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Does carbonate-associated sulphate record nutrition in lucinid and thyasirid bivalve shells from modern hydrocarbon seeps?

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CARBONATE-ASSOCIATED SULPHATE AND CHEMOSYMBIOSIS

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ABSTRACT

We test whether chemosymbiotic bivalves with sulphide-oxidizing bacteria record their nutritional strategy in the sulphur isotope composition of the carbonate-associated sulphate (CAS) in their shells, as a possible indicator of thiotrophic chemosymbiosis in the fossil record. The hypothesis rests on the possible incorporation of ^{34}S-depleted sulphate resulting from sulphide oxidation in sufficient quantity to affect the intra-shell sulphate-sulphur isotope mass balance and hence the isotopic composition of sulphate, which is incorporated into carbonate with little or no fractionation. We analysed shell material of lucinid (Lucinoma asapheus) and thyasirid (Thyasira vulcolutre) bivalves from active mud volcanoes in the Gulf of Cadiz. Our results show that the CAS-$\delta^{34}S$ values of the bivalve shells do not reflect the variety of sulphur sources present at hydrocarbon seeps, but instead only record seawater sulphate values. Low $\delta^{34}S$ values were, however, measured in the animals' soft tissues and shell organic matter (SOM), both displaying a strong influence of the depleted sulphide used as nutrition by the chemosynthethic bacteria. Given its potential for long-term preservation, SOM may therefore represent a more promising record of chemosymbiosis in the fossil record, while CAS from seep bivalves can be used to reconstruct local seawater sulphate.

INTRODUCTION

Carbonate-associated sulphate (CAS) is the trace sulphate incorporated into the lattice of carbonate minerals and has been shown to record the sulphur isotopic composition ($\delta^{34}S$) of the solution from which the carbonate is formed (Kampschulte & Strauss, 2004). Therefore, CAS from marine carbonates typically reflects the isotopic value of ambient seawater sulphate, currently $\delta^{34}S$ +20‰ Vienna Cañon Diablo troilite (VCDT) (Bottrell & Raiswell, 2000). However, at modern hydrocarbon seeps multiple pools of sulphur exist, which could be incorporated into carbonate. The purpose of this research is to determine the origin of seep-bivalve shell CAS, to evaluate its potential as a record of biogeochemical processes such as chemosymbiosis.

Hydrocarbon seeps are locations characterized by elevated concentrations of hydrocarbons, most commonly methane, that are emitted at an ambient temperature (Van Dover et al., 2002). This methane is produced deep within the seafloor and, when seeping upwards, the majority of the methane is anaerobically oxidized within the sediment. Anaerobic oxidation of methane (AOM) is coupled to high rates of microbial sulphate reduction, producing abundant sulphide. At sites of high methane flux, such as hydrocarbon seeps or mud volcanoes, the sulphide resulting from this reaction is depleted in ^{34}S relative to seawater sulphate by 20–40‰ (Deusner et al., 2014). The ^{34}S-depleted sulphide is utilized by sulphide-oxidizing (thiotrophic) bacteria that live in symbiosis with seep invertebrates such as bivalves, which house the bacteria in their gills. The bacteria oxidize sulphide to release energy for fixation of inorganic carbon from seawater and for the production of organic molecules to provide nutrition for the bacteria and their bivalve host (Vetter, 1991; Fisher, 1995). Transfer of sulphide to symbionts and subsequent sulphide oxidation to sulphate can occur via a number of pathways (Bruser, Lens & Truper, 2000; Taylor & Glover, 2000; Dreier et al., 2012), resulting in further ^{34}S-depletion, ranging from $\delta^{34}S$ 1–10‰ (Vetter & Fry, 1998). If this ^{34}S-depleted sulphate is incorporated into the carbonate shells of molluscs via the extrapallial fluid, CAS from chemosymbiotic bivalves will be depleted in ^{34}S relative to seawater sulphate. The presence of ^{34}S-depleted sulphate in CAS could therefore be construed as evidence of bacterial sulphide oxidation occurring within the shell, and thus a likely indicator of thiotrophic chemosymbiosis. To determine this, we analysed ^{34}S-values of the shell CAS and intercrystalline shell organic matter (SOM) of two species of deep burrowing thiotrophic bivalves belonging to the families Lucinidae (Lucinoma asapheus) and Thyasiridae (Thyasira vulcolutre) (Rodrigues et al., 2013).

MATERIAL AND METHODS

The specimens analysed were collected from active mud volcanoes in the Gulf of Cadiz (Table 1), that display relatively mild fluxes of methane and sulphide, with a methane-sulphate transition zone of

80 cm at the Mercator mud volcano (MV) and 20-55 cm at the Carlos Ribeiro MV (Van Rensbergen et al., 2005; Niemann et al., 2006). In the Gulf of Cadiz a large number of chemosymbiotic species have been identified, including 11 bivalve species from four families with chemosymbiotic members (Solemyidae, Lucinidae Thyasiridae and Mytilidae) (Rodrigues et al., 2010, 2013; Oliver et al., 2011). The collected specimens of Lucinoma asapheus and Thyasira vulcolutre show no evidence of any sulphide staining in either live or dead specimens. This is an important observation since it makes the contamination of CAS by sulphide oxidation during extraction less likely. Previously published soft-tissue values for our specimens are as follows: $\delta^{34}S$ lucinids = -15.96‰; thyasirids = -21.92 to + 1.03‰ (Rodrigues et al., 2013).

CAS and SOM extraction methods

All soft tissue was removed from the live collected specimens, and the shell material from these and dead shells was cleaned in deionised water in an ultrasonic bath for 10 min to remove surface contamination before drying overnight at 70 °C. The shell material was then powdered in an agate pestle and mortar and sieved to ensure all material was <150 µm. To extract sulphur bound to inter-crystalline organic compounds, powdered material was weighed and treated with a 5% (vol/vol) NaOCl solution overnight, before vacuum-filtering onto weighed glass-fibre filter papers (Whatman GFA). The powder was dried and the weight loss from the NaOCl extraction was determined. The sample was then dissolved in 50% (6 M) HCl to liberate the CAS. $BaSO_4$ for isotopic analysis was precipitated from both the NaOCl and HCl solutions by adjusting the pH to between 2.5 and 3 with either HCl or ammonium hydroxide before heating to about 70 °C and adding 10% of the volume of the sample solution of 100g/l BaCl solution. Whereas the HCl precipitate contains the CAS, the NaOCl precipitate represents the intercrystalline SOM. The proteinaceous SOM is present as an organic framework around and within the carbonate crystals, to guide nucleation and provide strengthening of the shell (Berman et al., 1990; Kamat et al., 2000; Marin et al., 2012). Because SOM is secreted by the mantle, it has been shown to reflect the isotopic composition of the animal's soft tissues (O'Donnell et al., 2003; Dreier et al., 2012). The amount of sulphur from each extraction was determined by gravimetry. Because $BaSO_4$ precipitates are sometimes impure, the concentration of sulphur contained in the BaSO4 precipitate was determined during the isotope analyses and used to correct the weight of sulphur recovered.

Isotope analyses were performed using a Eurovector 3028HT elemental analyser coupled to an Isoprime mass spectrometer at the University of Leeds. Between 250 and 400 µg of $BaSO_4$ were weighed into tin cups and combusted at 1020 °C in a pulse of pure oxygen (BOC, research grade N5.5) in a stream of helium (BOC, CP grade) at a flow rate of 80 ml/min. The stream of gas was passed through tungstic oxide, copper wire and magnesium perchlorate to ensure quantitative conversion to SO_2, and to remove excess oxygen and water, before passing through a 1-m

chromatographic column designed for sulphur analyses (Elemental Microanalysis, part no. E3002) held at 85 °C. The isotopic ratio of the sample gas was determined relative to a pulse of pure SO_2 reference gas (BOC, 99.9%) and calibrated to the international VCDT scale using a $BaSO_4$ internal laboratory standard SWS-3A, derived from seawater sulphate with a $\delta^{34}S$ value of +20.3‰ and an international chalcopyrite standard CP-1 with a $\delta^{34}S$ value of -4.56‰. Standards were run every 8-10 samples. The analytical precision is <0.3‰ (1 standard deviation). Sulphur isotopes are given as δ values in per mil (‰) relative to the VCDT standard.

RESULTS

The mean CAS-$\delta^{34}S$ values are +18.9 ±1.0‰ for the lucinids and +19.4 ±1.8‰ for the thyasirids (Table 2, Fig. 1), showing no significant difference between the two taxa (ANOVA: P = 0.737). A significant difference (P < 0.05) was found in the CAS concentrations (expressed as S in the whole shell powder) between the thyasirid shells (406 ± 75 ppm, mean ± one standard deviation) and the lucinid shells (206 ± 92 ppm) (ANOVA: P = 0.043). The lowest concentrations of CAS were produced by the dead collected shells of both Thyasira vulcolutre and Lucinoma asapheus, but there was no correlation between CAS concentration and CAS-$\delta^{34}S$ values. Both the amount of organic material removed from the shell by bleaching (T. vulcolutre = -1.5% mean weight loss, L. asapheus = -1.35%, ANOVA: P = 0.277) and the concentration of organic-S in the shell (ppm in untreated material: 111 ± 21ppm S for lucinids and 81 ± 67 ppm S for thyasirids, ANOVA: P = 0.159) were similar. The $\delta^{34}S$ of the SOM-bound sulphur released by the NaOCl leach for thyasirids ranged from +1.6 to +8.8‰ and for lucinids from -1.6 to +2.1‰ (no significant difference between the groups, P = 0.080), and was significantly different from CAS-$\delta^{34}S$ for both T. vulcolutre (ANOVA: P = 0.007) and L. asapheus (ANOVA: P = < 0.001). Soft tissue $\delta^{34}S$ values obtained from Rodrigues et al. (2013) (Table 1) are also distinct from the CAS-$\delta^{34}S$ values (thyasirids ANOVA: P = 0.043, lucinids ANOVA: P = 0.001) (Fig. 2).

DISCUSSION

$\delta^{34}S$ of CAS as a potential indicator of chemosymbiosis

Our results indicate that the $\delta^{34}S$-CAS in the carbonate shell of chemosymbiotic bivalves at active mud volcanoes is mostly derived from seawater sulphate and does not incorporate a significant proportion of ^{34}S-depleted sulphate from sulphide oxidation. Therefore, we conclude that $\delta^{34}S$-CAS from these bivalves does not record nutrition and cannot be used as an indicator of chemosymbiosis.

Our δ^{34}S-CAS values (range +17.6 to +21.3‰, mean +19.15 ± 1.4‰, n = 6) for the chemosymbiotic bivalves are mostly < +20‰, a little below the lower end (+20.1‰) of the range for modern bivalves reported by Kampschulte et al. (2001). It is possible that this indicates incorporation of depleted sulphate generated by symbionts, albeit a small contribution. Another possible explanation for this trend towards lower δ^{34}S-CAS values, rarely discussed in previous studies, is the incorporation of sulphur from intra-crystalline SOM. The intra-crystalline SOM is likely to share the depleted isotopic values found in soft tissues and inter-crystalline SOM and, unlike the inter-crystalline SOM, is not removed during the bleach step because it is bound within the lattice of the microcrystals.

While the organisms lived in sediments whose pore waters contained sulphate that is likely to have been affected by sulphate reduction and became enriched in ^{34}S relative to seawater, their active pumping of seawater through their burrows and shells makes it unlikely that this influences their CAS isotopic composition. This is evidenced by the relative isotopic depletion of shell CAS (+19.15 ± 1.4‰, n = 6) compared to seawater (+20.3‰). Hence, the sulphur isotopic mass balance within these chemosynthetic bivalves is determined by the relative supply of sulphate from seawater (related to shell pumping rate) and sulphate derived from symbiont-controlled oxidation of sulphide, which is in turn linked to sulphide supply and symbiont oxidation rates. Oxidation of the sulphide has a high oxygen requirement, and rapid and continuous uptake of oxygenated water have been suggested (Childress & Girguis, 2011). It is therefore very likely that the amount of ^{34}S-depleted sulphate generated by the chemosymbiotic bacteria is too small to be detected isotopically in the CAS, relative to the high concentrations of seawater sulphate. Alternatively, the isotopically depleted sulphate generated by the symbionts might not be transported through the mantle epithelia to the extrapallial fluid and therefore would not be available to become incorporated within the shell (Wilbur, 1964; Neff, 1972). While some metabolic ions are known to be incorporated (e.g. metabolic carbon can make up to 10% of the total shell carbon; Duperron et al., 2008), the biosynthetic pathways for sulphur incorporation are not well known. Instead of being conducted to the calcification site, the depleted sulphate could be treated as waste product and expelled via the posterior exhalant siphon (Lucinidae) or discharged into the sediment (Thyasiridae) (Jolly et al., 2004; Raulfs et al., 2004). A third explanation for the absence of ^{34}S-depleted sulphate is a scenario whereby the sulphide is not completely oxidized to sulphate by the bacteria, but is instead stored in the bacterial cells as elemental sulphur (Vetter, 1985; Lechaire et al., 2008) or excreted as a product of intermediate oxidation state.

δ^{34}S of SOM as a potential indicator of chemosymbiosis

Soft tissue δ^{34}S values below 5‰, as reported for the specimens analysed here, are interpreted to indicate a thiotrophic mode of nutrition (Rodrigues et al., 2013). The wide range of soft tissue δ^{34}S

values for Thyasira vulcolutre has been attributed to local and regional variability of the sulphur-isotope composition of the sulphide pool (Rodrigues et al., 2013). Previous studies have confirmed that SOM, which is secreted by the mantle, reflects the isotopic composition of the animal's soft tissues (O'Donnell et al., 2003; Dreier et al., 2012).

This study presents the first δ^{34}S-SOM values from bivalves living at active mud volcanoes, and from the Thyasiridae in general. Previously published SOM-isotope data from thiotrophic bivalves (Lucinidae and Vesicomyidae) living at hydrothermal vent sites and shallow reducing environments reported δ^{34}S-SOM values ranging from -26.7 to -2.5‰ (n = 8; Mae et al., 2007; Dreier et al., 2012, 2014) and are thus more negative than the results obtained in the present study (δ^{34}S -1.6 to 8.8‰). The isotopic difference between δ^{34}S-SOM and associated δ^{34}S-soft tissue from the published studies can be as high as 11.5‰, but with the SOM having in general isotopically lower values than soft tissue (-11.5 to -2.1‰, n = 3; Mae et al., 2007; Dreier et al., 2012), compared to the enrichment observed in this study (+7.4‰ and +23.5‰, n = 2). This difference does not correlate with variation in soft tissue δ^{34}S values and is thus unlikely to relate to isotopic differences in environmental sulphide sources. Therefore, the high δ^{34}S-SOM values obtained in this study and their enrichment compared to soft tissue data are more likely explained through species-specific biological effects or differences in SOM extraction methods, whereby fractionation could be caused by partial extraction of SOM or partial incorporation of the pool of soft tissue sulphur into SOM. Unfortunately, no published data exist on the transport mechanism of sulphur from soft tissues into SOM; with the (limited) available data it is not possible to distinguish between the two possibilities.

The δ^{13}C and δ^{18}O analyses of mollusc shell carbonate are well known to provide information about the isotopic composition of seawater dissolved-inorganic carbon and temperature, while the isotopic analyses of soft tissue and SOM provide information on nutrition. Our results conform to this pattern, as we show that δ^{34}S-CAS is only of use in reconstructing the isotopic composition of local seawater sulphate, even when analysing chemosymbiotic bivalves, but that thiotrophic nutritional strategies can be inferred from δ^{34}S-SOM of bivalves in a similar way to that of soft tissue. Given its potential for long term preservation, SOM therefore represents the more promising record of chemosymbiosis in the fossil record.

For future analyses it is important to understand the relationships between the CAS and SOM isotopic compositions and the concentrations of sulphur sources, and to develop methods of analysis that effectively separate the organic and inorganic sulphur fractions of the shell.

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Figure 1. The sulphur-isotopic composition of shell organic matter (SOM) and carbonate-associated sulphate (CAS) vs the concentrations of CAS in the shells of Thyasira vulcolutre and Lucinoma asapheus from the Gulf of Cadiz mud volcanoes. Seawater value from Bottrell & Raiswell (2000).

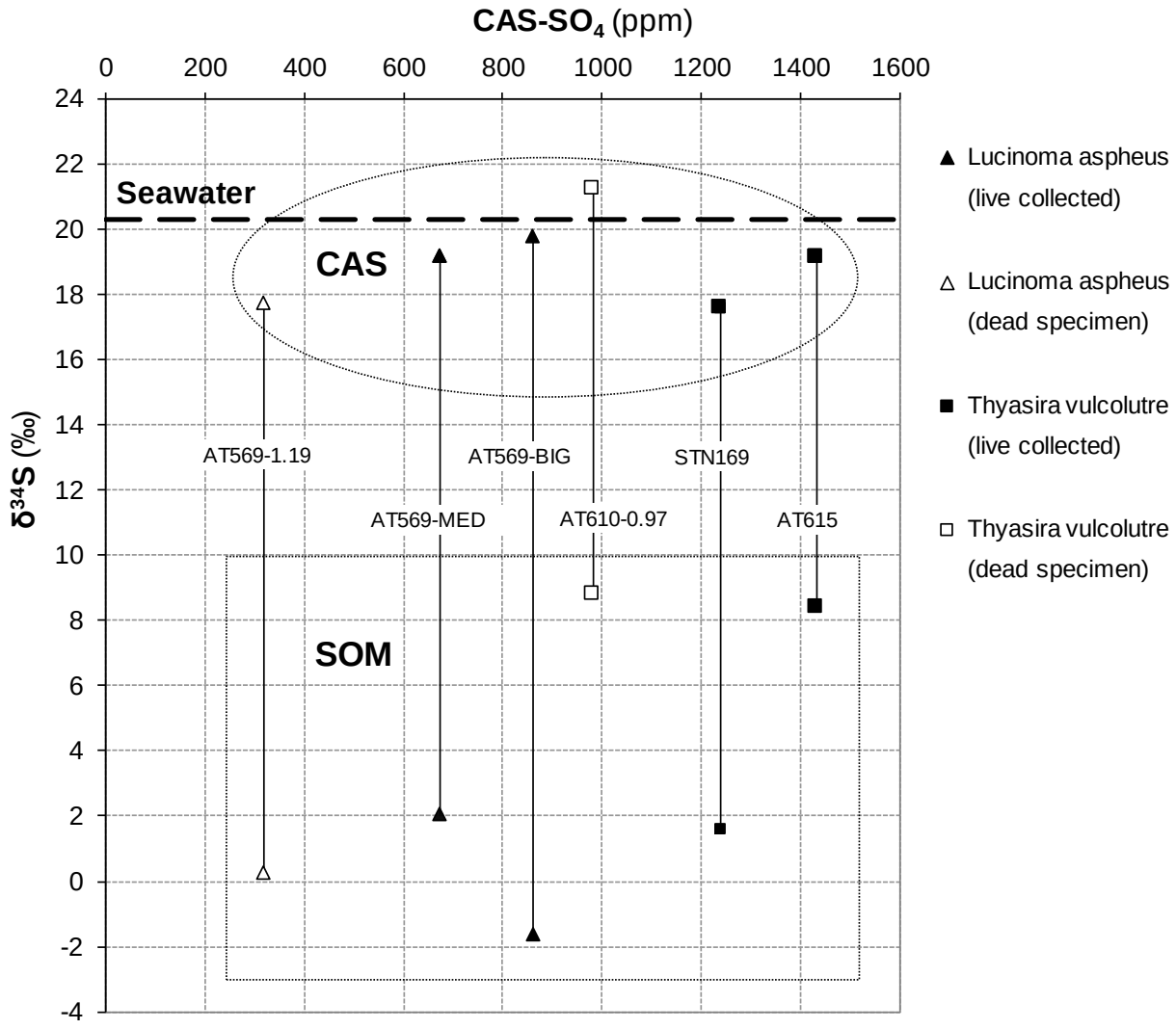
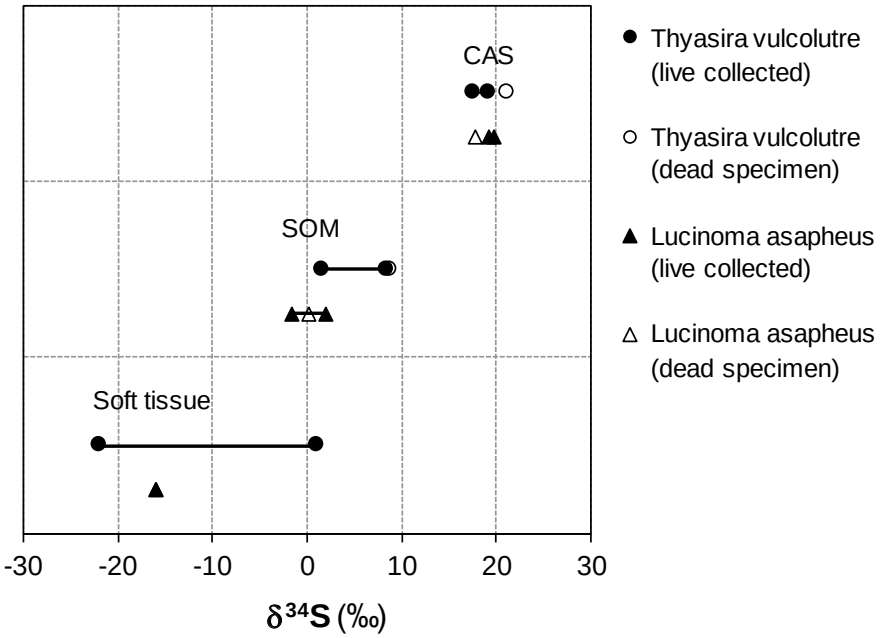


Figure 2. Range of values of CAS, SOM (both this study) and soft tissue values (Rodrigues et al., 2013) for Thyasira vulcolutre and Lucinoma asapheus from the Gulf of Cadiz mud volcanoes.



388 **Table 1.** Sample collection of shells from the Gulf of Cadiz used for this study, with associated $\delta^{34}S$
389 soft tissue values. Abbreviation: MV, mud volcano.
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Specimen code	**Species**	**Locality (depth)**	**Material**	**Weight (g)**	**Soft tissue value ($\delta^{34}S$) VCDT** (Rodrigues et al., 2013)
AT569-BIG	Lucinoma asapheus	Mercator MV, Gulf of Cadiz (358m)	Live collected specimen; both complete valves analysed	7.23	-15.96 (+/- 2.55)
AT569-MED	Lucinoma asapheus	Mercator MV, Gulf of Cadiz (358m)	Live collected specimen; single complete valve analysed	2.90	
AT569-1.19	Lucinoma asapheus	Mercator MV, Gulf of Cadiz (358m)	Dead specimen; single complete valve analysed	1.05	
AT615	Thyasira vulcolutre	Carlos Ribeiro MV, Gulf of Cadiz (2200m)	Live collected specimen; one complete and one incomplete valve analysed	0.70	1.03 (+/- 0.45)
STN169	Thyasira vulcolutre	Carlos Ribeiro MV, Gulf of Cadiz (2199m)	Live collected specimen; both complete valves analysed	0.96	-21.92 (+/- n/a)
AT615-0.97	Thyasira vulcolutre	Carlos Ribeiro MV, Gulf of Cadiz (2200m)	Dead specimen; single complete valve analysed	0.86	n/a

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Table 2. Sulphur and sulphate amounts present in the Lucinoma asapheus (L) and Thyasira vulcolutre (T) shells from the Gulf of Cadiz mud volcanoes and their carbonate-associated sulphate (CAS) and shell organic matter (SOM) sulphur isotopic values. See Table 1 for specimen codes.

Specimen code (species)	**% sample lost on bleaching**	**NaOCl-S (ppm in whole shell)**	**NaOCl-S (ppm in material removed)**	**δ^{34}S-SOM**	**CAS-S (ppm in whole shell)**	**CAS-SO_4 (ppm in whole shell)**	**δ^{34}S-CAS**
AT569-BIG (L)	1.43	107	7461	-1.6	287	861	**19.8**
AT569-MED (L)	1.73	93	5388	2.1	225	674	**19.2**
AT569-1.19 (L)	n/a	135	n/a	0.3	106	318	**17.8**
AT615 (T)	1.38	17	1260	8.4	478	1433	**19.2**
STN169 (T)	1.33	151	11418	1.6	413	1239	**17.6**
AT615-0.97 (T)	n/a	73	n/a	8.8	327	982	**21.3**