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Sulfur isotope analysis by MC-ICP-MS and application to small medical samples†

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We describe a technique of S isotope analysis in sulfate form with the first separation stage involving anion-exchange and the second stage of mass-spectrometric analysis by MC-ICP-MS using standard-sample-standard bracketing. Ammonium in 1 : 1 stoichiometric proportion with sulfate was used to improve transmission and stability and to avoid cone and membrane clogging by condensable species. The working resolution of ~9000 allowed the main interferences, notably ^{32}SH on ^{33}S, to be resolved. The matrix effect caused by phosphorus present in biological samples is negligible for S/P ratios ≥10: our chemical protocol allows S/P ≥ 150 to be routinely achieved. Replicate measurements of S standard solutions give values of isotopic abundances within errors of accepted values and demonstrate a reproducibility of ±0.10‰ for $\delta^{34}S$ and ±0.15‰ for $\delta^{33}S$ (2s). The technique is adequate for quantities as small as 10 nanomoles. We investigated the $\delta^{34}S$ of 110 samples of cancer patients and 10 samples of rheumatoid arthritis patients. We avoided the use of blood collection tubes with sulfate-containing heparin. Sulfur in serum is transported by albumin and fibrinogen. Most serum and plasma $\delta^{34}S$ values fall within a narrow interval of ~1‰ around a mean $\delta^{34}S_{VCDT}$ of ~6.0‰. The $\delta^{34}S$ values of total blood, serum, and plasma are very similar. Despite the short turnover time of albumin and fibrinogen, S is surprisingly well regulated. Subtle variations of 0.2–0.3‰ around the mean value can be assigned to sex and age, with sulfur in male and adult samples tending to be heavier than in their female and juvenile counterparts. This narrow range of variations across the spectrum of a large number of individuals not selected for controlled dietary habits seems paradoxical. In general, breast and prostate cancer and rheumatoid arthritis have very little effect on the average serum $\delta^{34}S$, but increase the scatter of values. We confirm that the serum of patients affected by liver cancer and other pathologies is depleted of albumin-born sulfur. While sulfur in the serum of patients with non-malignant liver pathologies tends to be isotopically light, the serum $\delta^{34}S$ of medicated hepatocellular carcinoma patients tends to be at the high end of control values.

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Introduction

Sulfur is a major element in the human body and the value of its isotopic variability as a biochemical marker of S coordination and pathways largely remains to be explored. Its turnover time, calculated as the ratio between body sulfur and diet requirement, is about 200 days, which attests that S is a fairly reactive biological component. Sulfur is present in two major amino acids, cysteine and methionine and in a critical aminosulfonic acid, taurine. Methionine is the only essential sulfur-bearing amino acid and allows cellular biosynthesis of cysteine and taurine. Taurine is the major sulfur-bearing compound of skeletal muscles. The disulfide bridge connects the two terminal S atoms of two cysteine residues. This ubiquitous bond which can be easily broken controls protein polymerization and stereochemistry. It is found in albumin, the most abundant serum protein, in fibrinogen involved in blood clotting, and in glutathione involved in intracellular redox controls. Cysteine also plays a role in intracellular metal storage through metallothionein. The sulfur concentration is ~0.3 wt% in the human body, 1200 mg l^{-1} in human serum, 300 mg l^{-1} in plasma and

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1700 mg l^{-1} in whole blood. Exploring the potential of S isotopes as a biomarker is motivated by observations: ^{34}S is enriched by a few parts per million in methionine relative to cysteine and cystine,[1] while S isotope compositions are significantly shifted in the blood of some liver cancer patients relative to controls.[2]

The standard technique for the measurement of sulfur isotope abundances, notably in geological materials, is mass-spectrometry with electron-ionization gas sources. Sulfur is first converted to SO_2 or SF_6 gas and subsequently analyzed by using a mass spectrometer. Continuous flow devices using a gas chromatograph with helium as the carrier, known as elemental analyzer-isotopic ratio mass spectrometers (EA-IRMS), have been developed, which are now considered a routine and commercially available technique.[3–7] Sample preparation is minimal, with no need for chemical separation. Precision for replicates of mineral isotopic standards measured is in the order of $\pm 0.2‰$ (2σ), but requires the extraction of a few mg of elemental sulfur.[5] It has only been in the last ten years that multiple-collector inductively coupled plasma mass spectrometry (MC-ICP-MS) has been used to measure the isotopic composition of sulfur,[8–11] improving precision over gas mass spectrometry and with only a few micrograms of elemental sulfur. Recently, Paris *et al.*[11] have shown that, by using a membrane desolvator and working at a mass resolution of 8000–10 000, $\delta^{34}S$ values of natural samples could be measured by MC-ICP-MS with a typical reproducibility of 0.08–0.15‰ (2sd) for sulfate amounts as low as 5 nmol sulfur.

For applications in biomedicine, the advantages of MC-ICP-MS, a method significantly more labor-intensive than EA-IRMS, can therefore be summarized in a few points:

(1) Small amounts of sulfur are required for a precise isotopic analysis. Smaller constituents of body fluids, such as membrane cells or the free-sulfur fraction of the plasma, can be analyzed after ultrafiltration.

(2) The $\delta^{33}S$ value can be determined with a useful precision. The correlation between $\delta^{33}S$ and $\delta^{34}S$ may be useful for identifying branched reactions and finite reservoir effects (Rayleigh distillation).[12]

(3) The possibility of measuring the isotope compositions of Fe, Cu, Zn, Ca, and S in small volumes of aliquots of serum collected during blood tests.

(4) When the isotope compositions are measured on elemental S, MC-ICP-MS does not entail uncertainties arising from the isotope composition of oxygen, or any other element, that would be part of the molecular compound, typically SO_2, analyzed by gas-source mass spectrometry.

The preliminary purpose of the present study was to develop an MC-ICP-MS method for sulfur isotope analysis as originally inspired by Craddock *et al.*,[9] which eventually turned out to be rather similar to that of Paris *et al.*[11] We strived, however, to replace elements that potentially clog the cones or the desolvator membrane (Na and Ag). A careful analysis of the results of international standards allowed us to assess the optimal fractionation law for both the instrumental bias and natural mass-dependent fractionation processes. We then proceeded to evaluate the potential of the MC-ICP-MS method for the analysis of biomedical samples, and in particular, to compare the performances of the present methods relative to those of EA-IRMS. Since the Na and K contents of blood samples are relatively high compared to the S content, a particular concern was the evaluation of the matrix effect.

We determined the S isotope compositions in over 300 human serum and plasma samples on a Neptune Plus MC-ICP-MS in the pseudo-high resolution mode, *i.e.*, a mode in which only the width of the source slit is reduced and the peak height is measured in a mass interval for which interfering species such as O_2 and SH are not significant. Samples included those from healthy 'control' individuals of both gender and of different ages. They also included samples from patients affected by different pathologies (rheumatoid arthritis, and liver, prostate, and breast cancer). We also present a limited set of results for blood and cell components. The accuracy of the method was compared with that of Paris *et al.*[11] and validated by measurements of the international standard reference materials. The typical reproducibility obtained for standard and sample solutions is similar to the one achieved by the IRMS method,[5] but the minimal amount of sulfur required for such measurements is one to two orders of magnitude lower. In this work, we also tested whether sulfur chemistry purification could be coupled with Cu, Zn, Ca, and Fe separations in order to determine the isotopic compositions of the four elements in a single sample of only 200 μl of serum or plasma.

Analytical techniques

Chemical purification

For bovine serum, human serum and plasma, the $\delta^{34}S$ values were determined in 200 μl of material. All biological samples were digested in a mixture of concentrated HNO_3 and H_2O_2 in a closed vessel on a hot plate (130 °C) for 12 hours. After evaporation to dryness the digestion was repeated to ensure full breakdown of organic matter. The four IAEA isotopic standards were dissolved in concentrated HNO_3. Sulfur was purified using one-step column chemistry on a strong anion-exchange resin according to a protocol established decades ago.[13–15] We chose anion-exchange resin to avoid the high sulfur blank caused by the sulfonic acid functional groups of the cation-exchange resin chosen by Craddock *et al.*[9] After digestion, the dried residues were taken up in diluted HNO_3 0.03 N and put through Bio-Rad columns filled with 0.8 ml of 200–400 mesh AG1-X8 anion-exchange resin in chloride form. The resin was initially washed with 10 ml of distilled water, 20 ml of HCl ± 6 N and rinsed with 40 ml of distilled water. After loading in HNO_3 0.03 N media, the matrix was removed with 10 ml of HNO_3 0.03 N and then sulfur eluted with 5 ml of HNO_3 0.5 N. 98 ± 2% of the sample sulfur is recovered after elution. The total procedural blank was ~15 ng S, which is to be compared with 50–200 μg of a typical serum sample. The effect of the chemistry blank on the results is therefore negligible. The main problematic species left after separation of sulfate from biological samples is phosphate and column fraction cuts and eluents were adjusted in such a manner that the weight S/P ratio is >20 and for the most part >100 (see below).

Mass-spectrometry measurements and data acquisition

The running conditions are described in Table 1. Sulfur isotope compositions were measured on a MC-ICP-MS Neptune Plus fitted with a Cetac Aridus II desolvator. Typical settings are described in Table 1. Sulfur has four stable isotopes, (^{32}S, ^{33}S, ^{34}S and ^{36}S), which are all affected by polyatomic isobaric interferents of higher mass than the isotopes of interest. Because of the major interference created by ^{36}Ar, ^{36}S could not be measured. Significant interference by O_2^+ occurs at all sulfur masses with a relative mass separation of $M/\Delta M$ of 1801, 1460 and 1296 relative to ^{32}S, ^{33}S and ^{34}S, respectively. Resolving the main interference $^{32}S^1H$ peak from the ^{33}S peak requires a resolution of 3908. Isotope ratios were therefore measured in the high-resolution mode ($R \sim 9000$). The central cup was positioned on the interference free plateau (Fig. 1).

The machine background was evaluated using the 'on-peak zero' protocol by measuring signal intensities for 40 s using a 0.05 N HNO_3 solution before each standard or sample analysis. The background at mass 32 is equivalent to 8 ppb S. Standard-sample-standard bracketing was used throughout to correct for instrumental mass bias using an elemental Alfa Aesar ammonium sulfate standard 'in-house' solution (batch number 61301051). Two recent studies have shown that, by using a desolvator system (DSN-100 or Aridus) coupled with a MC-ICP-MS, addition of a cation to the purified fraction of sulfur was critical to improve SO_4^{2-} transmission through the desolvation process. Paris *et al.*[11] added Na^+, while Han *et al.*[10] chose Ag^+. Here, in order to reduce the mineral load of the plasma and to minimize clogging of the cones and Aridus membrane, we opted for NH_4^+, which breaks down into non-condensable N and H. By adding variable amounts of NH_4^+, we kept the same conservative approach to determine the minimum amount of this reagent that would provide

Table 1 Neptune Plus parameters

Mass spectrometer settings	
R_f power	1200 W
Cool gas	15 l min^{-1}
Aux gas	~0.9 l min^{-1}
Sample gas	~1.1 l min^{-1}
Aridus settings	
Spray chamber temp.	110 °C
Desolvating membrane temp.	160 °C
Ar flow	~9 l min^{-1}
N_2 flow	~0.05 l min^{-1}
Nebulizer	Savillex 100 µl min^{-1}
Cones	Std sampler + H skimmer
Measurement settings	
Cup configuration	32S (L4), 33S (C), 34S (H4)
Resolution mode	High
Acquisition	40 cycles
Integration time	4 s
Uptake time	90 s
Quick wash	HNO_3 0.05 N
Quick wash time	30 s
Blank subtraction	Yes

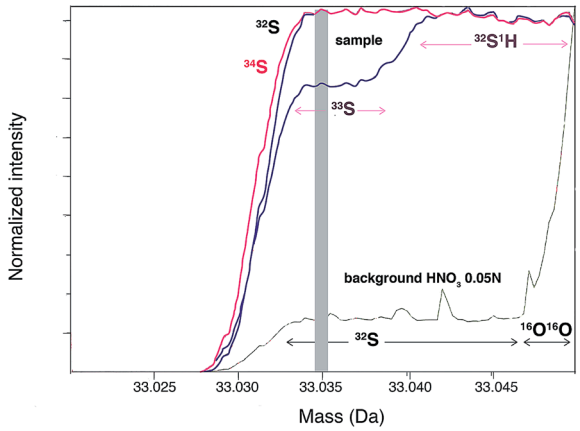

Fig. 1 Peak shapes for ^{32}S, ^{33}S and ^{34}S in the high-resolution mode for the in-house standard and the HNO_3 0.05 N cleanup solution. The shaded bar represents the interference-free mass at which isotopic ratios were measured.

reproducible and consistent $^{34}S/^{32}S$ values of our in-house standard relative to international standards. This minimum amount is obtained by adding high-purity ammonia solution in 1 : 1 NH_4 and SO_4 stoichiometric proportion (Fig. 2). Adding more ammonia would not alter the results but was found unnecessary. The concentration of sulfur and the matrix charge due to ammonium ions in sample solutions were matched with those of the bracketing standard after measuring the S contents in an aliquot of the processed samples by emission spectroscopy (Thermofisher ICAP). The reproducibility of S concentrations is <60 ppm. We analyzed solutions at a sulfur concentration of 8 mg l^{-1} with standard cones. Using the 'quick-wash' function of the Aridus II, a throughput of 48 samples in 12 hours was achieved.

In order to test the effect of the oxidation state of sulfur in the mass-spectrometer, a stoichiometric solution of ammonium persulfate (S^{7-}) solution was run without preliminary chemical separation and the results were compared with those of the same solution processed to turn sulfur into sulfate (S^{6-}). The

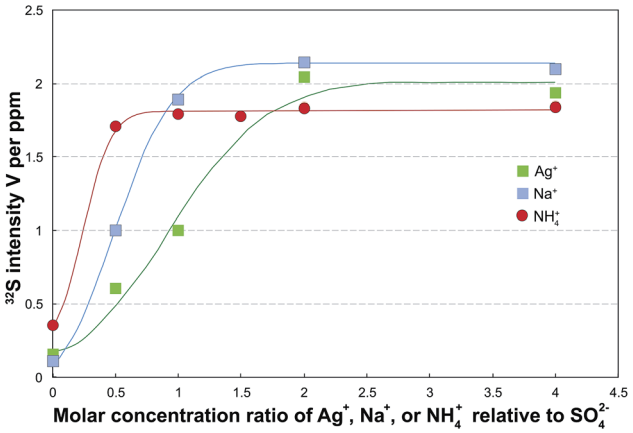

Fig. 2 Comparison of the effect of Na^+ and Ag^+ (ref. 10) and NH_4^+ (this work) on sulfate sulfur transmission by MC-ICP-MS. Equal molar proportions of ammonium and sulfate ensure the stability of the signal and make the result insensitive to a precise signal matching.

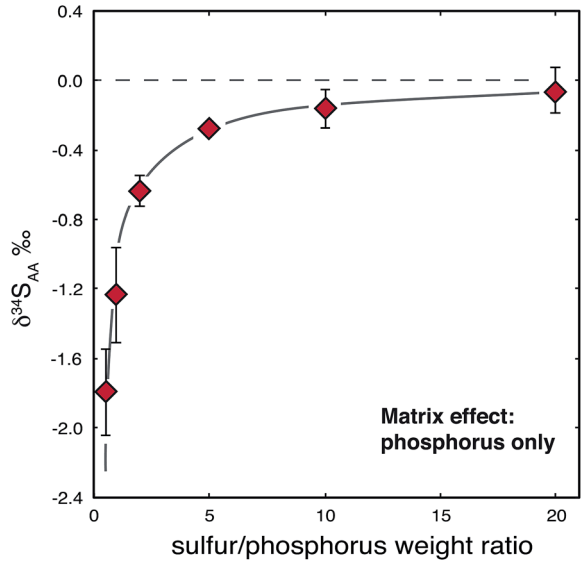

Fig. 3 Assessment of the matrix effect: ammonium phosphate was added to an AA standard solution to achieve variable weight S/P ratios. For S/P > 15, the effect on $\delta^{34}S$ is within the error limits of the measurement.

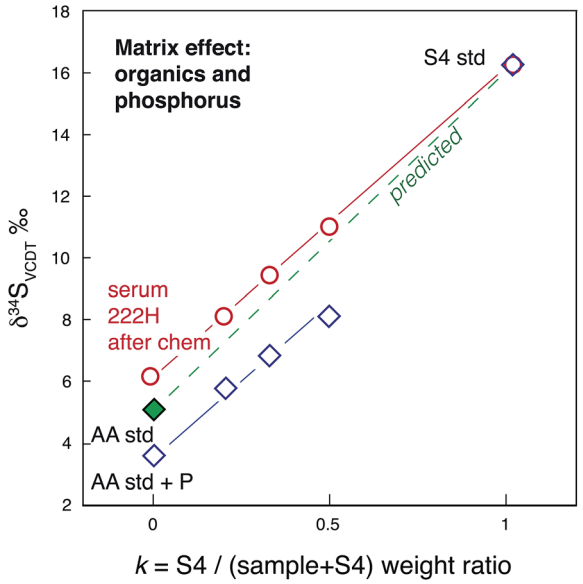

Fig. 4 Correlation between the isotope compositions of sulfur, expressed in $\delta^{34}S$, of sample serum 222H with standard reference IAEA S4 added in variable proportions ($R^2 = 0.999$). In order to assess whether a mass bias is present that would vary with the proportion of sample present, the matrix effect was exaggerated by adding ammonium phosphate to an AA standard solution used as a sample up to S/P = 1 and mixed with variable proportions of IAEA S4. The mass bias clearly departs from a linear relationship with this proportion. This observation suggests that the matrix does not affect the values measured for phosphate-free samples.

value for S^{7-} ($\delta^{34}S = 0.78 \pm 0.23‰$, $n = 5$) before chemistry was consistent with the value after chemistry ($\delta^{34}S = 0.55 \pm 0.16‰$, $n = 5$).

Phosphorus is abundant in biological materials. Since the addition of NH_4 beyond the stoichiometric proportions was found to have little effect, we set out to evaluate the effect of phosphate on the measured isotopic abundances by adding 20, 33, and 50 percent of ammonium phosphate to the Alfa Aesar standard. Severe deviation from the mixing line between $\delta^{34}S$ and the sulfur proportion was observed for S/P $\leq$ 10, which attests to the matrix effect of phosphate (see Fig. 3 and ESI†). The few samples, for which S/P < 20 was measured after the first purification step, were purified a second time, which resulted in S/P values >100.

Sulfur isotope compositions were first measured as $\delta^{34}S$ with respect to the in-house solution with $\delta^{34}S_{AA} = [(^{34}S/^{32}S)_{sample}/(^{34}S/^{32}S)_{AA} - 1]$. The $\delta^{34}S_{VCDT} = -4.89 \pm 0.14$ of the Alfa Aesar standard solution relative to the Vienna Canyon Diablo Troilite was calibrated with the reference materials IAEA S1, S2, S3 and S4 (Table 2). The complete method was used to run the standard Fetal Bovine Serum (FBS) and samples of human serum and plasma.

The effect of the biological matrix was assessed by first running different mixtures of inorganic reference material IAEA S4 and the serum sample processed for S purification before mixing. The measured S/P mass ratio of the processed serum sample 222H itself is 157. The array between $\delta^{34}S$ and the weight proportion $k = wt_{S4}/(wt_{S4} + wt_{222H})$ is linear (Fig. 4). Such a linear array would be compatible with an influence of the matrix only if the induced isotopic bias was proportional to $1 - k$. In order to assess this possibility, we exaggerated the matrix effect by adding ammonium phosphate to an AA standard solution used as a sample up to S/P = 1 and mixed with variable proportions of IAEA S4. In this experiment the matrix effect does not appear to depend on k. The linear trend defined by the mixtures of IAEA S4 and 222H therefore reflects a straightforward mixing line free of matrix influence.

We also investigated the performances of the technique when Jet cones are mounted for sulfur-poor sample applications. With these cones, the sensitivity dramatically increases to 20 V ppm^{-1} in HR so that the minimal amount required for a measurement with comparable uncertainties drops to ~10 nmol. A 0.1‰ spread of $\delta^{34}S$ values obtained for 0.5 mg l^{-1}

Table 2 Comparison between the accepted $\delta^{34}S/^{32}S_{VCDT}$ in ‰ of IAEA standards (http://nucleus.iaea.org/rpst/ReferenceProducts/ReferenceMaterials/Stable_Isotopes/34S32S/) with the values measured by MC-ICP-MS

Standard name	Accepted $\delta^{34}S/^{32}S_{VCDT}$	2s	Measured $\delta^{34}S/^{32}S_{V\text{-}CD}$	2s	N
IAEA S1 Ag_2S	−0.30	n.d.	−0.27	0.17	15
IAEA S2 Ag_2S	+22.7	0.40	+22.23	0.11	15
IAEA S3 Ag_2S	−32.30	0.40	−32.10	0.31	14
IAEA S4 S	+16.90	0.40	+16.70	0.23	16

standard solutions with the Jet cones is identical to that achieved with standard cones at 8 mg l^{-1}. We also confirmed that the isotope compositions of reference materials IAEA S1, S2, S3 and S4 measured with the Jet cones and standard cones are indistinguishable. We observed that the production of $^{32}S^{1}H$ hydride is higher when Jet cones are mounted, so that the instrument has to be run in the high-resolution mode (9000) if the value of $\delta^{33}S$ is needed. The samples analyzed in the present work are concentrated enough to be run with standard cones, but the method can be applied to sulfur-poor samples, such as cell cultures or even cell membranes, using the Jet cones with similar analytical performances.

A preliminary description of the technique may be found as a Thermo-Finnigan Application Note using the following link: http://www.thermoscientific.fr/content/dam/tfs/ATG/CMD/cmd-documents/sci-res/app/ea/icp-ms/AN-30300-ICP-MS-Sulfur-Isotope-Human-Serum-AN30300-EN.pdf.

Samples

In order to validate the technique without the problem of a biological matrix, we first analyzed inorganic IAEA reference material IAEA S1, S2, S3 (Ag_2S), and S4 (elemental S) (http://nucleus.iaea.org/rpst/ReferenceProducts/ReferenceMaterials/Stable_Isotopes/34S32S/). To this material were added two ammonium sulfate solutions used as in-house reference materials kindly provided by Dr Anne-Marie Desaulty from the Bureau de Recherches Géologiques et Minières in Orléans, France. For the sake of securing large quantities of homogeneous biological materials as in-house standards and for future interlaboratory comparison, we also analyzed two batches of Bovine Liver SRM 1577b and 1577c, soy (IA-RO-68) and tuna fish (IA-RO-69) proteins, and two batches of fetal bovine serum sold by Sigma Aldrich.

Serum samples from healthy donors were provided by Etablissement Français du Sang in Lyon. Serum samples of children were provided by Drs Justine Bacchetta et Tiphanie Ginhoux (Centre Hospitalo-Universitaire de Lyon) as part of the VITADOS project (EUDRACT number 2011-A01050-41). Serum samples from the longitudinal studies of breast and colon cancer were provided by the Centre de Ressources Biologiques of Centre Léon Bérard in Lyon. Treatment by using antineoplastic platinum drugs was ubiquitous for these samples and for those labeled GR, which belong to a longitudinal series collected from a single patient. Serum samples and resections of hepatitis and liver cancer patients were collected by the INSERM Hepatology Department in Lyon. The serum samples of rheumatoid arthritis patients were provided by the Centre Hospitalo-Universitaire Edouard Herriot de Lyon. The prostate cancer samples were provided by Hospital Lyon-Sud. The biospecimens referred to as 'Australian' were obtained from the Victorian Cancer Biobank, Australia. We analyzed by MC-ICP-MS 7 serum and 7 red blood cell samples of liver cancer patients previously analyzed by Balter *et al.*[2] by EA-IRMS. In order to test the effect of medication on sulfur isotopes, we also compared the serum $\delta^{34}S$ of patients with non-cancer pathologies and of patients already engaged in a cancer-treatment protocol. All samples received appropriate approval by the ethical study committee of each hospital.

In addition to the serum analysis, we also checked some points that potentially may have adverse effects on the results.

a. The incidence of the blood collection tubes used for analysis was investigated (see the spreadsheet labeled 'tubes' in the ESI†). Plasma was centrifuged out either in green tubes containing Li heparin through gel or in lavender tubes with EDTA and no gel. Serum was separated by centrifugation in three different ways (1) with powdered silica without (red) (2) or with gel (orange) and (3) in dry tubes (rose). Total blood was collected in either rose or lavender tubes. Because fibrinogen (M = 340 kDa) contains large concentrations of sulfur, we compared serum and plasma samples, *i.e.*, samples collected in dry tubes (red, 1 hour) and dry tubes with EDTA (lavender, 30 mn). We also tested for possible effects of blood collection tubes, notably for serum in gold tubes (gel + clotting agent) and plasma in green tubes (gel + Li heparin).

b. The difference between red blood cells and serum was also tested. Hemolysis of red blood cells in double-distilled water allowed cellular membranes, which contain measurable amounts of sulfate in the form of polysaccharides compounds such as heparan sulfate, to be separated out by centrifugation.

c. Sulfur in serum being largely concentrated in albumin (M = 66.5 kDa), we separated proteins on 50 kDa, 10 kDa, and 3 kDa filters and analyzed both the filtrate and the 50k retentate after dilution with solutions of either NaCl, PBS (phosphate buffer), or distilled water. The low molecular weight fraction, referred to as 'free sulfur', contains peptides, free amino acids and inorganic sulfate.

Ab initio calculations of relative ^{34}S enrichment by amino acids

In order to provide a framework for biochemical interpretations, we estimated equilibrium S isotope fractionation by density functional theory for sulfide, sulfate, cysteine, cystine, glutathione, methionine, and taurine (Table 3). Cysteine has a thiol end with an –SH moiety, while methionine has a thioether end with $-S-CH_3$. Sulfur in both glutathione and cystine is carried by cysteine residues. The calculations of compound

Table 3 Equilibrium $^{34}S/^{32}S$ enrichment in ‰ of different sulfur-bearing inorganic and organic species on a 1000 ln β scale (reduced partition function ratios) at 298 K. The calculations include the effect of one hydrate shell on SO_4^{2-}. $^{34}S/^{32}S$ fractionation between two species may be obtained in ‰ by subtracting their 1000 ln β values. See Fujii *et al.*[16] for details

HS^-	H_2S	Cysteine	Cystine	Glutathione	Methionine	Taurine	$SO_4^{2-}6H_2O$
4.75	11.42	16.11	17.12	15.67	20.21	71.59	73.94

structures, vibrational modes, and isotope fractionation have been adapted for S isotopes from Fujii *et al.*[16] Sulfur isotopes do not significantly fractionate between cysteine, glutathione, and cystine. Sulfur is ~4‰ heavier in methionine relative to cysteine. ^{34}S enrichment in taurine is extreme due to its sulfate moiety, as it is in the sulfate ion itself. Because of kinetic effects and composite pathways, equilibrium values cannot directly be used to infer the $\delta^{34}S$ of a particular compound, but should rather be considered a hint for the relative order of ^{34}S enrichment. Our results are in agreement with Oduro *et al.*'s[1] estimates of $^{34}S/^{32}S$ fractionation between cysteine, cystine, and methionine also obtained by using an *ab initio* technique.

Results and discussion

The complete results are reported in the ESI.† Table 4 reports the data on the standard materials.

Overall performance of the technique

Under the present conditions described in Table 1, a typical transmission of 2.5 V ppm^{-1} is achieved with standard cones in the high-resolution configuration (~9000). The two-sigma external dispersion (standard deviation) of $\delta^{34}S$ and $\delta^{33}S$ for 25 replicates of the in-house Alfa Aesar standard solution at 8 mg l^{-1} is ±0.10‰ and ±0.15‰ (2σ), respectively. The lower reproducibility of $\delta^{33}S$ relative to $\delta^{34}S$ is due to the small ^{33}S beam (~160 mV) and the narrow interference-free plateau. The reproducibility of biological samples is also affected to some extent by the presence of the remaining organic matter. Sample replicates indicate that the ±2-standard interval of replicates is typically <0.1‰ for both inorganic and biological samples (*e.g.*, FBS). The mean value of $\Delta^{33}S = \delta^{33}S - 0.515\delta^{34}S$ of the biological samples is 0.06‰ ± 0.13 ($n = 213$, 2s).

Table 5 Comparison of $\delta^{34}S$ results obtained on seven pairs of serum and red blood cell samples previously analyzed by EA-IRMS[2] and two other biomedical samples. EA-IRMS data by Iso-Analytical. 1 and 2 refer to replicate measurements

		MC-ICPMS		
Sample #	Nature	1	2	EA-IRMS
259	Serum	4.37	4.33	2.69
162	Serum	1.97	2.04	0.44
204	Serum	4.74	4.65	3.60
119	Serum	8.08	7.96	7.16
46	Serum	8.08	8.03	6.15
86	Serum	3.78	3.82	1.70
62	Serum	7.77	7.67	6.48
259	Red blood cells	4.14	4.26	3.05
162	Red blood cells	1.91	1.89	1.37
204	Red blood cells	4.04	4.02	3.23
119	Red blood cells	7.44	7.44	7.10
46	Red blood cells	6.90	6.97	5.57
86	Red blood cells	3.12	2.87	1.97
62	Red blood cells	7.42	7.52	6.93
109EA	Serum	5.63	5.58	5.70
EU	Urine	5.40	5.64	5.43

The $\delta^{34}S$ values of the biological reference material, NIST SRM 1577b, previously measured by EA-IRMS by Fry *et al.*[17] and Iso-Analytical Ltd (Crewe, UK) standards IA-RO-68 (soy protein) and IA-RO-69 (tuna fish protein) show that the MC-ICP-MS Lyon values are 0.75 to 1.0 per mil higher than EA-IRMS values (Table 5). We also regressed the values obtained by using the two techniques for 7 serum and 7 red blood cell samples[2] (Fig. 5). The slope of 1.0 shows the consistency of the results, but the IRMS values are lighter, with a mean-square shift of 1.2‰. The origin of the bias between the two methods for biological samples needs further investigation.

Table 4 $\delta^{34}S$ results on standard materials. *N* is the number of post-column replicates. For $N = 1$, the 2σ error is from standard reproducibility

Sample	Description	$\delta^{34}S_{VCDT}$	2σ	*N*
Alfa Aesar 91100979	Ammonium sulfate solution	3.76	0.20	5
Spex AF-14-163SY	Ammonium sulfate solution	14.54	0.22	5
PSA	Ammonium persulfate	0.78	0.23	5
	Same after S purification	0.55	0.16	5
SRM1577b[a]	Bovine liver	8.25	0.09	4
SRM1577c	Bovine liver	2.63	0.06	5
IA RO68[b]	Soy protein	6.01	0.13	3
IA RO69[c]	Tuna protein	20.31	0.11	5
FBS 0800211408	Fetal bovine serum	10.17	0.20	9
	Same after Cu–Zn–Fe chemistry	10.31	0.31	3
FBS 0800211408-2	Fetal bovine serum	10.11	0.11	3
FBS 0800211408-3	Fetal bovine serum	10.40	0.06	2
FBS 014M3399-1	Fetal bovine Serum	2.17	0.16	6
	Same after Cu–Zn–Fe chemistry	1.77	0.18	3
FBS 014M3399-2	Fetal bovine serum	2.15	0.03	2
	Same after Cu–Zn–Fe chemistry	2.00	0.06	2
BP3K 'free' S	$M < 3$ kDa	9.58	0.10	1
BR50K 'large proteins'	$M > 50$ kDa	9.81	0.10	1

[a] EA-IRMS $\delta^{34}S_{VCDT}$ values: 7.5 ± 0.2‰.[17] [b] EA-IRMS $\delta^{34}S_{VCDT}$ values: 5.25‰ (Iso-Analytical). [c] EA-IRMS $\delta^{34}S_{VCDT}$ values: 19.21‰ (Iso-Analytical).

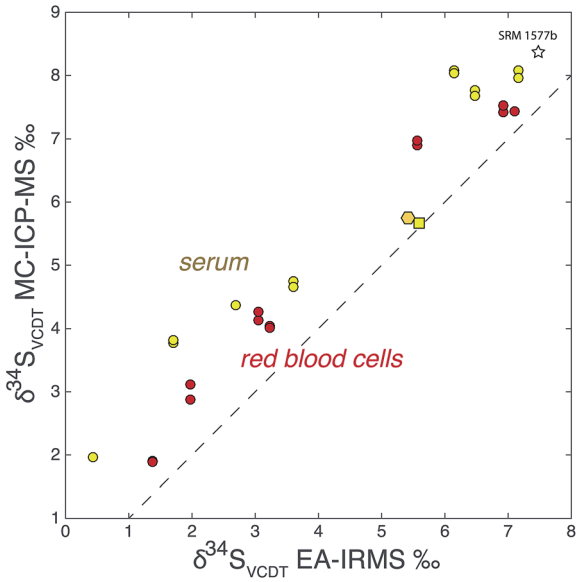

Fig. 5 Comparison between the $\delta^{34}S$ values obtained by EA-IRMS and by MC-ICP-MS. Yellow circles (serum) and red circles (red blood cells) from Balter *et al.*[2]

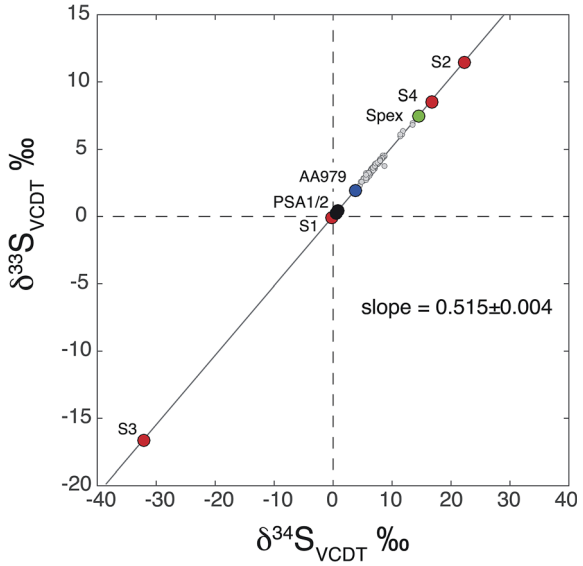

Fig. 6 Mass-dependent fractionation of the inorganic IAEA reference material (red circles), standard solutions (other colors) and biomedical samples (small grey-shaded circles). The slope is calculated from the data on inorganic samples.

For comparison, samples of fetal bovine serum were also run both before and after the column purification protocol used to purify Cu, Zn and Fe for isotope analysis and which involves anion-exchange macroporous resin.[18] The sulfur isotopic composition of the samples run after Cu–Zn–Fe separation is indistinguishable, within uncertainty, from the composition of the material that had not been submitted to separation (see Table 4 and ESI†). Prior chemical processing of the samples for Cu, Zn and Fe isotopic analysis therefore does not induce significant contamination or isotope fractionation of sulfur.

Inorganic reference materials

The four IAEA reference material samples encompass a very broad range of $\delta^{34}S$ (~60‰) but the results agree with accepted values to within 0.3‰, *i.e.*, within errors. In a $\delta^{33}S$ *vs.* $\delta^{34}S$ plot (Fig. 6), these reference samples, together with the AA979 and Spex results, define a straight-line with a slope of 0.515 ± 0.004 (at the 95 percent confidence level, $r^2 > 0.99$) indistinguishable from the literature value of the terrestrial fractionation line of modern geological materials.[19–21] Most values of $\Delta^{33}S = \delta^{33}S - 0.515\delta^{34}S$ fall in a range of ±0.05‰. Occasionally, large positive values of $\Delta^{33}S$ up to 0.2 and even 0.3‰ can be obtained, which attest to the presence of hydrides as a result of a minute drift of the magnetic field at the edge of the interference-free plateau in spite of the high resolution used for the measurement.

A related plot, $\ln(1 + \delta^{33}S)$ *vs.* $\ln(1 + \delta^{34}S)$, can be used to assess which physical process prevailed during the *natural* fractionation of the S isotopes.[22,23] The Generalized Power Law (GPL)[22] predicts that the slope $s = ({M_{33}}^n - {M_{32}}^n)/({M_{34}}^n - {M_{32}}^n)$ where n is the order of the GPL ($n = 0$ for the exponential or kinetic law, $n = -1$ for equilibrium fractionation, $n = 1$ for the power law). The slope obtained from the same samples (0.511 ± 0.004) corresponds to an apparent value of n of about −0.3 ± 0.4 (in between kinetic and equilibrium), which can be explained in different ways, notably the effect of branched reactions and Rayleigh distillation,[12] or a mixture of processes.

The effect of blood collection tubes

The effect (1 s.d.) of the choice of blood collection tubes on the measured S abundances seems to be limited to less than 0.15‰ for plasma and total blood and in most cases <0.1‰. For serum, the effect is ≤0.1‰. The tubes with heparin, a highly sulfated glycosaminoglycan used for its anti-coagulant properties, do not show any anomalous behavior.

Biomedical samples: preliminary observations

In spite of the very preliminary character of the dataset on blood components, we made the following observations:

(1) Plasma contains sulfur-rich fibrinogen while serum does not. They differ from each other and from total blood by no more than 0.3‰. The small variability of the difference may be due to the short half-life of fibrinogen in blood (4 days[24]).

(2) As previously noted by Balter *et al.*,[2] the difference in the $\delta^{34}S$ values of the seven pairs of serum and red blood cell (RBC) samples is quite small. The mean-square deviation of the difference is 0.6‰, with serum being isotopically heavier.

(3) The $\delta^{34}S$ values of RBC membranes are >1.5‰, lower than those of the cytoplasm liberated by osmotic choc in pure water.

(4) 'Free' sulfur in plasma (M < 3 kDa) is predominantly in the form of sulfate. It has isotopically 4–6‰ lighter $\delta^{34}S$ relative to sulfur from the large protein fractions (M > 50 kDa), which are dominated by albumin. The low $\delta^{34}S$ of the free sulfate suggests that it is derived from organic sulfur and not from absorbed mineral sulfate, which is expected to be much heavier. The $\delta^{34}S$ of M > 50 kDa is closer to the plasma value when the

diluting solution is NaCl and the difference is maximum for dilution with phosphate buffer (PBS).

(5) The few cases of healthy donors at hand suggest that $\delta^{34}S$ variability for a given donor varies from 0.21 to 0.85‰ for serum and 0.20 to 0.69‰ for plasma (1 s.d.)

In a $\delta^{33}S$ *vs.* $\delta^{34}S$ diagram, the biomedical samples define a straight-line with a slope of 0.519 ($r^2 = 0.94$), which is not significantly different from the slope defined by the inorganic standard solutions. The value of the slope confirms that any potential mass-independent effects should be negligible. The biomedical data scatter more than the inorganic samples do, partly because the overall spread is smaller, partly because the uncertainties in the measurement are larger.

Biomedical samples: healthy donors

Age and sex factors correlate with the $\delta^{34}S$ of healthy donors (Table 6). In general, $\delta^{34}S$ is slightly higher for younger subjects than for older subjects and for males than for females. Young donors (<17) show a large spread of $\delta^{34}S$ with values up to 7.5‰ that are not observed in other age groups. In spite of some substantial overlaps, males and females form different groups both for the young ($p = 0.02$) and the older ($p = 0.01$) donors. Young male donors rather poorly separate from their older counterpart ($p = 0.17$), in contrast with the young and older female donors ($p = 0.016$).

The explanation of these differences is unlikely to lie in the blood itself. The amino acid sequence of albumin ($M = 66.5$ kDa) contains 35 cysteine and 6 methionine residues[25] and is a major S carrier in the blood. It is synthesized in the liver and has a half-life of 19 days.[24] Fibrinogen ($M = 340$ kDa) only contains 8 cysteine and 10 methionine residues.[26]

Biomedical samples: patients

Overall, the impact of pathologies on $\delta^{34}S$ is small, although with exceptions (Table 6). Caution should be exercised when the groups consist of a very small number of samples. The most visible effect is not on the average $\delta^{34}S$ values of the populations but on standard deviations, which are doubled for breast and liver cancer and tripled for male patients with arthritis relative to controls. Serum samples from rheumatoid arthritis patients attest that, on average, inflammation has no appreciable impact. $\delta^{34}S$ values in serum samples of breast cancer patients are not significantly different ($p = 0.7$) from the values in the control group of female adults. The values of prostate cancer patients cannot be separated from those of male adult controls ($p = 0.13$). The same lack of the major isotopic shift is observed for patients affected by pre-cancer pathologies (cirrhosis, hepatitis B and C).

The $\delta^{34}S$ values of resections are substantially higher than the values of both serum and erythrocytes. One reason may be that the fraction of taurine in tissue is important relative to the fraction of amino acids[27] with taurine concentrating the heavier S isotopes (Table 3).

The *average* value of $\delta^{34}S$ in serum samples from liver cancer patients does not differ from that of the control group ($p = 0.3$). Balter *et al.*[2] reported EA-IRMS sulfur data on serum, erythrocytes, and total blood from un-medicated (naive) liver cancer patients and controls and found that the $\delta^{34}S$ values in the serum and red blood cells of some patients were significantly lower than those of the control group. A plot of $\delta^{34}S$ *vs.* S concentration (Fig. 7) elucidates some aspects of sulfur isotopes for these patients. It has long been known that albumin (~2 wt% S) is a sensitive marker of cancer progression either alone

Table 6 Statistics on the $\delta^{34}S$ values of each group of biomedical serum samples

Group	Average	2s	*n*
Control	6.1	0.9	59
Control adult	6.0	0.7	25
Control adult male	6.2	0.7	11
Control adult female	5.8	0.6	14
Control child	6.3	1.0	34
Control child male	6.4	1.1	14
Control child female	6.1	0.9	20
Arthritis	6.2	1.3	10
Arthritis male	6.2	1.9	3
Arthritis female	6.2	1.2	7
Liver cancer	6.5	1.7	45
Liver other pathologies	6.5	3.4	67
Prostate cancer	6.4	0.6	6
Breast cancer	5.9	1.3	16
GR	7.8	0.6	17
Australian	12.0	1.7	5
Total blood	6.0	0.6	7

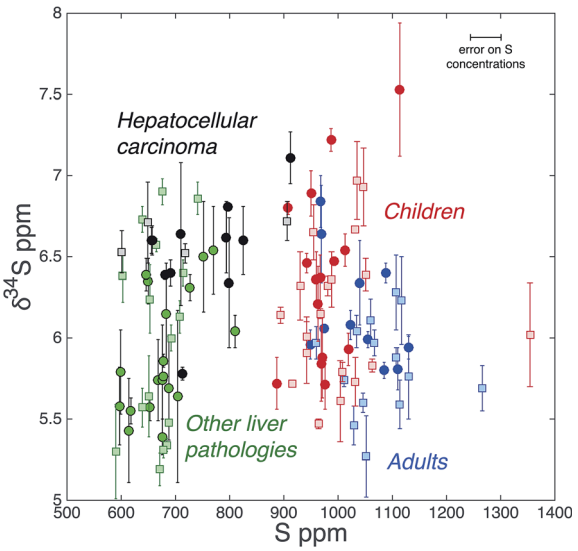

Fig. 7 Isotopic composition of sulfur *vs.* S content in the serum of patients affected by liver cancer. Errors on sulfur concentrations are smaller than the symbol size. The data are split according to three characteristics: green for hepatocarcinomes *vs.* black for other liver pathologies; for controls, blue stands for adults and red for children, while full circles refer to men and open squares to women. The low S levels in patients reflect the low albumin content. The values of $\delta^{34}S$ in the serum of hepatocellular carcinoma patients plot to the high end of 'normal' values, but the albumin levels are remarkably low relative to controls.

or together with bilirubin and prothrombin (Child-Turcotte-Pugh score):[28,29] an albumin cutoff value of 3.5 g dL^{-1} (~700 ppm S) is commonly adopted with low values being considered a marker of damage caused by liver cancer, the organ that produces and breaks down albumin. What is seen here is that the $\delta^{34}S$ of most liver patients, regardless of the pathology, tends to be at the lower end of the range of values observed for controls. In the small group of patients, $\delta^{34}S$ in serum and red blood cells of medicated cancer patients seems to plot at the high end of controls, but the serum S concentrations remain anomalous. If the $\delta^{34}S$ of cancer and arthritis patients in general does not look more abnormal, it is likely to be because most patients who are considered here are medicated and their albumin cycle restored, even if it remains overall less efficient.

We speculate that the origin of the present scatter of $\delta^{34}S$ must involve proteins that fractionate sulfur isotopes and therefore lies in the cellular balance of glutathione and methionine. Glutathione plays important roles in cancer cells:[30] it controls cellular metal cytoplasmic detoxification, protects cells from oxidative stress and maintains the redox balance between extracellular cystine and cysteine. Contrary to cysteine, which is synthesized from methionine, glutathione is synthesized from imported cystine, which depends on the x_c^- cystine/glutamate 'antiporter', a membrane transporter for the cellular uptake of cystine in exchange for intracellular glutamate.[30] With reference to the S isotope fractionation scale tabulated above, enhanced cycling of cystine relative to methionine should result in lower blood $\delta^{34}S$. How this excess is related to the type of liver disease remains to be determined.

Other anomalous but internally consistent data suggest, however, that either sample collection and preservation or treatment by platinum antineoplastic drugs, such as carboplatin, may have affected the results drastically. The $\delta^{34}S$ values of the serum samples from the small 'Australian' group are substantially higher than in any control sample.

Origin of the $\delta^{34}S = 6‰$ value

Overall, the narrow range of $\delta^{34}S$ in serum samples across hundreds of individuals with presumably variable diets (*e.g.*, French *vs.* Thai, as in Balter *et al.*[2]) represents a biochemical challenge. The range of 5‰ observed by Katzenberg and Krouse[31] for the human diet is not reflected in human blood. It is therefore likely that serum $\delta^{34}S$ is largely controlled by the food chain and that the uptake of methionine by the digestive tract is modulated by the biochemical pathways of cysteine and taurine biosynthesis in the body. Given the strong isotope fractionation caused by amino acids and taurine, the interesting possibility arises that some sulfur-containing units, possibly methionine, travel up the food web largely unchanged or fully regenerated from the primary producers to humans.

Conclusions

We described a technique of S isotope analysis involving anion-exchange separation followed by MC-ICP-MS determination. Replicate measurements of S standard solutions give values of isotopic abundances within errors of accepted values and demonstrate a reproducibility of ±0.10‰ for $\delta^{34}S$ and ±0.15‰ for $\delta^{33}S$ (2σ). The technique is adequate for quantities as small as 10 nanomoles.

Serum $\delta^{34}S$ values fall within a narrow interval of ~1‰ around a mean $\delta^{34}S_{VCDT}$ of ~6‰. In spite of the short turnover time (19 days[24]) of its main carrier, albumin, S is isotopically well regulated. Subtle variations of 0.2–0.3‰ around the mean value can be assigned to sex and age. Rheumatoid arthritis and breast and prostate cancer do not seem to impact the average serum $\delta^{34}S$ significantly but increase the scatter of values. We confirm, however, a substantial effect in the serum and the red blood cells of patients affected by liver pathologies[2].

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