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Assessing the uncertainties of $\delta^{13}C$- and $\delta^{15}N$-values determined by EA-IRMS for palaeodietary studies

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ABSTRACT

At the beginning of a program on palaeodiet, interest arose in how various uncertainty components propagate throughout the process of δ-value determination and how they affect the final uncertainties of the δ-values. The uncertainty components considered in this investigation arise from the precision of the measurement and from the uncertainties of the used isotope standards' δ-values. In the uncertainty analysis, correlations were also taken into account, since they most often lead to an increase of the final uncertainties. The applied procedure permits not only to calculate the overall uncertainty of the normalized δ-values, but also to estimate the contributions of the various sources of uncertainties to the final uncertainty value. It was therefore possible to trace the different uncertainty components throughout the entire evaluation process. The uncertainties of the $\delta^{13}C$-values determined in this study were mainly caused by the respective statistical components resulting from the measurement process, since the uncertainties of the certified $\delta^{13}C$-values of the used international reference materials are small compared to the measurement uncertainty. In contrast, the $\delta^{15}N$ values of the available nitrogen reference materials are less precise than those of the carbon isotope standards and thus more strongly affect the uncertainties of the determined $\delta^{15}N$-values. Consequently the uncertainties of $\delta^{15}N$-values can be underestimated, when only the statistical component is considered. Nevertheless, the final uncertainties obtained in this analysis are small in magnitude compared to the variation in δ-values relevant for diet interpretation. Although the investigated correlations had an enlarging effect on the uncertainties of the normalized δ-values, it could be shown that this effect is small and can be neglected in most palaeodietary studies.

1. Introduction

Carbon and nitrogen stable isotope ratios expressed as $\delta^{13}C$- and $\delta^{15}N$-values, which are defined as the deviation in parts per thousand (‰) of the respective stable isotope ratio of a sample from the ratio of the corresponding international reference material (Vienna Pee Dee Belemnite, VPDB and Ambient Inhalable Reservoir, AIR respectively), in bone collagen are widely used for palaeodietary reconstructions. For this purpose it is common practice to determine the $\delta^{13}C$- and $\delta^{15}N$-values of bone collagen by means of continuous flow elemental analyzer - isotope ratio mass spectrometry (EA-IRMS). This technique allows the determination of these δ-values with a precision in the 0.1‰ range e.g., a precision of ≤0.15‰ and ≤0.20‰ (1 standard deviation of 10 natural abundance samples with a constant quantity of 50 μg carbon and 100 μg nitrogen, respectively) is guaranteed for $\delta^{13}C$- and $\delta^{15}N$-determinations by the manufacturer for the EA-IRMS system used in the present study (*CE Instruments NC*2500 elemental analyser coupled to a *Micromass Optima* mass spectrometer). Under optimized conditions (sample amounts, tuning etc.) higher measurement precisions can be accomplished.

Recently a palaeodiet laboratory has been established at the Isotope Research Group of the University of Vienna. One of the requirements in the setup of this laboratory was the development of a suitable protocol for the EA-IRMS collagen measurements. Also performed was a detailed analysis of various contributions to the final uncertainty of the δ-values determined according to the protocol.

Traditionally for the normalization of the δ-value of a sample to the international isotope scale a so-called "single point anchoring" via a secondary reference material, a calibrated laboratory standard

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or the reference gas, which were tied to the primary standard, was used. Only some years ago, for better inter-laboratory agreement of the isotopic data it was recommended to normalize the determined δ-values to the international isotope scales by a linear function defined by the measured and known δ-values of two isotope standards (e.g., Coplen et al., 2006). Paul et al. (2007) found that using more then two anchor points for a linear regression ("multi-point normalization") resulted in smaller normalization errors compared to the two-point normalization method. The regression coefficients (slope and axis intercept) of the normalization equation are, however, correlated with each other, which makes the determination of uncertainties of the normalized δ-values more complex.

Isotope standards used to establish the normalization function are usually in-house standards, since commercially available isotope standards e.g., from the International Atomic Energy Agency (IAEA) or the United States Geological Survey (USGS) are in short supply and thus should not be used in measurements on a daily basis. Hence, the international reference materials are used for the calibration of laboratory isotope standards suited to the sample materials to be analyzed (e.g., Werner and Brand, 2001). Such standard materials are selected and calibrated by each laboratory individually. Due to the calibration of different in-house standards against the same reference materials, correlations between the calibrated δ-values of these laboratory standards arise. Such correlations influence the uncertainties of sample δ-values normalized against the calibrated laboratory standard values. An increase of the uncertainties can be expected, but it is not predictable to what extent. Since rather small differences in δ-values are generally interpreted as dietary changes e.g., the trophic level shift in $\delta^{13}C$ between a predator and its prey is known to be around 1‰ (e.g., Bocherens and Drucker, 2003), it was necessary to check whether the influence of the correlations leads to overall uncertainties of a magnitude to distort the interpretations of C and N stable isotope data. For a quantification of this effect, a detailed error propagation considering all the correlations arising during the determination of δ-values was performed.

Furthermore, δ-values of palaeodiet samples are in most cases results of multiple determinations and reported as mean values. Usually a set of mathematical transformations is applied to the measurement results in order to obtain the final δ-values of the samples. Considering the achievable precision range, it is of great importance to know the size of other possible uncertainty contributions to the final results in addition to the measurement uncertainty. Therefore, this study considered the propagation of various uncertainty components, associated with the standards and samples, during the entire process of δ-value determination, from the calibration of laboratory standards to the sample δ-values.

2. Methods

2.1. General overview of the applied mathematical procedures

In this approach, mathematical procedures well known in the analysis of experimental data were used. These were the calculation of weighted means, fitting a straight line to a set of experimental data and applying the rules of uncertainty propagation to each step of the data analysis. As the experimental results were not independent from each other, covariances had to be considered to perform the propagation of uncertainties correctly. The methods applied are given in several textbooks on data analysis (see e.g., Branham, 1990; Smith, 1991, and Brandt, 1999). The basic procedures will be described shortly in this section. Vectors and matrices help to formulate them in a very compact and concise manner and they are easily implemented in computer algebra systems and even in standard spread sheet programs, as all matrices needed in the course of this work were small. Typical matrix sizes for which operations like inversion or multiplication had to be performed were 3×3 or 4×4. In a few cases, matrices up to the size 10×10 had to be inverted.

2.1.1. Calculation of a weighted mean and its uncertainty

A set of measured independent data points $y_1, y_2, \ldots, y_n$ can be written as data vector $\mathbf{y}$, and with the help of the corresponding uncertainties $\Delta y_1, \Delta y_2, \ldots, \Delta y_n$, a $n \times n$ variance matrix is defined with the squares of the uncertainties (the variances) as diagonal elements and zero elsewhere. Often the quantities y_i are not independent from each other (i.e., correlated) e.g., if they are measured relative to the same standard, and the uncertainty of the standard contributes to the uncertainties of all measured values. Their uncertainties and dependencies are then described by a variance-covariance matrix, often simply called covariance matrix $\mathbf{C}_y$:

$$\mathbf{C}_y = \begin{pmatrix} \mathrm{var}(y_1) & \mathrm{cov}(y_1,y_2) & \cdots & \mathrm{cov}(y_1,y_n) \\ \mathrm{cov}(y_2,y_1) & & & \vdots \\ \vdots & & & \\ \mathrm{cov}(y_n,y_1) & \cdots & & \mathrm{var}(y_n) \end{pmatrix} \quad (1)$$

In the case of independent data, only the diagonal elements (variances) have to be considered. However, covariances (off-diagonal elements) can occur between quantities deduced from the independent data. These covariances can then be estimated according to the generalized rules of error propagation discussed below.

The covariance matrix is symmetrical ($\mathrm{cov}(y_i, y_j) = \mathrm{cov}(y_j, y_i)$) and frequently correlation coefficients are used to describe the dependency of y_i and y_j:

$$\mathrm{corr}\left(y_i,y_j\right) = \frac{\mathrm{cov}\left(y_i,y_j\right)}{\sqrt{\mathrm{var}(y_i)}\cdot\sqrt{\mathrm{var}\left(y_j\right)}} = \frac{\mathrm{cov}\left(y_i,y_j\right)}{\Delta y_i \cdot \Delta y_j} \quad (2)$$

If weights have to be applied to the measured data $\mathbf{y}$ e.g., to calculate a weighted mean value, a weight matrix $\mathbf{W}$, calculated as the inverted of the covariance matrix ($\mathbf{W} = \mathbf{C}_y{}^{-1}$) is used. With this weight matrix the weighted mean $\bar{y}$ is then calculated according to (following the notation given by Brandt, 1999):

$$\bar{y} = -\left(\mathbf{a}^{\mathrm{T}}\mathbf{W}\mathbf{a}\right)^{-1}\left(\mathbf{a}^{\mathrm{T}}\mathbf{W}\mathbf{y}\right) \quad (3)$$

The vector $\mathbf{a}$ in this case is a single column vector

$$\mathbf{a} = \begin{pmatrix} -1 \\ . \\ . \\ -1 \end{pmatrix} \quad (4)$$

and $\mathbf{a}^{\mathrm{T}}$ is its transposed. In the case of independent y_i the covariance matrix is a diagonal matrix with the squares of the uncertainties, Δy_i^2, as diagonal elements and hence its inverted, the weight matrix $\mathbf{W}$, consists of the inverses of the squares of the uncertainties as diagonal elements and is zero elsewhere. As easily can be seen, Equation (3) then becomes the well known formula

$$\bar{y} = \left(\sum_{i=1}^{n} \Delta y_i^{-2}\right)^{-1} \cdot \sum_{i=1}^{n}\left(\Delta y_i^{-2}\cdot y_i\right). \quad (5)$$

To check the consistency of the data, within their uncertainties and covariances, the reduced χ^2 value can be calculated according to Equation (6), where ϵ is the vector of the residuals (differences between the individual measurements y_i and the weighted mean) and ϵ^{T} its transposed. $\mathbf{W}$ is the weight matrix and f is the number of the degrees of freedom, which is $n-1$, if a mean of n measurement values is calculated.

$$\chi^2_{\mathrm{red}} = \frac{\boldsymbol{\epsilon}^{\mathrm{T}}\mathbf{W}\boldsymbol{\epsilon}}{f} \tag{6}$$

The square root of the reduced χ^2 can be interpreted as the ratio of the external – an uncertainty estimated from the scatter of the data – to the internal uncertainty – an uncertainty calculated from the individual uncertainties Δy_i – and should be approximately one. There does not exist a general rule how to proceed, if χ^2_{red} is significantly larger than unity, but individual solutions have to be found. The treatment of data with inconsistent uncertainties is discussed below. The internal uncertainty can be calculated using the weight matrix **W** and the vector **a** as given in Equation (4):

$$\Delta\bar{y} = \sqrt{\left(\mathbf{a}^{\mathrm{T}}\mathbf{W}\mathbf{a}\right)^{-1}} \tag{7}$$

2.1.2. Calculation of a linear fit through data points

The second procedure used was fitting a straight line or linear function to a set of data points (x_i,y_i) (linear regression). In this case, the linear regression yields a calibration function through data points consisting of certified and measured reference materials' δ-values. Standard least-squares procedures, as described e.g., by Brandt (1999), were used, where it is assumed that the uncertainties of the *y*-values or the covariance matrix $\mathbf{C}_y$ in case of correlated *y*-values are known or can be calculated, and that the *x*-values of the data points are known exactly (without uncertainties). The latter assumption is not valid in the application of this formalism; therefore the uncertainties of the *x*-values were transformed to *y*-value uncertainties (see 2.2). The fitting procedure results in a linear function described by the parameters *a* and *b*:

$$y = a + b\cdot x \tag{8}$$

The least-squares formalism provides a "best estimate" for the parameters *a* and *b* given by the following equation

$$\begin{pmatrix} a \\ b \end{pmatrix} = -\left(\mathbf{A}^{\mathrm{T}}\mathbf{W}\mathbf{A}\right)^{-1}\left(\mathbf{A}^{\mathrm{T}}\mathbf{W}\mathbf{y}\right) \tag{9}$$

Here the vector **y** contains the *y*-values of the measured data points, the weight matrix **W** is the inverted of the corresponding covariance matrix $\mathbf{C}_y$ and the matrix **A** contains the *x*-values of the measured data points.

$$\mathbf{A} = -\begin{pmatrix} 1 & x_1 \\ \vdots & \vdots \\ 1 & x_n \end{pmatrix} \tag{10}$$

The linear function parameters *a* and *b* are derived from measured data and therefore have also uncertainties and in general they are correlated, even if the input data y_i are independent. Their covariance matrix $\mathbf{C}_{(a,b)}$ is given by Equation (11).

$$\mathbf{C}_{(a,b)} = \left(\mathbf{A}^{\mathrm{T}}\mathbf{W}\mathbf{A}\right)^{-1} \tag{11}$$

To check the consistency of the data also for the linear fit, the reduced χ^2 value can be calculated according to Equation (6). The vector of the residuals ϵ is calculated as the differences of the *y*-values of the individual data points and the expected values according to the fitted linear function. The number of the degrees of freedom *f* is in this case $n-2$ with *n* the number of data points.

2.1.3. Propagation of uncertainties

If the data are not independent the rules of error propagation can be generalized to the propagation of variances and covariances in the following way: let *f* be a function depending on the variables $y_1, y_2, \ldots, y_n$. Then the variance (or square of the uncertainty) of this function is given by:

$$\mathrm{var}(f) = \Delta f^2 = \sum_{i=1}^{n}\sum_{j=1}^{n}\frac{\partial f}{\partial y_i}\frac{\partial f}{\partial y_j}\mathrm{cov}\left(y_i, y_j\right) \tag{12}$$

In the case of independent y_i (i.e., $\mathrm{cov}(y_i,y_j) = 0$, for $i \neq j$) Equation (12) becomes the usual "Gaussian error propagation law" as $\mathrm{cov}(y_i,y_i) = \mathrm{var}(y_i)$ and all terms with $i \neq j$ vanish.

$$\mathrm{var}(f) = \Delta f^2 = \sum_{i=1}^{n}\left(\frac{\partial f}{\partial y_i}\right)^2\cdot\mathrm{var}(y_i) = \sum_{i=1}^{n}\left(\frac{\partial f}{\partial y_i}\cdot\Delta y_i\right)^2 \tag{13}$$

When a second function *g* depending on the same variables y_1, $y_2,\ldots, y_n$ is introduced, the covariance between these two functions cov(*f*,*g*) can be calculated according to:

$$\mathrm{cov}(f,g) = \sum_{i=1}^{n}\sum_{j=1}^{n}\frac{\partial f}{\partial y_i}\frac{\partial g}{\partial y_j}\mathrm{cov}\left(y_i, y_j\right) \tag{14}$$

The covariance cov(*f*,*g*) will not be zero for independent y_i, as both *f* and *g* depend on the variables y_i and non-zero contributions to the sum in Equation (14) are present for $i = j$.

A major goal of this investigation was to trace the contributions of the initial (independent) sources of uncertainties throughout the process of δ-value determination. The uncertainties, which had to be considered, are the uncertainties of the reference materials' δ-values provided by the distributor and the measurement uncertainties introduced by the precision of the individual measurements. The contributions of these sources of uncertainties were calculated individually for any intermediate and final result according to the uncertainty propagation rules. Thus for any numerical value derived from these measurements the square of the uncertainty can be represented as a quadratic sum of independent uncertainty contributions.

2.2. Calibration of laboratory standards

In order to produce a laboratory standard, two organic substances (L-alanine, Merck; and fish gelatin, Sigma Aldrich) were calibrated against various international isotope reference materials (USGS40; IAEA: CH-3, CH-6, CH-7, N-1, N-2, NO-3). The δ-values and uncertainties of these reference materials as given by the distributor are listed in Table 1.

In the calibration procedure, several sample batches consisting each of 10 replicates of the material to be calibrated (laboratory standard) plus at least two reference materials, were measured in separate measurement runs (see Table 2). Since the EA-IRMS software can only provide δ-values determined with respect to a single standard or the reference gas, normalization has to be carried out "off-line", when more than one reference material is

Table 1
Consensus δ-values and uncertainties of the international reference materials used in this study (see IAEA Reference Materials Catalogue).

Reference material	$\delta^{13}C$ [‰]	Uncertainty	$\delta^{15}N$ [‰]	Uncertainty
IAEA-CH-3	−24.724	0.041		
IAEA-CH-6	−10.449	0.033		
IAEA-CH-7	−32.151	0.050		
USGS40	−26.389	0.042	−4.5	0.1
IAEA-N-1			0.4	0.2
IAEA-N-2			20.3	0.2
IAEA-NO-3			4.7	0.2

Table 2
Number of measurements carried out during the calibration procedure together with the number of times an individual reference material was present (in triplicate) in a measurement batch.

Standard	δ^{13}C-measurements	USGS40	IAEA-CH-6	IAEA-CH-3	IAEA-CH-7
L-alanine	8	8	8	3	1
Fish gelatin	7	5	7	7	4
Standard	δ^{15}N-measurements	USGS40	IAEA-N-1	IAEA-N-2	IAEA-NO-3
L-alanine	8	8	8	8	0
Fish gelatin	5	3	3	5	3

used. The certified δ-values of the reference materials (y-axis) were plotted against their measured values (x-axis) and the calibration function was considered to be linear as e.g., described in Paul et al. (2007) (see Fig. 1 and Fig. 2). If three or more reference materials were used, the calibration parameters a and b were derived by employing least-squares fitting procedures. It should be noted that the data points through which the calibration function is fitted, have uncertainties in their x-values (the measured values) as well as in their y-values (the certified values). Estimates of the measurement uncertainties were derived from the standard deviation of the 10 laboratory standard replicates present in the batch. The measurement uncertainties were transformed to the y-axis and added in quadrature to the given uncertainties of the reference materials to enable the application of standard least-squares procedures, which rely on the assumption that the abscissa data are known exactly and which use the ordinate data uncertainties to derive a weight matrix. Such a procedure has been widely adopted in nuclear reaction data measurements and evaluations, more general approaches have been extensively discussed in the metrological literature (see e.g., Krystek and Anton, 2007). The preliminary slope used to convert the measurement uncertainties was obtained by linear regression without considering data weights. With a set of three or four determined δ-values (x_i) and a corresponding set of certified δ-values (y_i) together with the combined uncertainties Δy_i, the parameters a and b of the linear calibration function and their variance-covariance matrix were derived according to the weighted standard least-squares fitting procedure given in 2.1.

In this case the covariance matrix contained only the variances of the determined δ-values y_i as diagonal elements i.e. the squares of the combined uncertainties, $(\Delta y_i)^2$, since the international standards are considered as independent. However, the calibration function parameters, i.e. the slope and the axis intercept in the linear equation, calculated according to Equation (9) are not

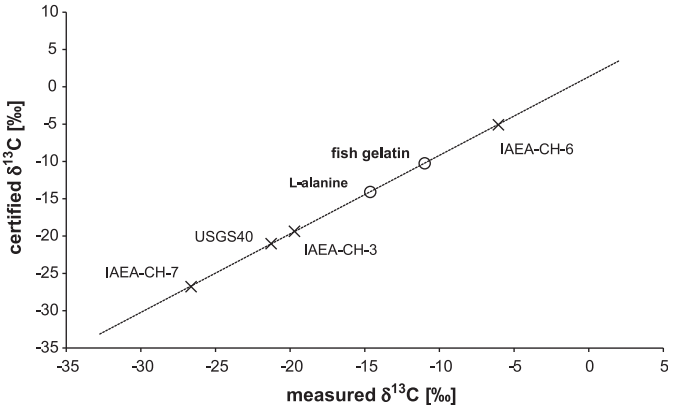

Fig. 1. Normalization of laboratory standard δ^{13}C-values.

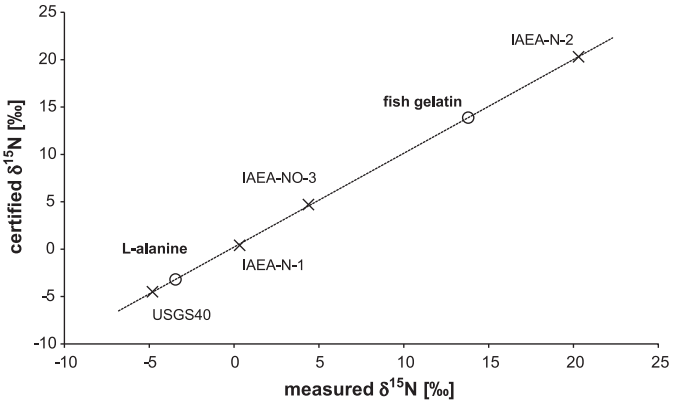

Fig. 2. Normalization of laboratory standard δ^{15}N-values.

independent and their covariance matrix $\mathbf{C}_{(a,b)}$ is given by Equation 11 according to the rules of uncertainty propagation. This matrix contains the uncertainties Δa and Δb of the parameters a and b as well as their covariance cov(a,b).

In the measurement runs where only two international isotope standards were measured together with the replicates of the laboratory standards, the calculation of the calibration function parameters is straightforward. It does not require any least-squares fitting procedures and the covariance matrix $\mathbf{C}_{(a,b)}$ can be calculated according to the generalized rules of uncertainty propagation as described in 2.1.

For each run, the calibration function was then applied to calculate the normalized δ-value of the laboratory standard. The overall uncertainty of the normalized δ-value (ΔS_L) was estimated by generalized uncertainty propagation as shown in Equation (15), where Δx_L is the uncertainty associated with the laboratory standard measurement, Δa the uncertainty of the axis intercept and Δb the uncertainty of the slope of the calibration function. Apart from cov(a,b), no other covariances arise, because a and b have been obtained from the δ-values of the international reference materials and thus are independent from the determined δ-value of the laboratory standard (x_L).

$$\Delta S_L^2 = \left(\frac{\partial S_L}{\partial x}\Delta x_L\right)^2 + \left(\frac{\partial S_L}{\partial a}\Delta a\right)^2 + \left(\frac{\partial S_L}{\partial b}\Delta b\right)^2 + 2\frac{\partial S_L}{\partial a}\frac{\partial S_L}{\partial b}\mathrm{cov}(a,b) \tag{15}$$

The square of the overall uncertainty (ΔS_L^2) was also expressed as quadratic sum of independent uncertainty components. The primary (independent) sources of the uncertainties are the measurement uncertainty of the laboratory standard Δx_L, the measurement uncertainties of the n isotope reference standards, which have been transformed to the y-axis, ΔS_{im} and the given δ-value-uncertainties of the reference materials ΔS_{ic}. Alternatively to Equation 15 the overall uncertainty of the normalized δ-value (ΔS_L) can also be calculated by applying the "Gaussian error propagation law":

$$\Delta S_L^2 = \left(\frac{\partial S_L}{\partial x}\Delta x_L\right)^2 + \sum_{i=1}^{n}\left(\frac{\partial S_L}{\partial S_i}\sqrt{\Delta S_{im}^2 + \Delta S_{ic}^2}\right)^2 \tag{16}$$

with S_i denoting the certified δ-value of an individual reference material. As the uncertainty components stemming from the measurement uncertainties (Δx_L and ΔS_{im}) do not contribute to covariances amongst the individual laboratory standard measurements, they can be combined to a "statistical" (sometimes called "random") component ΔS_{Ls}. Thus Equation 16 was re-arranged and

Table 3
$\delta^{13}C$- and $\delta^{15}N$-values of the calibrated laboratory standards with their 1σ-uncertainties and the different independent uncertainty components, which add up quadratically to the 1σ-uncertainty. For the uncertainty components four positions after the decimal point are shown so that the smallest contribution, which lies in the range of 10^{-4}, can be displayed.

			Uncertainty components				
Standard	$\delta^{13}C$ [‰]	1σ-Uncertainty	Statistical	USGS40	IAEA-CH-6	IAEA-CH-3	IAEA-CH-7
L-alanine	−19.45	0.04	0.0331	0.0169	0.0127	0.0058	0.0005
Fish gelatin	−15.61	0.05	0.0415	0.0045	0.0197	0.0088	0.0005
			Uncertainty components				
Standard	$\delta^{15}N$ [‰]	1σ-Uncertainty	Statistical	USGS40	IAEA-N-1	IAEA-N-2	IAEA-NO-3
L-alanine	−3.19	0.10	0.0406	0.0797	0.0374	0.0032	–
Fish gelatin	13.88	0.14	0.0253	0.0065	0.0193	0.1307	0.0371

the total uncertainty for the result of any of the laboratory standard measurements could be written as:

$$\Delta S_L^2 = \left(\frac{\partial S_L}{\partial x}\Delta x_L\right)^2 + \sum_{i=1}^{n}\left(\frac{\partial S_L}{\partial S_i}\Delta S_{im}\right)^2 + \sum_{i=1}^{n}\left(\frac{\partial S_L}{\partial S_i}\Delta S_{ic}\right)^2 = \Delta S_{Ls}^2 + \sum_{i=1}^{n}\left(\frac{\partial S_L}{\partial S_i}\Delta S_{ic}\right)^2 \quad (17)$$

From these independent uncertainty components, the variance-covariance matrix for each set of individual measurements of the two laboratory standards (alanine and fish gelatin) was calculated according to Equation (14).

For the final δ-values of the laboratory standards a weighted mean of the results of the individual measurements was calculated according to Equation (3), with the vector **y** containing the δ-values of the laboratory standard from the single measurements.

The reduced χ^2 was calculated according to Equation (6). Its numerical value should be approximately one indicating that the internal and the external uncertainty are about the same size, but for both δ-values of the alanine laboratory standard and for the $\delta^{13}C$ of the fish gelatin it was significantly larger than one. A common procedure to deal with this problem, especially if the input data are uncorrelated, is to multiply the entire variance-covariance matrix by a scaling factor $s = \chi^2_{red}$ to force agreement between internal and external uncertainties. It must be noted that this procedure is not based on any theoretical principle, but is nothing more than increasing those uncertainties, which seem to be underestimated and a careful inspection of the data is inevitable (see e.g., the discussion given in Smith, 1991; Section 12.1 and the comment of Brandt, 1999; Section 9.1). As no "questionable" data or outliers were identified, it was decided to enhance only the measurement uncertainties by applying a suitable scaling factor to generate agreement between external and internal uncertainties. This is justified by the analysis of the δ-values, which were determined in different runs performed over a longer time period (several months). This analysis indicated larger variations on a long-term scale compared to the variation of 10 identical samples in a single measurement run, which was initially used as the estimate of the measurement uncertainty.

The overall uncertainty of the weighted mean of the laboratory standards was calculated using the rules of uncertainty propagation (Equation 12) and afterwards split into contributions of the different independent uncertainty components (measurement and reference materials' uncertainties).

As a result of the calibration procedure, the δ-values of the laboratory standards are correlated with each other and with those of the international reference materials, because the same reference materials were used for the calibration of both laboratory standards. From the uncertainty components caused by the reference materials, covariances can be calculated according to Equation (14), which are needed for the calibration of palaeodiet samples described in the next section.

2.3. Evaluation of the uncertainties of palaeodiet samples

In the course of a palaeodiet study, the $\delta^{13}C$- and $\delta^{15}N$-values of a large number of bone collagen samples from different archaeological sites in Austria were determined by EA-IRMS. The instrument was mainly operated in the so-called "peak jump" mode, i.e. after the measurement of the nitrogen isotope ratios the mass spectrometer was switched to carbon and the carbon isotopes were measured subsequently in the same sample. In a measurement sequence (batch) for the determination of the $\delta^{13}C$- and $\delta^{15}N$-values of the investigated collagen samples, 10 replicates of the alanine laboratory standard were included to monitor the instrument performance in each measurement run and to obtain an estimate for the measurement uncertainty. Due to the demand for multiple-point normalization there were three replicates of at least another isotope standard (an international reference material or fish gelatin) present in each sample batch. The N-2 reference material was frequently used in the $\delta^{15}N$ determinations before the fish gelatin was found a suitable candidate for a $\delta^{15}N$ laboratory standard material ($\delta^{15}N$-value: 13.88‰) for palaeodiet, and calibrated as such. In addition to alanine and fish gelatin, the plan is to calibrate cane sugar (C_4 plant) in order to obtain a laboratory standard in the $\delta^{13}C$-range of collagen samples from consumers of marine (or C_4) based diets as well.

Throughout the entire palaeodiet study, different combinations of standards were used, but each combination was selected to encompass the expected range of the samples' δ-values. Every collagen sample was measured three times with each subsample present in a different measurement batch.

The normalization of the determined δ-values followed basically the same procedure as described for the laboratory standards. As shown above, the laboratory standards are correlated with each other and with the international standards used for their calibration. Therefore the off-diagonal elements of the weight matrix for

Table 4
Correlation coefficients between the $\delta^{13}C$-values of the laboratory standards and the international reference materials in percent. Only the upper triangle of the symmetric matrix is displayed.

	L-alanine	Fish gelatin	USGS40	IAEA-CH-6	IAEA-CH-3	IAEA-CH-7
L-alanine	100	20	43	32	15	1
Fish gelatin		100	10	42	19	1
USGS40			100	0	0	0
IAEA-CH-6				100	0	0
IAEA-CH-3					100	0
IAEA-CH-7						100

Table 5
Correlation coefficients between the $\delta^{15}N$-values of the laboratory standards and the international reference materials in percent.

	L-alanine	Fish gelatin	USGS40	IAEA-N-1	IAEA-N-2	IAEA-NO-3
L-alanine	100	12	82	39	3	0
Fish gelatin		100	5	14	94	27
USGS40			100	0	0	0
IAEA-N-1				100	0	0
IAEA-N-2					100	0
IAEA-NO-3						100

calculating the parameters of the linear normalization function for the unknown samples are different from zero.

The standard deviation of the δ-values of all L-alanine laboratory standards measured throughout the period of about one year was used as an estimate of the measurement uncertainty for the sample runs. This long-term variation (0.11‰ for $\delta^{13}C$- and 0.09‰ for $\delta^{15}N$-values) was taken as the minimum measurement uncertainty for every sample run. The standard deviation determined for the 10 laboratory standard replicates in a measurement batch was used when it was larger than the long-term variation.

The overall uncertainty for a single δ-value was estimated according to the generalized rules of error propagation and split into the different components as described above.

The final δ-value of a sample was calculated as the weighted mean of the three measurement runs according to Equation (3). Again, its overall uncertainty was split into the different mutually uncorrelated components.

3. Results and discussion

3.1. Laboratory standards

Table 3 shows the results of the calibration procedure of both laboratory standards. The given uncertainty components are independent (uncorrelated) and thus add up quadratically to the overall uncertainty. The component denoted as "statistical" includes the measurement uncertainties of the laboratory standard replicates and the international reference materials used in the calibration procedure and does not contribute to the covariance between the two laboratory standards. Other components in columns named after the individual reference materials denote the contribution from the uncertainties of the certified values provided by the distributor. The $\delta^{15}N$-values of both laboratory standards show larger uncertainties than the respective $\delta^{13}C$-values. This is caused by the larger uncertainties of the certified values of the available $\delta^{15}N$ reference materials (see Table 1) compared to those of the $\delta^{13}C$ reference materials.

The magnitudes of the individual contributions are largely influenced by the position of the laboratory standard's δ-value on the regression line relative to that of the reference materials. The closer it is to the δ-value of a specific reference material, the larger is the contribution of this reference material. Another influencing factor, besides the "certified uncertainty", is the number of times a certain reference material was used in the calibration process. The more often an international standard is present in the measurements, the larger is its contribution to the total uncertainty of the calibrated laboratory standard value (compare Table 3).

It can be seen from this table that the statistical component accounts for the major part of the overall $\delta^{13}C$-uncertainties. Whereas in the case of the $\delta^{15}N$-uncertainty of the L-alanine standard it is only the second largest contribution after the USGS40-component and the $\delta^{15}N$-uncertainty of fish gelatin is mainly caused by the IAEA-N-2 due to a $\delta^{15}N$-value close to that of the fish gelatin (see Fig. 2).

The correlations between the individual isotope standards determined from the "non-statistical" uncertainty components given in Table 3 according to Equations (14) and (2) are displayed in Table 4 ($\delta^{13}C$) and Table 5 ($\delta^{15}N$). The numerical value of a correlation strongly depends on the same parameters, which determine the magnitude of the uncertainty components (the position of the laboratory standard's δ-value on the regression line, the number of times a reference material is used during the calibration process and the certified uncertainty of a reference material).

Table 6
Final $\delta^{13}C$- and $\delta^{15}N$-values of typical samples with their 1σ-uncertainties and the respective independent uncertainty components, which add up quadratically to the overall 1σ-uncertainty. For the uncertainty components four positions after the decimal point are shown so that the smallest contribution, which lies in the range of 10^{-4}, can be displayed.

Sample-ID	$\delta^{13}C$ [‰]	1σ-uncertainty	Statistical	L-alanine	Fish gelatin	USGS40	IAEA-CH-6	IAEA-CH-3	IAEA-CH-7
31	−22.29	0.08	0.0690	0.0174	–	0.0267	0.0075	0.0030	0.0003
156	−22.12	0.09	0.0724	0.0397	0.0070	0.0210	0.0075	0.0084	0.0007
116	−21.32	0.09	0.0731	0.0401	0.0109	0.0204	0.0091	0.0069	0.0006
151	−20.89	0.08	0.0706	0.0350	0.0073	0.0187	0.0099	0.0077	0.0006
109	−20.75	0.08	0.0719	0.0374	0.0096	0.0192	0.0102	0.0067	0.0006
28	−19.87	0.07	0.0682	0.0166	–	0.0208	0.0121	0.0029	0.0002
138	−19.29	0.08	0.0690	0.0290	0.0077	0.0156	0.0129	0.0067	0.0005
55	−18.16	0.07	0.0679	0.0135	0.0071	0.0156	0.0153	0.0039	0.0003
133	−17.76	0.08	0.0682	0.0231	0.0081	0.0127	0.0159	0.0058	0.0004
50	−17.58	0.07	0.0684	0.0158	–	0.0152	0.0164	0.0028	0.0002
51	−16.27	0.07	0.0688	0.0153	–	0.0120	0.0189	0.0027	0.0002
Sample-ID	$\delta^{15}N$ [‰]	1σ-uncertainty	Statistical	L-alanine	Fish gelatin	USGS40	IAEA-N-1	IAEA-N-2	IAEA-NO-3
28	4.01	0.11	0.0567	0.0120	–	0.0514	0.0356	0.0616	–
31	4.80	0.11	0.0568	0.0113	–	0.0483	0.0355	0.0680	–
116	5.82	0.11	0.0557	0.0192	0.0133	0.0410	0.0279	0.0705	0.0196
109	6.23	0.11	0.0557	0.0182	0.0139	0.0393	0.0274	0.0735	0.0204
156	7.25	0.11	0.0585	0.0158	0.0155	0.0349	0.0264	0.0812	0.0227
151	8.05	0.12	0.0588	0.0139	0.0166	0.0315	0.0255	0.0871	0.0244
133	9.49	0.12	0.0595	0.0104	0.0188	0.0253	0.0240	0.0979	0.0275
51	9.56	0.13	0.0580	0.0074	–	0.0293	0.0349	0.1065	–
55	10.38	0.13	0.0573	0.0049	0.0117	0.0221	0.0312	0.1075	0.0172
138	11.36	0.14	0.0606	0.0060	0.0215	0.0173	0.0220	0.1118	0.0316
50	11.44	0.14	0.0587	0.0058	–	0.0218	0.0347	0.1217	–

3.2. Palaeodiet samples

Table 6 shows a few typical examples of final δ-values of palaeodiet samples determined as weighted means of three measurement results. The component called "statistical" includes the uncertainty associated with the measurements of the sample and the standards in these determinations. "L-alanine" and "fish gelatin" are the contributions of the measurement uncertainties of the laboratory standards and of the international reference materials originating from the respective laboratory standard calibration. Here these two components have to be treated as non-statistical contributions as they contribute to the uncertainties of all paleodiet samples calibrated against them. The components in columns named after the international reference materials comprise the contributions of the respective standard's "certified uncertainty" originating from the sample measurement and the contributions of the "certified uncertainties" propagating from the laboratory standard calibration process. From the uncertainty components given in Table 6, often called an error matrix, covariances between individual sample results can be derived according to Equation (14). This might be of interest, if further statistical procedures are applied, as some statistical procedures require that correlations within the data set studied are small.

Similar to the findings in the laboratory standard calibration, the overall $\delta^{13}C$-uncertainties of the samples were mainly caused by the "statistical" component and the biggest contribution to the $\delta^{15}N$-uncertainties came from the uncertainty of the certified value of an international reference material. The reference material that contributed to the highest degree to the $\delta^{15}N$-uncertainties of the samples was the IAEA-N-2 standard. Although the $\delta^{15}N$-value of the NO-3 standard matches best with the $\delta^{15}N$-values of the samples, no significant contribution was generated by this standard, as it was never directly employed in the sample measurements and in the calibration of L-alanine. Further it was seldom used in the calibration of the fish gelatin. In contrast, the N-2 standard was present in many sample measurements and used as an anchor point in the calibration of both laboratory standards. Consequently this standard has the biggest influence on the sample uncertainties, with the magnitude of the contribution depending on the numerical $\delta^{15}N$-value of the sample. The closer a sample's $\delta^{15}N$-value matches the value of the N-2 standard, the larger is the magnitude of the N-2 contribution, which is also reflected in the final uncertainties. This is depicted in Fig. 3, where the final uncertainties of the samples from four "measurement series" are plotted against their $\delta^{15}N$-values. A series comprises 3 determinations of a sample batch, for which the same combination of isotope standards was used. From Fig. 3 it is evident, that the lower $\delta^{15}N$-values as e.g., found in herbivore collagen, were determined with smaller uncertainties than the higher ones (closer to that of the N-2 standard) in the range of omnivore/carnivore collagen. This finding is a consequence of the chosen measurement protocol and is mainly caused by the selection of the reference materials used for the normalization of the laboratory standards and the δ-values of the samples to the international isotope scales.

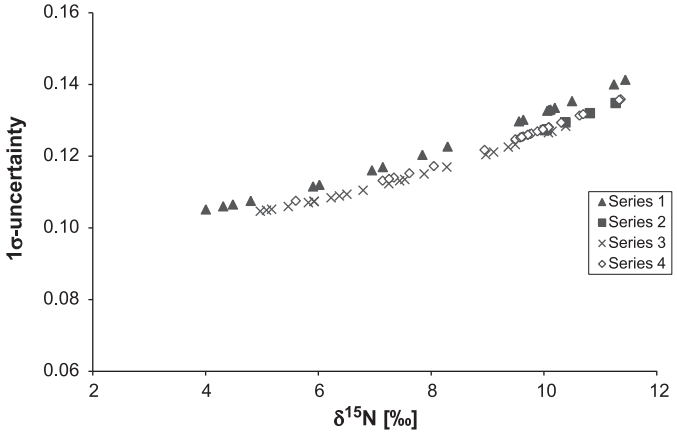

Fig. 3. Dependency of the final uncertainty of a sample on its numerical $\delta^{15}N$-value, found in the data of this study. The samples combined in one batch were measured three times on different days using the same combination of isotope standards. The final $\delta^{15}N$-value of a sample, plotted in this diagram, is a weighted mean of the three determinations.

4. Conclusions

An in-depth study of the uncertainties as they arise in the determination of $\delta^{13}C$- and $\delta^{15}N$-values, when multi-point normalization of the δ-values is used, was performed. This study investigated the influence of correlated uncertainties, which are a consequence of the calibration and normalization procedures, on the final uncertainties of sample δ-values.

For the assessment of the uncertainties of δ-values determined in collagen samples for a palaeodiet study, a method was applied which allowed the tracing of different uncertainty components arising from the precision of the measurement and from the uncertainties of the used isotope standards' δ-values, throughout the entire evaluation process. The contributions of the individual uncertainty components were investigated, from the calibration of the laboratory standards to the calculation of the weighted means of triplicate δ-value determinations of real samples.

At each step of this process, numerical values for the correlations between the standards, which had been introduced due to normalization to the same reference materials, and also between the different measurement runs for a sample batch were calculated. They were considered during the normalization and the calculation of weighted means of the δ-values. The combination of isotope reference materials chosen for the calibration of laboratory standards influences the final uncertainty of the δ-values of the samples normalized to these laboratory standards. Furthermore, the uncertainty of a δ-value is affected by the position of the δ-value on the regression line with respect to the anchor points.

The uncertainties of the $\delta^{13}C$-values are mainly caused by the respective statistical components resulting from the measurement process, since the certified δ-values of the used international $\delta^{13}C$ reference materials are very precise. However, the δ-values of the available $\delta^{15}N$ reference materials show clearly larger uncertainties and thus more strongly affect the uncertainties of the determined $\delta^{15}N$-values. Consequently the uncertainties of $\delta^{15}N$-values can be underestimated, when only the statistical component is considered. However, the final uncertainties obtained in this analysis are small in magnitude compared to the variation in δ-values relevant for diet interpretation. The investigated correlations had an enlarging effect on the uncertainties of the normalized δ-values, but this effect was small. Hence, the overall uncertainties of the sample δ-values can in most cases be neglected for further statistical examination like the comparison of group means, as it is frequently employed in the area of palaeodietary studies. For certain applications it is though useful to have accurate estimates of uncertainties e.g., when reproducibility of sample δ-values within a laboratory or between different laboratories is investigated, since the underestimation of uncertainties might imply a false disagreement of the measured values.

References

Brandt, S., 1999. Data Analysis: Statistical and Computational Methods for Scientists and Engineers, third ed. Springer Verlag, New York, Berlin, Heidelberg.

Branham Jr., R.L., 1990. Scientific Data Analysis, An Introduction to Overdetermined Systems. Springer Verlag, New York, Berlin, Heidelberg.

Bocherens, H., Drucker, D., 2003. Trophic level isotopic enrichment of carbon and nitrogen in bone collagen: case studies from recent and ancient terrestrial ecosystems. International Journal of Osteoarchaeology 13, 46–53.

Coplen, T.B., Brand, W.A., Gehre, M., Gröning, M., Meijer, H.A.J., Toman, B., Verkouteren, R.M., 2006. New guidelines for $\delta^{13}C$ measurements. Analytical Chemistry 78, 2439–2441.

IAEA Reference Materials Catalogue. Stable Isotopes. http://nucleus.iaea.org/rpst/ReferenceProducts/ReferenceMaterials/Stable_Isotopes/index.htm (accessed 28.05.11).

Krystek, M., Anton, M., 2007. A weighted total least-squares algorithm for fitting a straight line. Measurement Science and Technology 18, 3438–3442.

Paul, D., Skrzypek, G., Fórizs, I., 2007. Normalization of measured stable isotope compositions to isotope reference scales–a review. Rapid Communications in Mass Spectrometry 21, 3006–3014.

Smith, D.L., 1991. Probability, Statistics, and Data Uncertainties in Nuclear Science and Technology. American Nuclear Society, LaGrange Park.

Werner, R.A., Brand, W.A., 2001. Referencing strategies and techniques in stable isotope ratio analysis. Rapid Communications in Mass Spectrometry 15, 501–519.