# Assessment of Enzymatic Methods in the $\delta^{18}$O Value Determination of the L-Tyrosine $p$-Hydroxy Group for Proof of Illegal Meat and Bone Meal Feeding to Cattle

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**ABSTRACT:** The $\delta^{18}$O value of the $p$-hydroxy group of L-tyrosine depends on the biosynthesis by plants or animals, respectively. In animal proteins it reflects the diet and is therefore an absolute indicator for illegal feeding with meat and bone meal. The aim of this investigation was to perform the positional $^{18}$O determination on L-tyrosine via a one-step enzymatic degradation. Proteins from plants, herbivores, omnivores, and carnivores were characterized by their $\delta^{13}$C, $\delta^{15}$N, and $\delta^{18}$O values, the latter for normalizing the positional $\delta^{18}$O values. Their L-tyrosine was degraded by tyrosine phenol lyase to phenol, analyzed as (2,4,6)-tribromophenol. Degradation by tyrosine decarboxylase yielded tyramine. The $\delta^{18}$O values of both analytes corresponded to the trophic levels of their sources but were not identical, probably due to an isotope effect on the tyrosine phenol lyase reaction. Availability of the enzyme, easy control of the reaction, and isolation of the analyte are in favor of tyrosine decarboxylase degradation as a routine method.

**KEYWORDS:** L-tyrosine, positional $\delta^{18}$O value, plant origin, animal origin, tyrosine phenol lyase reaction, tyrosine decarboxylase reaction, meat and bone meal, bovine spongiform encephalopathy

## ◼ INTRODUCTION

In 1994, the suspicion arose that bovine spongiform encephalopathy ("mad cow disease", related to scrapie in sheep and Creutzfeldt−Jakob disease in humans) could be transferred among ruminants by meat and bone meal as a protein source in their diet. As a consequence, in December 2000 the European Commission banned the use of this diet additive from the feed of livestock, especially cattle.[1] Since then, the majority of meat and bone meal is burned in power stations or cement factories, yet it is still available as organic fertilizer. Meanwhile, a European-wide control system for the identification of prions as the inducing agent of the disease in slaughtered cattle has been established and is routinely practiced.[2] However, so far no method exists to prove the illegal feeding of meat and bone meal to ruminant livestock.

Isotope ratio abundance determinations have been considered as a suitable basis. From many investigations on food chains it is known that the $\delta^{15}$N value of proteins is influenced by the diet and is raised by ~+2‰ per trophic level. This offers, for example, the opportunity to reconstruct prehistoric nutrition or to distinguish between omnivores and vegetarians.[3] In an experiment with chicken, a correlation of the $\delta^{15}$N values of the animals' protein to the relative amount of meat and bone meal in their feed was shown.[4] In a systematic study with cattle, a Spanish group[5] found that the $\delta^{15}$N value of hair and milk proteins from animals grown under conditions of intensive farming was raised by >+6.4‰ relative to those of a control group and postulated that this was a basis for a method to prove meat and bone meal feeding. However, as a relative enhancement of the $\delta^{15}$N value

can also be due to feeding with "organic" food, it cannot be an absolute indication for feeding with meat and bone meal. Bahar et al.[6] demonstrated that the isotope characteristics of beef are not absolutely indicative for the diet of the animals and do even not permit a discrimination between products from conventional and organic farming, respectively. Therefore, the $\delta^{15}$N value cannot provide an absolute indication for feeding with meat and bone meal, especially when the very first trophic level of the food chain, the plant material, is unknown.

In contrast, we have been able to show that the $\delta^{18}$O value of the phenolic hydroxyl group of L-tyrosine is an absolute indicator for the origin of the amino acid from plant or animal biosynthesis.[7] In plants, L-tyrosine is synthesized via the shikimic acid pathway. Here the oxygen atom in the *para*-position of the aromatic ring of the amino acid originates from water via erythrose and has, due to an equilibrium isotope effect, a $\delta^{18}$O value of ~+27‰ above that of leaf water.[8] On the other hand, only animals and some microorganisms are capable of synthesizing L-tyrosine from L-phenylalanine, introducing the oxygen atom in question by a monooxygenase reaction from $O_2$; a kinetic isotope effect leads to a $\delta^{18}$O value of ~+7‰.[8] Therefore, the $\delta^{18}$O value of the $p$-OH group of L-tyrosine from any protein must be an absolute indicator for its (partial) origin from animal production.

Any determination of this $\delta^{18}O$ value demands the conversion of the amino acid into a derivative, in which the oxygen atoms of the carboxyl group are eliminated. This positional isotope analysis has originally been performed by means of a five-step chemical degradation with a total yield of 60%, starting from 3 g of the amino acid.[7] This method is quite laborious for a routine assay and demands large amounts of starting material. Therefore, the aim of the present investigation was to develop a (one-step) enzymatic process and to check the establishment of the procedure as a routine assay. Potential candidates were seen in the tyrosine phenol lyase and the tyrosine decarboxylase reactions. Problems anticipated with both methods were the isolation of a few milligrams of the corresponding analyte from a large volume of incubation medium. In addition, for the first reaction, a possible oxygen exchange of enzyme intrinsic water with a postulated keto-quinoid intermediate of the reaction[9,10] had to be excluded. With the second reaction, the oxygen isotope ratio analysis of the N-containing analyte tyramine could be especially problematic, yet this problem has quite recently been overcome.[11]

## ■ MATERIALS AND METHODS

**Protein Samples, Enzymes, and Chemicals.** Despite many attempts to obtain protein samples from meat and bone meal fed animals or cattle infected with bovine spongiform encephalopathy, such samples could not be obtained. It was also not possible to provide a "100% animal originating L-tyrosine", which would have to be synthesized by animal L-phenylalanine hydroxylase. Hence, we had to verify the principle of our method and the reliability of the methodology on the basis of proteins, the origin and history of which were typical for relative amounts of plant- and/or animal-originating L-tyrosine, respectively. For this reason, the samples to be analyzed were selected according to the following criteria: (a) plant proteins as starting material in the food chain, representing the most dominant cattle feed and belonging to the main photosynthetic plant groups; (b) samples from herbivores, mainly ruminants, with different controlled diets; and (c) samples from omnivores and carnivores representing different trophic levels with respect to the L-tyrosine source.

Wheat gluten, corn protein (zein), and soy protein were sourced from local agricultural commerce and beef and turkey meat from a local supermarket. Dog and cat meat samples were provided from the Veterinarian Department and meat from a crocodile and its feed by M. Starck, Department of Biology II, both of the Ludwig Maximilians Universität, München, Germany. Milk from cattle (mixtures from many cows) with controlled diet (i, meadow grazing only; ii, 50% grass, 20% hay, 30% concentrate, mainly from wheat; iii, 40% concentrate of wheat, corn, soy, rape, and molasses, 35% corn silage, 25% grass silage) was provided by D. Weiss, Freising, Germany. Goat milk and meat from a suckling kid goat, exclusively nourished by this milk, as well as the fodder (meadow, hay, some concentrate) and the drinking water of the goat were obtained from a local farm. A hare muscle sample was provided by a local hunter, and a pike was provided by J. Lamina from the Animal Science Department of the Technische Universität München, Freising, Germany.

Tyrosine phenol lyase (EC 4.1.99.2, from *Citrobacter freundii*, $2 \times 40$ U/0.5 mL phosphate buffer) was a kind gift of R. S. Phillips, Department of Chemistry, University of Georgia, Athens, GA. Tyrosine decarboxylase (EC 4.1.1.25, apoenzyme from *Streptococcus faecalis* NCTC 6783, 250 mg of 0.30 U/mg and 250 mg of 0.56 U/mg) was purchased from Worthington Biochemical Corp., Freehold, NJ. Lactate dehydrogenase (EC 1.1.1.27, 508 U/mg), formate dehydrogenase (EC 1.2.1.2, 0.47 U/mg), pyridoxal phosphate, NAD$^+$-dihydrate, and (2,4,6)-tribromophenol from Fluka, Steinheim, Germany, and TLC cellulose plastic sheets and L-tyrosine from E. Merck, Darmstadt, Germany, were purchased from a local representative. $^{18}O$-enriched water (10 atom % $^{18}O$) was

sourced from Aldrich, Taufkirchen, Germany, and tin and silver capsules for the isotope ratio analysis were bought from IVA Analysentechnik, Meerbusch, Germany. L-Tyrosine from chicken feathers and from human hair was kindly provided by G. Fronza from the Politecnico di Milano, Milan, Italy. Any other ordinary chemicals were purchased locally in the highest purity available.

**Sample Pretreatment, Protein Hydrolysis, and Isolation of L-Tyrosine.** The demand of oxygen for the isotope ratio determined the amount of sample required. Usually, $10-15$ g of dry protein was needed. One hundred grams of meat was cut into pieces of $\sim$2 cm$^3$ and freeze-dried for 24 h. Then it was ground in an ordinary laboratory mill and defatted by Soxhlet extraction with petroleum ether (bp $40-60$ °C) for at least 6 h. Milk ($0.5-1$ L) was skimmed by centrifugation, and the casein was precipitated at pH 4.3 with 1 N HCl. The precipitate was centrifuged and washed three times with deionized water and then dried by lyophilization. Aliquots of all proteins were preserved for isotope ratio measurements.

Ten grams of dry protein was suspended in 150 mL of 6 N HCl and 4 mL of thioglycolic acid in a 250 mL two-necked flask with a reflux condenser and gas inlet. The mixture was heated to reflux temperature for 24 h under occasional rinsing with N$_2$. The content was transferred to a 500 mL round-bottom flask and evaporated to dryness in a rotatory evaporator. The residue was resuspended three times in 250 mL of water and dried using a rotatory evaporator. Then it was dissolved in 250 mL of water and adjusted to pH $\sim$2.5 with 5 N NaOH. After the addition of 5 g of charcoal, the suspension was heated to 80 °C for 15 min under stirring.[12] Then it was filtered through filter paper and the charcoal rinsed with 60 mL of hot water.

The combined filtrates were adjusted to pH 5 with 5 N NaOH and concentrated until the onset of crystallization, which was completed overnight in the refrigerator at 4 °C. The precipitate was collected by filtration, rinsed with cold water, and once recrystallized from hot water. The purity of the product was checked by TLC [cellulose on plastic foils, *n*-butanol/acetic acid/water (v/v/v 50:20:30), 0.3 mg ninhydrin in 100 mL *n*-butanol] and by elemental analysis in tandem with the isotope ratio determination. Most products had a purity of >90% and were used without further purification for incubation. A possible isotope fractionation of the process was controlled by determining the $\delta^{13}C$ and $\delta^{15}N$ values of a sample of commercial L-tyrosine before and after recrystallization; they agreed within 0.1‰.

**Enzymatic Fission of L-Tyrosine by Tyrosine Phenol Lyase, Isolation, and Derivatization of Phenol.** As the activity of tyrosine phenol lyase is inhibited by Na$^+$ and Li$^+$,[10] any buffers were prepared from KH$_2$PO$_4$ and KOH. L-Tyrosine equivalent to 30 mg of phenol (0.33 mmol = 60 mg of L-tyrosine) was incubated, and the volume of the incubation medium was adapted to the low solubility of the substrate. The incubation procedure[13,14] was modified, in that formate dehydrogenase, lactate dehydrogenase, and formate were used in excess relative to tyrosine phenol lyase; NAD$^+$ was added at $^1/_{10}$ equivalent. To 140 mL of 0.55 mM postassium phosphate buffer (pH 8.3) in a 250 mL flask was added 7 mg of thioglycolic acid, and the pH value was adjusted to 8.0 (solution A). To 15 mL of this solution were added 2 mg of pyridoxal phosphate and 2 U of tyrosine phenol lyase (25 $\mu$L of a solution of 40 U/0.5 mL in phosphate buffer), and the solution was preincubated at 27 °C for 30 min (solution B). To the remaining 125 mL of solution A were added $60-90$ mg of L-tyrosine, 36 $\mu$L of formic acid, and 23 mg of NAD$^+$-dihydrate, and the solution was adjusted to pH 8.0. Then 2 U each of formate dehydrogenase and lactate dehydrogenase were added, and the solution was brought to 27 °C. It was combined with solution B, and after 5 min of gentle stirring, the L-tyrosine was almost completely dissolved. The incubation in a closed flask was 48 h at 27 °C, the enzymes being supplemented, units as above, after 24 h.

This reaction time had been elaborated by the turnover control of a representative sample. One milliliter of incubation medium samples was

taken for HPLC control of substrate and product concentrations at times 0, 24, and 48 h. Thirty-four microliters of 5 M trichloroacetic acid was added to the samples. The precipitate formed was removed by centrifugation. Eighty-five microliters of the supernatant was diluted with water to 1 mL. This solution was injected into a Dionex HPLC system BioLC, consisting of an AminoPac PA-10 analytical column, 2 × 250 mm, with an AminoPac PA 10 Guardpre column, 2 mm (10−32), equipped with an ED 50 electrochemical detector, a GS 50 gradient pump, and an AS 50 autosampler (Dionex GmbH, Idstein, Germany). One milliliter of each of a 20 $\mu$M solution of L-tyrosine (substrate) and a 420 $\mu$M solution of phenol (product) was used as standard. After 48 h, L-tyrosine had disappeared, and this time was used for any other incubations.

After the incubation, the pH value of the medium was adjusted to 3.5 by acetic acid. The precipitate was eliminated through centrifugation. For the isolation of the phenol the supernatant was filtered for solid phase extraction through a 12 mL Giga Tube with 1 g Strata X 33 $\mu$m polymeric sorbent (Phenomenex Inc., Torrance, CA),[15] conditioned with 5 mL of methanol and 5 mL of deionized water. The column was dried with $N_2$, and the bound phenol was eluted with 20 mL of dichloromethane. The phenol was extracted with 20 mL of 0.5 N NaOH, and the solution was acidified with 6 N HCl. A solution of 0.5 mL of $Br_2$ in 10 mL of acetic acid was added dropwise until the supernatant remained slightly yellow. The precipitate of (2,4,6)-tribromophenol was isolated by filtration, washed with ice-cold water, dried by lyophilization, and weighed into silver capsules for the oxygen isotope ratio analysis. The yields of this product relative to the applied L-tyrosine did not exceed 70%.

To check the isolation procedure for isotope fractionations, we determined the $\delta^{18}O$ value of a sample of phenol and then prepared a solution of ∼120 mg of this phenol in 200 mL of $H_2O$ and divided it into two equal parts. In one of them, the derivatization to (2,4,6)-tribromophenol was directly performed; the other part was submitted to the solid phase and solvent extraction procedures prior to precipitation. The $\delta^{18}O$ values of the two (2,4,6)-tribromophenol samples were identical within 0.1‰, but 0.5‰ more negative than that of the original phenol. This is above the SD of the isotope ratio measurement (±0.2‰) but within the overall reproducibility of the complex procedure (∼±1.0‰).

For the control of a possible oxygen exchange of the keto-quinoid intermediate with water, in some experiments 0.625 mL of the incubation medium was replaced by $^{18}O$-enriched water (10 atom % $^{18}O$). Five milliliters of the labeled medium was preserved for the O-isotope ratio analysis. After the isolation of the phenol, the solution was adjusted to pH 9.3 and the water was recovered by distillation. The distillate was cleared with 5 g of charcoal and, after the addition of another 0.045 mL of 10% $^{18}O$-labeled water, reused for the preparation of another labeled incubation medium.

### Enzymatic L-Tyrosine Decarboxylation and Isolation of Tyramine.

With reference to Ziadeh et al.[16] 100 mg of L-tyrosine was added to 100 mL of 0.1 M of acetate buffer (pH 5.5). Twenty milligrams (6.0 or 11.2 U) of the tyrosine decarboxylase preparation (a raw dry powder of a bacterial homogenate) and 0.5 mg of pyridoxal phosphate were preincubated in another 5 mL of the same buffer at 37 °C for 15 min. Afterward, the two solutions were combined in a closed incubation vessel, the gas phase of which was connected via an S-shaped glass tube to a water-filled calibrated cylinder to control the turnover of the substrate by volumetric measurement of $CO_2$ production. The medium was stirred at 37 °C. After 20 h another 9 U of enzyme were added and at maximum 36 h the gas production indicated the quantitative conversion of the substrate (calculated as ∼14.0 mL for 100 mg of L-tyrosine). The medium was centrifuged to eliminate the solid parts of the enzyme preparation. The supernatant was transferred to a 300 mm × 10 mm i.d. column with 10 g of Dowex 1X8 anion exchange resin, 200−400 mesh, in Cl⁻ form, retaining the acetate and pyridoxal phosphate ions. The product was eluted with 100 mL of water (identified by ninhydrin for

tyramine and $AgNO_3$ for Cl⁻). The tyramine chloride containing fractions were combined and lyophilized. The remainder contained unknown impurities from the enzyme preparation and had to be submitted to preparative HPLC for further purification by a Knauer HPLC preparative system with a Nucleosil 100 C18 column, 250 mm × 20 mm i.d. (Macherey-Nagel, Düren, Germany), a K-1800 WellChrom preparative pump, and a K2501 UV detector (Knauer, Berlin, Germany). As tyramine is only partially soluble in water, the procedure had to be repeated in up to 18 portions, dissolved in a few milliliters of $H_2O$ under the addition of some drops of 6 N HCl. Tyramine was eluted with deionized water (40 mL/min at 3 MPa). The fractions containing tyramine were identified by their UV absorption at 254 nm. In total, the product had to be isolated from several hundred milliliters of eluate by lyophilization. The residue, a mixture of tyramine, tyramine hydrochloride, and NaCl, was directly used for isotope ratio analysis.

### Isotope Ratio Analysis of Proteins, L-Tyrosine, Tyramine, (2,4,6)-Tribromophenol, and Water.

The indications of the isotope ratios are given, relative to international standards from the International Atomic Energy Agency (IAEA) in Vienna, Austria (VPDB for carbon, AIR for nitrogen, VSMOW and IAEA 600, 601, and 602 for oxygen), in $\delta$ value notations, based on isotope ratios $R = $ [heavy isotope]/ [main isotope], for example, for carbon:

$$\delta^{13}C = (R_{Sample}/R_{VPDB} - 1) \times 1000 \text{ (‰)} \tag{1}$$

The determinations of the $\delta^{13}C$ and $\delta^{15}N$ values of the proteins and the L-tyrosine samples were performed in a multielement isotope ratio analyzer.[17] The device simultaneously provided the elemental concentrations of C and N. Three milligrams of the samples in tin capsules were measured against a casein laboratory standard ($\delta^{13}C_{VPDB} = -23.4‰$ and $\delta^{15}N_{AIR} = 6.2‰$); the analytical precision limits were for $\delta^{13}C$ ±0.1‰ and for $\delta^{15}N$ ±0.2‰. The $\delta^{18}O$ values of the proteins and most of the tyramine samples were determined at ETH Zürich, and some tyramine samples were analyzed by H.-J. Kupka, Elementar Analysensysteme GmbH, Hanau, Germany.[11] Laboratory references calibrated against VSMOW and other international standards (IAEA 600, IAEA 601, IAEA 602) were used for control, and the SD was ±0.2‰.

For the $\delta^{18}O$ value determination of (2,4,6)-tribromophenol at the Max-Planck-Institute for Biogeochemistry, Jena, Germany, samples corresponding to 0.3−1.0 mg of O in silver capsules were converted by high-temperature conversion at 1400 °C. The CO formed was analyzed in a mass spectrometer against laboratory standards, calibrated to VSMOW and other international standards. Error limits are given with the results. The $\delta^{18}O$ value measurements of the incubation water were performed by Hydroisotop GmbH, Schweitenkirchen, Germany. Five milliliters of the medium was equilibrated with $CO_2$, the $\delta^{18}O$ value of which was determined relative to a laboratory standard, calibrated to VSMOW and other international standards. The error limit was 0.2‰.

## ■ RESULTS AND DISCUSSION

### Origins and Isotopic Characteristics of the Proteins, $\delta^{13}C$ and $\delta^{15}N$ Values.

The $\delta^{13}C$ values of the plant proteins (Table 1) are typical for $C_3$- and $C_4$-plants. As expected, the $\delta^{13}C$ values of the meat and casein from cattle and goat, which had been fed exclusively with hay and grass, and of the meat of the hare, living on local $C_3$-plants, are nearly identical or quite close to those of these plants. Any other animal proteins show a distinct $^{13}C$ enrichment, probably due to a more or less greater direct or indirect presence of corn (products) in the diets. The diet influence must be overlapped by a trophic shift (usually 1−2.5‰ per level).[3] The unexpected $^{13}C$ depletion of the crocodile meat relative to the beef feed can be explained only

**Table 1. Origin, History, and Isotope Characteristics of Protein Samples Used for the Proof of a Correlation between the Trophic State of L-Tyrosine and the $\delta^{18}$O Value of Its $p$-OH Group**

| protein, name, origin[a] | history, diet[b] | $\delta^{13}C_{VPDB}$ (‰) | $\delta^{15}N_{AIR}$ (‰) | $\delta^{18}O_{VSMOW}$ (‰) | $\Delta\delta^{18}O_{VSMOW}{}^{c}$ (‰) |
|---|---|---|---|---|---|
| wheat gluten, comm | cattle feed, tec qual | −28.0 | +1.3 | +25.1 | |
| zein, comm | cattle feed, tec qual | −14.8 | +2.0 | +22.5 | |
| soy protein, comm | cattle feed, tec qual | −25.2 | +0.6 | +18.9 | |
| chopped beef, comm | unknown | −22.1 | +4.6 | +10.4 | ±0 |
| cow's milk casein, comm | unknown | −23.2 | +5.4 | +9.7 | −0.7 |
| cow's milk casein 1 | exclusively grass | −27.1 | +5.4 | +10.2 | −0.2 |
| cow's milk casein 2 | grass, hay, conc | −27.3 | +5.3 | +9.1 | −1.3 |
| cow's milk casein 3 | corn + grass silage, conc | −21.3 | +5.6 | +10.8 | +0.4 |
| goat's milk casein | grass, hay, conc | −26.7 | +5.2 | +8.5 (−10.3)[d] | −1.9 |
| kid goat (muscle) | exclusively goat milk | −25.7 | +6.5 | +8.7 | −1.7 |
| hare (muscle) | local plants | −28.2 | +7.9 | +10.8 | +0.4 |
| turkey (muscle), comm | unknown | −23.5 | +1.3 | +10.1 | −0.3 |
| dog (muscle) | unknown | −21.8 | +7.5 | +11.5 | +1.1 |
| cat (muscle) | unknown | −22.0 | +7.3 | +9.9 | −0.5 |
| pike (muscle), local river | unknown | −30.1 | +16.5 | +8.7 (−11.1)[d] | −1.7 |
| crocodile (muscle), terra | beef | −23.4 [−21.5][e] | +5.4 [+4.7][e] | +12.6 | +2.2 |

[a] comm, commercial; terra, terrarium. [b] tec qual, technical quality; conc, concentrate. [c] $\delta$ value shift of the sample relative to the average $\delta^{18}$O value of the animal proteins (+10.4‰). [d] Data in parentheses are of the corresponding drinking water. [e] Data in square brackets are for the diet beef. The SE is ≤0.3‰ for all isotope measurements.

by the assumption that the sample investigated was not representative for the ordinary diet of the animal.

With regard to the nitrogen data, the low $\delta^{15}$N value of the soy protein is typical for a legume; any other $\delta^{15}$N values are probably dominated by the actual fertilization. A trophic shift can be seen in the $\delta^{15}$N values from plants to cattle products and carnivores (usually 2−4‰ per trophic level)[3] and even between the goat's milk casein and the kid goat meat. The unexpected high $^{15}$N enrichment of the hare muscle protein can tentatively be attributed to autocoprophagy, recorded for these animals, or simply to the possibility that a main part of the animal's diet was plants from a field fertilized with organic manure. This underscores our reservation against the suitability of the $\delta^{15}$N value as an indicator for proof of feeding meat and bone meal.

**Origins and Isotopic Characteristics of the Proteins, $\delta^{18}$O Values.** The $\delta^{18}$O values of the proteins are most interesting in the present context. Proteins contain approximately 25% O. The majority of the oxygen atoms are bound in the carbonamide function, a smaller part being bound in carboxyl (Glu, Asp) and hydroxyl groups (Ser, Tyr, Hypro) of side chains. Free carboxyl groups show, at equilibrium, an $^{18}$O enrichment of ∼+19‰ relative to the surrounding water.[8] The oxygen atoms of peptide groups are expected to be more enriched, probably up to ∼+24‰, due to an isotope effect on the amino acid activation for the protein biosynthesis. The $\delta^{18}$O values of the OH groups of Ser and plant-originating Tyr should be identical to those of carbohydrates, namely ∼+27‰ above water. Taking these facts into account, we estimate the average $^{18}$O enrichment of plant proteins relative to the mean $\delta^{18}$O value of leaf water to be ∼+23‰. Yet this value is plant-typical and underlies (local) climatic, seasonal, diurnal, and plant physiological parameters.[18,19] As an example, the mean value of the leaf water of a *Rhus typhina* tree from this area was −2.2‰.[20] Average values for grain plants in our surroundings are between −3 and +2‰ (A. Rossmann, private communication), and for soy plants with lower transpiration a value of ∼−5‰ can be assumed. From this, $\delta^{18}$O values
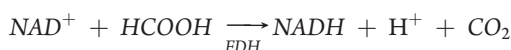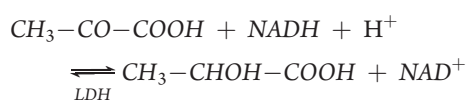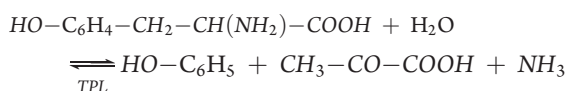
from +20 to +26‰ can be expected for the plant proteins investigated in this work. The experimental $\delta^{18}$O values in Table 1 agree excellently with this expectation. An estimation of the $\delta^{18}$O values of carbohydrates and hence of the $p$-OH group of plant L-tyrosine (shift +27 ± 2‰ above water) suggests $\delta^{18}$O values between +25 and +29‰.

A similar estimation of the $\delta^{18}$O value of animal proteins is by far more complex. The source of the major oxygen component, the peptide oxygen, is the "cell water", a product of the local drinking water, the diet, and oxidation water. No data exist about correlations between the $\delta^{18}$O values of animal tissue water, as isolated, for example, by meat squeezing, and body proteins. Aside from the drinking water, mainly the diet has a large influence on the $\delta^{18}$O value of tissue water,[21] also manifested by differences between tissue water of different species from the same area.[22] These observations are confirmed by the finding that the $\delta^{18}$O value of cattle hair from animals grazing in grassland in our area was +11 ± 1‰, but +7 ± 1‰ in winter, when the animals' diet was hay and grass silage.[23] Collagen should be slightly "lighter" due to its content of Hypro with the OH group introduced by a monooxygenase reaction. Our data in Table 1 are, for most herbivores (mean value +10.4 ± 1‰), in line with these results. Some differences may be inferred from the water source. That of the crocodile might have been water from the terrarium, $^{18}$O enriched by evaporation. As to the relative depletion of the pike protein ($\delta^{18}$O value of river water −11.1‰), one has to take into account that the $\delta^{18}$O value of dissolved oxygen in an aquatic system, hence the oxidation water, depends on the photosynthetic activity in and the oxygen saturation of the water and can be quite different from that of atmospheric $O_2$.[21,24] The $\delta^{18}$O value of the goat products cannot be explained.

An estimation of the $\delta^{18}$O values of animal proteins on the basis of the drinking water alone ($\delta^{18}$O value −10.5 ± 0.5‰ in our area) or of known data on tissue water (−4 ± 2‰)[22,25] would lead to unsatisfactory results. Therefore, our data must definitely in part be caused by organic material of the diet of the

animals. This provides an opportunity to correct the uncertainties on the experimental $\delta^{18}O$ values of L-tyrosine contributed by the natural $\delta^{18}O$ value range of the original plant diet ($\pm 2‰$ on 27‰ for carbohydrates and the phenolic group of L-tyrosine). They not only directly influence the plant-originating L-tyrosine $p$-OH group but also indirectly influence the protein $\delta^{18}O$ values. Hence, in reverse, the shifts of the latter from the average value +10.4‰ ($\Delta\delta$ values) (Table 1) should be a basis for the "normalization" of the experimental $\delta^{18}O$ values of the $p$-OH group of L-tyrosine, obtained by hydrolysis of the proteins.

**Assessment of the Tyrosine Phenol Lyase Reaction for the Positional $\delta^{18}O$ Value Determination.** Tyrosine phenol lyase ($\beta$-tyrosinase, EC 4.1.99.2) is a bacterial pyridoxal phosphate dependent enzyme, for example, from *Escherichia intermedia* A-21[13] or from *Citrobacter freundii*.[9,10] The catalyzed reaction, degrading the amino acid into phenol, pyruvate, and ammonia, is reversible, mainly due to the relatively low solubility of L-tyrosine in water at pH 7.0. Hence, the enzyme has been used to synthesize isotopically labeled (substituted) L-tyrosine from (substituted) phenol, pyruvate, and ammonia.[26] To force the reaction to a quantitative fission of L-tyrosine, we coupled it to an irreversible enzymatic system, eliminating pyruvate by its reduction to lactate with NADH, regenerated by the formate dehydrogenase (FDH) reaction (TPL = tyrosine phenol lyase, LDH = lactate dehydrogenase):

$$HO-C_6H_4-CH_2-CH(NH_2)-COOH + H_2O$$
$$\underset{TPL}{\rightleftharpoons} HO-C_6H_5 + CH_3-CO-COOH + NH_3$$

$$CH_3-CO-COOH + NADH + H^+$$
$$\underset{LDH}{\rightleftharpoons} CH_3-CHOH-COOH + NAD^+$$

$$NAD^+ + HCOOH \underset{FDH}{\longrightarrow} NADH + H^+ + CO_2$$

L-Tyrosine samples from different origins were incubated at pH 8.0 with the enzyme under these conditions for 48 h at 27 °C. The phenol formed was isolated and analyzed as (2,4,6)-tribromophenol.

**Reaction Mechanism and Possible Oxygen Exchange of the Product with Water.** The mechanism of the tyrosine phenol lyase reaction[9,10] includes the formation of a substrate keto-quinoid intermediate in the enzyme—substrate complex. As the latter contains also two molecules of water in the active site, it would be of importance to study whether the intermediate can exchange oxygen atoms with this water. Therefore, the reaction was first studied in the presence of oxygen-18-labeled water (Table 2). The data demonstrate unequivocally that the $^{18}O$ content of the medium water does not affect the $\delta^{18}O$ value of the phenolic OH group of L-tyrosine. The differences between the samples obtained in normal and $^{18}O$-labeled water suggest that they originate from experimental errors due to the complex analyte preparation.

For the interpretation of the result with respect to the structure of the enzyme and the mechanism of the catalyzed reaction,[9,10] the following three alternatives have to be discussed: (i) The two water molecules in the active site do not equilibrate with the water of the medium. A definite decision about this possibility cannot be drawn from our result. (ii) The water molecules are fixed at their position and do not come into contact with the oxygen atom of the carbonyl group of the intermediate. This is a

**Table 2. Dependence of the $\delta^{18}O$ Value of the $p$-OH Group of L-Tyrosine from Degradation by Tyrosine Phenol Lyase on the Incubation Medium**

| protein and origin[a] | $\delta^{18}O_{VSMOW}$ (‰) incubation water | $\delta^{18}O_{VSMOW}$(‰)$p$-OH L-tyrosine[b] |
|---|---|---|
| casein, comm | −10.0 | +20.4 ± 0.9 |
| | +238.5 | +22.6 ± 0.2 |
| wheat gluten | −10.0 | +27.3 ± 0.1 |
| | +156.2 | +24.0 ± 0.2 |
| beef comm | −10.0 | +23.9 ± 0.1 |
| | +132.1 | +22.4 ± 0.7 |

[a] comm, commercial. [b] The SD relates to the isotope ratio measurement.

quite probable alternative, as the water molecules are situated closer to the side chain than to the *para*-position of the aromatic ring of the substrate.[9] (iii) The half-life of the intermediate is far below that of the exchange reaction ($>CO + HOH \leftrightarrow >C(OH)_2$). As a matter of fact, the minimum half-life of oxygen exchange between carbonyl or quinoid groups and water is on the order of minutes or seconds,[27,28] whereas that of the keto-quinoid intermediate in question must be on the order of milliseconds. Furthermore, the "concentration" of the two reactants is certainly not in favor of a fast exchange reaction. In conclusion, our results are in agreement with the structure of the active site of the enzyme and the kinetic probability of the exchange process. Therefore, the tyrosine phenol lyase reaction is suitable for our purpose, but this had to be experimentally proved.

**Phenol as the Analyte for the Positional Oxygen Isotope Analysis of L-Tyrosine.** First, the selected L-tyrosine samples were characterized by their bulk $\delta^{13}C$ and $\delta^{15}N$ values (Table 3) and compared with the original proteins (Table 1). With two exceptions, all amino acids are depleted in $^{13}C$ by a few ‰ relative to the corresponding proteins. This is in line with general experiences for products of the shikimic acid pathway.[29] Whereas the $\delta^{15}N$ values of most plant and herbivorous L-tyrosine samples are quite similar to those of the corresponding proteins, samples from the omnivorous and carnivorous groups show positive and negative $^{15}N$ shifts relative to their protein, respectively. This can be attributed to their different origins and the complex metabolism of L-tyrosine.

Most informative in the present context are the differences between the positional $\delta^{18}O$ values of L-tyrosine (Table 3) and the bulk $\delta^{18}O$ values of their proteins (Table 1). As outlined above, the positional $\delta^{18}O$ values of the plant L-tyrosine samples, correlated to leaf water, are expected to be $+27 \pm 2‰$, confirmed by the experimentally found value of 27.5‰. Leaf water is also the main source of the protein oxygen; however, the expected shift for plant proteins is $+23 \pm 2.5‰$, also experimentally confirmed (Table 1). In contrast, most positional $\delta^{18}O$ values for L-tyrosine from animal-originating samples differ from those of their proteins by 5−14‰. This is in line with the expected origin of the oxygen in the animal proteins from "cell water" and diet and of the phenolic OH group of L-tyrosine in the proteins from plant leaf water, animal diet, and $O_2$. Among the L-tyrosine samples of casein from milk of cows with controlled diet, the $\delta^{18}O$ value of the product from animals with corn and grass silage

**Table 3.** $\delta^{13}$C and $\delta^{15}$N Values of L-Tyrosine Samples and $\delta^{18}$O Values of Its $p$-OH Group, Analyzed on (2,4,6)-Tribromophenol from Phenol by the Tyrosine Phenol Lyase Degradation of L-Tyrosine

| protein | | L-tyrosine | | L-tyrosine ($p$-OH group) | | | |
| --- | --- | --- | --- | --- | --- | --- | --- |
| | | | | | $-\Delta\delta^{18}$O$_{VSMOW}$ | | |
| name and origin[a] | history, diet[b] | $\delta^{13}$C$_{VPDB}$ (‰) | $\delta^{15}$N$_{AIR}$ (‰) | $\delta^{18}$O$_{VSMOW}$ (‰), exptl[c] | values (‰) from Table 1 | $\delta^{18}$O$_{VSMOW}$ (‰), norm[e] | $f \times 100$ (%), for $f$ see eq 2 |
| wheat gluten, comm | cattle feed, tec qual | −27.8 | +5.1 | +27.3 ± 0.1 | | +27.3 | |
| zein, comm | cattle feed, tec qual | −13.2 | +2.6 | +27.6 ± 0.1 | | +27.6 | |
| chopped beef, comm | unknown | −26.4 | +2.3 | +23.9 ± 0.1 | ±0 | +23.9 | 15.5 |
| cow's milk casein, comm | unknown | −25.8 | +6.8 | +23.5 | +0.7 | +24.2 | 14.0 |
| cow's milk casein 1 | exclusively grass | −30.0 | +5.6 | +19.3 ± 0.8 | +0.2 | +19.5 | 37.5 |
| cow's milk casein 2 | diet grass, hay, conc | −30.3 | +5.5 | +19.3 ± 0.8 | +1.3 | +20.6 | 32.0 |
| cow's milk-casein 3 | corn + grass silage, conc | −25.1 | +5.5 | +22.2 ± 0.8 | −0.4 | +21.8 | 26.0 |
| goat's milk casein | grass, hay, conc | −29.8 | +5.4 | +18.4 ± 1.1 | +1.9 | +20.3 | 33.5 |
| kid goat (muscle) | exclusively goat milk | −30.5 | +5.5 | +18.0 ± 1.8 | +1.7 | +19.7 | 36.5 |
| turkey (muscle), comm | unknown | −26.7 | +3.0 | +21.2 ± 0.2 | +0.3 | +21.5 | 27.5 |
| chicken (feathers), China | unknown | −21.6 | −2.0 | +24.9 ± 0.5 (+18.9 ± 0.4)[d] | ? | +24.9 | 10.5 |
| human (hair), China | unknown | −28.3 | +1.4 | +19.6 ± 0.4 (+17.8 ± 0.2)[d] | ? | +19.6 | 37.0 |
| dog (muscle) | unknown | −26.2 | +5.9 | +18.3 ± 0.6 | −1.1 | +17.2 | 49.0 |
| cat (muscle) | unknown | −26.5 | +5.5 | +17.7 | +0.5 | +18.2 | 44.4 |
| crocodile (muscle), terra | beef | −27.0 | +6.9 | +17.7 ± 1.1 | −2.2 | +15.5 | 57.5 |

[a] comm, commercial; terra, terrarium. [b] conc, concentrate; tec qual, technical quality. [c] exptl, experimental. [d] Values by chemical degradation (see ref 7). [e] norm, normalized.

diet is the most positive one, indicating the highest percentage of "plant L-tyrosine". This result excludes a potential contribution of "animal L-tyrosine" from the silage feed but in contrast suggests that the silage process and/or the rumen fermentation may even contribute to the "plant L-tyrosine". In addition, the result again confirms the influence of the diet on the $\delta^{18}$O value of the samples' OH group and justifies their correction by "normalization".

The individual fractions $f$ of "animal L-tyrosine" ($\delta^{18}$O value +7‰) in the proteins can be calculated from the normalized $\delta^{18}$O values ($\delta^{18}$O$_{norm}$) of the L-tyrosine of the samples. With an average $\delta^{18}$O value of "plant L-tyrosine" (fraction $1 - f$) of +27‰, $\delta^{18}$O$_{norm}$ is

$$\delta^{18}O_{norm} (‰) = [(1 - f) \times 27 + f \times 7](‰)$$

$$f = (27 - \delta^{18}O_{norm})/20 \qquad (2)$$

The expression of $f$ in % ($f \times 100$) (Table 3) shows that the proteins from herbivores and some omnivores contain between 15 and 40% "animal L-tyrosine", among them the goat kid at the upper limit, whereas the carnivorous proteins attain values >40%. This result is convincing proof for the correctness of the principle of the method. The L-tyrosine samples from human hair and chicken feathers are from China, and their preparation is unknown. The difference between the data found by biochemical and chemical analyte preparation is tentatively attributed to an isotope effect on one of them.

**Assessment of the Tyrosine Decarboxylase Reaction for the Positional $\delta^{18}$O Value Determination, Tyramine as Analyte.** Tyrosine decarboxylase (EC 4.1.1.25) is a pyridoxal phosphate dependent enzyme occurring in plants, insects, and mammals; enzymes from different origins have different substrate specificities. The enzyme from animals, involved in the metabolism of neurotransmitters, degrades only aromatic amino acids.[30] The

enzyme from *S. faecalis* used in this work decarboxylates L-tyrosine and L-3,4-dihydroxyphenylalanine, but is inactive versus L-phenylalanine and L-tryptophan. The turnover rate of the catalyzed reaction (TDC = tyrosine decarboxylase)

$$HO-C_6H_4-CH_2-CH(NH_2)-COOH + H_2O$$
$$\xrightarrow{TDC} HO-C_6H_4-CH_2-CH_2-NH_2 + CO_2$$
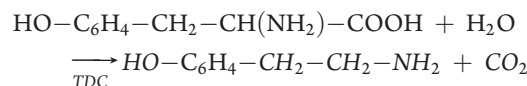
can, at the incubation pH of 5.5, easily be controlled by measuring the volume of the produced $CO_2$. Ziadeh et al.[16] used enzymes from *S. faecalis* for the selective conversion of L-phenylalanine and L-tyrosine into the corresponding amines for a selective $\delta^{15}$N value determination. Although the authors found no difference between the $\delta^{15}$N values of the amino acids and the extracted amines, we do not believe that the applied ethanol extraction of tyramine at pH 5.5 is quantitative, because the compound is amphoteric. Therefore, we tested anion exchange chromatography, as we assumed that this method would be suited to isolate the analyte tyramine in one step. However, impurities from the raw enzyme preparation forced us to add a second purification step, preparative HPLC, which in its turn demanded, because of the low solubility of tyramine in water, the manipulation of large solvent amounts. The undefined composition of the resulting analyte, a mixture of tyramine, tyramine hydrochloride, and NaCl, demands the provision of a purer enzyme preparation for the application of the method as a routine assay with ion exchange chromatography as the sole purification step. A general serious problem has been so far the oxygen isotope ratio determination of N-containing organic analytes, which had only been overcome by high-temperature conversion. The isotope ratio determinations of the tyramine samples have been performed with this method at ETH Zürich. However, on the basis of a recent

**Table 4. $\delta^{13}$C and $\delta^{15}$N Values of L-Tyrosine Samples and $\delta^{18}$O Values of Its $p$-OH Group, Analyzed on Tyramine from the Tyrosine Decarboxylase Degradation of L-Tyrosine**

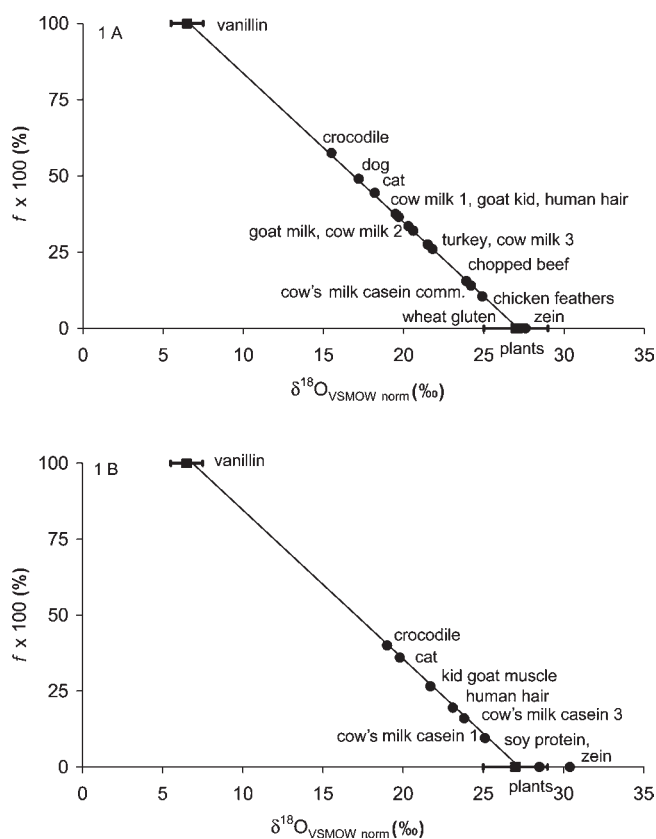| | protein | | L-tyrosine | L-tyrosine ($p$-OH group) | | | | |
| | | | | | | | $f \times 100$ (%),[c] for $f$ see eq 2 | |
| name and origin[a] | history/ diet[b] | $\delta^{13}C_{VPDB}$ (‰) | $\delta^{15}N_{AIR}$ (‰) | $\delta^{18}O_{VSMOW}$ (‰), exptl | $-\Delta\delta^{18}O_{VSMOW}$ (‰) values from Table 1 | $\delta^{18}O_{VSMOW}$ (‰), norm[e] | TDC | TPL |
|---|---|---|---|---|---|---|---|---|
| zein, comm | cattle feed, tec qual | −13.2 | +2.6 | +30.4 ± 0.3 | | +30.4 | | |
| soy protein, comm | cattle feed, comm | −28.0 | +0.8 | +28.5 ± 0.5 | | +28.5 | | |
| cow's milk casein 1 | exclusively grass | −30.0 | +5.6 | +24.9 ± 0.7 | +0.2 | +25.1 | 9.5 | 37.5 |
| cow's milk casein 3 | corn + grass silage, conc | −25.1 | +5.5 | +24.2 ± 0.2 | −0.4 | +23.8 | 16.0 | 26.0 |
| kid goat (muscle) | exclusively goat milk | −30.5 | +5.5 | +20.0 ± 0.4 | +1.7 | +21.7 | 26.5 | 36.5 |
| human (hair), China | unknown | −28.3 | +1.4 | +23.1 ± 0.5 | ? | +23.1 | 19.5 | 37.0 |
| cat (muscle) | unknown | −26.5 | +5.5 | +19.3 ± 0.8 | +0.5 | +19.8 | 36.0 | 44.4 |
| crocodile (muscle), terra | beef | −27.0 | +6.9 | +21.2 ± ? | −2.2 | +19.0 | 40.0 | 57.5 |

[a] comm, commercial; terra, terrarium. [b] tec qual, technical quality; comm, commercial; conc, concentrate. [c] exptl; experimental. [d] TDC, by tyrosine decarboxylase; TPL, by tyrosine phenol lyase, Table 3. [e] norm, normalized.

methodological development,[11] a simpler and more reliable performance will be available in the future.

We incubated preferably samples that had already been analyzed by the tyrosine phenol lyase method. Again, the general agreement of the results (Table 4) with the theory confirms the correctness of the concept and the suitability of the method. Only the positional $\delta^{18}$O value of L-tyrosine from zein exceeds the expected limit for the amino acid from plants (+27 ± 2‰). As also the relatively high $\delta^{18}$O value of the casein from milk of cows fed corn silage (cow's milk casein 3) is in line with this result (Table 1), these results may be due to the special water management of C₄-plants, again demonstrating the influence of the organic part of the diet on the $\delta^{18}$O values of the animal products. These findings will be taken into account in the future for the normalization of the experimental $\delta^{18}$O values. Also, any other data in Table 4 appear to be reasonable.

However, the absolute $\delta^{18}$O values by the tyrosine decarboxylase process are generally at least by 2‰ more positive and the $f$ values more than 10% below those obtained by the tyrosine phenol lyase method (Table 3) for the same samples. The suspicion arises that a systematic error in one of the methods or between them might be the reason. As the L-tyrosine conversions had been quantitative and the isolation methods of the analytes do not imply, as far as could be controlled, isotope fractionations, the only explanation that we can offer for this discrepancy is an oxygen isotope effect on the tyrosine phenol lyase reaction itself, probably on the conversion of the of the COH bond into a CO bond at the formation of the keto-quinoid intermediate.

**Proposal for Practical Application.** The practical check of the content of "animal L-tyrosine" in unknown samples should be performed by means of a graph showing the relative fraction "$f \times 100$" in percent as a function of the normalized $\delta^{18}$O value of the sample (Figure 1). A straight line between the extreme value for a 100% "animal L-tyrosine", +7‰ for any oxygen atom introduced by a monooxygenase reaction as represented by that of vanillin,[8] and the mean theoretical and experimental value for plant originating L-tyrosine, +27‰, will permit the normalized experimental data to be assigned to the "degree of carnivorous feed". Independent from proof of the absolute correctness of the data, the most complete results actually exist from the tyrosine phenol lyase method, and they will be used here for a demonstration of the practical check of meat and bone meal feeding on unknown samples.



**Figure 1.** Relative fraction $f \times 100$ (%) of animal originating L-tyrosine from individual protein samples as a function of the normalized $\delta^{18}$O values of the $p$-OH group of the amino acid: data from L-tyrosine degradation by tyrosine phenol lyase (A) and by tyrosine decarboxylase (B), respectively. The theoretical $\delta^{18}$O values for the extreme values are symbolized by solid squares. That of "animal L-tyrosine" (+7 ± 1‰) is represented by vanillin, and that of "plant L-tyrosine" (+27 ± 2‰) is identical with several experimental data. Comm, commercial. For space reasons the names of some samples are abbreviated.

As the actual ($f \times 100$) values for herbivores, mainly cattle, and some omnivores are below 40% and those for carnivores between

40 and 55%, values for cattle above 40% would be suspicious. A $\delta^{18}O$ value shift of +2‰ corresponds to an increase of 10% in animal protein in the feed source. However, at present this is also near the experimental error limit, but we are convinced that an optimization will soon be possible.

**Summary.** The present results confirm the correctness of the principle of using the $\delta^{18}O$ value of the *p*-OH group of L-tyrosine from animal proteins, normalized by means of their $\delta^{18}O$ values, as an absolute indication of the protein source in the animals' diet. Therefore, they provide proof of the illegal use of meat and bone meal in feed. They also demonstrate that a one-step enzymatic method is realistic for the positional oxygen isotope ratio analysis on the amino acid. Of the two methods tested, the tyrosine decarboxylase process offers the best prospect to be implemented as a routine method. The enzyme is commercially available and, provided the provision of a purer product, the tyramine isolation can be simplified to a one-step procedure. A special advantage is also the easy volumetric turnover control of the reaction. Finally, a recent advance in the oxygen isotope ratio determination of N-containing analytes[11] eliminates any problem in the oxygen isotope ratio analysis of tyramine.

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## ■ ABBREVIATIONS USED

AIR, air (nitrogen); EC, Enzyme Commission (number); NCTC, National Collection of Type Culture; VPDB, Vienna PeeDee Belemnite; VSMOW, Vienna Standard Mean Ocean Water.

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