MT mixed disulfides. ALM, and its probable precursor S-adenosylmethionine, were not elevated in these patients. Both ALM (see above) and DLM are elevated in plasma of patients with both highly elevated levels of methionine and S-adenosylmethionine (DLM in CBS deficiency: $0.8–19.5 \mu M$; DLM in glycine N-methyltransferase deficiency: $29.9 \mu M$ [72]). No DLM was detected in rat tissues.

Some reports relate L-methionine toxicity with elevated transamination, leading to elevated MT levels [74,75]. Toxicity of MT has been ascribed to inhibition of certain enzymes [76,77].

3.2.3. DMS in blood, serum and tissues

Due to its neutral nature, DMS is stable in blood. Free DMS is the only VSC detected in blood of normal persons in very low concentrations (<7 nM), close to the GC detection limit [28]. Elevated blood DMS levels were found in cirrhotics (7–50 nM, [28,78]), in a patient with isolated persistent hypermethioninemia due to hepatic MAT deficiency (96 nM, [10]), in patients and with extra-oral halitosis (10–80 nM, [38]) and in cystinotic patients due to cysteamine treatment (298–617 nM, [70]). All these patients suffered from blood-borne extra-oral halitosis due to the presence of DMS in their alveolar air. No DMS was detected in tissues of rats.

3.3. VSCs in urine

Various reports describe the GC profiling of volatile compounds in urine and their application to metabolic investigations [79] but besides our research, little is known about the presence of VSCs in urine. "Normal" concentrations of free gaseous H_2S, MT and DMS are very low and generally below the GC detection limit of 3 nM. Elevated concentrations of DMS up to 1645 nM were found in patients with isolated persistent hypermethioninemia [10,36]. Elevated concentrations of MT (56 nM) and DMS (70 nM) were found in a patient with extra-oral halitosis [38], in cystinotic patients due to cysteamine treatment (MT: 15–160 nM; DMS: 160–659 nM, [70]), and in patients with CBS-deficiency (MT: 22–389 nM; DMS: 8–120 nM). Huge elevations of MT (about 11 μM) and DMS (8–23 μM) have been reported in the urine of humans after eating asparagus [80]. This urine also contained elevations of

free H_2S (own observations) and of DMDS. The pungent urinary odor was mainly ascribed to MT and was not present in all humans after eating asparagus.

ALS and DLS were absent in urine of normals. The urine always contained ALM (reference range: 1.4–5.2 mmol/mol creatinine) and DLM (1.2–4.6 mmol/mol creatinine). Elevations of ALM and DLM in blood or serum also resulted in elevated concentrations in urine: CBS deficiency with concomitant high plasma methionine: ALM: 7.5–107, DLM: 2.4–128 mmol/mol creatinine; glycine N-methyltransferase deficiency [72]: ALM: 120, DLM: 256 mmol/mol creatinine; MAT deficiency [10,36]: ALM: normal values, DLM: 4.9–992 mmol/mol creatinine.

Elevations of ALM and DLM in urine are much more pronounced than elevations in blood or serum. Storage of urine samples at $-20\,^\circ C$ showed that these forms of MT are stable for many years. Analyses in urine are therefore a powerful tool to explore and confirm metabolic disorders in methionine metabolism.

3.4. VSCs in breath

VSCs in breath are present in only the free gaseous forms. Halitosis or bad breath is a concern to millions of people [37,81]. The VSCs H_2S, MT and DMS play the main role in the cause of halitosis. Tonzetich was the great pioneer in this field [82]. The odor threshold concentrations of VSCs are extremely low, in fact they are the lowest of all chemicals known [37,40]. The thresholds of objectionability amount to 4 nM for H_2S, 0.5 nM for MT and 1 nM for DMS (1 nM = 24 ppb) [38].

The three primary methods for measuring halitosis are organoleptic measurements (sniffing), sulfide monitors and GC [83]. Research in this field got an enormous boost by the development of the Halimeter [84], a sulfide monitor which measures the sum of the VSCs. However, the Halimeter cannot differentiate between the various VSCs and is almost insensitive to DMS [38]. GC with FPD detection is by far the method of choice in halitosis research [34,37,85]. However, a standard GC is an expensive apparatus, needs trained personnel and is therefore not suited for the clinical practice. Recently, a portable gas chromatograph OralChroma™ has been marketed, to detect the VSCs [86]. This apparatus is relatively cheap ($6000) and is very easy to use. It only needs electricity and the apparatus can be used everywhere. It might become the apparatus of choice in the field of halitosis. It is a very sensitive apparatus, it can detect all three VSCs and it perfectly differentiates between intra- and extra-oral halitosis [87].

There are two forms of real halitosis, viz. intra-oral halitosis (oral malodor) and extra-oral halitosis. Most reports now agree that the most frequent sources of halitosis (80–90%) exist within the oral cavity and include bacterial reservoirs such as the dorsum of the tongue, saliva and periodontal pockets, where anaerobic bacteria degrade sulfur containing amino acids to produce the foul smelling VSCs, in particular H_2S and MT. Comparison of the concentrations of H_2S and MT in breath [38] and in the more concentrated mouth air [88] with their thresholds of objectionability lead to the conclusion that MT is the predominant causative factor of intra-oral halitosis, where patients have bad breath from the mouth but not from the nose. In extra-oral halitosis or blood-borne halitosis [37], patients have bad breath from both the mouse and the nose (alveolar air). While intra-oral halitosis is largely caused by MT and to a lesser extent by H_2S, these compounds can hardly be found in extra-oral blood-borne halitosis, where DMS is often the main cause of halitosis.

In vitro experiments have shown [15] that the thiol MT, containing a free–SH group, immediately reacts with whole blood within seconds, resulting in irreversible binding and oxidation by red blood cells, thereby preventing transportation of MT from the blood into alveolar air and thus into breath. The same holds for H_2S. Only a short transient existence of free MT in blood may appear when blood is subjected to large doses of MT. Small amounts of free MT were detected in blood (5–9 nM) and alveolar breath (2–3 nM) of cystinotic patients, 1–3 h after oral cysteamine intake [70]. Huge breath DMS levels, up to 83 nM, were measured in these patients. DMS is a neutral molecule which is stable in whole blood and can be transported from blood into alveolar air and breath. Extra-oral halitosis due to elevated breath DMS was also found in a patient with isolated persistent hypermethioninemia due to MAT deficiency (DMS: 5.86 nM; normals: 0.13–0.65 nM) [10]. In fact, the bad breath in this patient, visiting the clinic for his bad breath, put us on the scent of the new metabolic disorder of isolated persistent hypermethioninemia [36]. We recently found a new form of extra-oral blood-borne halitosis caused by DMS, probably due to a hitherto unknown metabolic disorder (breath DMS: 0.5–2.5 nM) [38]. Halitosis due to elevated DMS in the breath of cirrhotics (0.5–14.1 nM) is known as fetor hepaticus [78].

Some other volatiles found in blood-borne halitosis, e.g. acetone in diabetes, trimethylamine in the rare fish odor syndrome and allyl methyl sulfide after garlic consumption [37], are also stable in whole blood.

Breath analysis by GC or GC coupled with mass spectrometry (MS) is a non-invasive diagnostic tool which might give valuable information in some systemic diseases, metabolic disorders, cancer and some medications [37,89–91].

3.5. VSCs in feces

All three VSCs H_2S, MT and DMS are present in feces. Moore et al. [92] found that one of the major fecal odorants was MT. H_2S is the most predominant VSC in feces. It is produced in the human large intestine through bacterial metabolism, firstly by reduction of sulfate-reducing bacteria [5] and secondly by the fermentation of sulfur-containing amino acids, cysteine and methionine [4–7]. H_2S can also be formed out of MT, probably catalyzed by methanethiol oxidase [16]. Levitt's group [12,13] found that in cecal mucosa MT was first demethylated to H_2S and H_2S was then oxidized to thiosulfate and sulfate. They stated that in cecal mucosa formation of H_2S out of MT is more pronounced than methylation of MT to DMS by the enzyme thiol S-methyltransferase [11,93]. This latter enzyme is also capable of converting H_2S into MT.

The presence of the very toxic H_2S in the colon has been implicated in the pathogenesis of ulcerative colitis by Roediger et al. [94] and Levine et al. [95], although this was later disputed by the group of Roediger [96]. However, their analysis method is subjected to criticism (see below). An accurate and sensitive method for the determination of H_2S in feces is required in order to evaluate its role in bowel pathophysiology.

H_2S exists in feces in its free form and in the bound form, probably as sulfides of iron or other metal ions, to the insoluble particulate fraction of feces [97,98]. The bound H_2S is acid labile and is quantitatively released under strong acidic conditions. The free toxic form of H_2S in feces amounts to less than 5% of total H_2S [97,98] (mean total H_2S: 0.8 µmol/g wet weight [98]), resulting in a free H_2S concentration of less than 0.04 µmol/g. Moore et al. [96] reported falsely elevated free fecal H_2S concentrations of about 50% of total H_2S. For free H_2S estimation, they extracted feces with 1 M NaOH solutions and used the basic supernatant fraction, following centrifugation of the NaOH slurries, for the free H_2S estimation. The same was done by Florin [99], also leading to falsely elevated H_2S values in feces. We found that such a procedure also extracts large amounts of fecal bound H_2S (50% or more) into the NaOH solution and thus into the free H_2S fraction. In vitro in rat and human colonocytes, Roediger et al. [94] have shown that HS^- (or free H_2S) at 2 mM

inhibits butyrate oxidation and Christl et al. [100] showed that HS$^-$ at 1 mM significantly increases cell proliferation rates. These concentrations are 25–50 times higher than the experimentally found free H_2S concentration of less than 0.04 μmol/g [98], corresponding to about 0.04 mM. Under normal conditions, the free H_2S concentration is probably too low to exert a toxic effect in the colon. However, the free H_2S concentration might become elevated at high fecal sulfide levels, as was shown by us [98]. In vitro incubation of a fecal suspension with 50 mM cysteine resulted in high total H_2S levels of 66 μmol/g wet weight. Now, only 2.3 μmol/g (3.5%) was present in the bound fraction and 62.5 μmol/g (96.5%) as free H_2S. It was concluded that the bound fraction was probably limited to 2.3 μmol/g. This binding limit is dependent on the number of binding places in feces, e.g. of the amount of iron and other sulfide binding substances.

Three methods have been described for the determination of H_2S in feces, *viz.* the methylene blue (MB) method [99,101], microdistillation with ion chromatography [60] and GC with specific sulfur detection [98,102]. The spectrophotometric MB method was first developed to measure sulfide in water [103]. Florin [99] used this method for feces and claimed to distinguish between the free and bound H_2S. He found a mean total sulfide level of 0.66 μmol/g and a falsely elevated (see above) free H_2S level of 0.17 μmol/g (26% of total). This method is not suited for distinguishing between the two H_2S forms. Moreover, the MB method in fecal material is subjected to several interferences, such as the presence of interfering colored substances and the turbidity caused by particulate fecal matter. Fecal slurries were diluted 25–50 times, to circumvent this problem. Strocchi et al. [101] developed a modified MB method and diluted the fecal sample 200 times, which also lowered the sensitivity. In our hands, both methods gave a fecal sulfide detection limit of 0.4 μmol/g feces, which is by far not sensitive enough. Many fecal samples have total sulfide levels below 0.4 μmol/g. Richardson et al. [60] used microdistillation with ion chromatography. This method only measured total sulfide levels in a group of 15 volunteers (0.11–0.72 μmol/g wet weight). As expected, production of VSCs in feces is largely dependent on the diet [104,105]. Intake of protein from meat elevated total sulfide 2–4 times [60]. GC is by far the preferred method to detect very low H_2S concentrations in feces. Using our dynamic GC technique, it was possible to distinguish between free (less than 0.04 μmol/g) and bound H_2S (0.8 μmol/g) [98]. The detection limit of this GC method for fecal H_2S amounted to 0.5 nmol/g, which is about 800 times more sensitive than the MB method. Furne et al. [102] also used the reliable GC technique. Using equilibrium headspace injection, they measured a mean total H_2S value of 1.38 ± 0.46 μmol/g in fecal samples of ten healthy subjects.

Although focus in fecal VSC research is completely on the toxic H_2S, we also measured the less toxic MT and DMS in fecal material. MT also exists in feces in its free and acid-labile bound form. Although fecal total MT concentrations are smaller than those of H_2S (in 2 experiments: 3 and 12 times smaller), its free concentration (22 and 54% of total MT) was about 3 times higher than that of H_2S in both experiments. This was also seen in headspace samples above fresh fecal human material. The difference between the behavior of H_2S and MT in feces must be explained by the difference in binding strength. H_2S binds much stronger as sulfides to metal ions of, e.g. iron and zinc than MT does. This is the reason why MT has a much higher percentage of the free form (22 and 54% of total MT) than H_2S (less than 5% of total H_2S). The neutral DMS is only present in its free form. Its free concentration may vary, just as those of H_2S and MT, and is dependent on the diet used. The DMS concentration in headspace samples above fresh fecal human material ($n = 6$) was about 2 times smaller than that of MT. Small concentrations of DMDS, the oxidation product of MT, were also seen in fecal headspace samples.

Future research in this field should focus on reliable measurements of both free and bound H_2S. Thus far, the dynamic GC method is the only method to achieve these requirements [98].

3.6. VSCs in flatus

There are some excellent reviews about flatulence or flatology [106,107]. The flatus has a bad reputation because of its sound and especially of its odor. It receives more attention in the popular scatological literature than in the scientific one, as is evident from the following phrase about its noise: "Beans, beans, the musical fruit, The more you eat, the more you toot".

The total 24-h volume of flatus produced varied widely among subjects from 476 to 1491 ml. The volume of one individual flatus emission varied between 33 and 125 ml [108] or between 17 and 375 ml [109]. Most scientific publications have focused on the quantitatively important (>99%) odorless gases (oxygen, nitrogen, carbon dioxide, hydrogen, methane). The odor results from trace components. In the past, skatole and indole, ammonia, short chain fatty acids, H_2S [3] and mercaptans have been stated to contribute to the odor [92]. In a GC-MS analysis of the odor of human flatus, Moore et al. [92] found that MT was one of the major fecal odorants. In a semi-quantitative analysis of VSCs in human flatus [110], we found that H_2S, MT and DMS were present in most emissions, in concentrations high above their recognition threshold odor concentrations [40], especially for MT and DMS. Small concentrations of DMDS were present in a few emissions. It was concluded that a combination of sulfur volatiles is probably the major cause of the odor of human flatus. Concentration and composition of VSCs in the flatus of the same person varied widely, probably dependent on the diet used. Suarez et al. [111] developed a quantitative GC method to measure the VSCs in human flatus. Flatus samples were collected via a rectal tube that was connected to a gas-impermeable bag. The tube was inserted in the rectum and each passage was collected in a separate bag. H_2S was the predominant sulfur gas in flatus (1.06 ± 0.2 μM), followed by MT (0.21 ± 0.04 μM) and DMS (0.08 ± 0.01 μM). They found that H_2S showed the strongest correlation with odor intensity, followed by MT and DMS. The odors of H_2S, MT and DMS were respectively described as "rotten eggs", "decomposing vegetables" and "sweet".

We developed a bathtub method for quantitative flatus sampling (Fig. 2). This flatus is known in the popular literature as the "bathtub fart" and is the only flatus you can see by the bubble or bubbles. The VSCs in the flatus sample were measured by GC. The results are shown in Table 2.

The mean concentrations of all three VSCs in flatus were high above their perception threshold and also above their 100% recognition threshold levels. The flatus concentrations were about 200–1000 times higher than those in breath of patients with halitosis [38]. Luckily, flatus becomes normally diluted before it reaches the nose of the observer. Taking into account this dilution, it appears that the concentrations of H_2S and DMS were in most instances below their flatus perception threshold values. In contrast, the concentration of MT in flatus was mostly much higher than its flatus perception threshold. Experimental gas mixtures with an MT level above the flatus perception threshold of 50 nM had a similar pungent odor as most of the flatus emissions. It is therefore concluded that MT is mainly responsible for the foul pungent odor of flatus. In their study, Suarez et al. [111] found that H_2S had the strongest correlation with odor intensity. Their mean H_2S value was about 5 times higher than that of MT, whereas in our study these VSCs had about the same concentrations, with MT mostly somewhat higher. The composition of VSCs in flatus in our study agrees well with that found in fecal headspace (see above), also showing higher values for free MT. Possibly, the use of a diet rich in pinto beans in the Suarez study might have elevated H_2S levels. Nevertheless, the

Table 2
Odor characteristics of the VSCs in human flatus.

	H$_2$S	MT	DMS
Concentration ($n = 28$)	149 ± 56	216 ± 44	144 ± 24
(range)	(0–995)	(3–1066)	(4–166)
Flatus volume (ml, mean ± SEM)	84 ± 16 (range: 5–300 ml)		
Perception threshold[a]	0.01	0.0004	0.008
100% Recognition threshold[b]	42	1.5	4.2
Threshold of objectionability[c]	4	0.5	1
Theoretical flatus perception threshold[d]	400–800	16–32	320–640
Experimental flatus perception threshold[e]	750	50	750

Data are expressed in nM (mean ± SEM, 1 nM = 24 ppb), except for flatus volume (ml). The VSCs have been measured as described in Section 2.5.

[a] The lowest threshold from Ref. [40].
[b] Concentration at which 100% of an odor panel defined the odor as being representative of the odorant.
[c] Threshold concentration of an odorant producing an objectionable smell [38].
[d] Perception threshold times a flatus dilution factor of 4–8×10^4; see Section 2.5.
[e] See Section 2.5.

odor characteristics of H$_2$S and MT are such that even at a 5 times lower concentration for MT, MT has still a stronger foul odor than H$_2$S and should still be mainly responsible for the foul odor.

The size of the first morning flatus (193 ± 69 ml, range: 70–300 ml, $n = 7$) was significantly larger than a flatus during the day (45 ± 37 ml, range: 5–130 ml, $n = 21$), indicating that during the night, there is a considerable buildup of flatus gas in the large intestine.

Flatulence is a medical matter of no small import, e.g. in patients with a stoma where flatus is discharged uncontrolled from a stoma. Knowing the compounds responsible for the foul odor has lead to the development of devices to reduce flatus odor [112].

4. Conclusions

Sulfur is a biologically active element. The biological significance of sulfur volatiles, especially H$_2$S, has received great attention. The foul odor of the sulfur volatiles is a striking characteristic and plays a major role in bad breath, feces and flatus. Detection of these VSCs might give valuable information in some systemic diseases and metabolic disorders. Despite its highly toxic properties, H$_2$S has been lately recommended to become a new gasotransmitter (blood-borne signaling molecule), based on high concentration found in healthy tissues. However, there is much doubt about the reliability of the assay methods used. Determination of VSCs, especially that of H$_2$S, is a cumbersome procedure. Analysis of H$_2$S is often hindered by numerous artifacts, as there are: the instability of sulfide, its high volatility, its great susceptibility to oxidation, its adherence to various materials (e.g. glass), and the erroneous release of sulfide out of some rubbers used or out of the often used reagent DTT. This might lead to artificially elevated or lowered levels and explains the large discrepancy among the various reports. Moreover, most reports do not differentiate between the three most important forms: free H$_2$S, acid-labile and dithiothreitol-labile sulfide. These three forms probably have completely different biological functions and methods should distinguish between these forms. Therefore, new reliable methods should be used to measure these three forms and to elucidate the chemical structure of labile sulfide, in order to get more insight in the physiological role of the various forms. Gas chromatography can be the method of choice to measure the VSCs.

5. Abbreviations used

VSCs	volatile sulfur compounds
H$_2$S	hydrogen sulfide
MT	methanethiol
DMS	dimethyl sulfide
DMDS	dimethyl disulfide
ALS	acid-labile sulfide
DLS	dithiothreitol-labile sulfide
GC	gas chromatography
DTT	dithiothreitol
ALM	acid-labile methanethiol
DLM	dithiothreitol-labile methanethiol
HPLC	high-performance liquid chromatography
FPD	flame photometric detector
CBS	cystathionine β-synthase
MAT	methionine adenosyltransferase
MS	mass spectrometry
MB	methylene blue

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