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In Vitro Quantification of Hydrolysis-Induced Racemization of Amino Acid Enantiomers in Environmental Samples Using Deuterium Labeling and Electron-Impact Ionization Mass Spectrometry

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D-Amino acids indicate aging, bacterial origin, and pathogenic properties of peptides in the environment, but the reliable assessment of D-enantiomers must account for a yet unknown formation during hydrolyses. Here, we introduce a method for the in vitro determination of the hydrolysis-induced racemization (HIR) of amino acids in environmental samples. It involves hydrolyses with hydro- and deuteriochloric acid (6 M, 12 h, 105 °C), desalting, and selective detection of chiral mass fragments of amino acid-*N*-pentafluoropropionyl derivatives. D-Amino acids formed in ^{2}HCl incorporated deuterium into their C_{α} position. This resulted in a relative signal loss of the nondeuterated fragment compared with the ^{1}HCl hydrolysate. Mathematically evaluating the relative target signal intensities of both hydrolysates allowed the quantification of the proportion of D-amino acids formed during sample processing. Side-chain incorporations of deuterium were no limitations for this method as they could be estimated from that of the respective L-enantiomers. In soil and litter samples, between 0 (D-glutamic acid) and 85% (D-allo-isoleucine) of the detected D-amino acids were formed upon hydrolysis (standard error, 5−11%). For a given amino acid, the HIR varied by a factor of 2−10 between samples, thereby confirming that HIR must be individually assessed for samples from different environments.

Life on earth almost exclusively uses levorotatory, or left-handed amino acids (L-enantiomers), rather than D-enantiomers. Nevertheless, with improved analytical methods, D-amino acids have been detected in a variety of peptides of multicellular organisms in the past few years. With increasing gene decoding of the peptides, it became clear that the presence of D-amino acids may be crucial for cell functioning.[1,2] D-Amino acids are used for cross-linking in the bacterial peptidoglycan, probably to protect them from their own proteases.[3] Antibiotics and antimicrobial peptides may often only develop an antiseptic function when containing D-amino acids.[4] Determination of D-amino acids was also used for assessing the nutritional value of food[5,6] and for characterizing the aging of metabolically stable proteins such as in dentin,[7] marine sediments,[8−10] or buried soils.[11] Moreover, the assessment of stereoisomer ratios has been recommended as a tool for reconstruction of earth temperatures[12] or for the identification of extraterrestrial C sources.[13−15] The studies underline the increased interest in a reliable quantification of D-enantiomers in complex matrixes. However, as concluded in the method review of Scaloni et al.,[16] developing methods for reliable assessment of D-amino acids in complex matrixes is still an urgent research demand.

In many environmental studies, only free, rather than protein-bound amino acid enantiomers were investigated.[13,14,17] This might be due to amino acid racemization upon hydrolysis[18,19] or amino acid losses during desalting of environmental samples.[20] Usually, hot 6 M HCl is used to release amino acids from proteins. Both the high temperatures and low pH catalyze the conversion of the L-amino acids into their D-form.[7,21] The opposite way may be neglected in naturally occurring proteins, due to low concentrations of the respective D-amino acids.[22] Subjecting free L-amino

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acids to acid hydrolysis is inadequate for assessing the hydrolysis-induced racemization (HIR) of the L-amino acids in proteins. Racemization of protein-bound amino acids may either be faster (usually at 2−4-fold rate) or slower than that of free amino acids.[7,23,24] This depends on the presence of catalysts, the protein structure, the position of the amino acid within the protein, and, possibly, protein size and folding.[21,25,26] Especially high racemization rates were found for N-terminal amino acids, which racemize 100 times faster than those at the C-terminal end.[7]

Recently, Amelung and Zhang[27] introduced a procedure that allowed reliable removal of a variety of interfering compounds using cation exchange chromatography and oxalate for metal complex formation. Nevertheless, the racemization rate of amino acids upon hydrolysis still remained unclear for environmental proteins with an often unknown structure. To be able to understand the role of D-amino acids in environmental proteins, a method is required that allows us to account for method-induced racemization reactions.

For amino acid racemization, the hydrogen bound to the α-carbon atom is removed and another proton is added from the reverse side. Performing this reaction in a deuterated or tritiated medium leads to a direct labeling of the inverted molecules.[18,24,28] These labeling techniques were yet restricted to the HIR determination of optically pure proteins[18,28] and of lysinoalanine that is not biologically produced in the environment.[24] In addition, these labeling techniques were thought not to be suitable to estimate HIR of protein-bound aspartic and glutamic acids. As these compounds also incorporated deuterium or tritium into their side chains, their D-enantiomer concentration formed upon hydrolysis was not proportional to the amount of heavy hydrogen incorporated.[18,28] Since D-aspartic acid was the best indicator among the D-amino acids for characterizing protein aging,[10,21] and D-glutamic acid content could be an excellent indicator for microbial cell wall residues in the environment,[3] assessing HIR of these two compounds for environmental samples is urgently required.

In general, assessing HIR of amino acids in environmental samples is more difficult than in pure proteins. This is due to higher D-amino acid concentrations being present in aged proteins[10,21,29] as well as natural ^{13}C, ^{15}N, and ^{2}H abundances resulting in isotopic shifts in the respective mass spectra.[30] Such isotope effects can be mathematically accounted for[24] provided that (i) all target ions in the mass spectra are linearly detected and (ii) fragmentation effects do not change relative amino acid ion abundance. For most naturally occurring amino acids both requirements are not fulfilled. (i) Mass detection of D-amino acids in the low-mass range was frequently nonlinear,[27] possibly due to mass fragmentation not following first-order kinetics. (ii) Fragmentation effects were evident in proton-transfer reactions, resulting in additional ion masses by 1 or 2 atomic mass units.[30,31] The amount of incorporated deuterium may therefore not be estimated from the relative intensity of the $m/z + 1$ target ion signal.

This study presents a procedure that allows in vitro assessment of HIR of protein-bound amino acids in naturally occurring proteins, including for the first time those that incorporate deuterium into their side-chain positions. As amino acids were completely recovered upon purification, the method allows reliable quantification of amino acid enantiomers in environmental samples.

MATERIALS AND METHODS

Samples. We used soil and litter samples for the method evaluation since they cover a wide range of proteins from different origins and at different stages of decomposition. The samples comprised three taxonomically different soil types from different climatic regions with different land use and amino acid concentrations. In addition, amino acid enantiomers were quantified in recent (<0.5 year) and decomposed (~2 year) litter samples that do not contain minerals potentially catalyzing HIR (Table 1S, Supporting Information).

Sample Processing. Amino acids were liberated by acid hydrolysis of samples containing 3−4 mg of organic C in either 6 M ^{1}HCl or 6 M ^{2}HCl in $^{2}H_2O$ (12 h, 105 °C). In addition, an L-amino acid standard solution (containing 100 μg of each compound) was treated in the same manner. After the hydrolysis, 200 μL of internal standard solution containing 88 μg of L-norvaline was added to the hydrolysates. Further sample processing was conducted as outlined by Amelung and Zhang.[27] This involved adsorption of the amino acids on cation exchange resins that were rinsed with oxalic acid for metal removal and later with NH_4OH for amino acid elution.

To determine the recovery of incorporated deuterium, the purification steps were also conducted by using a deuterated amino acid mixture as reference solution. It contained >98% deuterium at the α position of each of 100 μg of D/L-alanine-2-$^{2}H_1$, D/L-aspartic acid-2,3,3-$^{2}H_3$, and D/L-glutamic acid-2,4,4-$^{2}H_3$. The first two compounds were purchased from Promochem GmbH (Wesel, Germany); deuterated glutamic acid was obtained from Dr. Ehrensdorfer GmbH (Augsburg, Germany). For the control, 100 μL of this reference solution was freeze-dried and solely subjected to derivatization. To test whether interfering compounds affected deuterium recovery, we also spiked 100 μg of each compound in the deuterated reference solution to a selected mineral soil and litter hydrolysate (nos. 3 and 4, respectively; Table 1S; Supporting Information).

For derivatization, amino acids were converted to *N*-pentafluoropropionyl isopropyl esters as outlined by Frank et al.[19]

Gas Chromatography/Mass Spectrometry. The GC analysis was carried out on a model 6890 gas chromatograph (Hewlett-Packard, Palo Alto, CA) equipped with a Hewlett-Packard model 5973 mass spectrometer (Hewlett-Packard) as the detector. We used a 30-m capillary column (25 m prolonged by 5 m, 0.25-mm i.d., 0.12-μm film thickness) coated with Chirasil-L-Val (Chrompack, Middelburg, The Netherlands) as stationary phase. The system was operated at ramped flow conditions (0.8 mL min^{-1} for 14 min,

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Table 1. Recovery of Spiked Amino Acid Standard Solutions (100 μg of Each Enantiomer) and Proportions of D-Enantiomers in Different Environmental Samples[a]

amino acid	recovery ± SE,[b] % of spike	% D-enantiomer (of respective L-enantiomer)					
		Haplocryoll	Paleustoll	Plinthustalf	fresh litter	decomposed litter	proteins[c]
D,L-alanine	100 ± 3.1	13.8	12.1	8.0	1.9	3.3	0.5−1.3
D,L-valine	105 ± 0.8	1.4	1.6	1.1	1.2	0.6	0.2−0.7
D,L-threonine	95 ± 4.6	0.7	0.7	1.1	0.4	0.5	<0.1−0.9
D,L-isoleucine	112 ± 1.5	1.7	1.5	1.5	1.0	1.2	nd[g]
D-*allo*/L-isoleucine[d]	ns[f]	0.7	0.9	0.6	0.7	0.6	0.3−1.0
D,L-leucine	101 ± 1.6	3.2	4.5	3.4	1.1	1.5	1.7−3.0
D,L-proline	95 ± 1.1	1.4	1.3	2.1	0.7	1.0	0.8−1.5
D,L-serine[e]	102 ± 6.5	3.5	7.0	9.1	0.1	0.4	0.4−2.2
D,L-aspartic acid	88 ± 3.7	16.5	16.4	12.3	1.9	3.0	1.7−5.0
D,L-phenylalanine	97 ± 2.8	3.4	4.7	3.0	1.0	2.0	0.1−1.5
D,L-glutamic acid	88 ± 3.5	11.7	11.0	21.6	1.5	3.4	1.9−3.3
D,L-tyrosine	98 ± 4.9	4.5	4.7	3.7	1.1	1.6	1.4
D,L-lysine	104 ± 5.4	2.6	2.8	3.0	0.6	1.6	1.8−3.0

[a] For details concerning the samples, see Table 1S. [b] Recovery data for the soil types were taken from Amelung and Zhang[27] with the standard error (SE) over the soils and both D- and L-enantiomers ($n = 6$). [c] Percentages of D-enantiomers generated from L-amino acids in proteins as reviewed and assessed by Frank et al., ≥22 h hydrolysis; the rates were estimated from subjecting the proteins to different hydrolysis times. [d] Epimerization of L-isoleucine to D-*allo*-isoleucine. [e] Quantification difficult, due to strong interactions of D-serine with the glass walls of the injection device. [f] ns, not spiked. [g] nd, not determined.

Table 2. Hydrolysis-Induced Racemization of Protein-Derived Amino Acids in Soil and Litter Samples ($n = 2$, SE in Parentheses)

amino acid	% of total D-amino acid concentration detected in soil					% of L-amino acid
	Haplocryoll	Paleustalf	Plinthustalf	fresh litter[a]	decomposed litter	range
D-alanine	5.4 (3.1)	4.5 (2.7)	6.1 (0.1)	35.0 (8.4)	11.4 (3.1)	0.4−0.7
D-valine	26.6 (2.5)	11.5 (1.7)	29.4 (3.7)	43.8 (6.3)	64.1 (2.0)	0.2−0.5
D-threonine	nd[b]	nd	nd	nd	nd	nd
D-*allo*-isoleucine	46.8 (3.2)	48.6 (0.9)	51.5 (2.0)	85.1 (1.0)	70.5 (0.0)	0.7−0.9
D-isoleucine	0	5.5 (1.2)	0	5.4 (0.2)	0	<0.1
D-leucine	20.0 (2.5)	6.1 (1.1)	20.9 (0.9)	50.9 (1.8)	46.0 (0.8)	0.3−0.7
D-proline	50.4 (2.2)	62.3 (9.3)	71.3 (4.2)	61.2 (8.1)	70.8 (2.3)	0.4−1.5
D-serine	nd	nd	nd	nd	nd	nd
D-aspartic acid	16.4 (3.2)	12.3 (2.6)	29.8 (8.7)	15.9 (5.2)	0	0.0−3.7
D-phenylalanine	2.8 (10.5)	4.5 (0.6)	28.3 (0.5)	30.8 (4.4)	24.0 (0.7)	0.1−0.8
D-glutamic acid	0	0.7 (2.3)	0	14.9 (7.2)	0	0.0−0.2
D-tyrosine	30.0 (3.2)	21.7 (1.5)	22.0 (5.5)	46.9 (7.9)	41.9 (7.5)	0.5−1.4
D-lysine	40.3 (0.3)	33.5 (1.4)	23.8 (5.0)	64.0 (5.8)	47.6 (1.7)	0.4−1.0

[a] $n = 3$. [b] nd, not determined (deuterium is reexchanged during mass fragmentation).

1.2 mL min^{-1} for 9 min (rate, 20 mL min^{-1}), and then again 0.8 mL min^{-1} (rate, 0.4 mL min^{-1})). The split ratio was 100:1. Helium was used as the carrier gas. The temperature program was identical to that of Amelung and Zhang.[27] The injector temperature was 200 °C, and the temperature of the MS interface was 230 °C.

For the quantification of amino acid enantiomers, the peak area of a single specific target ion per substance was used. For peak identification, one to three further characteristic ions (qualifiers) per substance were monitored (Table 2S, Supporting Information). Identification of target ions that contained the hydrogen at the C_α position was performed by evaluation of ion mass patterns obtained at EI-SCAN mode with and without deuterium labeling along with comparing relative ion abundances with those reported for the heptafluorobutyl esters.[31] After identification of the target and qualifier ions, the system was operated in the SIM mode. The GC/MS system was calibrated daily with 3−7 point calibration functions of derivatized, external amino acid enantiomer standards.[27]

RESULTS AND DISCUSSION

Precision, linearity, and limit of detection of the determination of D-amino acids using the proposed sample cleanup have already been elucidated by Amelung and Zhang.[27] The lower limit of detection was 0.7 pg (except for serine, for which the detection limit depended on liner quality); the coefficient of variation for replicate analysis was 12% for alanine and ≤10% for all other amino acids using MS quantification. Linearity was frequently not achieved over the whole concentration range; thus, D- and L-enantiomers were quantified with independent calibration functions.

D-Amino Acids in Soil and Litter Samples. The sample hydrolysates contained significant proportions of D-amino acids (Table 1). In soil, high D-enantiomer contents were especially found for alanine and glutamic acid, which are known to occur in bacterial cell walls.[3] High D/L ratios were also found for aspartic acid, which is most prone to racemization.[19] Less D-amino acids were detected in the litter samples. The fresh litter samples

revealed significantly lower D/L ratios than the older, decomposed litter samples (Table 1). Our results, therefore, support previous assumptions that the accumulation of D-amino acids in environmental samples might be a useful indicator for the age, decomposition state, or microbial origin of the organic constituents.[10,32,33]

According to Kimber et al.,[34] the presence of rare D-amino acids that are no part of the microbial protein complex, such as D-proline, D-valine, and D-phenylalanine, could indicate abiotic protein aging in the terrestrial environment. Nevertheless, abiotic racemization is a slow process that may take thousands of years at environmental temperatures.[7,21] Soil organic matter age in the mineral surface soil was approximately only 400−500 years.[35] The litter samples were even much younger (<0.5 years for the German beech litter, ~2 years for the British grass litter layer). This gives rise to the question of whether some of the detected D-amino acids really occurred in the soil prior to hydrolysis.

In general, hydrolysis-induced racemization of free L-amino acid standards may only account for ≤0.5% of D-amino acids.[19,27] In optically pure proteins, however, up to 5% of the L-amino acids inverted during hydrolysis into their D-forms,[19] partly exceeding the amounts of D-amino acids in terrestrial soil and, especially, fresh litter samples (e.g., D-proline, Table 1). As terrestrial samples comprise a mixture of different proteins with yet unknown structure and composition, HIR of the protein-bound D-amino acids is likely different from that of model proteins. As outlined in the introduction, this racemization has not yet been evaluated for environmental samples. Furthermore, it could not be determined for dicarboxylic amino acids that exhibited high D-enantiomer proportions in the soil. The following sections describe a new approach to assess this method-induced racemization. For this purpose, samples and pure standards were hydrolyzed in ^{1}HCl and ^{2}HCl.

Identification of Deuterium Incorporation. Treating an L-amino acid standard solution with hot 6 M HCl results in a slight production of D-amino acids.[19] Their mass spectra were similar to those of the respective L-enantiomers (Figure 1a,b, shown for alanine), suggesting that there were no significant stereochemical effects on fragmentation. In the case of alanine, the highest ion abundance occurred for the fragment m/z = 190 u. It was produced after cleavage of the propionic ester group (Table 2S, Supporting Information; Figure 1). Nevertheless, intense signals were also observed for the qualifier ion masses Q_1 = 191 and Q_2 = 192, amounting to 30−35% of total peak area in this mass range. This was much more than can be attributed to isotope effects.[30] The low natural abundance of ^{13}C (1.1%) and ^{15}N (0.37%) may only explain a relative area intensity of ~6% at the Q_1 mass (for 5 C and 1 N in the T_0 fragment of alanine; Table S2, Supporting Information), and effects on Q_2 (e.g., caused by ^{18}O natural abundance) are even much lower. We, therefore, conclude that the signal intensity discovered at the atomic mass T_0 + 1u (= Q_1 for alanine) did not result from isotope but from fragmentation effects, i.e., from a protonation of the target masses at the electron impact ionization.[30,31]

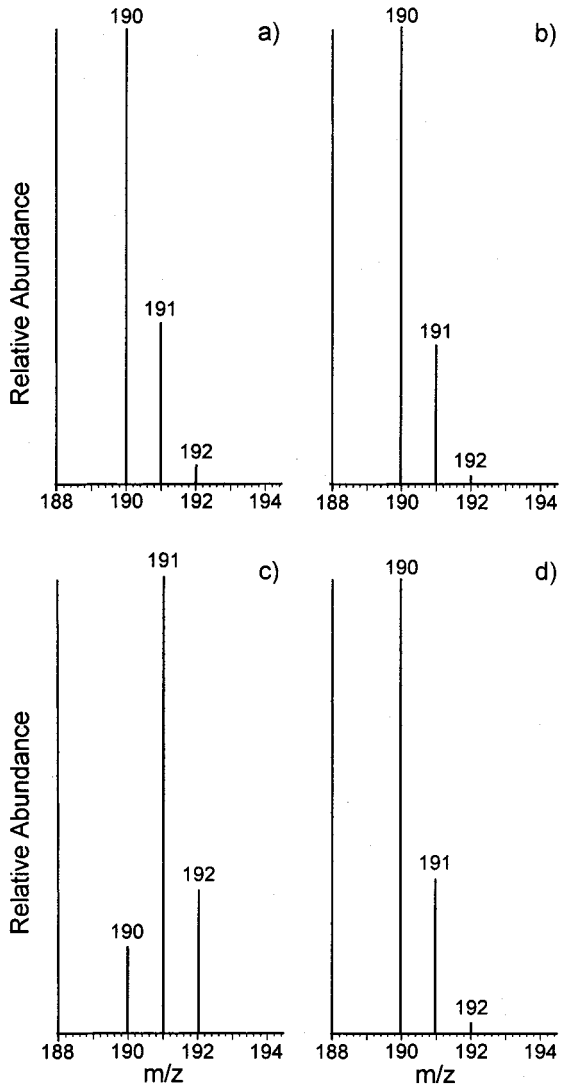

Figure 1. Section of the mass spectrum of *N*-PFP-D-alanine isopropyl esters after subjecting an L-alanine standard solution to hot hydrolysis (12 h, 105 °C) in (a) 6 M ^{1}HCl and (c) in 6 M ^{2}HCl. The spectra of the corresponding L-alanine derivatives are shown on the right-hand side, i.e., after treating the L-alanine standard with (b) hot 6 M ^{1}HCl and (d) hot 6 M ^{2}HCl, respectively. All spectra were recorded using selective ion monitoring of target and qualifier ions.

When hydrolysis was performed in ^{2}HCl, relative ion intensities of the $m/z = T_0 + 1u$ fragments increased at the expense of those of the T_0 ions for the D-enantiomers. The respective mass spectrum of D-alanine was thus dominated by the ion mass m/z = 191 u (Figure 1c). As no such effects were observed for the L-enantiomer (Figure 1d), deuterium was incorporated from the reverse side only at the C_α position during the inversion of the L-amino acid to the D-enantiomer. This is consistent with observations by Frank et al.[24,28,36]

Although the hydrolysis of the L-amino acid standards was performed in high-purity deuteriochloric acid, the respective mass spectra of the D-enantiomer showed evidence of remaining T_0 signal intensity (Figure 1c). This indicated that some of the target mass fragments were not deuterated. This target ion corresponds

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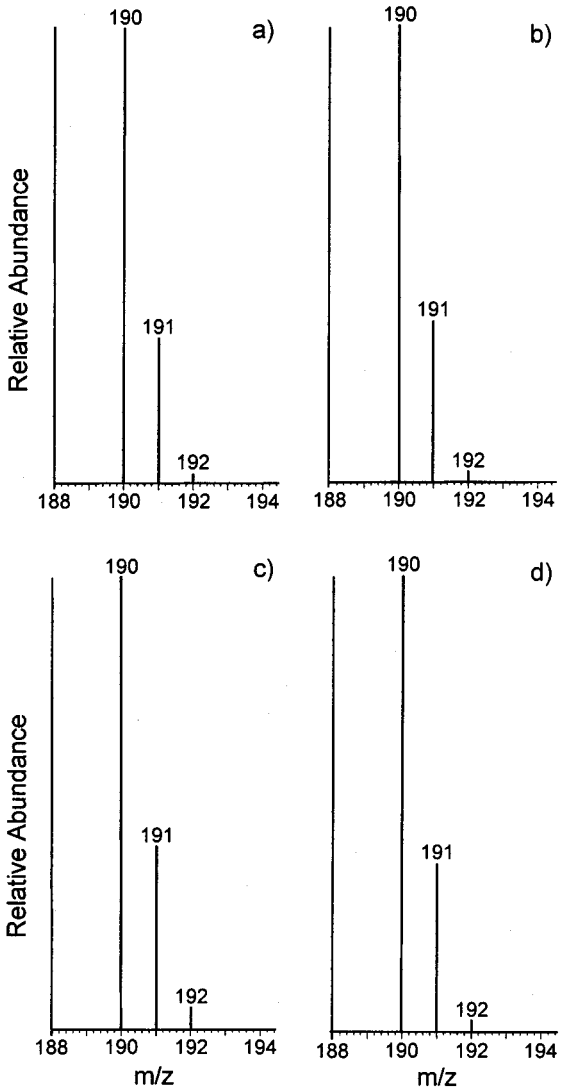

Figure 2. Section of the mass spectrum of *N*-PFP-D-alanine isopropyl esters released from soil (Plinthustalf) with (a) hot 6 M ^{1}HCl and (c) with 6 M ^{2}HCl. The spectra on the right-hand side are those of the corresponding L-alanine derivatives, obtained when treating the soil with (b) hot 6 M ^{1}HCl and (d) hot 6 M ^{2}HCl, respectively. All spectra were recorded using selective ion monitoring of target and qualifier ions.

to the amount of D-amino acids formed upon derivatization (<0.4% of L-alanine racemized upon derivatization[27]). For many environmental samples, such effects may be neglected, due to the higher proportions of D-amino acids found in soil and litter than formed upon derivatization. If in exceptional cases such effects cannot be neglected, they can be mathematically corrected, because the amino acids in environmental hydrolysates exist in free forms; i.e., derivatization-induced racemization is similar to that in derivatization of free standard solutions.

Figure 2 shows the results of a similar experiment conducted with soil instead of a pure L-amino acid solution. Again, mass spectra for L-alanine were similar for the respective ^{1}HCl and ^{2}HCl hydrolysates (Figure 2b,d). And again, racemization upon hydrolysis with ^{2}HCl induced a relative increase of the $m/z = 191$ u signal due to the deuterium incorporation at the C_α (Figure 2a,c). This increase, however, was small, suggesting that the proportion of D-alanine formed during hydrolysis was small. Thus, higher

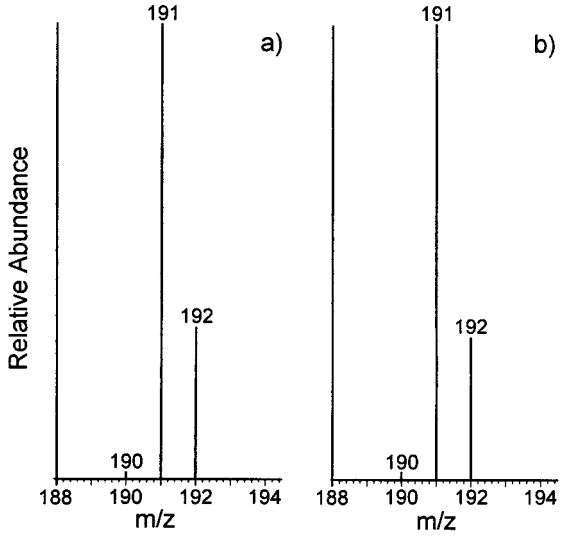

Figure 3. Section of the mass spectrum of prelabeled *N*-PFP-D-alanine (2-$^2H_1$) isopropyl ester (a) after sample cleanup and derivatization and (b) without sample cleanup, solely subjected to derivatization.

signal proportions were detected for the remaining T_0 fragment compared with the pure standard solution. This remaining T_0 signal was too large to solely attribute to D-alanine formed upon derivatization. Therefore, it mainly indicated the detection of soil-inherent D-alanine. This corroborates the finding that D/L ratios of alanine found in the soil hydrolysates largely exceeded those detected after HIR of L-alanine in pure standards or after hydrolytic release of L-alanine in optically pure proteins[19,27] (see also Table 1).

Deuterium Recovery. To ascertain that the incorporated deuterium really remained in the amino acid with further sample processing, we processed racemic alanine that was prelabeled with deuterium at the α position. The respective mass spectra showed that no extra T_0 (190) mass fragment was detected after derivatization (Figure 3b). If deuterium was lost during sample processing, this would have resulted in a loss of one atomic mass unit that would have been indicated by a detection of T_0. Nevertheless, an identical mass spectrum was obtained when spiking prelabeled D,L-alanine (2-$^2H_1$) to a 6 M HCl solution prior to sample processing (Figure 3a). Spiking prelabeled D-alanine to soil and litter also did not result in a significant increase of T_0 signal intensity (spectra not shown). The results implied that deuterium at the C_α position was completely recovered during sample processing. Hydrolysis-induced racemization may therefore be reliably estimated from the proportion of deuterium that is incorporated into D-amino acids after hydrolyzing environmental proteins with hot 6 M ^{2}HCl.

Mathematical Estimation of Deuterium Incorporation. Usually, the relative abundances of the deuterated mass fragments were calculated from the increase of the target ion by one atomic mass.[24,28,36] Nevertheless, our results clearly show that this is not possible using EI-MS due to the high initial abundance of the $m/z = T_0 + 1$u mass fragments (Figure 1c). In addition, the relative signal intensity of the $T_0 + 1$u mass fragment (Q_1 for alanine) is affected not only by a gain in one atomic mass unit due to deuterium incorporation but also by a loss in intensity caused by the formation of the $T_0 + 2$u mass fragment ($= Q_2$ for alanine; Figure 1). The latter corresponds to protonation of the deuterated

mass fragment. We conclude that the degree of deuterium incorporation into any amino acid may not be estimated from the relative signal intensity of the deuterated fragments, i.e., singly hydrolyzing in ^{2}HCl is insufficient for estimating HIR of amino acids in environmental samples. Instead, we recommend continuing quantification of the T_0 target ions in comparative analyses of both ^{1}HCl and ^{2}HCl hydrolysis treatments.

Incorporation of ^{2}H at the C_α position during inversion results in a shift of signal intensity from the T_0 to the $T_0 + 1$u mass fragment. The concentration of D-amino acid generated from the respective L-configuration upon hydrolysis ($C_{D,rac}$) is given by the intensity loss of the T_0 signal after ^{2}HCl hydrolysis relative to the T_0 intensity after ^{1}HCl hydrolysis (eq S1, Supporting Information). Relating this concentration to the total D-amino acid concentration in the ^{2}HCl hydrolysate (not assessable via T_0) gives an estimate of the proportion of D-amino acids formed during hydrolytic treatment of environmental proteins (eq 1), where $C_{D,rac}$ is the

$$\frac{C_{D,rac}}{C_{D^{total},^2HCl}} = \frac{(C_{D,^1HCl} - C_{D,^2HCl}) - \Delta E}{C_{D^{total},^2HCl}} \quad (1)$$

concentration of the D-amino acid racemized upon hydrolysis, $C_{D,^1HCl}$ and $C_{D,^2HCl}$ and the measured concentrations of the nondeuterated D-amino acid after ^{1}HCl and ^{2}HCl hydrolysis, quantified via the signal intensity of the mass fragment T_0, $C_{D^{total},^2HCl}$ is the total D-amino acid concentration in the ^{2}HCl hydrolysate, and ΔE is an error term. It should be noted that eq 1 uses real amino acid concentrations determined from T_0 signal intensities using independent calibration functions and selective ion monitoring of T_0. The mere utilization of changing T_0 signal intensities is not possible for HIR assessment, as the signal response to changing amino acid concentration was frequently nonlinear.

When the ^{2}HCl hydrolysate is used as a correct reference, analytical errors are only reflected in the exact assessment of $C_{D,^1HCl}$. Since it must be assured that the difference in T_0 signal intensities of the ^{1}HCl and ^{2}HCl hydrolysates solely reflects the degree of deuterium incorporation and not any systematic errors in different sample processing, the measured signal intensity of $C_{D,^1HCl}$ should be adjusted to similar sample workup yields as in the ^{2}HCl treatment. This adjustment is indicated by the error term ΔE. As different sample processing of the ^{1}HCl and ^{2}HCl hydrolysates affects not only the D- but also the L-enantiomer concentrations, the analytical error is indicated by different L-enantiomer concentrations in the ^{1}HCl and ^{2}HCl hydrolysates. For error adjustment, the concentration of the D-amino acids in the ^{2}HCl hydrolysate may be upgraded if the respective L-amino acid has been lost and, vice versa, downgraded if the respective L-amino acid concentration was higher than in the ^{1}HCl treatments (see Supporting Information for mathematical details concerning formula development). Thus, the proportion of D-amino acids formed upon hydrolysis may be estimated as

$$\text{HIR (\%)} = C_{D,rac}/C_{D^{total},^2HCl} \times 100 = [1 - (C_{D,^2HCl}/C_{D,^1HCl})\,(C_{L,^1HCl}/C_{L,^2HCl})] \times 100 \quad (2)$$

where HIR is hydrolysis-induced racemization. The same formula is obtained using $C_{D,^1HCl}$ as reference and correcting the $C_{D,^2HCl}$ signals for analytical errors.

According to eq 2, estimation of HIR in environmental samples only requires a quantification of the T_0 signal of the respective D- and L-enantiomers. The equation is valid for all amino acid enantiomers that incorporate one deuterium into their C_α position upon inversion. Equation 2 assumes that the ratio ($C_{L,^2HCl}/C_{L,^1HCl}$) solely reflects analytical errors between the workups of the ^{1}HCl and ^{2}HCl hydrolysates because the incorporated deuterium is solely embedded from the reverse side of the C_α atom and is not lost again upon further sample processing (see above). Theoretically, this assumption is only correct if the sample is free of D-amino acids. Considering racemization from D to L, $C_{L,^1HCl}$ always contains a fraction of L-enantiomer that is derived from this conversion. $C_{L,^2HCl}$ includes the exact same fraction of L-form, but labeled with deuterium. By measuring only the nondeuterated ion fragment, $C_{L,^1HCl}$ should exceed $C_{L,^1HCl}$ in samples with D-amino acids. Nevertheless, this was not the case in any of our samples ($P > 0.05$; aspartic and glutamic acid not tested due to ^{2}H incorporation into the side chains). Hence, the contribution of the racemization of the D-amino acids to $C_{L,^1HCl}$ is not evident, and its effect on HIR may be neglected as proposed by Child et al.[22] Even in the worst case, when the amino acids consist of up to 20% D-forms (Table 1), of which up to 5% may racemize during hydrolysis (Table 2), conversion of D to L only adds 1% signal intensity to $C_{L,^1HCl}$; i.e., violating the assumption that the sample is free of D-amino acids underestimates HIR by a factor of <1.01.

The increase in one atomic mass unit due to the incorporation of deuterium was observed for all amino acids except for the acidic amino acids (see below) and for the D-serine that lost its C_α proton during mass fragmentation (Table 2S, Supporting Information). Our method, therefore, could not be applied to estimate HIR of serine. Uncertainties also remain for threonine due to an unknown proton extraction from the respective target ion (Table 2S, Supporting Information). The formation of D-*allo*-threonine upon hydrolysis of a standard mixture was too small to allow reliable assessment of the pathways of deuterium incorporation above the detection limit. For sensitivity enhancement, it might be advisable to work with chemical ionization-mass spectrometry as this may help to reduce fragmentation effects.[36]

Monoaminodicarboxylic Acids. As aspartic and glutamic acids incorporate deuterium or tritium label into the C_β or C_γ positions of their side chains, it was suggested that labeling techniques may not be used for quantification of HIR.[18,28] Our study confirms that treating pure standard mixtures and soil with ^{2}HCl results in an increase in atomic mass unit larger than unity for these compounds (Figure 4, Figure 1S; Supporting Information). Nevertheless, the respective mass spectra of aspartic and glutamic acids were not similar to those obtained for C_α and side-chain-labeled D/L-aspartic acid-2,3,3-$^2H_3$ and D/L-glutamic acid-2,4,4-$^2H_3$. These spectra were dominated by the $T_0 + 3$u signal (Figure 2S; Supporting Information, not shown for glutamic acid). Our results therefore indicate that side-chain labeling with deuterium was incomplete. Similar to D,L-alanine-2-$^2H_1$, the mass spectra of both prelabeled monoaminodicarboxylic acids remained unchanged when spiking the compounds to the hydrolysate prior to purification. All of the incorporated deuterium was therefore

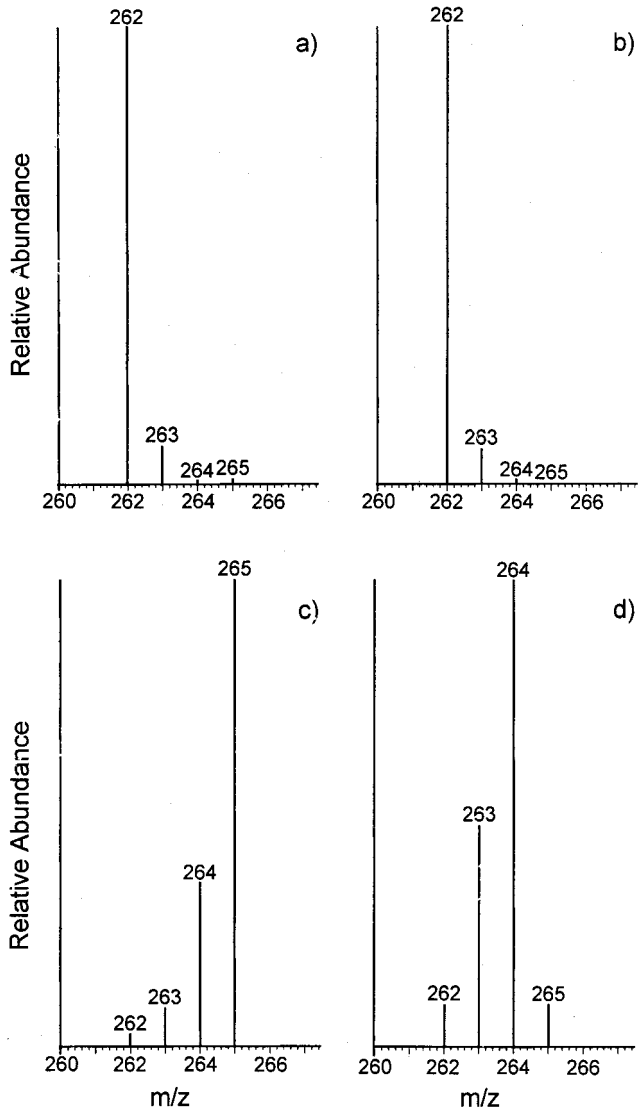

Figure 4. Section of the mass spectrum of *N*-PFP-D-aspartic acid diisopropyl ester of a pure standard after subjecting an L-aspartic acid standard solution to hot hydrolysis (12 h, 105 °C) in (a) 6 M ^{1}HCl and (c) in 6 M ^{2}HCl. The spectra b and d correspond to those of the remaining L-aspartic acid derivatives, i.e., after treating L-aspartic acid with (b) hot 6 M ^{1}HCl and (d) hot 6 M ^{2}HCl, respectively. All spectra were recorded using selective ion monitoring of target and qualifier ions.

completely recovered after sample workup (Figure 2S; Supporting Information). Spiking D/L-aspartic acid-2,3,3-$^2H_3$ and D/L-glutamic acid-2,4,4-$^2H_3$ to the soil and litter did not significantly increase T_0 signal intensity.

Any side-chain incorporation of deuterium results in an additional shift from T_0 to higher molecular masses that goes along with a reduction of T_0 signal intensity by an unknown factor S. To estimate HIR, the measured T_0 signals have to be multiplied with the factor S^{-1} to be able to estimate deuterium incorporation into the C_α position. Since deuterium side-chain reactions occur at both D- and L-amino acids, eq 3 results in the following form:

$$\mathrm{HIR}\ (\%) = 100C_{\mathrm{D,rac}}/C_{\mathrm{D,^1HCl}} = 100[1 - (C_{\mathrm{D,^2HCl}}S_{\mathrm{D}}{}^{-1}/C_{\mathrm{D,^1HCl}})\,(C_{\mathrm{L,^1HCl}}/C_{\mathrm{L,^2HCl}}S_{\mathrm{L}}{}^{-1})] \quad (3)$$

where S_D and S_L are the factors by which the T_0 signals are reduced due to the incorporation of deuterium into the side chains of the D- and L-amino acids, respectively.

We know little about the kinetics of deuterium exchange reactions at side-chain positions of the two amino acids. Assuming that the respective enantiomers exhibit a similar degree of proton replacement in their side chains during hydrolysis, we may write $S_D \approx S_L$; i.e., they cancel out of the fraction, and HIR can be calculated similarly to the other amino acids using eq 2. With the mathematical formalism employed here, side-chain incorporation of deuterium does not limit the estimation of HIR. Analysis showed that S_L remained constant for the different samples (17.2 ± 1.9% for aspartic acid, 28.5 ± 1.3% for glutamic acid), suggesting that side-chain labeling was not affected by the presence of catalysts or different protein nature in environmental samples.

To test that stereochemistry of the amino acids does not significantly affect deuterium labeling at side-chain positions, we need to consider the mechanism of the respective inversion reaction. According to Frank et al.,[19] aspartic acid racemizes fast under acidic conditions due to a stabilization of an enolized intermediate by double bond conjugation. The reaction implies that one of the two protons at the C_β position is actively involved in the inversion reaction of aspartic acid. Since any enolization is a proton exchange reaction, it seems reasonable to assume that the "active" proton is additionally exchanged by deuterium in those D-aspartic acid molecules that are formed during hydrolysis in ^{2}HCl. There is no reason to assume that protons in soil-inherent D-aspartic acid or at the "passive" part of the side chain are replaced more by deuterium than in the respective L-enantiomer. For aspartic acid, S_D could slightly exceed that of S_L, i.e., $S_D{}^{-1} \leq S_L{}^{-1}$. According to eq 3, this results in a slight risk of underestimating HIR of D-aspartic acid. However, when hydrolyzing an L-aspartic acid standard in ^{2}HCl and correcting the signal intensity of the respective D-enantiomer signal for derivatization-induced racemization, signal losses in T_0 intensity relative to a corresponding ^{1}HCl hydrolysate was not significantly more pronounced for the D- than for the L-enantiomer ($P > 0.05$). This suggests that, for free aspartic acid, the assumption $S_L \approx S_D$ is fulfilled. Little is known about the effect of protein structure on the ^{2}H incorporation at side-chain proton positions. However, since the side chains are not part of protein bondings, such effects may be assumed to be negligibly small.

In contrast to aspartic acid, glutamic acid racemizes slowly in HCl, due to lacking potential of the side-chain protons participating in the enolization reaction during inversion. Thus, stereochemical differences in the hydrogen replacement at side-chain positions are unlikely. Hydrolysis of L-glutamic acid standards in ^{1}HCl and ^{2}HCl confirm this assumption (data not shown). Hence, eq 2 gives a correct estimate for the HIR of glutamic acid in environmental samples.

Hydrolysis-Induced Racemization in Environmental Samples. In the right column in Table 2, less than 5% of the L-amino acids converted to the D-form during the 12-h hydrolysis. Relative to the hydrolysis of free standard solutions reported by Amelung and Zhang,[27] higher D-contents were produced by proline, aspartic acid, phenylalanine, and lysine, suggesting that racemization of these amino acids can be favored in environmental proteins. Yet, the degree of conversion varied up to a factor of 8 (phenylalanine) between samples, which may be attributed to

catalysts, different protein structure, or both. Hence, HIR must be individually assessed for a given sample set.

As the significance of HIR for data interpretation depends on the inherent D-content of the amino acids, we expressed HIR relative the total D-amino acid content detected in the samples (eq 2), which confirmed considerable variability of HIR between the different amino acids and the different samples (Table 2). Overall, between 0 and 85% of the D-amino acid concentration found in the hydrolysates of environmental samples must be attributed to HIR. For all amino acids, the mean absolute deviation did not exceed 11% of the D-amino acid concentration in the hydrolysate, i.e., HIR can be estimated with a coefficient of variation of 5−11% of the means, depending on the respective quality of the calibration function.

The HIR contributed relatively little to the concentrations of the bacterial biomarkers D-alanine and D-glutamic acid. Also, the concentration of D-aspartic acid in soil and litter samples may be explained by less than 30% with racemization during hydrolysis. The high D-contents of these amino acids in the environment (Table 1) indicate that, next to amino sugars,[37] other microbial cell wall N residues can be detected in soil. Moreover, the results indicate that protein aging processes that produce D-aspartic acid are evident not only in the abiotic[21,29] but also in the biotic environment (see also Ingrosso and Perna[38]).

Miller et al.[12] determined the epimerization ratio of D-*allo*-isoleucine to L-isoleucine in emu eggshells to reconstruct earth temperatures of the Southern Hemisphere. Our results indicate that a similar approach would not easily work for soil, because more than half of the detected D-*allo*-isoleucine was formed upon hydrolysis (Table 2). In contrast, HIR of L-isoleucine at both asymmetric centers was negligibly small. Care must also be taken when using the presence of D-proline in soil hydrolysates as a marker for cell aging.[29,34] This is because more than 70% of the D-proline concentration is not present in the samples prior to hydrolysis.

We could not quantify HIR of threonine and serine, because the deuterium label was lost during mass fragmentation at the EI mode (70 keV). Modifying their fragmentation pattern, e.g., by using chemical ionization, other derivatization reagents, or by applying LC−MS and MS−MS couplings might warrant further attention. However, since we also observed strong liner sensitivity of D-serine,[27] we conclude that, at present, the determination of this compound in complex environmental samples is not reliably possible.

For a given amino acid, the degree of HIR varied by a factor of 2 (D-*allo*-isoleucine) to 10 (D-phenylalanine) between the different samples (Table 2). When comparing the D/L ratios of a given amino acid in different environmental samples, we strongly recommend quantifying HIR, at least for the key and edge points of each sample set. Here, we only investigated five samples. The present data indicate that, for most of the investigated amino acids, HIR was larger for the litter than for the soil samples due to the lower D/L ratios found in the litter relative to the older organic matter in soil (Table 1). In this context, not only increasing D/L ratios but also decreasing HIR could indicate aging processes in the living environment.

CONCLUSIONS

Reliable quantification of amino acid enantiomers in environmental samples required correction for HIR. This was achieved by simple mathematical evaluation of T_0 signal losses due to deuterium incorporation into the C_α position. The advantage over other recommended procedures, the presented method (i) is not restricted to free amino acids but applicable also to protein-bound amino acids in complex matrixes, (ii) is not affected by isotopic contributions to signal intensities, (iii) is not restricted to optically pure proteins, and (iv) allows the estimation of HIR of amino acids that also incorporate deuterium into side-chain positions for the first time.

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SUPPORTING INFORMATION AVAILABLE

Additional information as indicated in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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