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δ^{34}S-Value Measurements in Food Origin Assignments and Sulfur Isotope Fractionations in Plants and Animals

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The δ^{34}S values of biological material, especially food commodities, serve as indicators for origin assignments. However, in the metabolism of higher plants sulfur isotope fractionations must be expected. As a matter of fact, the δ^{34}S values of the sulfate- and organic-S, respectively, of Brassicaceae and *Allium* species vegetables showed differences between 3 and 6‰, and differences in glucosinolates were between 0 and 14‰. δ^{34}S-value differences of total-S between individual tissues of the same plant were ~3‰. It is believed that these relatively small and variable fractionations are due to the partition of individual S-metabolism steps to different plant compartments, where they may occur independently and quantitatively. The δ^{34}S values of herbivore muscle meat and milk relative to the diet and between an animal and its child had trophic shifts of ~1.5‰. ^{34}S enrichments of up to 4‰ were observed for hair, hooves, and horn, an isotope fractionation of −5‰ between the diet sulfate and cartilage. Therefore, the reported agreements between δ^{34}S value of biomass and primary S sources are true for only bulk material and not for individual compounds or tissues.

KEYWORDS: Sulfur isotopes; isotope effects; isotope fractionations; δ^{34}S values; origin assignment; glucosinolates; Brassicaceae; *Allium* species; animal tissues; organic-S; sulfate-S

INTRODUCTION

Sulfur is an important bioelement, involved in many fundamental functions of life. The element is a key building block of several vitamins and coenzymes. In the form of disulfide bridges, it is essential for the secondary and tertiary structure of many proteins, it is a functional part in the active site of many enzymes, and it is indispensable in several electron transport systems. Nevertheless, its global concentration in plants is < 1% (*1*), and its average concentration in animal biomass does not exceed 2%. The largest part (90%) of the "organic-S" in plants is concentrated (via proteins) in the two amino acids cysteine and methionine (*1*), which are also the sulfur sources for most other S-containing molecules. Among these, some plants do have relatively high concentrations of secondary S-containing natural compounds with functions as repellents or attractants, often also being precursors of vegetable aromas (e.g., mustard oils) and fragrances; most well-known are the glucosinolates of Cruciferae, for example, Brassicaceae, and the disulfides and sulfoxides of *Allium* species.

Sulfur has also acquired an important role in the context of origin assignments of food and food commodities by means of isotope ratio measurements (*2*). The corresponding analyses of plant material are normally restricted to the determination of the bulk δ^{34}S value and to that of the "soluble protein"; in the case of animal samples, defatted muscle meat is the preferred material. According to the general experience, the bulk plant sulfur is depleted by only 1−2‰ relative to its primary sources, soil and sea spray sulfate or SO_2 from the atmosphere (*1*,*3*−*5*). {The ratio R of the sulfur isotopes' concentrations $^{34}S/^{32}S$ in a sample is expressed in the δ-notation relative to an international standard V-CDT (Vienna Canion Diablo Troilit) with $R = 0.0441509$ and defined as $\delta^{34}S_{sample}$ (‰) = $[(R_{sample}/R_{V\text{-}CDT}) - 1] \times 1000$.} From plant material the δ^{34}S signal is transferred to animals and not essentially altered in food chains (*6*, *7*); therefore, it is even used in investigations in nutrition research (*8*−*10*). Hence, perfunctorily seen, the plants' assimilatory sulfate reduction, the provision of "organic sulfur" from sulfate, and the sulfur metabolism in animals seem to proceed without important sulfur isotope fractionations.

However, the reactions of the plants' S-assimilation are more or less identical to those of the dissimilatory sulfate reduction (*11*), the use of sulfate as oxidant in anaerobic bacterial energy metabolisms, which is known to be accompanied by large S-isotope fractionations (*12*). This discrepancy can be understood only when the sulfate taken up by plants is either completely reduced or, in the case of incomplete reduction, partially excreted or stored, respectively (*11*). An excretion of sulfate by plants has so far not yet been observed, but Krouse et al. (*13*) found sulfate in deciduous trees, conifers, and grass, which was enriched in ^{34}S relative to the organic-S by 5−6‰. On the other hand, some plants excrete H_2S, ^{34}S-depleted relative to the S source by −15‰ (*1*).

Therefore, the role of sulfur as an indicator of origin in plant and animal biological material must be considered more carefully,

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especially with respect to the $\delta^{34}S$ values of defined fractions and compounds. Thus, the aim of the present work was to study whether and where S-isotope fractionations occur in higher plants and animals. For this purpose, samples from sources that are known to have relatively high sulfur contents or are of importance in the characterization of food commodities and in nutrition chains were investigated. The examples from plants were tissues from Brassicaceae and *Allium* species and glucosinolates; the latter compounds are of special interest in the present context, as they contain sulfur in the oxidized and in the reduced form in the same molecule. The examples chosen from animals were different tissues from beef, goat, and fish, among them cartilage, containing collagen as a carrier of "organic-S" and chondroitin sulfate as a carrier of "sulfate-S".

MATERIALS AND METHODS

Chemicals and Samples. Chondroitin sulfate (from beef trachea), sodium taurocholate, taurine, and sinigrin (from white mustard seed) originated from Fluka, Steinheim, Germany, glucotropaeolin (plant origin unknown) from Calbiochem, Bad Soden, Germany, glucoiberin (from iberis) from Cfm Oskar Tropitzsch e.K., Marktredwitz, Germany, any further glucosinolates such as gluconapin, epi-progoitrin, and progoitrin (origin and quality unknown; for details see **Figure 2**) were a kind gift of Prof. Dr. W. H. Schnitzler and Dr. G. Nitz, Lehrstuhl für Gemüsebau der Technischen Universität München. Myrosinase (EC 3.2.1.147), 262 U/g from Sigma-Aldrich, and any other chemicals originated from local sources in the highest purity available. All vegetables investigated were purchased at local markets, a rape plant was from a local field, and cress from commercial seed was grown on filter paper with distilled water. A 10-week-old kid goat (*Capra aegagrus hircus*), which had been nourished exclusively on its mother's milk, samples (details see preparations) from a 3-year-old ox (*Bos primigenius taurus*), and samples of the animals' drinking water and feed were provided from a local farm. The fish was a pike (*Esox lucius*) from the Moosach, a small tributary of the river Isar, obtained from Prof. Dr. J. Lamina, Forschungsdepartment Tierwissenschaften der Technischen Universität München.

Isolation of Vegetable Fractions for S Content and $\delta^{34}S$-Value Determinations. For the determination of the total-S content and the dry matter of the vegetables and their $\delta^{34}S$ values, ~50 g of the edible parts were, in order not to activate myrosinase or to lose volatile S compounds, cut into pieces of ~2 cm^3, which were immediately frozen and lyophilized. The dried matter was weighed and homogenized, and the powder was used directly for isotope ratio measurement and sulfur elemental analysis (see later).

For the isolation of the vegetable fractions, samples of several 100 g of the vegetable were, after the addition of some water, homogenized in an ordinary kitchen blender. The sludge was kept overnight at room temperature and then brought to pH 2.0 with 6 N HCl to complete the hydrolysis of the glucosinolates; then it was centrifuged. The precipitate, after a two-fold extraction with water, was discarded. The clear supernatants were combined, adjusted with 6 N HCl to pH 2.0, and then heated in a boiling water bath for 0.5 h. The precipitate ("soluble protein") formed was collected by centrifugation, washed three times with ~50 mL of H_2O, dried by lyophilization, and then ground for isotope ratio and integrated S elemental analysis. Although this soluble protein fraction is certainly not the total protein of the vegetable, we presume that its $\delta^{34}S$ value is representative of the whole protein of the samples (all leaf proteins are globular and must contain approximately identical Cys and Met amounts). To the supernatant of the protein fraction was added 20 mL of a 0.5 N $BaCl_2$ solution; the precipitate of $BaSO_4$ was collected by centrifugation, washed twice with a few milliliters of 6 N HCl and H_2O, respectively, and then dried with acetone. The precipitate was ground and used for the isotope ratio determination of the sulfate-S. The data of the samples together with some deduced values are compiled in **Table 1**.

Bulk and Sulfate $\delta^{34}S$ Values of Glucosinolates by Incubation with Myrosinase. For the determination of the bulk $\delta^{34}S$ value of the glucosinolates ($\delta^{34}S_T$), 0.4 mg of the sample was directly analyzed. Myrosinase (EC 3.2.1.147) is a β-glucosidase; the hydrolysis of glucosinolates by the enzyme yields unstable intermediates, which decay into sulfate and, depending on the pH and the original amino acid, isothiocyanates, thiocyanates, or nitriles and elemental-S (*15*). About 15 mg of glucosinolate was incubated in 10 mL of a 33 mM phosphate buffer, pH 6.5, for 1 h at 37 °C with 0.5 U of myrosinase. To stop the enzymatic hydrolysis, 6 N HCl was added to attain pH 2.0. To this solution was added 10 mL of 0.5 N $BaCl_2$ solution; the mixture was allowed to stand overnight, and the precipitate of $BaSO_4$ was collected by centrifugation, washed twice with a few milliliters of 6 N HCl and H_2O, respectively, and then dried with acetone. The precipitate was ground and used for the isotope ratio determination of the sulfate-S ($\delta^{34}S_S$). As the isothiocyanates are, depending on their structure, more or less volatile, the $\delta^{34}S$ value of the organic-S ($\delta^{34}S_O$) was calculated from the difference ($2\delta^{34}S_T = \delta^{34}S_S + \delta^{34}S_O$).

Glucoiberin has a third S atom in the amino acid originating side chain (S_{O2}). To determine the $\delta^{34}S$ value of all three of them, the enzymatic degradation of this glucosinolate was performed at pH 4.5, yielding sulfate (S_S), elemental-S from the thioglucose-S (S_{O1}), and the sulfur (S_{O2}) of the aglucone as a nitrile (*15*, *16*), in the present case a nonvolatile water-soluble sulfoxide. Thirty milligrams of the glucosinolate in 10 mL of 0.3 M KH_2PO_4 solution, pH 4.5, was incubated for 3 h with 0.8 U of myrosinase at 37 °C. Then the reaction was stopped by the addition of 5 mL of 2 N HCl, and the colloidal elemental S formed could be separated by centrifugation but, due to the very little amount of precipitate, not be used for a direct determination of the $\delta^{34}S$ value ($\delta^{34}S_{O1}$). The sulfate formed was precipitated, isolated, and analyzed as before. From the supernatant, the myrosinase, which also contained some sulfur and which had not been precipitated by the addition of HCl, was eliminated by centrifugation together with the residual colloidal S. For this purpose, the solution was centrifuged (Primofuge Bio R, Haereus) through a Falcon-tube (Millipore, MA), retaining any particles with MW 10000. The aqueous filtrate was lyophilized, and aliquots of the remainder (salts containing the 4-[methylsulfoxibutyl]nitrile) were submitted to isotope ratio analysis ($\delta^{34}S_{O2}$). The $\delta^{34}S$-value of the β-thioglucosinolate sulfur ($\delta^{34}S_{O1}$) resulted from the difference: $3\delta^{34}S_T = \delta^{34}S_S + \delta^{34}S_{O1} + \delta^{34}S_{O2}$.

Preparation of Animal Feed and Tissue Samples for Isotope Ratio Measurements. *Animal Feed.* To 1 L of drinking water, adjusted with 6 N HCl to pH 2.0, was added 50 mL of a 0.5 M $BaCl_2$ solution. After 12 h at 4 °C, the $BaSO_4$ formed was collected by centrifugation, dried, and used for the determination of the $\delta^{34}S$-value. Twenty milliliters of goat milk was lyophilized, the remainder being directly used for isotope analysis. Another liter of the milk was centrifuged, and the fat was skimmed off. The defatted milk was adjusted to pH 4.3 by the addition of 6 N HCl, and the solution was heated in a boiling water bath for 30 min. The precipitated

Table 1. Sulfur Amounts in Fractions from Different Vegetables (One from a "Non-Sulfur" Plant, One from *Allium* Species, and Three from Brassicaceae)[a]

		dry matter of total sample				dry matter of sample protein				$BaSO_4$ from sample		
		dry wt		sulfur (S_T)		dry wt		sulfur (S_O)		wt	sulfur (S_S)	
vegetable	fresh matter (g)	%	g	%	mg	%[b]	g	%	mg	g	%	mg
paprika (*Capsicum annuum*)	460	8.6	39.5	0.13	51	1.3	6.0	0.49	29	0.125	13.7	17
leek (*Allium porrum*)	617	13.1	80.8	0.39	315	2.2	13.6	1.49	203	0.070	13.7	10
Brussels sprouts (*Brassica oleracea* var. *gemmifera*)	549	15.8	86.7	0.49	425	4.5	24.7	0.64	158	0.323	13.7	44
broccoli (*Brassica oleracea* var. *silvestris*)	437	11.2	48.9	0.46	226	3.6	15.7	0.83	130	0.666	13.7	91
cauliflower (*Brassica oleracea* var. *botrytis*)	1121	6.3	70.6	0.39	276	2.5	28.0	0.61	171	0.347	13.7	48

[a] The total dry matter of the vegetable was determined by lyophilization, and the dry matter of the protein fraction was calculated on the basis of its concentration in fresh matter given by ref *14* (pp 764−765). [b] The sulfur contents of the samples were measured by elemental analysis simultaneously with the isotope ratio determinations (see there).

casein was isolated by centrifugation, washed twice with water, dried, and weighed in tin capsules for the S-isotope ratio analysis. The supernatant was adjusted to pH 10.0 with 5 N NaOH, and the precipitated albumin was isolated by centrifugation. After acidification of the remaining liquid with 6 N HCl, about 1 mL of 0.5 M $BaCl_2$ was added, and the $BaSO_4$ formed was isolated as before. The feed of the ox (hay and commercial concentrate) was lyophilized, ground, and homogenized; the resulting powder was used for isotope ratio analysis.

Keratin and Collagen. Twenty grams of muscle meat of the animals (ox, Musculus masseter; kid goat, Musculus biceps femoris) was minced and dried by lyophilization; the dry matter was defatted by a 6 h extraction with petroleum ether in a Soxhlet device. From the defatted residue four samples of 3 mg were used for the δ^{34}S-value analysis. Hair samples of the mother goat, the kid, and the ox were rinsed with water and then with a mixture of CH_3OH/CH_2Cl_2 (2:1). The dried material was cut by means of scissors, and 2 mg samples were analyzed. The hooves of the animals were brushed under hot water, and horn samples were cut from different sites and rasped. The chippings were used for analysis. Samples from the horn were obtained accordingly. The exact sampling sites are given in **Figure 4**. For the isolation of collagen from bones and the ox trachea (private communication from C. Lehn) crudely chopped pieces of the material were dried in vacuo and afterward defatted by a 6 h extraction with petroleum ether in a Soxhlet device. For demineralization, 10 g of the remaining ground material was stirred for 45 min in 200 mL of 1 N HCl at 20 °C. The material was precipitated by centrifugation and washed with water until neutralization. Afterward, it was digested overnight in 200 mL of 1 N HCl at 90 °C. The hot solution was cleared by filtration with a pressure-filtration system Antlia (Schleicher & Schuell, Dassel, Germany) and two layers of filters (glass fiber filter and a filter AE 98, Schleicher & Schuell). The filtrate was lyophilized and the remainder used for the determination of the δ^{34}S value. Individual fish scales were dried and analyzed as such, and fish muscle (myomers of the trunk musculature) was minced, dried by lyophilization, defatted, and ground for isotope analysis.

For the determination of the δ^{34}S value of chondroitin sulfate, cartilage from the hip joint of the goat kid and the trachea of the ox, respectively, was minced and lyophilized. The collagen of the sample was isolated and analyzed as above. The δ^{34}S value of chondroitin sulfate was obtained from the difference between that of the dried cartilage and the collagen of the sample isolated as above. [The S content of mammalian collagen is ~0.2% (*17*) and that of chondroitin sulfate, 7.4%; cartilage contains at maximum 40% of the carbohydrate (*18*). Hence, maximal 95% of the cartilage-S is sulfate-S.]

Body Fluids. The urine of the animals was collected after slaughter from the bladder by means of a syringe. After adjustment to pH 2.0 with 5 N HCl and the addition of 0.2 mL/mL 0.5 M $BaCl_2$ solution, it was kept overnight at 4 °C. The $BaSO_4$ was isolated by centrifugation, washed with water, dried with acetone, and used for the isotope ratio analysis. The bile fluid was isolated correspondingly; it was lyophilized and the remainder directly used for the ratio isotope analysis of taurocholate.

Isotope Ratio and Elemental Analysis Measurements. All samples were weighed into tin capsules (Elementar Analysensysteme GmbH, Hanau, Germany); the sample amount was adjusted, based on the expected S content, to provide approximately 30 μg of S. Each measurement was repeated four to six times, depending on the available sample amount and the reproducibility of the result; the error limits were ±0.3‰. The capsules were combusted in a device from a Vario EL III elemental analyzer (Elementar Analysensysteme GmbH) with an isotope ratio mass spectrometer (IRMS) IsoPrime of GV Instruments, Manchester, U.K., as described in ref *19*. The device provided simultaneously the elemental sulfur content of the sample via the response of a thermoconductivity detector (TCD), calibrated by means of a reference compound (Elementar Standard 05000959, error ± 5% rel). Laboratory standards (casein, barium sulfate), which had been calibrated to each other and to V-CDT, were used throughout. A correction of the δ^{34}S values for ^{18}O (*20*) was not necessary, as under the applied instrumental conditions (a pulse of an excess O_2 is injected simultaneously with the drop of the sample into the furnace) the oxygen on the SO_2 formed from any analyte originated exclusively from the combustion gas of the device. We proved this experimentally as follows: a sample of 0.5 mL of SO_2Cl_2 was hydrolyzed in 80 mL of H_2O (δ^{18}O −10‰) and a second sample in water labeled with $H_2{}^{18}O$ (δ^{18}O ≥ +120‰). The H_2SO_4 formed in both samples was precipitated as $BaSO_4$, which was then analyzed for δ^{34}S as any other samples. No difference was found between the two sulfate samples, proving that oxygen isotope characteristics were exclusively determined by the O_2 from the combustion process.

RESULTS AND DISCUSSION

Assimilatory Sulfate Reduction and S-Isotope Fractionation in Plants. *Fundamentals*. As outlined in the Introduction, the assimilatory sulfate reduction is the transformation of sulfur from sulfate [S_S] into organic-S [S_O] by reduction. After the uptake of the ion, which is assumed to not imply an isotope fractionation (*21*), the assimilation starts with the activation of SO_4^{2-} by its binding to AMP and additional phosphate transfer. The product 3′-phosphoadenosine-5′-phosphosulfate (PAPS = "active sulfate") is the starting material for the synthesis of sulfate esters and for the sulfate transfer to a carrier protein, where it is reduced to S^{2-} in two steps, which is then transferred to form

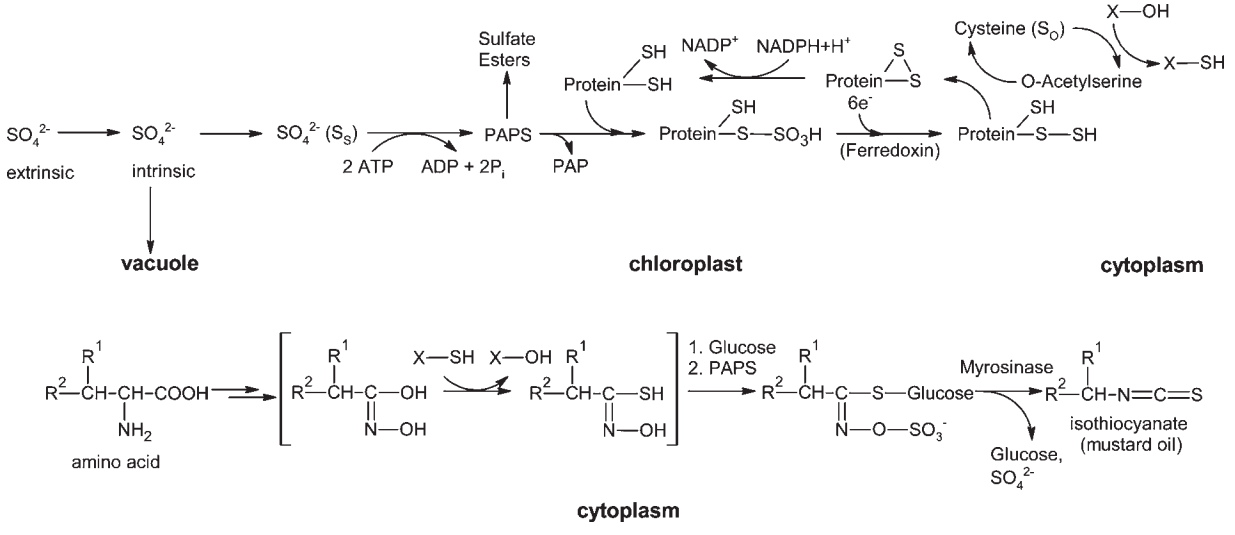

Figure 1. Main reactions of the assimilatory sulfate reduction (after ref *22*) and origin of the two sulfur atoms in glucosinolates (after ref *18*, Vol. 1, pp 390−391). S_S = sulfate sulfur, S_O = "organic (reduced) sulfur", PAPS = 3′-phosphoadenosine-5′-phosphosulfate ("active sulfate"), PAP = 3′-phosphoadenosine-5′-phosphate. X-SH is cysteine; the sulfur transfer yields (via serine?) pyruvate + NH_3, here symbolized as X−OH. Isotope fractionations are mainly expected for the sulfate activation and the consumption of active sulfate.

Table 2. Sulfur Amounts in the Total (S_T), Protein = Organic (S_O), and Sulfate (S_S) Fractions of Different Vegetables (Calculated from Data of **Table 1**) and Corresponding δ^{34}S Values$_{V\text{-}CDT}$[a]

	S amount in fraction (mg/100 g of fresh matter)				δ^{34}S values$_{V\text{-}CDT}$ (‰)			isotopic shifts ($\Delta\delta_{V\text{-}CDT}$) (‰)	
vegetable	S_T	S_O	S_S	SO_4^{2-} reduction (%)	$\delta^{34}S_T$	$\delta^{34}S_O$	$\delta^{34}S_S$	$\delta^{34}S_S - \delta^{34}{}_T$	$\delta^{34}S_O - \delta^{34}S_T$
paprika (*Capsicum annuum*)	11.2	6.3	3.7	66.9	2.5	0.3	3.2	0.7	−2.2
leek (*Allium porrum*)	51.0	33.9	1.6	96.9	7.6	6.4	12.6	5.0	−1.2
Brussels sprouts (*Brassica oleracea* var. *gemmifera*)	77.0	28.8	8.0	89.6	5.7	3.3	6.7	1.0	−2.4
broccoli (*Brassica oleracea* var. *silvestris*)	51.7	29.6	20.8	59.8	8.9	5.1	10.1	1.2	−3.8
cauliflower (*Brassica oleracea* var. *botrytis*)	24.7	15.3	4.2	83.0	7.6	4.5	9.1	1.5	−3.1

[a] Calculations of the turnover (reduction) rate and the isotopic shifts.

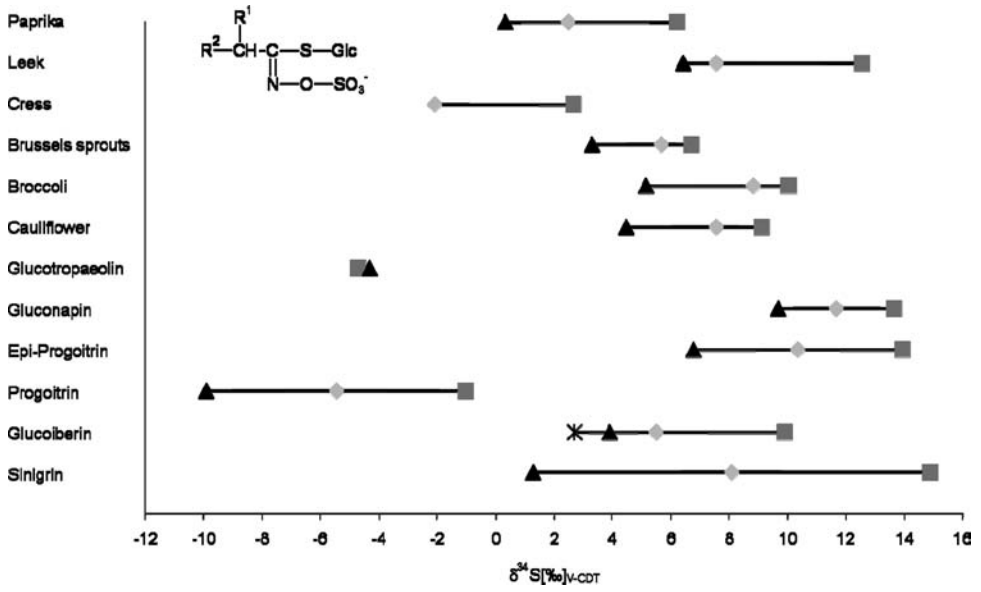

Figure 2. δ^{34}S values$_{V\text{-}CDT}$ (‰) of total sulfur ($\delta^{34}S_T$, gray tilted squares), sulfate sulfur ($\delta^{34}S_S$, gray squares), and soluble protein/organic sulfur ($\delta^{34}S_O$, black triangles) of vegetables and glucosinolates, respectively. For glucoiberin the "normal" (thioglucosidal) $\delta^{34}S_{O1}$ is 3.9, that of the side chain $\delta^{34}S_{O2}$ (∗) 2.7‰. Structures and main sources (in parentheses) of glucosinolates (R^1 = H): glucotropaeolin (cress), R^2 = benzyl-; gluconapin (rape), R^2 = 3-butenyl-; epi-progoitrin (broccoli), R^2 = 2(*S*)-hydroxy-3-butenyl-; progoitrin (broccoli), R^2 = 2-hydroxy-3-butenyl-(2*R*)-; glucoiberin (*iberis*), R^2 = 3-methylsulfinylpropyl-; sinigrin (black mustard, Brussels sprouts), R^2 = 2-propenyl-.

cysteine (**Figure 1**). Cysteine is the direct or indirect precursor of any other organic-S.

On the basis of the above reaction scheme (**Figure 1**), one can assume that mainly two reactions must be responsible for in vivo isotope fractionations, provided they comprise kinetic isotope effects and are located at metabolic branching points with incomplete turnover (*23*). The first one is the activation of sulfate, which could leave ^{34}S-enriched residual sulfate, the second one must be connected to the two competing transfers of sulfate from PAPS, one to the formation of sulfate esters, the other to the reduction process. In addition, some further S transfers from cysteine to secondary products may imply S-isotope fractionations and thus provide ^{34}S-differences between the amino acid and these secondary compounds.

S-Isotope Fractionations in Vegetables. As already indicated, in plant material origin assignments by means of δ^{34}S-value determinations, the samples are the bulk matter and the so-called "soluble protein fraction". It is neither worthwhile nor possible to isolate other defined organic fractions in this context because of their low concentrations, especially as a part of the low molecular weight S-containing fractions is volatile. Therefore, we concentrated our investigations on the δ^{34}S measurements of the bulk matter and the soluble protein fractions. However, the simultaneous availability of the elemental contents and the δ values of S, C, and N by our analytical method and device (*19*) gave us additional information.

In all samples, the sulfur in the soluble protein is ^{34}S-depleted (**Table 2**; **Figure 2**) and that in the residual sulfate ^{34}S-enriched relative to the bulk material; the fractionations are between 2.9 and 6.2‰. These differences meet those described by Krouse et al. (*13*) for sulfate-S and organic-S of tree leaves (5−6‰). From cress, a protein fraction could not be isolated. For the Brassicaceae, the δ^{34}S value of the sulfate, for the other plants the δ^{34}S value of the organic-S, is closer to that of the bulk material. This makes sense, as in Brassicaceae, sulfate is not only surplus starting material but also an essential part of the glucosinolates (**Figure 1**). In line with this is the observation that among the "sulfur-plants" (paprika with its very low S_T-content is out of the discussion in this context), leek shows a higher degree of reduction than the Brassicaceae, accompanied by the highest shift between $\delta^{34}S_S$ and $\delta^{34}S_T$ (**Table 2**). Nevertheless, this shift is quite small and does not at all correspond to that expected from the correlation between a maximum expected kinetic isotope effect ($k_{32}/k_{34} = \sim 1.02$) (*24*) and the turnover rate (96.9%), from which a shift of ∼ 75‰ can be calculated (*25*).

Another aspect that cannot be neglected in this context concerns local and seasonal differences of the concentrations of individual compounds within a plant or plant parts (*16*, *26*). For example, we have found for the $\delta^{34}S_T$ value of stem and silique material of a rape plant 5.5‰, whereas that of the seed was 3.6‰, and from cress seed with $\delta^{34}S_T = -2.1$‰, plants grew with

$\delta^{34}S_T = -0.5‰$. This must not be the expression for an isotope fractionation but simply be due to a shift of the ratio of S-containing ingredients with different oxidation states and $\delta^{34}S$ values. Further information is expected from the S-isotope pattern analysis on glucosinolates.

S-Isotope Differences of Sulfate and Organic-S in Glucosinolates. Glucosinolates are the most common natural compounds with two or more S atoms in the same molecule, one in the sulfate and the others in the reduced oxidation state. The enzymatic hydrolysis of the compounds yields, depending on the pH value of the reaction and the structure of the aglucone, glucose, sulfate, and different and often volatile organic products (*15*). Therefore, the ^{34}S-pattern analysis of glucosinolates must be indirect, in that the $\delta^{34}S$ value of the organic-S (S_O) has to be calculated from the difference of the $\delta^{34}S$ values of the total-S (S_T) and the sulfate-S (S_S). In most cases, the relative amounts of S_O and S_S are 1:1 but 2:1 for glucoiberin; in the latter case we call the "normal" organic-S of the thioglucoside position S_{O1}, the one originating from the amino acid residue S_{O2}. The difference between the $\delta^{34}S$ values of these two organic S atoms (**Figure 2**) confirms our expectation that, after the reduction of sulfate, additional S-isotope fractionations can occur in the subsequent S-metabolism.

In **Figure 2**, the results of our measurements are displayed together with the $\delta^{34}S$ values of the above-described vegetables. As already found for the latter, none of the isotopic shifts observed attains those observed for bacterial sulfate reductions. The measured isotopic shifts between S_S and S_O vary from ~0‰ (glucotropaeolin) to 13.8‰ (sinigrin). Assuming that for the biosynthesis of sinigrin in a closed compartment both sulfur atoms would originate from the same sulfate pool ($\delta^{34}S = \delta^{34}S_T$), 50% of this one would be reduced, and from the above shift a "pro forma kinetic isotope effect" $k_{32}/k_{34} = 1.010$ could be calculated (*25*).

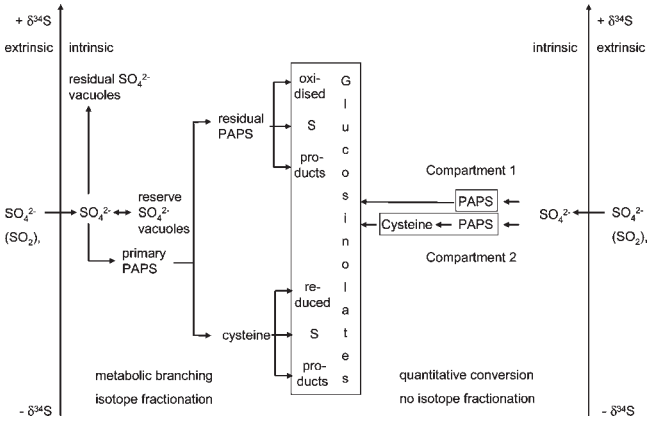

Figure 3. Potential reasons for the relatively small and variable S-isotope fractionation in plants, mainly in context with the glucosinolate biosynthesis. The online formation and reduction of PAPS (see **Figure 1**) with metabolic branching imply isotope fractionations (left part). The partition of individual biosynthesis steps to different plant compartments, where they can proceed without branching and with quantitative turnover, prevents isotope fractionations (right part).

Discussion of the Results with Plant Systems. Maximal ^{34}S-kinetic isotope effects expected for S-binding fissions are ~1.02 (*24*); those in vitro measured on organic chemical reactions are from 1.015 to 1.018 (*27*). For bacterial systems, maximal $\delta^{34}S$-value shifts of ~50‰ have been observed, from where a kinetic isotope effect of 1.024 has been assumed (*21*). Fry reports even fractionations of 74‰ (ref *4*, p 256). However, in both cases, a turnover rate is not given; therefore, a real kinetic isotope effect cannot be calculated. Furthermore, one has to keep in mind that in the case of the assimilatory sulfate reduction, at least two reactions in sequence can contribute to isotope fractionations (see **Figure 1**). The facts that the fractionations observed in the present study are relatively low and not correlated to the sulfate reduction rate and that they depend on the plant species suggest that the conditions in the "system plant" are quite different from those in in vitro and in bacterial systems.

What are the special characteristics of the synthesis system plant? First, in the plant the substrate sulfate is continuously supplied and parts of the product may even get lost; second, and most important, is that a plant is a multicompartment system with different tissues and cell parts, implying the possibility that parts of biosyntheses can occur independently and perhaps quantitatively at different sites. Concerning glucosinolates, it is known for glucosinolates that the plant tissues or compartments with the highest product concentration must not be identical with the site of their biosynthesis (*16*, *28*, *29*). The core or main pathway of the glucosinolate biosynthesis is cytosolic, whereas the site of a chain elongation of the amino acids is the chloroplast (*28*, *29*). In this organelle, also the ferredoxin-dependent sulfate reduction and the sulfate activation are located (*22*); the latter may perhaps also take place independently in other compartments.

Provided a common intermediate for different products, for example, PAPS, is synthesized from a part of the starting material SO_4^{2-} in a given compartment of an organism and then distributed to different sinks (left part in **Figure 3**), these processes imply isotope fractionations. In contrast, when the intermediate is independently synthesized by quantitative conversion of the starting material at different sites and then either quantitatively converted (e.g., reduced) or transferred for a subsequent step in the next compartment (right part in **Figure 3**), no isotope fractionation will result. Intermediates from various sites may afterward be converted in another compartment to a final product, which can thus have optional isotope characteristics. In a plant with various compartments (organs, cells, organelles), many S-containing products are synthesized from several common intermediates, and they are deposited in different sites with temporal and local concentration variations. Therefore, in the multicompartment system plant, despite the existence of defined chemical processes with implied in vitro isotope effects, their realization as isotope fragmentations will finally be dictated by

Table 3. $\delta^{34}S$ Values of Fractions from Animals Bred under Controlled Diet[a]

	feed (S_O, S_S)[b]	water (S_S)	muscle meat (Cys/Met)	collag cartil (Met)	collag bone (Met)	hoof/horn kerat (Cys)[e]	hair kerat (Cys)	bile liquid (taurochol)	urine (S_S)	cartilage (chondr-S_S)[f]
ox	4.8	4.3	5.4	3.1	4.3	6.0−6.4 5.6−7.1	8.1	3.9 [0.0]	10.4	2.5 [3.5]
kid goat/mother	5.3[c]	8.3[d]	5.5	3.5	6.0	5.9−6.3	6.4 (5.7)	4.4	7.4	2.4

[a] All data are mean values from four to six measurements, SD = 0.3‰. The indications in parentheses following the fractions designate the main or unique S-containing compound in this fraction. S_S = sulfate-sulfur; S_O = organic sulfur, nm = not measured. Values in square brackets are data from commercial products. [b] The $\delta^{34}S$ value of the cattle feed is a bulk mean value of hay and concentrate taking into account their relative amounts in the diet; the $\delta^{34}S$ value of the kid goat feed is the bulk value of the milk. [c] $\delta^{34}S$ value of the whole lyophilized milk; the $\delta^{34}S$ value of the casein was 4.9‰ and that of the whey proteins 4.6‰. [d] The $\delta^{34}S$ value of the sulfate of the milk water is given under water; its sample amount was sufficient for only one measurement. [e] For details see **Figure 4**. [f] Calculated from the bulk $\delta^{34}S$ values of cartilage and the corresponding collagen.

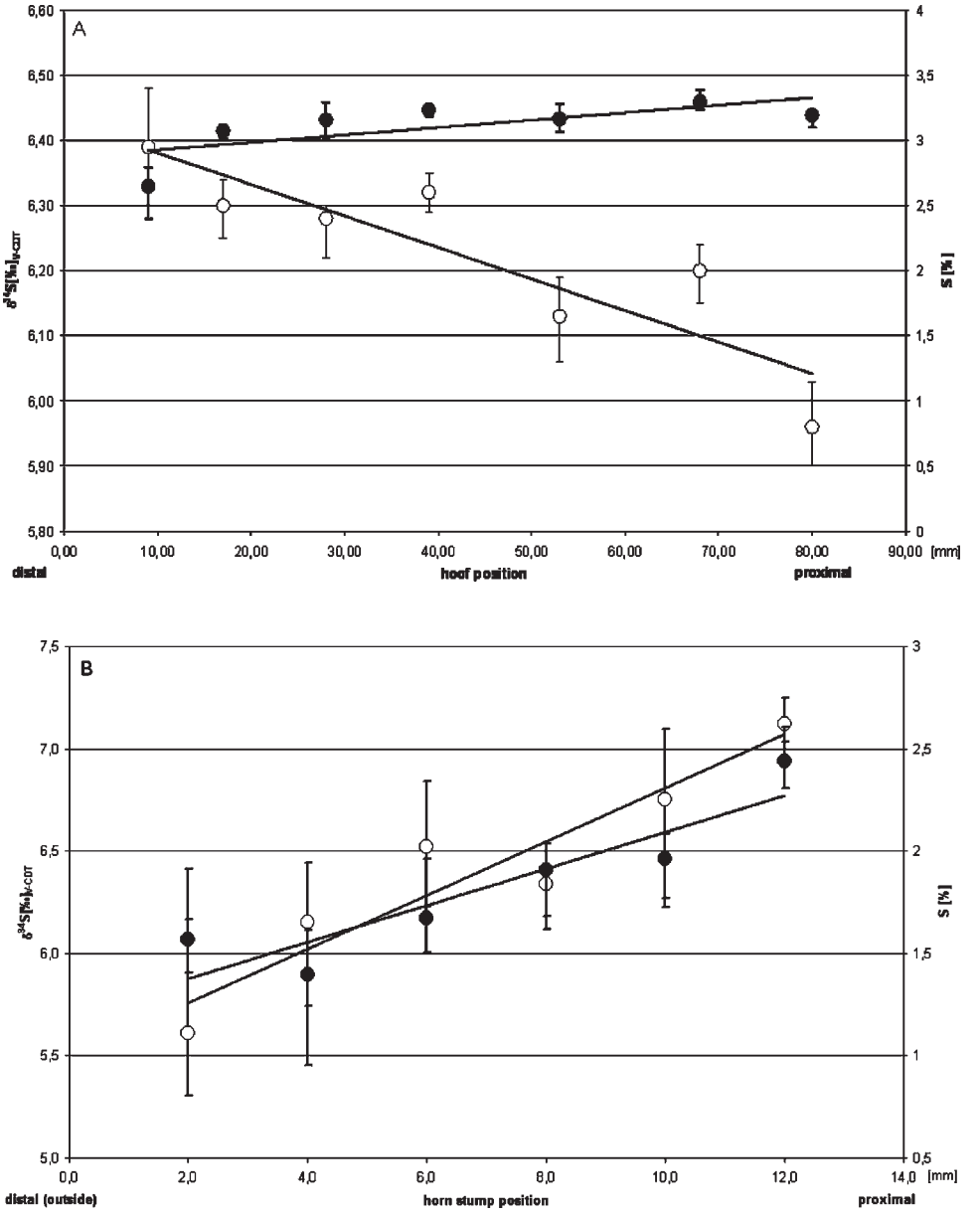

Figure 4. δ^{34}S values$_{V\text{-}CDT}$ (‰) (○) and S concentration (%) (●) of hoof (**A**) and horn stump (**B**) tissues of the ox in dependence of sampling site.

the organism's anatomy and physiology and by the metabolite fluxes between its compartments and, hence, will depend on the species and its metabolic conditions.

Possible S-Isotope Fractionations in Animal Metabolism. *Introduction and Results*. There is no indication in the literature that animals are capable of reducing sulfate and synthesizing complex S-containing compounds. As heterotrophic organisms they must receive most sulfur compounds via their diet, and even the amino acids methionine and (partially) cysteine are essential for most animals. Sources of sulfate are plant matter and drinking water; "organic sulfur" in any form is provided from plant and animal nutrients. From the preceding results we can assume that the sulfur supply of herbivores consists of 10−40% in the form of relatively ^{34}S-enriched sulfate and 60−90% of relatively ^{34}S-depleted organic-S.

Most of the S-containing organic products in animals occur at very low concentrations and can therefore not be isolated in amounts sufficient for an isotope analysis. However, certain organs or tissues are representative for defined compounds and will therefore be the subject of analysis in the present investigation. So, for example, >90% of the S content of collagen (in total ~0.2% S) occurs in the form of methionine (*17*), and therefore the δ^{34}S value of this protein is representative for that of this amino acid. Correspondingly, α-keratin in hair, nails, and horn (up to 4% S) is representative of cysteine, which is responsible for >90% of the protein's sulfur (ref *18*, Vol. 2, p 10). The dry matter of cartilage contains up to 40% chondroitin sulfate (ref *18*, Vol. 1, p 196); as the S content of this carbohydrate is 7.4%, a maximum of 95% of the cartilage-S is sulfate-S. Furthermore, it can be assumed that the S content and δ^{34}S value of bile liquid is indicative for taurine, as taurocholic acid is practically the sole S-containing compound in this product. **Table 3** and **Figure 4** contain the results obtained by the S-isotope ratio determination of such representative tissues/products from an ox, a suckling kid goat, and its mother and their assignments to the main S carrier.

These data permit the following statements:

(1) The bulk δ^{34}S values of the whole milk and the ox muscle meat indicate a <1‰ ^{34}S enrichment relative to that of the animal's diet; this confirms the justifiability of the normal use of bulk δ^{34}S values for these animal products in origin assignments.

(2) On the other hand, one has to keep in mind that the δ^{34}S value of the organic fraction in the ox's diet is distinctly below the

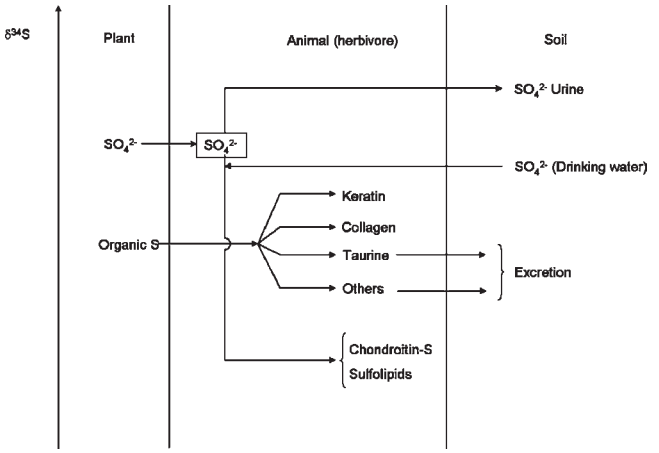

Figure 5. Sulfur supply, isotope fractionations, and relative sulfur isotope abundances in animal tissues and their typical S-containing ingredients.

bulk δ^{34}S value (4.8‰). Therefore, muscle meat, horn, and hair of the ox are ^{34}S-enriched relative to the organic fraction of the diet. This could be an expression for a general secondary ^{34}S-enrichment in the food chain as observed with trophic levels of other elements (*30*), although it is not as distinct as that for nitrogen of the same compounds (δ^{15}N values measured in this work: feed concentrate, 4.1‰; hay, 2.7‰; mother goat hair, 5.1‰; milk, 4.9‰; casein, 5.2‰; kid goat hair, 7.4‰; muscle meat, 6.5‰). The trophic shift can also be seen by comparison of corresponding fractions from mother and kid goat (milk/hoof or hair/hair). Nevertheless, it is also possible that the observed ^{34}S-enrichment could be caused by a partial degradation of S_O compounds of the diet by the rumen flora or intestinal bacteria of the animals (e.g., under formation of ^{34}S-depleted H_2S).

(3) A comparison of the δ^{34}S values of the organic fractions keratin and collagen of the investigated animals suggests an isotope fractionation between cysteine and methionine, perhaps already at the level of the amino acids' biosynthesis in plants (compare difference between the two "organic" S atoms in glucoiberin). Earlier findings of Krouse (*5*) for salmon showed such corresponding differences (δ^{34}S-values: inner flesh, 16−18‰; skin, 21‰), whereas our own results on the pike are contrary to expectation (δ^{34}S values: muscle ≙ Cys/Met = 3.4‰, scale ≙ Cys = 1.9‰). At present, we do not have an explanation for this result. In this context one has to point out the large δ^{34}S-value difference between the products from freshwater and seawater fish, respectively, which is typical for their origins [δ^{34}S value of freshwater sulfate, −22 to +20‰, and of seawater sulfate, +21‰ (ref *4*, p 43)]. Finally, isotope fractionations in the metabolism of cysteine are also suggested by the observation that the oxidation product taurine is relatively depleted in ^{34}S.

(4) The δ^{34}S values and the S concentrations of the ox hoof and horn (**Figure 4**) show significant dependencies on the site of sample collection. In the case of the hoof, the S concentration is slightly higher and the δ^{34}S value lower for the proximal (younger) part, whereas in the horn both data are distinctly higher in the center (older part?) of the organ. However, it must be said that the animal had originally been dehorned and the "horn" was a small stump, about the growth of which we do not have any knowledge. Also, for the kid goat's hoof a trend of the S concentration and δ^{34}S value was found: both were relatively higher toward the distal (tip) site. Actually, we do not have a satisfactory interpretation for these results, but we believe that they are, apart from diet effects, an expression for a change of the relative concentration of methionine and cysteine (containing proteins) in the tissue.

(5) The sulfate in the ox urine and in the milk water are distinctly ^{34}S-enriched relative to the input; on the other hand, cartilage and chondroitin sulfate are relatively depleted in ^{34}S. This suggests an isotope fractionation on sulfate in the animals' metabolism, which can be assigned to the partial activation of the ion (see PAPS in **Figure 1**) for the synthesis of proteoglycanes and sulfolipids (sulfatides), probably the sole larger sulfur isotope fractionation in the animal metabolism.

Discussion of the Results with Animal Systems. As summarized in **Figure 5**, the isotopic characteristics of inorganic- and organic-S in animals are predominantly caused by origin and diet, but certain isotope fractionations seem also to be implied in the animal metabolism itself. The first aspect is in line with a statement by Krouse (*5*), who found that "in human teeth and kidney stones sulfide and sulfate extracts are sometimes isotopically similar, but in a few cases, they are significantly different. The differences are difficult to explain but might reflect incorporation of sulfur of different isotopic composition at different times." The second aspect is evident in an observation by Richards et al. (*10*): the authors found a ^{34}S depletion between diet and hair of horses under optimal protein supply of the animal but an enrichment under protein deficiency. They believe that this effect was caused by the mobilization of intrinsic protein sources.

Our results permit the conclusion that both aspects are valid: the δ^{34}S values of animal samples basically reflect the diet but show small ^{34}S enrichments relative to it, depending on the animals' nutrition state, the trophic level, and the individual tissue. This is similar but not so distinctly expressed as for the nitrogen isotopes (*30*). A real isotope fragmentation seems to occur in context with the proteoglycane and sulfolipid biosyntheses.

Conclusion. The generally described slight ^{34}S depletion, expressed by the bulk δ^{34}S value of plant material relative to the primary S sources in the literature (*1*, *3*−*5*), cannot be explained so far; it may be due to a partial leaching of (^{34}S-enriched) sulfate by rainwater. Although the reactions of the assimilatory sulfate reduction by plants and the kinetic S-isotope effects on them must be identical to those of the bacterial dissimilatory sulfate reduction, the S-isotope fractionations in plants are by far smaller. We assume that this is caused by a local and temporal separation of the biosynthesis of individual parts of S-containing plant products with partial quantitative turnover rates of intermediates. The differences between sulfate-S and organic-S in plants are mostly between 3 and 6‰ but can reach up to 14‰. Due to local separations of these fractions, individual plant parts and products may attain δ^{34}S values differing distinctly from the bulk value.

The trophic shift of the sulfur isotopes for muscle tissue of herbivores relative to plant diet is 1−2‰, that of hoof and hair 2−5‰. As the muscle tissue is practically free of sulfate, the trophic effect can compensate for the depletion of the plant diet relative to primary sulfur sources. On the other hand, chondroitin sulfate and cartilage can have quite distinct depletions in ^{34}S (5−6‰) relative to the diet, due to an isotope effect on the proteoglycan biosynthesis. As the ^{34}S-enriched sulfate is excreted in the urine, for these tissues a real ^{34}S fractionation exists. Thus, like with plants, individual animal tissues can have δ^{34}S values distinctly different from the bulk value.

With the restrictions by these exceptions, main animal tissues and their bulk δ^{34}S values are practically identical to those of the corresponding primary S source and can be used for origin assignments and trophic level investigations.

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