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Original Contribution

# Isotope dilution mass spectrometry for the quantification of sulfane sulfurs

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ABSTRACT

Sulfane sulfurs are one type of important reactive sulfur species. These molecules have unique reactivity that allows them to attach reversibly to other sulfur atoms and exhibit regulatory effects in diverse biological systems. Recent studies have suggested that sulfane sulfurs are involved in signal transduction processes regulated by hydrogen sulfide ($H_2S$). Accurate and reliable measurements of sulfane sulfurs in biological samples are thus needed to reveal their production and mechanisms of actions. Herein we report a convenient and accurate method for the determination of sulfane sulfur concentrations. The method employs a triphenylphosphine derivative (**P2**) to capture sulfane sulfurs as a stable phosphine sulfide product, **PS2**. The concentration of **PS2** was then determined by isotope dilution mass spectrometry, using a $^{13}C_3$-labeled phosphine sulfide, **PS1,** as the internal standard. The specificity and efficiency of the method were proven by model reactions. It was also applied to the measurement of sulfane sulfurs in mouse tissues including brain, kidney, lung, liver, heart, spleen, and blood.

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Reactive sulfur species (RSS) are a family of sulfur-containing molecules that exist widely in biological systems. Representative examples are biothiols (such as cysteine and glutathione), S-modified cysteine adducts (such as *S*-nitrosothiols and sulfenic acids), hydrogen sulfide ($H_2S$), persulfides, and polysulfides. Many of these species play important roles in physiological and pathophysiological processes [1–3]. Among them, reactive sulfane sulfurs have received increased attention, as some of these compounds are suggested to be involved in signal transduction processes mediated by $H_2S$ [4–12]. Sulfane sulfur refers to a sulfur atom with six valence electrons but no charge (represented as $S^0$) [13–16]. Biologically relevant sulfane sulfur compounds include persulfides (R–S–SH), hydrogen polysulfides ($H_2S_n$, $n > 1$), and polysulfides (R–S–$S_n$–S–R), as well as protein-bound elemental sulfur ($S_8$) (Fig. 1). $H_2S$ and sulfane sulfurs are interchangeable; therefore they always coexist. From a chemistry perspective, sulfane sulfurs can be the metabolites of $H_2S$; they can also be the precursors of $H_2S$. Because of this property, some mechanisms of action that were originally attributed to $H_2S$ may actually be mediated by sulfane sulfurs. For example, recent work by Kimura and co-workers suggests that $H_2S$-derived sulfane sulfurs may be the actual signaling molecules [8,10]. Moreover it has been long appreciated that sulfane sulfurs have unique regulatory effects in diverse biological systems: posttranscriptional modification of transfer RNA, synthesis of sulfur-containing cofactors and vitamins, activation or inhibition of enzymes, etc. [4].

Despite the increased interest in sulfane sulfurs, fundamental questions regarding their production and functions remain to be clarified. To this end, accurate and reliable measurements of sulfane sulfurs in biological samples are needed. Currently, several methods for sulfane sulfur detection have been reported (Fig. 2). The traditional method, i.e., cyanolysis, is based on the reaction between sulfane sulfurs and cyanide ($CN^-$) under basic pH (8.5–10) to form thiocyanate ($SCN^-$), which can be further converted to ferric thiocyanate and measured by its characteristic UV absorbance at 460 nm [17]. Another method is to convert sulfane sulfurs into $H_2S$, upon treating with reductants such as dithiothreitol (DTT). The resultant $H_2S$ can be determined by chromatography methods or trapped with monobromobimane and then analyzed to deduce the concentrations of sulfane sulfurs [13]. It should be noted that monobromobimane-based methods have been used to detect individual sulfane sulfur species [18], as well as the whole sulfane sulfur pool [19]. Recently specific fluorescent probes, such as SSP2, were developed by our laboratory [20]. This method is based on a sulfane sulfur-mediated cyclization to turn on fluorescence signals. It allows the detection of sulfane sulfurs by convenient and nondestructive fluorescent measurements.

To develop other new and efficient methods for sulfane sulfur detection we have initiated a program to study the reactions of

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sulfane sulfurs. It is known that triarylphosphines ($PAr_3$) can rapidly react with sulfane sulfur species to form triarylphosphine sulfides ($S{=}PAr_3$) [21]. However this reaction has not been well-appreciated in sulfane sulfur detection. One report by Sörbo and colleagues employed a gas chromatographic method analyzing $S{=}PAr_3$ generated from sulfane sulfurs in tissue samples [22]. This work indicated the possibility of using this reaction for sulfane sulfur detection, but the method requires complicated chromatographic pretreatment of samples. We envisioned that isotope dilution mass spectrometry would be a useful application of the reaction for sulfane sulfur detection. Isotope dilution is a widely applied method for analyzing chemical substances in biological samples [23–26]. The method comprises the addition of isotope-enriched substances (as internal standards) to samples containing non-isotope-labeled analytes. Unlike traditional analytical methods, which rely on signal intensities, isotope dilution utilizes the ratios between internal standards and analytes to determine the concentrations. Another advantage of mass spectrometry is that the method does not need complex sample extraction and separation; therefore it is easy for to operate [27]. Because of these advantages, isotope dilution has been applied in the quantification of proteins, nucleosides (derived from DNA or RNA), vitamins, and other bioactive molecules [24–29]. Yet the application to sulfane sulfurs has not been reported.

Herein we propose an isotope dilution strategy for sulfane sulfurs (Fig. 3). We expected that triarylphosphine reagents could effectively react with sulfane sulfurs in biological samples to form phosphine sulfide **A.** After the reaction was completed, isotope-labeled phosphine sulfide **B** (exemplified as $^{13}C$ isotopes) would be spiked into the sample as internal standards. **A** and **B** should show identical chemical/physical properties (such as solubility, extraction efficiency, sensitivity to ionization in mass, etc.). When the mixture was subjected to mass analysis, the comparison of mass peak intensities corresponding to **A** and **B** should allow us to determine the concentration of **A** and in turn determine the original concentration of the sulfane sulfurs.

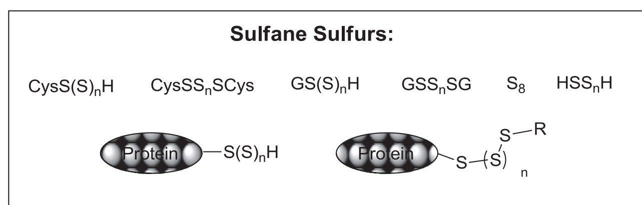


**Fig. 1.** The pool of biological sulfane sulfurs.

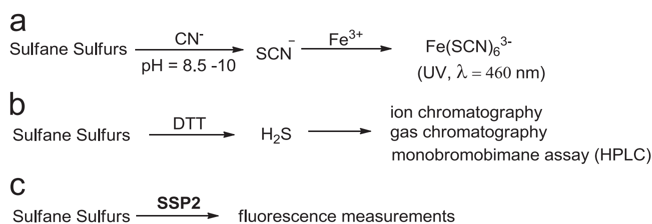


**Fig. 2.** Current methods for sulfane sulfur detection.

## Materials and methods

### *Chemicals*

All solvents and chemicals were reagent grade. L-Alanine, thionyl chloride, and Fmoc-Ala-OH-$^{13}C_3$ were purchased from Sigma–Aldrich; 4-dimethylaminopyridine and 1-hydroxybenzotriazole were purchased from Acros Organics; N,N'-dicyclohexylcarbodiimide, methyl iodide, and piperidine were purchased from Alfa Aesar. 2-(Diphenylphosphino)benzoic acid (**3**) was prepared according to a literature protocol [30].

### *Synthesis of phosphine **P1** and **P2***

Phosphine **P2** was prepared from L-alanine methyl ester through a standard coupling procedure with **3** in 54% yield [31]. $^1H$ NMR (300 MHz, $CDCl_3$) δ 1.27 (d, $J$ = 7.2 Hz, 3H), 3.72 (s, 3H), 4.64 (t, $J$ = 7.5 Hz, 1H), 6.54 (d, $J$ = 6.9 Hz, 1H), 6.95 (m, 1H), 7.41–7.25 (m, 12H), 7.64 (m, 1H); $^{13}C$ NMR (75 MHz, $CDCl_3$) δ 173.6, 168.4, 137.2, 137.1, 136.8, 134.4, 134.3, 134.0, 133.9, 130.6, 129.1, 129.0, 128.9, 128.8, 128.1, 128.0, 52.7, 48.6, 18.6; $^{31}P$ NMR δ −8.6; HRMS $m/z$ 392.1428 $[M+H]^+$. Calcd for $C_{23}H_{23}NO_3P$: 392.1416. IR 3269, 3067, 2946, 1723, 1567, 1465, 1249, 1053, 747; mp 123–124 °C.

The $^{13}C$-labeled phosphine **P1** was synthesized from $^{13}C$-labeled alanine methyl ester using the same procedure as for **P2**. The $^{13}C$-labeled alanine methyl ester was prepared from Fmoc-Ala-OH-$^{13}C_3$ in two steps: esterification with methyl iodide [32] and deprotection to remove the Fmoc group [33]. The overall yield was 23%. $^1H$ NMR (300 MHz, $CDCl_3$) δ 1.06 (m, 1.5H), 1.49 (m, 1.5H), 3.72 (d, $J$ = 3.9 Hz, 3H), 4.39 (m, 0.5H), 4.86 (m, 0.5H), 6.54 (d, $J$ = 6.6 Hz, 1H), 6.95 (m, 1H), 7.60–7.25 (m, 12H), 7.66 (m, 1H); $^{31}P$ NMR δ −8.6; MS ($ESI^+$) $m/z$ 433.3 $[M+K^+]$. IR 3270, 3057, 2946, 1728, 1565, 1460, 1249, 1052, 747; mp 124–125 °C.

### *Sulfane sulfur compounds*

Elemental sulfur and sodium tetrasulfide were purchased from Alfa Aesar. Sodium tetrathionate was purchased from Sigma–Aldrich. Other sulfane sulfur compounds such as **5, 6,** and **7** and sodium disulfide were prepared using methods described previously [18]. Compound **7** was a 1:1 mixture of trisulfide and tetrasulfide.

### *Time-dependent reactions of **P2** with sulfane sulfurs*

To a solution of **P2** (29.4 mg, 0.075 mmol) in $CH_3CN$ (20.0 ml) and phosphate buffer (5.0 ml, 100 mM, pH 7.4) was added **7** or $S_8$ (0.025 mmol). The mixture was stirred at room temperature. A 0.5-ml sample of the mixture was taken out at various time

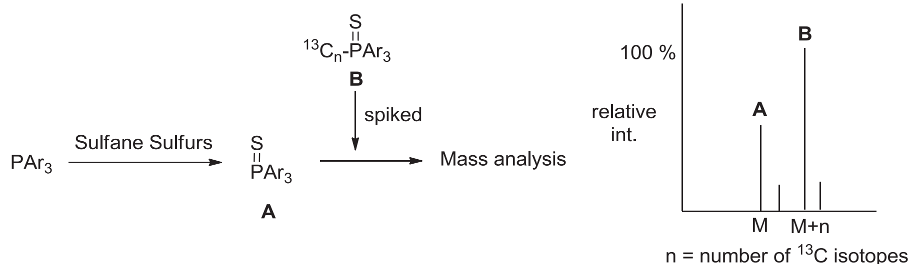


**Fig. 3.** Proposed isotope dilution mass spectrometry method for sulfane sulfur detection.

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