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## New frontiers for sulfur isotopic analyses

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### Abstract

Measurements of sulfur stable isotope ratios ( $^{34}\text{S}/^{32}\text{S}$ ) have suffered in the past from technical difficulties in analyzing low-S materials reducing their use despite their undeniable scientific interest. The measurement of  $^{34}\text{S}/^{32}\text{S}$  ratios is a powerful tool for deciphering problems such as determining the sources of environmental pollutants, to detect adulteration, tracking the evolution of the redox state of the oceans, and quantifying the role of the bacterial activity in sulfide minerals genesis. We have used an innovative high-precision, easy and rapid method of simultaneous analysis of carbon ( $^{13}\text{C}/^{12}\text{C}$ ), nitrogen ( $^{15}\text{N}/^{14}\text{N}$ ) and sulfur ( $^{34}\text{S}/^{32}\text{S}$ ) isotope ratios as well as elemental concentrations using a new combination of elemental analyzer and isotopic ratio mass spectrometer. This new technique provides multiple isotopic signatures on smaller sample aliquots with high precisions especially for sulfur determinations. The technique described here is Isotopic Ratio Mass Spectrometry (IRMS) on line in continuous flow mode with an Elemental Analyzer (EA) based on "purge and trap" technology rather than conventional packed-GC gas separation. Emphasis is put on the efficiency of the system to reliably combust sulfur-bearing compounds of both organic and inorganic origin with high conversion yields. This new technique demonstrates the high quality of  $^{34}\text{S}/^{32}\text{S}$  measurements for samples with S concentrations lower than 1 wt%. International calibrated references of diverse sulfur-bearing materials were used to calibrate two low (<1%) S-bearing phosphorites used as compositional reference material for future use as isotopic references: BCR32 and NBS120c. High quality of  $^{34}\text{S}/^{32}\text{S}$  determinations is illustrated using various international reference materials. Working calibrated material has been selected and characterized for all three isotopic signatures as well in order to fully use the capacities of the system in future work. The possibilities of such a system to reliably measure S isotopes as well as N and C within the same aliquot of sample opens up new fields of investigation in many domains where multi-isotopic approaches are required.

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## 1. Introduction

Sulfur isotopic analyses have suffered for a long time from their high degree of technical difficulties compared to carbon or nitrogen isotopic analyses<sup>1</sup>. But beyond those technical difficulties, the interest of sulfur is that it occurs under different oxidation states (-2 to +6) and it is present under various organic and inorganic species. Sulfur at natural abundance shows four isotopic species <sup>32</sup>S (95.02%), <sup>33</sup>S (0.75%), <sup>34</sup>S (4.21%) and <sup>36</sup>S (0.02%). Due to those technical difficulties most studies concentrate on <sup>34</sup>S/<sup>32</sup>S isotopic fractionation. To illustrate the complexity of the sulfur cycle we have reported in figure 1 a schematic outline of the natural sulfur cycle from Wynn et al 2008<sup>2</sup> and P. Wynn personal communication.

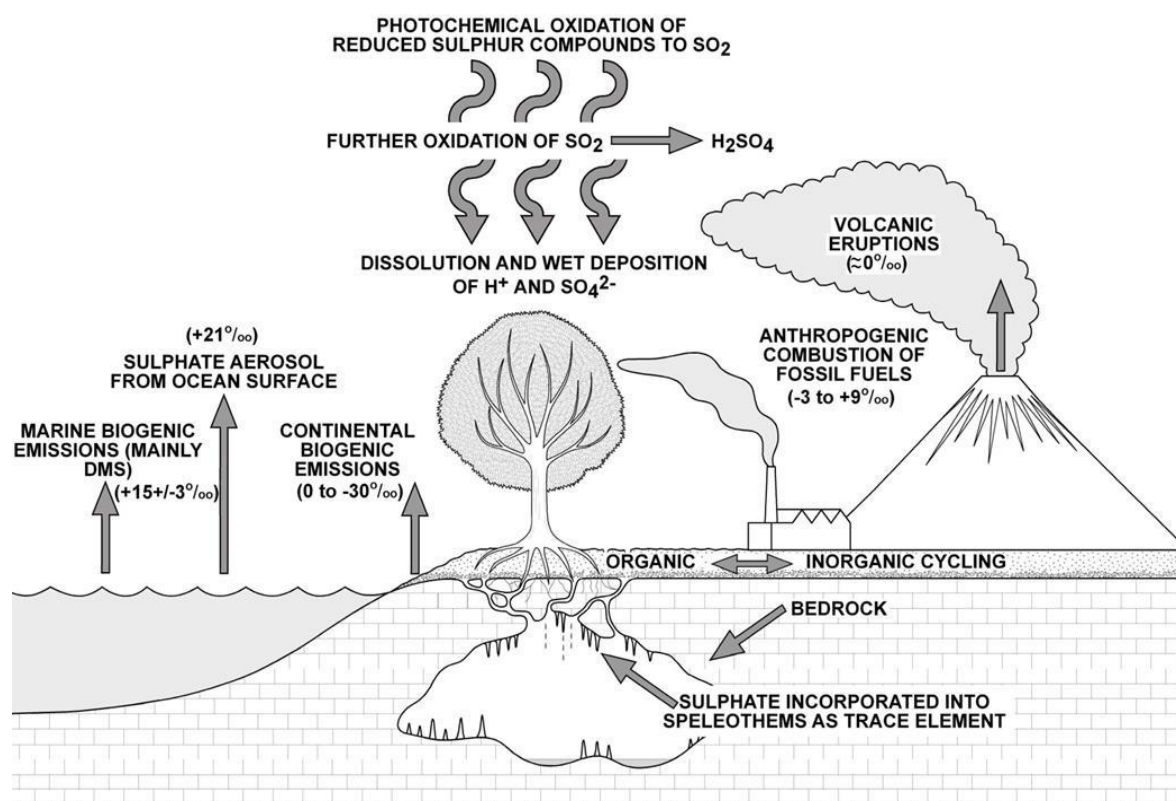


Figure 1 : From Wynn et al. 2008<sup>2</sup> : Environmental sulfur cycle schematic illustrating the complexity of the different isotopic fractionations occurring naturally.

This particular behavior of sulfur, once the technical aspect can be overcome, makes it a unique tool providing different or complementary information from conventional  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  measurements.  $\delta^{15}\text{N}$ ,  $\delta^{13}\text{C}$  and  $\delta^{34}\text{S}$  are accessible through continuous flow Elemental Analyser Isotopic Ratio Mass Spectrometry (EA-IRMS) techniques

since the early 90's usually in two steps: first the determination of  $\delta^{15}\text{N}$ ,  $\delta^{13}\text{C}$  then the determination of  $\delta^{34}\text{S}$  using different analytical setup and separating the analyte gases with a packed gas chromatography (GC) column.

The technique we present here is significantly different and uses “purge and trap” technology to separate the analyte gases which first improves significantly their separation and then allows the simultaneous determination of both  $\delta^{15}\text{N}$ ,  $\delta^{13}\text{C}$  and  $\delta^{34}\text{S}$  isotopic signatures as well as N, C and S elemental concentrations within 12 minutes.

## 2. Analytical techniques

The technical setup for NCS analyses using purge and trap technology is extensively described in Fourel et al 2014<sup>3</sup> and is illustrated in figure 1.

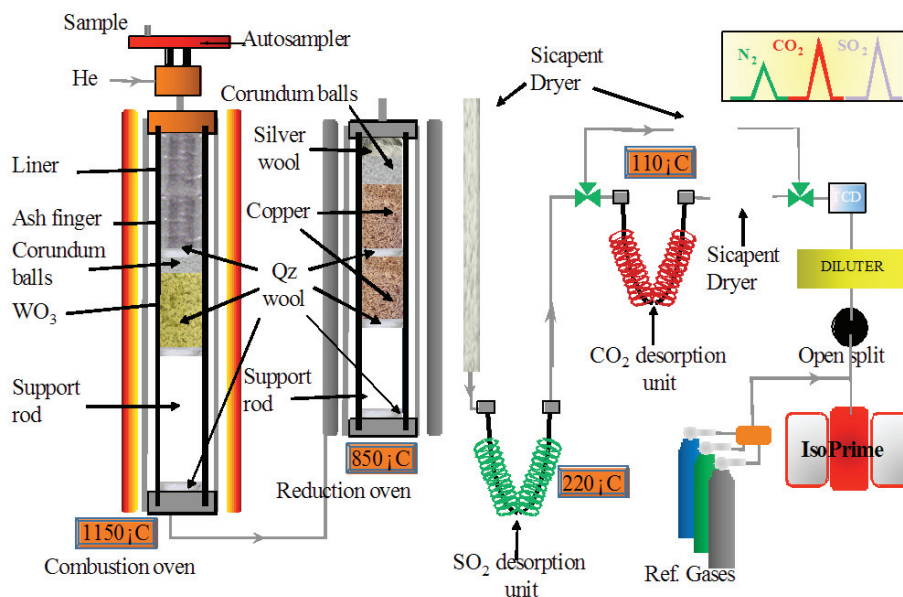


Figure 2 : Schematic principle of purge and trap EA technology configured in NCS mode. Compared to conventional EA techniques, the chromatographic column is replaced by 2 traps operating at room temperature which can then be heated to release the trapped gases.

The system we used is an Elementar Pyrocube that consists of a combustion oven using tungsten oxide as an oxidant operating at 1150°C, then a reduction oven filled with reduced copper operating at 850°C connected to a chemical water trap filled with sicapent dryer. This is similar to traditional EA setup. After the water trap, the analyte gases are no longer separated on a packed GC column but enter the original purge and trap module. N<sub>2</sub> is not trapped and is then introduced first in the source of the IRMS. CO<sub>2</sub> and SO<sub>2</sub> are then trapped respectively in 2 separate traps. The CO<sub>2</sub> trap is then heated at 110°C to release CO<sub>2</sub> followed by the SO<sub>2</sub> trap which is then heated at 220°C. Analyte gases are then introduced in the source of an IsoPrime IRMS through an open split in parallel with pulses of N<sub>2</sub>, CO<sub>2</sub> and SO<sub>2</sub> calibration gases. The resulting chromatogram is shown in figure 3.

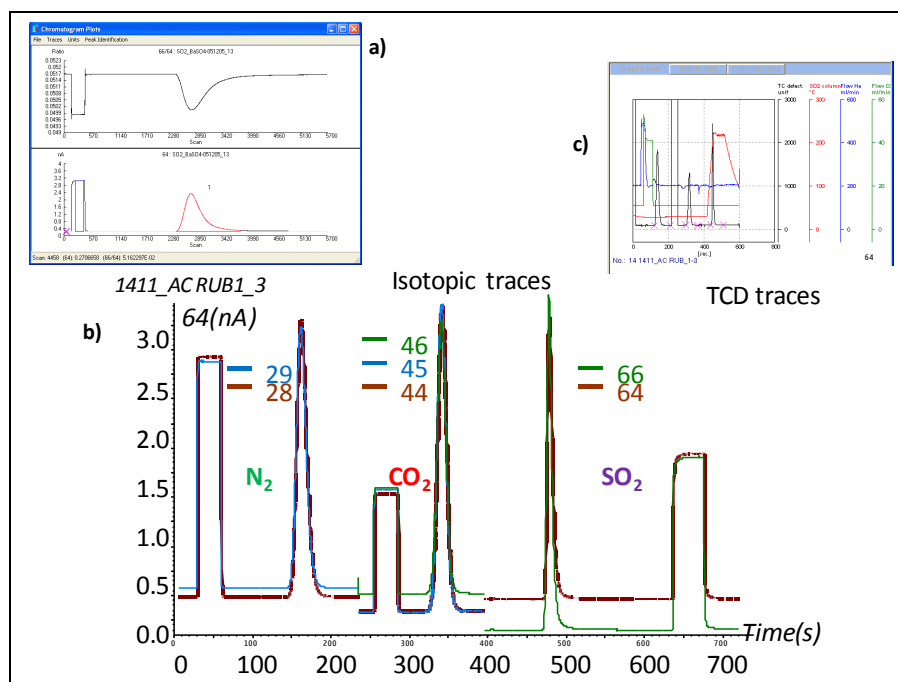


Figure 3 : Chromatograms for a typical NCS analysis using a Pyrocube purge and trap system comparing the SO<sub>2</sub> peak shape from a) conventional EA setup isotopic traces with b) purge and trap EA setup isotopic traces. SO<sub>2</sub> chromatographic peaks from masses 64 and 66 show significant improvement on the purge and trap chromatogram. C) shows the TCD traces measured from the Pyrocube software allowing the determinations of N, C and C compositions. The sample analysed here is our working calibrated material: rubeanic acid.

What is noticeable from figure 3 is the quality of the SO<sub>2</sub> chromatographic peak resulting from the purge and trap technology which has a direct impact on the quality of the isotopic measurements. This chromatogram was generated from the combustion of one of our working calibrated material: rubeanic acid1 (RUB1: C<sub>2</sub>H<sub>4</sub>N<sub>2</sub>S<sub>2</sub>). Another advantage of this technique concerns the timings for heating the traps that are user defined. It means that the quality of the chromatography is not altered in the case of samples with high C/S or C/N ratios, thus opening up new possibilities for low sulfur content samples.

### 3. Results

#### 3.1. <sup>34</sup>S/<sup>32</sup>S measurements with purge and trap EA-IRMS

Using the setup described above, we have first evaluated the quality of <sup>34</sup>S/<sup>32</sup>S measurements performed with this system. The capacity of such a system to reliably combust compounds for sulfur isotopic determinations has been evaluated and reported in Fourrel et al 2014<sup>3</sup>. Both organic and inorganic sulfur samples were mixed in various proportions to test the conversion yields. We decided to use IAEA-S1 (Silver sulfide Ag<sub>2</sub>S) and one of our working standard Ac. Rub.1 (rubeanic acid C<sub>2</sub>H<sub>4</sub>N<sub>2</sub>S<sub>2</sub>). We analysed sample amounts representing between 30μg and 350μg of S and calculated the yields based on the theoretical sulfur contents for Ag<sub>2</sub>S and C<sub>2</sub>H<sub>4</sub>N<sub>2</sub>S<sub>2</sub>. The data calculated from the TCD recording gave us yields of respectively 99.1% (SD=1.7; n=11) and 101.2% (SD=2.0, n=15) for IAEA-S1 and Ac Rub 1. This demonstrates the capacity of the system to combust S-bearing samples into SO<sub>2</sub> as well as the linearity of such combustion yields, which is an important parameter when trying to tackle samples with low S concentrations.

Three batches of isotopic measurements were performed using IAEA-S1 (Silver sulfide  $\text{Ag}_2\text{S}$ )  $\delta^{34}\text{S}_{\text{CDT}} = 0.30\%$ , IAEA-S2 (Silver sulfide  $\text{Ag}_2\text{S}$ )  $\delta^{34}\text{S}_{\text{CDT}} = 22.70\%$ , IAEA-S4 (native sulfur S)  $\delta^{34}\text{S}_{\text{CDT}} = 16.90\%$ , IAEA-S1 (Silver sulfide  $\text{Ag}_2\text{S}$ )  $\delta^{34}\text{S}_{\text{CDT}} = 0.3\%$ , IAEA-SO5 (Barium sulfate  $\text{BaSO}_4$ )  $\delta^{34}\text{S}_{\text{CDT}} = 0.5\%$ , IAEA-SO6 (Barium sulfate  $\text{BaSO}_4$ )  $\delta^{34}\text{S}_{\text{CDT}} = -34.10\%$ , and NBS127 (Barium sulfate  $\text{BaSO}_4$ )  $\delta^{34}\text{S}_{\text{CDT}} = 20.3\%$  (SD=0.4) (IAEA TECDOC-825 1995) international reference standards to evaluate the technique. The reference material was selected in order to offer isotopic values bracketing the expected values for most studied samples.

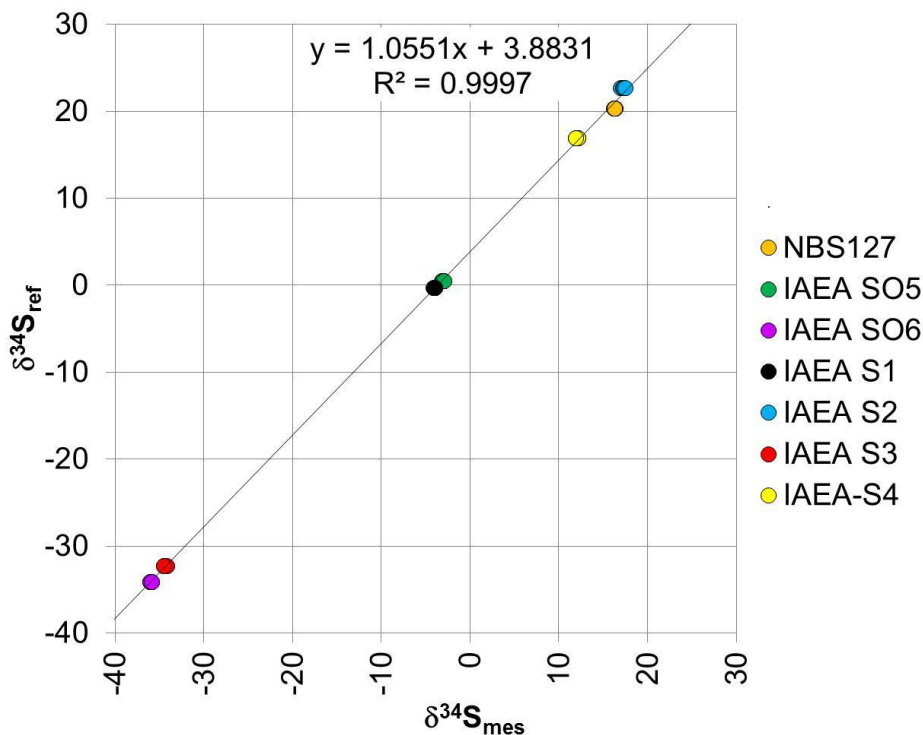


Figure 4 :  $\delta^{34}\text{S}_{\text{CDT}}$  Calibration run for NBS120c and BCR32. Working calibrated sample is Ac RUB-1 (rubeanic acid  $\text{C}_2\text{H}_4\text{N}_2\text{S}_2$  admitted value  $\delta^{34}\text{S}_{\text{CDT}} = 4.43$  (SD=0.28).

The quality of the results presented here for samples with less than 1 wt% of S allowed us to propose  $\delta^{34}\text{S}_{\text{CDT}}$  values of 18.16‰ (SD=0.31, N=23) and 18.33‰ (SD=0.39, n=20) for BCR32 and NBS120c, respectively.

### 3.2. $^{15}\text{N}/^{14}\text{N}$ , $^{13}\text{C}/^{12}\text{C}$ , $^{34}\text{S}/^{32}\text{S}$ simultaneous measurements with purge and trap EA-IRMS

An important feature of the purge and trap system is the capacity of the system to measure simultaneously the  $\delta^{13}\text{C}$ ,  $\delta^{15}\text{N}$  and  $\delta^{34}\text{S}$  values of the same sample aliquot. Up to now there has been no reference calibrated material available for such multi-isotopic analyses. In order to evaluate the quality of these simultaneous isotopic measurements, samples of aspartic acid (an amino acid of chemical formula  $\text{C}_4\text{H}_7\text{NO}_4$ ), which is used as an internal laboratory N and C calibration material, were mixed with samples of barium sulfate itself used as a calibration chemical compound for sulfur isotopic measurements (Figure 5)<sup>4</sup>. It is worthy to note that the aspartic acid had been previously calibrated against international reference material (IAEA-N1, IAEA-N2, IAEA-CH6, IAEA-CH-7, IAEA-C3, IAEA-C4). This test revealed that the isotopic reproducibility for the three isotopic ratios measured simultaneously compares well with the conventional individual determinations of  $\delta^{13}\text{C}$ ,  $\delta^{15}\text{N}$  and  $\delta^{34}\text{S}$  values (Table 1). Within the run, before and after the aliquots of our test mixture our two laboratory standards were also run individually with no significant differences between the values obtained as individual samples or mixed together.

	$\delta^{34}\text{S}_{\text{Mean}}$	$\text{SD}_{\delta^{34}\text{S}}$	N	$\delta^{15}\text{N}_{\text{Mean}}$	$\text{SD}_{\delta^{15}\text{N}}$	N	$\delta^{13}\text{C}_{\text{Mean}}$	$\text{SD}_{\delta^{13}\text{C}}$	N
WS Mixture	20.22	0.24	12	-6.49	0.18	16	-24.80	0.12	16
Ref. Val.	20.30			-6.45			-24.85		

Figure 5 : Reproducibility for  $\delta^{13}\text{C}_{\text{VPDB}}$ ,  $\delta^{15}\text{N}_{\text{AIR}}$  and  $\delta^{34}\text{S}_{\text{CDT}}$  values simultaneously determined from working laboratory calibration material (WS Mixture = aspartic acid  $\text{C}_4\text{H}_7\text{NO}_4$  mixed with NBS127 barium sulfate  $\text{BaSO}_4$  in proportion 2:1) used for N and C determinations and used for S determinations mixed together. SD = standard deviation. N = number of aliquots analysed. “Ref. Val.” are the admitted values for aspartic acid and barium sulfate calibrated samples.

### 3.3. Multiple Isotopic Approach to investigate diets of ancient Egyptians

A significant application for the multi-isotopic approach was demonstrated when we investigated diets of ancient Egyptians<sup>5</sup>. Ancient Egypt emerged at the end of the Neolithic period (6000B.P.) and is particularly renowned for its exceptional longevity. This means that throughout their evolution, the Egyptian civilization underwent various major changes such as military conquests, climatic changes such as drought and flooding, revolutions, that might have influenced the day to day life and especially nutrition of the Egyptian people. Diets for those populations are difficult to estimate as the main sources of information are figurative depiction while food remains from graves are not necessarily fully representative of the whole Egyptian population. Stable isotope analyses of human tissues can generate significant information regarding diets of ancient Egyptians. For instance, it can indicate the various proportions of plant and animal proteins, the proportions of terrestrial or aquatic food or the proportions of C3 and C4 plants.

During this study<sup>5</sup> we have determined  $\delta^{13}\text{C}$  values of hairs from ancient Egyptians. Those data are coherent with diets consisting of low amounts of C4 plant-derived food products as well as for inhabitants who lived in the Nile valley or oases despite different agricultural techniques. EA-IRMS  $\delta^{13}\text{C}$  values of hair are also coherent with  $\delta^{13}\text{C}$  determinations obtained from apatite carbonates analyzed by using acid digestion techniques. The lower  $\delta^{13}\text{C}$  values obtained for Coptic mummies might be explained by the consumption of olive oil introduced in Egypt during the Roman period.

Moreover,  $\delta^{15}\text{N}$  and  $\delta^{34}\text{S}$  values of human and animal tissues from the studied area have been represented in figure 6.  $\delta^{15}\text{N}$  variations recorded in antelopes and gazelles are interpreted as resulting more from aridity than a specific diet. Thus, the use of  $\delta^{15}\text{N}$  alone may not be sufficient for reconstructing paleo-diets but it has to be coupled with another proxy such as  $\delta^{34}\text{S}$ . Indeed, combinations of  $\delta^{15}\text{N}$  and  $\delta^{34}\text{S}$  obtained from various human and animal samples tend to illustrate that the consumption of fish such as the Nile perch remained limited as indicated by the data obtained from hairs of Coptic mummies. This study appears as a good example of the importance of the multi-isotopic approach in the field of archeology.

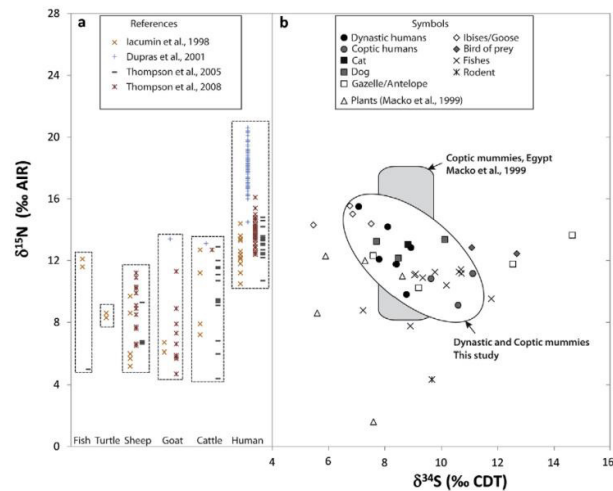


Figure 6 :  $\delta^{15}\text{N}_{\text{AIR}}$  and  $\delta^{34}\text{S}_{\text{CDT}}$  values of samples of human and animal tissues from Egypt (after Touzeau et al 2014 <sup>5</sup>).

### 3.4. High precision $\delta^{34}\text{S}$ measurements for medical applications

Another field of application for high precision  $\delta^{34}\text{S}$  determinations for samples with low (<1%) sulfur contents was investigated with natural stable isotope variations providing insights into copper and sulfur dysregulation in liver cancer <sup>6</sup>. Copper isotope determinations were done with MC-ICPMS techniques and are beyond the purpose of this article. Sulfur stable isotope compositions ( $^{34}\text{S}/^{32}\text{S}$ ) were performed using the purge and trap technology previously described. Blood and plasma samples analysed here represent another challenge for conventional isotopic NCS determinations as S concentrations are low (around 1 wt% of S) and the C concentrations are high (40 wt% of C) leading to high C/S ratios that are difficult to overcome with traditional chromatographic separations. The sulfur isotopic data are reported in figure 7.

The decrease of the sulfur isotope composition in blood of hepatocellular carcinomas HCC patients can be explained by the enhanced formation of sulfides, which  $\delta^{34}\text{S}$  values can drop down to -20‰. Tumor-derived hydrogen sulfide and allosteric disulfide bonds in cancer-related proteins are the more likely candidates, but it is still unclear how prevalent is the formation of these compounds. Considering that the sulfur content in proteins is stoichiometric and therefore constant, the blood  $\delta^{34}\text{S}$  value of HCC patients ( $\delta^{34}\text{S}_{\text{HCC}} = 4.0\text{‰}$ ) can be considered to be a mixture of the normal sulfur isotope composition ( $\delta^{34}\text{S}_{\text{N}} = 5.4\text{‰}$ ) and of total sulfides ( $\delta^{34}\text{S}_{\text{TS}} = 20\text{‰}$ )

Our data indicate that about 6% of the sulfur in the blood of HCC patients originate from tumor-derived sulfides. The use of natural variations of stable isotope compositions, a technique originating from Earth sciences, provides new insights on tumor microenvironment and allows quantifying the imbalances that are produced in the body.

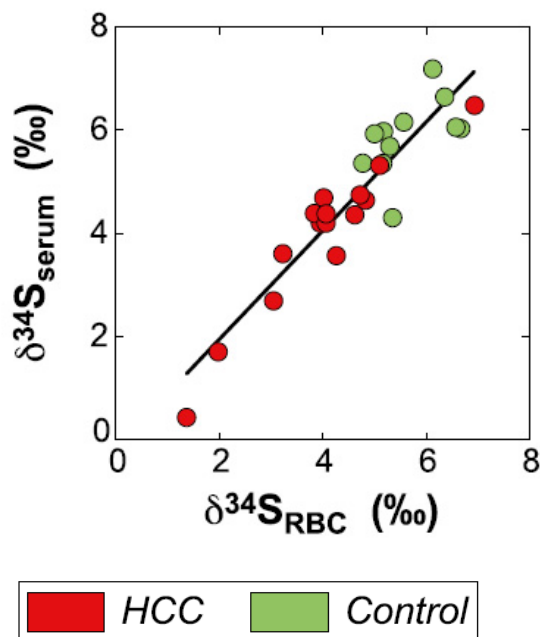


Figure 7 Sulfur isotope compositions in serum as a function of that of red blood cells. The black line stands for the least square correlation for all samples ( $y=1.05x-0.16$ ,  $R^2=0.86$ ) from Balter et al 2014 <sup>6</sup>.

#### 4. Conclusion

The results reported in this study demonstrate the capacities of the PyroCube Elemental Analyzer coupled online with an IRMS instrument to determine simultaneously  $\delta^{13}\text{C}$ ,  $\delta^{15}\text{N}$  and  $\delta^{34}\text{S}$  values from the same sample aliquot with high-levels of precision for the three isotopic systems ( $\pm 0.2\text{‰}$ ;  $\pm 0.2\text{‰}$ ;  $\pm 0.4\text{‰}$ ; respectively). This instrument opens up new possibilities for a multi-isotopic analysis of both organic and inorganic compounds without increasing either the analytical time or cost. Moreover, the results we are reporting in this study illustrate the capacities of the PyroCube system online with an IRMS instrument to reliably measure  $\delta^{34}\text{S}_{\text{CDT}}$  values of phosphorite rocks (BCR32 and NBS120c) with low S concentrations, (0.4 to 0.7 wt%), although relative large aliquots (5 to 8 mg) are required to ensure enough precision ( $\pm 0.3\text{‰}$ ). The capacities of this technique are illustrated in two different fields of applications: paleo-diet reconstruction for archaeology and for medical applications to investigate liver cancer.

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