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Establishing collagen quality criteria for sulphur isotope analysis of archaeological bone collagen

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Abstract Sulphur isotope measurements of bone collagen from archaeological sites are beginning to be applied more often, yet there are no clear criteria to assess the quality of the collagen and therefore the validity of the sulphur isotope values. We provide elemental data from different methods (DNA sequences, amino acid sequences and mass spectrometric measurements) which are used to establish a reliable system of quality criteria for sulphur isotope analyses of bone collagen. The difference in the amount of sulphur from fish and mammalian collagen type I led to the suggestion to use different criteria to assess the in vivo character of the collagen between these two categories. For establishing quality ranges, the bone collagen of 140 modern animals were analysed. The amount of sulphur in fish and mammalian bone collagen is 0.63±0.08% and 0.28 ±0.07%, respectively. Based on these results we define for mammalian bone collagen an atomic C:S ratio of 600±300 and an atomic N:S ratio of 200±100, and for fish bone an atomic C:S ratio of 175±50 and an atomic N:S ratio of 60± 20. These quality criteria were then applied to 305 specimens from different archaeological contexts.

Keywords Sulphur isotope · d34S · Bone collagen · Quality marker · Sulphur content

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Introduction

Sulphur isotope analysis on archaeological bone material has been increasingly applied in archaeology (Richards et al. 2001; Craig et al. 2006; Privat et al. 2007), largely due to technical advances in mass spectrometry, namely on-line continuous flow (Giesemann et al. 1994) which allowed the use of relatively small amounts of collagen. Sulphur isotopes of human bone collagen can be used to address questions of archaeological importance. For example, sulphur isotope ratios can be used to distinguish between freshwater and terrestrial ecosystems, especially when measured together with carbon and nitrogen isotope ratios. Also, sulphur isotope ratios can discriminate between the consumption of foods from different geographical regions, and so can be used within a given population to identify immigrants, again when used in conjunction with other isotopic measurements.

It is therefore important to establish criteria to validate the results of sulphur isotopic measurements of bone collagen, especially as the method is applied more often. In bone, collagen type I is the most abundant protein and commonly used for reconstructing palaeodiet (Ambrose 1990). Collagen type I is a triple helix (Ramachandran et al. 1968; Bornstein and Traub 1979; Piez 1976) and contains two alpha 1 and one alpha 2 helices (Piez 1984). The alpha 1 and alpha 2 chains differ only slightly and consist mainly of triplets with the order Gly-X-Y (where X stands for proline and Y for hydroxyproline); all other amino acids are less well represented (Piez 1976). The intracellular translated and post-translationally modified collagen fibres are transported into the intercellular space and are then organised into fibrils (Reddi 1984). In bone, the extracellular collagen is embedded in the mineral phase (Veis 1984) and there it can be preserved for a long time either

unaltered or only slightly altered (Ambrose 1990; DeNiro and Weiner 1988). For archaeological studies, it is necessary to determine the integrity of the analysed material. DeNiro (1985) introduced the use of the atomic C:N ratio (2.9–3.6) as a measure of quality for carbon and nitrogen analyses of bone collagen. Ambrose (1990) and DeNiro and Weiner (1988) analysed modern species to generate acceptable ranges of carbon and nitrogen contents. Acceptable quality markers for ancient bone collagen were also published by van Klinken (1999). If a bone collagen has a ratio outside of this acceptable range, this is likely due to the loss of glycine (the most abundant amino acid in collagen) or the addition of nitrogen due to microbial processes. Therefore, most palaeodietary studies rely on these values to indicate the unaltered nature of the extracted bone collagen for carbon and nitrogen isotopic measurements. There are similar issues for sulphur isotope analysis. First, a possible source of additional sulphur is from the burial environment itself. Inorganic sulphates or sulphur compounds from the soil (e.g. gypsum nodules, pyrites or sulphates) can add sulphur to the extracted biomolecules which therefore result in higher sulphur contents. Another source of alteration can be the microbial catalysis of sulphur-containing amino acids of the organic matrix and resulting loss of sulphur. For the isotopic ratios, additional sulphur from the soil is much more of a concern because of a possible shift towards the sulphur isotope ratio of the burial environment meaning that the measured value will not represent the in vivo value. Therefore, we propose the use of a similar measurement as is used in carbon and nitrogen isotope analysis, specifically the reporting of the amount of sulphur in a sample as well as a comparison between the sulphur amounts in the extracted bone collagen compared with the amounts of carbon and nitrogen (C:S and N:S ratios). Although few published studies address the problem of quality control of sulphur isotope analysis, there is still neither an accepted range for sulphur contents nor other reliable markers available. Additionally, the number of published analysed specimen is statistically very low (less than 150 in total). Here, we summarise these previous studies and provide results from our own unpublished studies to suggest quality control indicators for sulphur isotope measurements of archaeological bone collagen.

Previous research

There have been only a few published studies that have assessed the amount of sulphur in bone collagen as an indicator of the preservation quality of the analysed material (Richards et al. 2001; Craig et al. 2006; Privat et al. 2007). Richards et al. (2001) presented the first measurements of direct analysed ancient bone collagen using continuous flow mass spectrometry. Richards et al. (2001) argued for the use of C:S (and N:S) ratios to verify the integrity and in vivo character of the collagen molecules. Their measurements yielded C:S ratios of 494± 128. Based on calculations using published amino acid sequences, they determined the amount of sulphur in bone collagen to be 0.16%, which was also the value determined by Leach (2003). Another approach for calculating theoretical sulphur percentages in collagen type I was suggested by Craig et al. (2006). Here, published DNA sequences from genetic databases of different mammalian species were used to calculate the amount of sulphur containing amino acids and the percentage of sulphur. The theoretic average ratio found for dogs (*Canis familiaris*) and humans (*Homo sapiens*) was 548 and 605 for the C:S ratio and 177 and 189 for the N:S ratio, respectively. The analysed animals (dog, grey seal and red deer) had sulphur amounts of 0.22±0.02%, and therefore had C:S and N:S ratios of 496±39 and 148±12, respectively. Privat et al. (2007) analysed modern (humans, cow and fish) and ancient species for comparison and assessment of sulphur amounts. The resulting C:S and N:S ratios for the modern specimens were 278±104 and 88±33, and for the archaeological samples 394±117 and 121±36, respectively. Therefore, they argue that samples with sulphur amounts above 0.60% and C:S ratio below 200 and N:S ratio below 60 should be excluded. The aim of this present study is to obtain exact ranges for the quality control of sulphur measurements using many more modern samples from a range of species.

Methods

Bone collagen was extracted following the procedures described in Richards and Hedges (1999) and Brown et al. (1988). The bone surface was cleaned by air abrasion with Al_2O_3, cut into a piece with a mass of 500-700 mg and then demineralised in 0.5 M HCl for several days at 4°C. The demineralised bone was heated at 70°C for 48 h and then EZEE filtered and ultrafiltered (cut-off 30 kDa). The resulting filtrate was freeze-dried. This procedure was applied to all analysed bone samples, both modern and ancient.

Approximately 0.5 mg collagen was weighed into a tin capsule for carbon and nitrogen isotopic measurements. The sample was combusted in a Flash EA 2112 coupled to a Delta XP (Thermo-Finnigan®, Bremen, Germany) at the Max-Planck Institute for Evolutionary Anthropology in Leipzig (Germany). The resulting isotopic ratio has an error better than ±0.1‰ and the error for the calculated amount percent is less than 5%.

To analyse sulphur isotopes 10 mg of mammalian/avian bone collagen and 6 mg of fish bone collagen were

weighed out and mixed with 1 mg of V_2O_5 (Microanalysis, U.K.) to catalyse the combustion and reduce the variability (Morrison et al. 2000). The material was then combusted in a Heka EuroVector elemental analyser (HeKaTech, Wegberg, Germany) and analysed in a Thermo-Finnigan Delta V plus (Giesemann et al. 1994; Kester et al. 2001). The combustion takes place at 1,010°C and the resulting gases are reduced over hot copper (~800°C) to minimize the amount of SO_3 (Ueda and Krouse 1986). The gases are transported via a helium flow of ~95 ml/min and separated on a GC column (Poropak 0.8 m at 84°C; see Yun et al. 2005) and then channelled into the mass spectrometer via a ConFlo III. The N_2 and CO_2 gases are diluted out with helium gas and only SO and SO_2 gases are analysed at the masses 48, 50, 64 and 66. The $\delta^{34}S$ value is measured against a sulphur gas standard and corrected for oxygen isotope mass variations (Coleman 2004; Fry et al. 2002). A daily run of ten collagen samples is accompanied by the inorganic international standards NBS127 (20.3‰), IAEA-S1 (-0.3‰), IAEA-S2 (21.5‰) and IAEA-SO-5 (0.5‰) (Coplen and Krouse 1998) and two organic standards: NIST bovine liver 1577 b (7.5‰) (Fry et al. 2002) and IVA protein casein (6.3‰). The precision of the analyses for the standards (organic and inorganic) are better than ±0.4‰ with a standard deviation (σ) of better than 0.3‰. Four internal bone collagen standards (several extractions from the same bone of two pigs and two cows) were run daily within the runs and resulted in a standard deviation of ±0.8‰ (n= 123). Within a daily run, only ten samples of bone collagen can be analysed to avoid biases in the isotopic results, because of the increase of background noises in the mass spectrometer and heavy usage of the filament. The bone collagen samples run in duplicates on different days have differences of ±0.6‰ or better. The amount of sulphur was calibrated daily against the inorganic standards with a resulting error of better than 10%.

Materials

To calculate the theoretical sulphur contents (weight percent) of collagen type I from different species, several published amino acid profiles and DNA/protein sequences were collected and the weight percent of the elements of interest were calculated based on the amino acid contents (see Appendix for details). The DNA sequence for collagen type I of modern collagen should not vary much from the collagen sequences of ancient specimens. The protein database Swiss-Prot contains nine complete modern collagen type I sequences (separate sequences of alpha 1 and alpha 2 chains) of different species (as of August 2008). Table 1 lists the species by Latin name with their taxonomic class and family with the Swiss-Prot ID number. There are minor post-

Table 1 Amounts of carbon, nitrogen, and sulphur calculated from DNA sequences of different species taken from protein/DNA database Swiss-Prot

Common name	Class	Family	Latin name	Amount C (wt %)	Amount N (wt %)	Amount S (wt %)	Atomic C:N	Atomic C:S	Atomic N:S	Sequence ID
Zebrafish	Actinopterygii	Cyprinidae	*Danio rerio*	46.31	17.68	0.45	3.1	274	90	BAA29028; BAA22380
Chicken	Aves	Phasianidae	*Gallus gallus*	46.85	17.58	0.24	3.1	521	167	P02457; P02467
Rat	Mammalia	Muridae	*Rattus norvegicus*	46.69	17.55	0.22	3.1	566	182	P02454; NP_445808
Mouse	Mammalia	Muridae	*Mus musculus*	46.66	17.54	0.23	3.1	541	174	NP_031768; NP_031769
Dog	Mammalia	Canidae	*Canis familiaris*	46.76	17.56	0.23	3.1	542	175	NP_001003090; NP_001003187
Cattle	Mammalia	Bovidae	*Bos taurus*	46.82	17.59	0.19	3.1	657	212	AAI05185; NP_776945
Macaca	Mammalia	Cercopithecidae	*Macaca mulatta*	46.87	17.57	0.18	3.1	694	223	XP_001096194; XP_001097735
Human	Mammalia	Hominidae	*Homo sapiens*	46.80	17.62	0.20	3.1	624	201	P02452; P08123
Human	Mammalia	Hominidae	*Homo sapiens*	46.82	17.60	0.20	3.1	624	201	NP_000079; NP_000080

Table 2 Amounts of carbon, nitrogen, and sulphur calculated from amino acid sequences of different modern species, sequence data taken from listed references

Common name	Class	Family	Latin name	Material	Amount C (wt %)	Amount N (wt %)	Amount S (wt %)	Atomic C:N	Atomic C:S	Atomic N:S	*n*	References
Sturgeon	Actinopterygii	Acipenseridae	*Acipenser transmontanus*	Skin; swim bladder	41.93	15.54	0.30	3.1	380	121	3	Eastoe 1957; Kimura 1992
Japanese eel	Actinopterygii	Anguillidae	*Anuilla japonica*	Muscle	41.84	15.58	0.38	3.1	310	99	2	Sato et al. 1989
Common horse mackerel	Actinopterygii	Carangidae	*Trachurus trachurus*	Skin; muscle	41.43	15.64	0.31	3.1	362	117	2	Yata et al. 2001
Carp	Actinopterygii	Cyprinidae	*Cyprinus carpio*	Skin; muscle; swim bladder; scale; bone	41.84	15.43	0.37	3.2	305	96	9	Kimura 1983; Kimura et al. 1991; Mizuta et al. 1998; Sato et al. 1988; Sato et al. 1989
Grass carp	Actinopterygii	Cyprinidae	*Ctenopharyngodon idella*	Skin	42.04	15.65	0.41	3.1	273	87	1	Zhang et al. 2007
Cod	Actinopterygii	Gadidae	*Gadus morhua*	Skin	41.31	15.73	0.52	3.1	214	70	2	Piez et al. 1963; Gomez-Guillen et al. 2002
Alaska pollack	Actinopterygii	Gadidae	*Pollachius pollachius*	Skin	41.11	15.60	0.48	3.1	228	74	1	Kimura and Ohno 1987
Lingcod	Actinopterygii	Hexagrammidae	*Ophiodon elongatus*	Skin	41.30	15.57	0.50	3.1	220	71	1	Bracho and Haard 1995
Longnose gar	Actinopterygii	Lepisosteidae	*Lepisosteus osseus*	Skin	41.79	15.43	0.35	3.2	318	101	1	Kimura 1992
European hake	Actinopterygii	Merlucciidae	*Merluccius merluccius*	Skin	41.74	15.78	0.44	3.1	253	82	1	Gomez-Guillen et al. 2002
Blue grenadier	Actinopterygii	Merlucciidae	*Macruronus novaezelandiae*	Skin	41.29	15.52	0.39	3.1	282	91	1	Ramshaw et al. 1988
Japanese flounder	Actinopterygii	Paralichthyidae	*Paralichthys olivaceus*	Muscle; skin	41.30	15.54	0.41	3.1	269	87	2	Nishimoto et al. 2005
Bigeye snapper	Actinopterygii	Pricanthidae	*Priacanthus tayenus*	Skin; bone	42.24	15.49	0.35	3.2	337	106	5	Jongjareonrak et al. 2005; Kittiphattanabawon et al. 2005; Zhang et al. 2007
Chum salmon	Actinopterygii	Salmonidae	*Oncorhynchus keta*	Skin; muscle	41.25	15.60	0.45	3.1	248	80	2	Matsui et al. 1991
Rainbow trout	Actinopterygii	Salmonidae	*Oncorhynchus mykiss*	Skin	41.44	15.58	0.50	3.1	221	71	1	Saito et al. 2001
Black drum	Actinopterygii	Sciaenidae	*Pogonia cromis*	Bone; scale	41.70	15.60	0.36	3.1	314	101	2	Ogawa et al. 2004
Common mackerel	Actinopterygii	Scombridae	*Scomber scombrus*	Skin	41.70	15.56	0.41	3.1	271	87	1	Kimura 1983
Megrim	Actinopterygii	Scophthalmidae	*Lepidorhombus boscii*	Skin	41.55	15.90	0.39	3.0	284	93	1	Gomez-Guillen et al. 2002
Catfish	Actinopterygii	Siluriformes	n.d.	Skin	42.78	15.63	0.40	3.2	285	89	1	Liu et al. 2007
Dover sole	Actinopterygii	Soleidae	*Solea vulgaris*	Skin	41.50	15.96	0.30	3.0	369	122	1	Gomez-Guillen et al. 2002
Sheepshead	Actinopterygii	Sparidae	*Archosargus probatocephalus*	Bone; scale	41.68	15.58	0.38	3.1	297	95	2	Ogawa et al. 2004
Yellow sea bream	Actinopterygii	Sparidae	*Dentex tumifroms*	Skin; muscle	41.48	15.52	0.43	3.1	269	86	2	Yata et al. 2001
Lizard fish	Actinopterygii	Synodontidae	*Squirida elongata*	Muscle	41.76	15.58	0.12	3.1	928	297	1	Sato et al. 1989
Ocellate puffer	Actinopterygii	Tetraodontidae	*Takifugu rubipes*	Skin; muscle	41.41	15.49	0.44	3.1	254	81	4	Nagai et al. 2002; Yata et al. 2001; Zhang et al. 2007
Chicken	Aves	Phasianidae	*Gallus gallus*	Bone	42.12	15.32	0.23	3.2	488	152	1	Francois and Glimcher 1967
Cattle	Mammalia	Bovidae	*Bos taurus*	Bone; skin; muscle	42.32	15.28	0.18	3.2	643	199	5	Eastoe 1955; Eastoe and Leach 1958; Kawaguchi 1993; Li et al. 2004; Zhang et al. 2007
Bison	Mammalia	Bovidae	*Bison*	Bone	42.36	15.17	0.19	3.3	595	182	1	Ho 1967
Camel	Mammalia	Camelidae	*Camelus*	Bone	42.53	15.17	0.19	3.3	597	182	1	Ho 1967
Coyote	Mammalia	Canidae	*Canis latrans*	Bone	42.29	15.17	0.19	3.3	594	182	1	Ho 1967
Horse	Mammalia	Equidae	*Equus caballus*	Cartilage; bone; muscle	42.46	16.20	0.14	3.1	845	277	3	Ho 1966; Todhunter et al. 1994

Bobcat	Mammalia	Felidae	*Lynx rufus*	Bone	42.37	15.23	0.20	3.2	565	174	1	Ho 1967
Human	Mammalia	Hominidae	*Homo sapiens sapiens*	Bone; dentine; skin	42.63	15.58	0.19	3.2	609	190	13	Eastoe 1955; Eastoe et al. 1973; Miller and Rhodes 1982; Miller 1984; Seyer et al. 1977
Rabbit	Mammalia	Leporidae	n.d.	Muscle	41.88	15.32	0.21	3.2	532	167	1	Van Klinken and Mook 1990; Sato et al. 1989
Mouse	Mammalia	Muridae	*Mus musculus*	Skin; bone	41.79	15.30	0.29	3.2	389	122	2	Nawrot and Campbell 1977
Rat	Mammalia	Muridae	*Rattus norvegicus*	Tail	42.71	14.92	0.20	3.3	569	171	1	Robinson 1997
Armadillo	Mammalia	n.d.	n.d.	Bone	42.12	15.22	0.17	3.2	661	205	1	Ho 1967
Walrus	Mammalia	Odobenidae	*Odobenus rosmarus*	Bone	42.28	15.13	0.14	3.3	805	247	1	Matsumura 1973
Pig	Mammalia	Suidae	*Sus scrofa*	Muscle; skin	42.53	15.26	0.18	3.3	660	203	3	Katafuchi et al. 2007; Zhang et al. 2007
Black bear	Mammalia	Ursidae	*Ursus americanus*	Bone	42.33	15.16	0.21	3.3	538	165	1	Ho 1967

translational changes in structure (Piez 1984) which are not taken into account for the calculation of the weight percentages. Therefore, differences may occur between sequences from DNA compared to amino acid contents of extracted collagen type I from animal tissues. For comparison, amino acid sequences of collagen type I were collected of three classes of modern animals (Actinopterygii, Aves, and Mammalia). Thirty-nine modern and 15 Pleistocene species were collected (all 54 amino acid sequences are listed in Tables 2 and 3 with references). The calculated amounts of carbon, nitrogen and sulphur were used for comparison with data obtained by isotopic mass spectrometry of modern and ancient bone collagen.

A range of modern and ancient bone collagen of different species was analysed by mass spectrometry. The results from the modern samples are listed in Tables 4 and 5 show the mean values per species of the ancient samples grouped into time periods.

Results and discussion

The calculated amounts of carbon, nitrogen and sulphur from DNA sequences and published amino acid sequences are listed in Tables 1, 2 and 3. For the DNA sequences the average amount of carbon and nitrogen is 46.7 and 17.6 wt %, respectively. However, the amount of sulphur differs between these specimens at the taxonomic level where the mean value of fish collagen type I (Actinopterygii) is 0.45 wt %, for birds (Aves) it is 0.24 wt %, and for mammals (Mammalia) it is 0.21 wt % (Fig. 1). This is also evident in amino acid sequences taken from the literature (modern specimens). Here the amount of carbon and nitrogen is generally lower (carbon 42.1 wt %, nitrogen 15.4 wt %), but the difference in the amount of sulphur remains (fish 0.38 wt %, birds 0.23 wt %, mammals 0.18 wt %), shown in Fig. 2.

As can be seen (Fig. 3), there are signs of collagen degradation in the ancient bone collagen and the average sulphur amount decreases from the modern to prehistoric samples. The number of methionine residues is greatly decreased, and sometimes no methionine was found at all, and therefore the results are no more reliable than for the in vivo character of the (bone) collagen type I.

Most collagen type I does not contain the sulphur-containing amino acid cysteine; therefore, the amount of sulphur originates only from methionine. Neumann (1949) concluded that the amount of methionine in collagen type I differs between mammals and fish. Methionine is an essential amino acid for fish, birds and mammals as it is converted within liver tissue to sulphur-adenosylmethionine, which is the main methyl donor in animal tissues (Cantoni 1975). Small parts of the pool of methionine are metabolised

Table 3 Amounts of carbon, nitrogen, and sulphur calculated from amino acid sequences of different Pleistocene species, sequence data taken from listed references

Common name	Class	Family	Latin name	Material	Amount C (wt %)	Amount N (wt %)	Amount S (wt %)	Atomic C:N	Atomic C:S	Atomic N:S	*n*	References
Bison	Mammalia	Bovidae	n.d.	Bone	41.96	15.23	0.09	3.2	1,307	406	2	Ho 1967
Western Camel	Mammalia	Camelidae	n.d.	Bone	41.96	15.22	0.07	3.2	1,598	497	1	Ho 1967
Camel	Mammalia	Camelidae	n.d.	Bone	41.91	15.24	0.12	3.2	931	290	1	Ho 1967
Dire wolf	Mammalia	Canidae	n.d.	Bone	41.89	15.29	0.12	3.2	1,019	319	2	Ho 1967
Coyote	Mammalia	Canidae	n.d.	Bone	41.74	15.27	0.14	3.2	795	249	1	Ho 1967
Mammoth	Mammalia	Elephantidae	n.d.	Bone	41.61	14.95	0.03	3.2	3,699	1,139	1	Dungworth et al. 1976
Columbian mammoth	Mammalia	Elephantidae	*Mammuthus columbi*	Bone	42.35	15.13	0.21	3.3	538	165	1	Schaedler et al. 1992
Western Horse	Mammalia	Equidae	n.d.	Bone	42.05	15.38	0.10	3.2	1,147	360	3	Ho 1967
Saber toothed cat	Mammalia	Felidae	n.d.	Bone	41.97	15.26	0.14	3.2	808	252	3	Ho 1967
Lion-like cat	Mammalia	Felidae	n.d.	Bone	41.99	15.27	0.16	3.2	715	223	3	Ho 1967
Gopher	Mammalia	Geomyidae	n.d.	Bone	41.89	15.17	0.01	3.2	11,171	3,467	1	Ho 1967
Ground sloth	Mammalia	n.d.	n.d.	Bone	41.93	15.15	0.12	3.2	932	289	1	Ho 1967
Browsing ground sloth	Mammalia	n.d.	n.d.	Bone	42.41	15.03	0.11	3.3	1,028	312	1	Ho 1967
Walrus	Mammalia	Odobenidae	*Odobenus rosmarus*	Bone	45.57	14.10	0.08	3.8	1,519	403	1	Dungworth et al. 1976
Bear	Mammalia	Ursidae	n.d.	Bone	42.05	15.28	0.12	3.2	934	291	1	Ho 1967

n.d. No data

Table 4 Carbon, nitrogen, and sulphur contents of modern species and calculated atomic ratios of these elements

Common name	Class	Family	Latin name	Material	Amount C (wt %)	Amount N (wt %)	Amount S (wt %)	Atomic C:N	Atomic C:S	Atomic N:S	*n*
Pollock	Actinopterygii	Gadidae	*Pollachius pollachius*	Bone	43.40	16.34	0.58	3.10	200	64	1
Haddock	Actinopterygii	Gadidae	*Melanogrammus aeglefinus*	Bone	43.15	16.18	0.63	3.11	183	59	1
Cod	Actinopterygii	Gadidae	*Gadus morhua*	Bone	43.28	16.50	0.63	3.06	187	61	89
Perch	Actinopterygii	Percidae	*Perca fluviatilis*	Bone	44.28	17.03	0.75	3.03	157	52	1
Zander	Actinopterygii	Percidae	*Sander lucioperca*	Bone	44.44	16.77	0.80	3.09	148	48	1
Salmon	Actinopterygii	Salmonidae	n.d.	Bone	44.08	15.95	0.73	3.22	162	50	4
arctic char	Actinopterygii	Salmonidae	*Salvelinus alpinus*	Bone	43.31	15.46	0.64	3.27	180	55	1
Right whale	Mammalia	Balaenidae	*Eubalaena glacialis*	Bone	47.04	16.94	0.26	3.24	482	149	1
Humpback	Mammalia	Balaenopteridae	*Megaptera novaeangliae*	Bone	46.48	16.12	0.26	3.36	477	142	1
Cattle	Mammalia	Bovidae	*Bos taurus*	Bone	44.76	16.53	0.29	3.16	422	134	2
Scimitar-horned oryx	Mammalia	Bovidae	*Oryx dammah*	Bone	44.03	15.97	0.31	3.22	379	118	1
Sheep	Mammalia	Bovidae	*Ovis aries*	Bone	44.04	15.43	0.21	3.34	575	172	3
Goat	Mammalia	Bovidae	*Capra aegagrus*	Bone	43.37	16.75	0.33	3.02	350	116	1
Alpaca	Mammalia	Camelidae	*Vicuna pacos*	Bone	43.94	16.08	0.26	3.19	451	141	1
Llama	Mammalia	Camelidae	*Lama glama*	Bone	43.56	15.94	0.22	3.19	529	166	2
Dog	Mammalia	Canidae	*Canis familiaris*	Bone	43.70	16.07	0.25	3.17	466	147	2
Fox	Mammalia	Canidae	*Vulpus vulpus*	Bone	44.35	16.63	0.28	3.11	434	139	2
Mara	Mammalia	Caviidae	*Dolichotis*	Bone	43.16	15.31	0.32	3.29	360	109	1
Guinea pig	Mammalia	Caviidae	*Cavia porcellus*	Bone	44.35	15.62	0.45	3.31	263	79	1
Squirrel monkey	Mammalia	Cebidae	*Simia sciurea*	Bone	43.71	16.01	0.24	3.19	486	152	1
Common marmoset	Mammalia	Cebidae	*Callithrix jacchus*	Bone	43.77	15.43	0.23	3.31	507	153	1
Deer	Mammalia	Cervidae	*Cervus elaphus*	Bone	43.80	15.70	0.31	3.25	377	116	1
Fallow deer	Mammalia	Cervidae	*Dama dama*	Bone	44.40	16.04	0.17	3.23	696	216	1
Chinchilla	Mammalia	Chinchillidae	n.d.	Bone	45.07	17.04	0.63	3.09	191	62	1
Killer whale	Mammalia	Deplhinidae	*Orcinus orca*	Bone	43.56	17.01	0.29	2.99	401	134	1
Horse	Mammalia	Equidae	*Equus caballus*	Bone	44.39	16.15	0.23	3.21	516	161	2
Puma	Mammalia	Felidae	*Puma concolor*	Bone	44.79	16.26	0.26	3.21	459	143	1
Cat	Mammalia	Felidae	n.d.	Bone	44.50	16.21	0.32	3.20	371	116	1
Rabbit	Mammalia	Leporidae	n.d.	Bone	43.62	15.72	0.35	3.24	339	105	2
Seal	Mammalia	Phocidae	n.d.	Bone	44.80	16.41	0.26	3.19	472	148	3
Raccoon	Mammalia	Procyonidae	*Procyon lotor*	Bone	45.16	16.58	0.27	3.18	446	140	1
Sus	Mammalia	Suidae	*Sus*	Bone	44.67	16.15	0.28	3.23	432	134	6
Brown bear	Mammalia	Ursidae	*Ursus arctos*	Bone	44.53	16.35	0.29	3.18	409	129	1
Bearded dragon	Sauropsida	Agamidae	*Pogona vitticeps*	Bone	46.45	15.32	0.91	3.54	136	38	1

n.d. No data

Table 5 Carbon, nitrogen, and sulphur contents of ancient samples and the calculated atomic ratios of these elements sorted by age

Common name	Class	Family	Latin name	Material	Amount C (wt %)	Amount N (wt %)	Amount S (wt %)	Atomic C:N	Atomic C:S	Atomic N:S	*n*
Historic											
Sturgeon	Actinopterygii	Acipenseridae	n.d.	Bone	41.65	15.75	0.41	3.09	273	88	3
Cod	Actinopterygii	Gadidae	*Gadus morhua*	Bone	42.39	15.46	0.62	3.20	196	61	39
Bird	Aves	n.d.	n.d.	Bone	44.71	15.96	0.29	3.27	417	128	5
Sheep/goat	Mammalia	Bovidae	n.d.	Bone	44.95	16.33	0.26	3.21	521	163	26
Cattle	Mammalia	Bovidae	*Bos taurus*	Bone	44.82	16.42	0.25	3.18	503	158	8
Dog	Mammalia	Canidae	*Canis familiaris*	Bone	43.01	15.73	0.23	3.19	507	159	23
Cat	Mammalia	Felidae	n.d.	Bone	42.75	15.39	0.23	3.24	494	152	7
Human	Mammalia	Hominidae	*Homo sapiens sapiens*	Bone	45.35	16.74	0.23	3.16	536	170	12
Pig	Mammalia	Suidae	*Sus*	Bone	45.55	16.41	0.32	3.24	401	124	4
Ungulates	Mammalia	n.d.	n.d.	Bone	43.71	15.86	0.23	3.22	524	163	33
Prehistoric											
Bird	Aves	n.d.	n.d.	Bone	43.73	16.20	0.21	3.15	555	176	1
Cattle	Mammalia	Bovidae	*Bos taurus*	Bone	42.58	15.48	0.18	3.21	626	195	16
Dog	Mammalia	Canidae	*Canis familiaris*	Bone	43.63	16.00	0.21	3.18	554	174	1
Deer	Mammalia	Cervidae	*Cervus elaphus*	Bone	44.03	16.10	0.18	3.19	643	202	5
Human	Mammalia	Hominidae	*Homo sapiens sapiens*	Bone	42.56	15.21	0.21	3.27	554	170	134
Sea lion	Mammalia	Otariidae	n.d.	Bone	44.91	15.98	0.26	3.28	461	140	1
Wild boar	Mammalia	Suidae	*Sus*	Bone	44.46	16.15	0.18	3.21	649	202	3
Pleistocene											
Bison	Mammalia	Bovidae	n.d.	Bone	33.68	12.20	0.64	3.22	140	44	1
Sika deer	Mammalia	Cervidae	n.d.	Bone	44.12	16.45	0.21	3.13	560	179	1
Mammoth	Mammalia	Elephantidae	n.d.	Bone	43.76	15.91	0.49	3.21	238	74	1
Horse	Mammalia	Equidae	*Equus caballus*	Bone	43.65	15.57	0.23	3.27	540	166	25
Wild cat	Mammalia	Felidae	n.d.	Bone	43.71	15.98	0.20	3.19	583	183	1
Human	Mammalia	Hominidae	*Homo sapiens sapiens*	Bone	43.26	15.49	0.19	3.26	607	186	1
Herbivore	Mammalia	n.d.	n.d.	Bone	43.35	15.90	0.19	3.18	610	192	2

n.d. No data

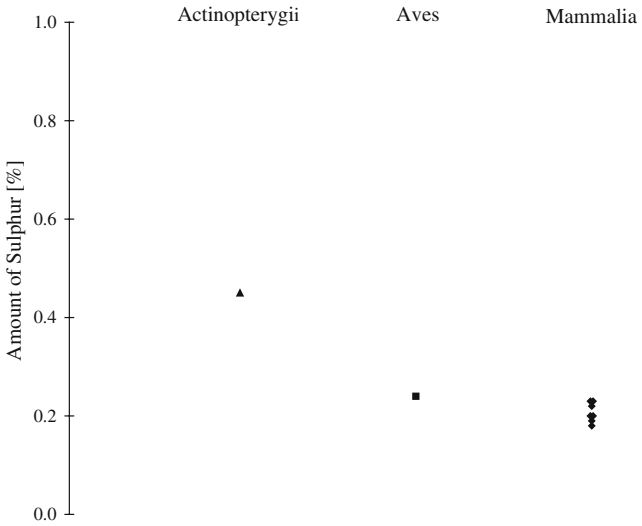

Fig. 1 Theoretical amount of sulphur in collagen type I calculated from DNA sequences based on amino acid composition of chain α1 (2x) and α2 (1x)

to cysteine or other sulphur-containing molecules (Dziewiatkowski 1962; Finkelstein and Martin 1984; Finkelstein et al. 1988), but within a balanced diet the incorporated methionine should be added to the methionine pool without much loss. Therefore, the imbedded methionine in collagen type I originates from dietary methionine (dietary protein; Ambrose and Norr 1993), but the amount of methionine residues within the collagen triple helix is determined by the genetic sequence. In accordance with the genetic sequences, the amino acid sequences of the modern specimens show a similar pattern in the amount of sulphur between fish and mammalian collagen type I (Table 6). Additionally, the amount of carbon and nitrogen differs between these sources of information (DNA sequences vs amino acid sequences). The calculated amount of carbon and nitrogen is consistently higher in sequences from DNA databases than in amino acid sequences. This seems to be a methodological artefact, but the general pattern holds. Although the differences between the methods are significant, taking into account the analytical error for amino acid analysis the absolute values differ only minorly. Both theoretical datasets are problematic; the DNA sequence will be altered post-translationally, and the extraction of collagen type I for the amino acid sequences adds another systematic error of gain or loss of collagenous material. Furthermore, the data of published amino acid sequences are biased as there is a huge variety of extraction methods for collagen type I. But, even with all these uncertainties, the data support the absolute differences in the amount of sulphur between collagen type I of mammals and fish. As shown in Table 6, there are differences between the theoretical and measured data possibly due to methodological errors. Reasons for the observed effect might be additional water bound to the molecule or post-translational changes which are not calculable from DNA sequences.

To avoid methodological biases, we tested if these theoretical considerations hold true by applying mass spectrometry to modern bone collagen extracted following the standard archaeological protocol (see above). The results are given in Table 4, and a summary by species, mean values per class, method and dating are given in Table 6. Most specimens were run at least in duplicate. Figure 3 shows the results of the mass spectrometric sulphur measurements for each specimen. Archaeological bone collagen type I was analysed in comparison to modern bone collagen, to estimate the influence of the burial environment onto the sulphur content in the organic part of the bone. For modern bone collagen from fish, birds and mammals, the median values of the amount of sulphur are 0.61, 0.27 and 0.27 wt %, respectively. Bird and mammalian bone collagen are very similar and therefore will be combined for further analyses of the data, because there are only a few samples of birds, too few for a statistical analysis. The only sample of a saurian species (bearded dragon) was excluded, but shows very interesting results, as discussed below. There is no significant difference in the amount of carbon in fish and mammalian bone collagen, but the amount of nitrogen and sulphur differs significantly (Table 7). The sensitivity to outliers makes the statistical analysis problematic and, because we are not dealing with Gaussian distributed results, all significances are biased. The Mahalanobis distance as a robust measure for the difference (De Maesschalck et al. 2000) can detect statistic significant variance within a dataset with a number of outliers. The analyses of the measured modern samples revealed very small differences in carbon and nitrogen, but not in sulphur. Below a certain threshold, the difference is not significant in a robust sense; this is the case for the amounts of carbon and nitrogen. The amount of sulphur differs signifi-

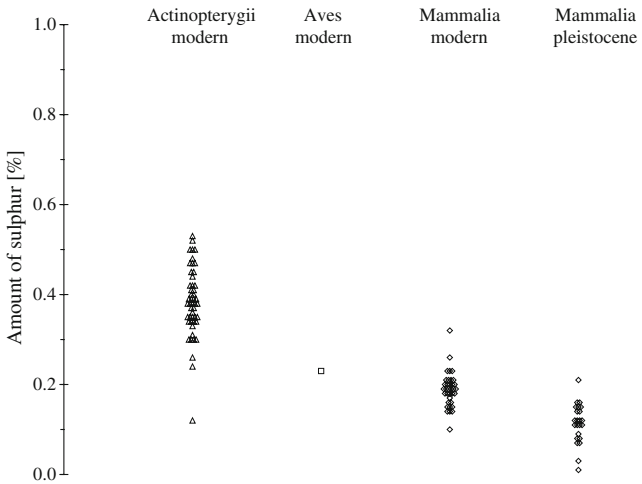

Fig. 2 Theoretical amount of sulphur in collagen type I calculated from amino acid sequences of published chemical protein analyses

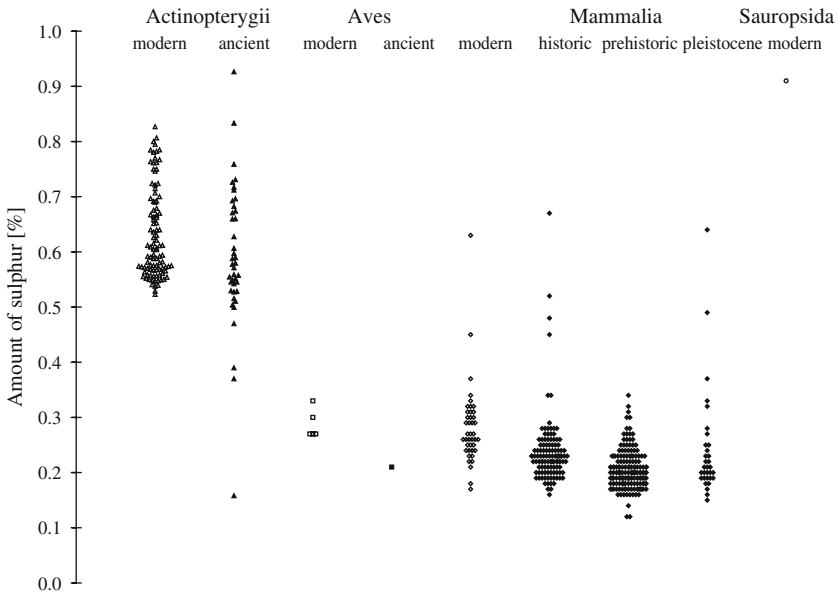

Fig. 3 Amount of sulphur in bone collagen analysed by mass spectrometry. Results are grouped by class of animals and the time periods they originate from

cantly and consistently between fish and mammalian bone collagen; therefore, it is necessary to validate the sulphur data of fish and mammalian bone collagen differently.

For the modern mammals, the amount of sulphur in bone collagen ranges absolutely from 0.17 to 0.63 wt %, but the 95% confidence interval ranges only from 0.19 to 0.32 wt % (average: 0.28±0.07). Because of possible loss or gain of material in archaeological bone collagen, this range should be expanded from 0.15 to 0.35 wt % (Fig. 3). The suggested range is taken from the 95% confidence interval combined with the average value and standard deviation in order to ensure adaptability to many different extraction methods and equipment between different laboratories. The calculation of atomic C:S and atomic N:S ratios (suggested by Richards et al. 2001, following DeNiro 1985) result in acceptable ranges for modern mammalian bone collagen from 313 to 696 and 111 to 216, respectively, when specimens with atomic C:N ratios below 2.9 or above 3.6 (the acceptable ranges for bone

Table 6 Mean values of the amount of carbon, nitrogen, and sulphur in fish (Actinopterygii) and mammals (Mammalia) determined by different methods (calculated from DNA and amino acid sequences and measured by mass spectrometry). Results are divided into time periods and origin

Time period	Element	Method	Actinopterygii	Mammalia
Modern	Carbon	DNA	46.31% (1)	46.78± 0.07% (8)
		Amino acid	41.70±0.40% (49)	42.43±0.92% (36)
		Mass spectrometry	43.34±0.43% (98)	44.41±0.86% (41)
	Nitrogen	DNA	17.68% (1)	17.58±0.03% (8)
		Amino acid	15.56±0.12% (49)	15.44±0.46% (36)
		Mass spectrometry	16.47±0.42% (98)	16.15±0.51% (41)
	Sulphur	DNA	0.45% (1)	0.21±0.02% (8)
		Amino acid	0.38±0.08% (49)	0.19±0.04% (36)
		Mass spectrometry	0.63±0.08% (98)	0.28±0.07% (41)
Historic	Carbon	Mass spectrometry	42.34±1.53% (42)	44.11±2.11% (113)
	Nitrogen	Mass spectrometry	15.48±0.57% (42)	16.07±0.83% (113)
	Sulphur	Mass spectrometry	0.60±0.15% (42)	0.24±0.07% (113)
Prehistoric	Carbon	Mass spectrometry	n.d.	42.67±2.68% (160)
	Nitrogen	Mass spectrometry	n.d.	15.29±1.09% (160)
	Sulphur	Mass spectrometry	n.d.	0.21±0.04% (160)
Pleistocene	Carbon	Amino acid	n.d.	42.14±0.75 (23)
		Mass spectrometry	n.d.	43.32±2.04% (32)
	Nitrogen	Amino acid	n.d.	15.19±0.27% (23)
		Mass spectrometry	n.d.	15.54±0.78% (32)
	Sulphur	Amino acid	n.d.	0.11±0.04% (23)
		Mass spectrometry	n.d.	0.24±0.10% (32)

n.d. No data

Table 7 Statistical analysis of the amount of carbon, nitrogen, and sulphur of fish (Actinopterygii) and mammalian (Mammalia) bone collagen. Results of the Wilks' Lambda test of distribution and Mahalanobis distances tested between the groups Acterinopterygii and Mammalia

	Wilks' Lambda test			
Element	λ	*F* value	p value	Mahalanobis distance
Carbon	0.994	3.071	0.080	0.031
Nitrogen	0.942	29.940	< 0.0001	0.300
Sulphur	0.162	2496.019	< 0.0001	25.043

collagen; DeNiro (1985)) are excluded (Fig. 4). In archaeological material, a similar range from 600±300 and 200±100, respectively, corresponds to valid data in accordance to the range of the amount of sulphur (Figs. 5 and 6). These ranges expand the ones suggested by Richards et al. (2001) (C:S=463±176) or Craig et al. (2006) (C:S=496±39, N:S=148±12) on a much broader range of animal species and number of specimens. The comparison with Privat et al. (2007) is problematic because there is no distinction between fish and mammalian samples, which confuses the mean values for the atomic C:S and N:S ratios.

By applying these quality criteria for mammalian and bird bone collagen to archaeological material, it can be observed that only a few samples tend to have higher amounts of sulphur than acceptable. In general, it can be seen that the amount of sulphur in the archaeological samples tends to be decreased compared to modern samples. There are three historic samples in the dataset with too much (by comparison to modern samples) sulphur, and therefore the atomic C:S and N:S ratios are too low. These results are excluded in the further process of analysing the dataset. Within the prehistoric samples, no obvious outliers were detectable; however, in the Pleistocene samples, again there are two samples outside of the established range. Nevertheless, there is no difference between the archaeological samples, but in the modern samples the amount of sulphur is higher.

The higher amount in modern samples might be a result of additional chondroitin sulphate attached to lysine residues of the modern collagen fibrils (Öbrink et al. 1975). Chondroitin sulphates are polysaccharides and bound in the endoplasmic reticulum to the protein chain. Each molecule can add sulphur to the collagen, when it is not removed completely during the extraction. The chondroitin sulphate connects the mineral phase with the organic collagen fibres (Burger et al. 1962; Schneiders et al. 2008). After demineralising the bone, the chondroitin sulphate can still be bound to the collagen molecules and the additional sugars would be detectable with individual amino acid analysis. During the analysis of carbon and nitrogen of bulk collagen, the contamination with carbon and nitrogen from chondroitin sulphate is not detectable because it is minor. Sulphur analysis is much more sensitive to contamination with additional material, because of the very small amount and direct measurement of one single amino acid (methionine). But no similar effect seems to be visible for archaeological material. During the time of being buried, the short protein attachments are degraded and therefore no longer detectable or measurable. The pH-value of the soil and other environmental influences may disconnect the bonding of chondroitin sulphate and collagen and therefore the small chondroitin sulphate molecules will be lost during ultrafiltration.

The bone collagen of modern fish (class: Actinopterygii) have amounts of sulphur from 0.52 to 0.83 wt % (Fig. 3). Based on this, an acceptable range for archaeological

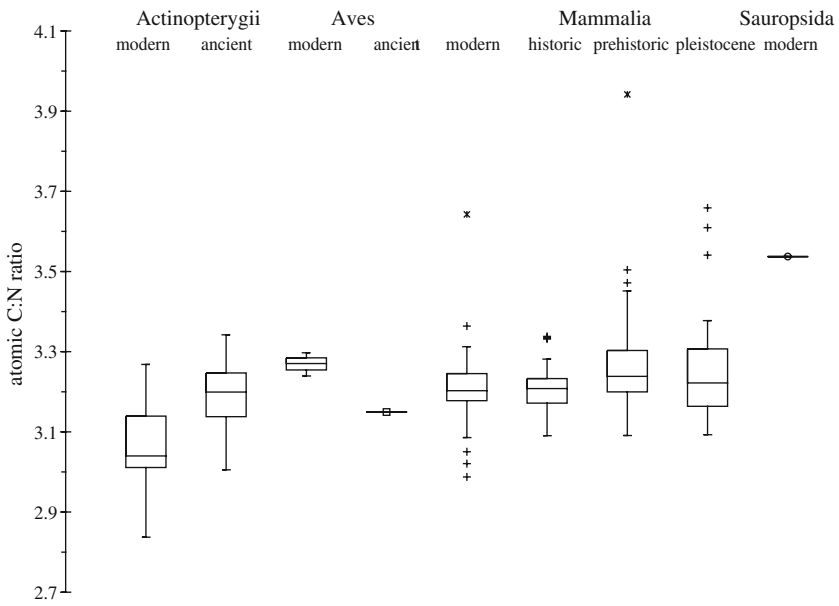

Fig. 4 Box plots of the C:N ratios of samples analysed by mass spectrometry. Results are grouped by class of animals and the time periods they originate from

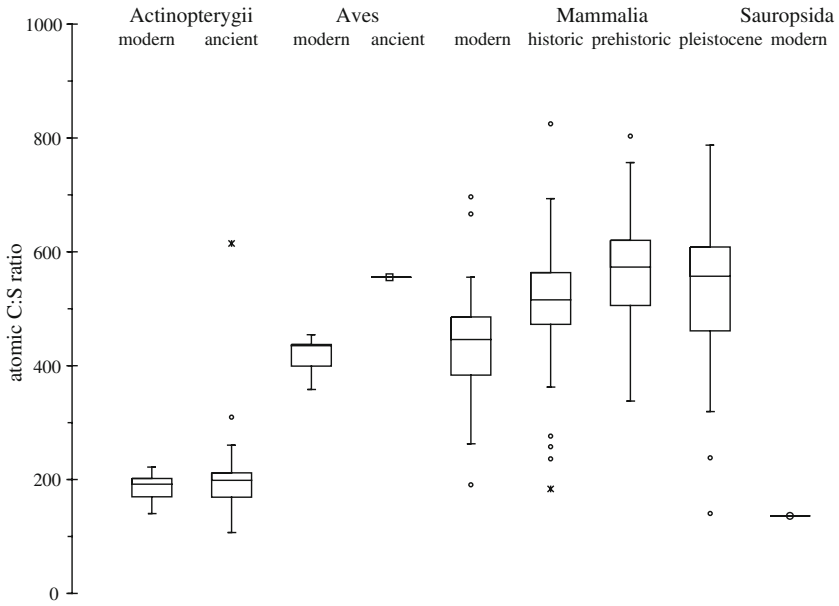

Fig. 5 Box plots of the C:S ratios of samples analysed by mass spectrometry. Results are grouped by class of animals and the time periods they originate from

material would be 0.40–0.85 wt %; see above for reasons for expanding the modern range. Comparing the atomic C:N ratios of modern fish (Actinopterygii) and mammalian (Mammalia) bone collagen reveal that there is a slight tendency towards lower atomic C:N ratios in fish bone collagen. The analysed bone collagen from modern fish bone tends to cluster on the lower end of the acceptable range (DeNiro 1985). The following quality criteria were applied only to specimens within the accepted range of the atomic C:N ratio and although the outliers are shown they are excluded from the estimation of acceptable ranges. The calculated atomic C:S and N:S ratios range from 175±50 and 60±20, respectively (Figs. 5 and 6). Within the historic fish samples, there are five specimens with sulphur values either too high or too low, and therefore their atomic C:S and N:S ratio ranges are outside the established range. The comparison of the amounts of sulphur in modern and historic samples shows that there is less variation in the modern samples than in the historic ones. Obviously then, the burial environment greatly influences the sulphur containing amino acids in fish bone collagen. Fish bones are less mineralised and tend to be more easily degraded than mammalian bone collagen. This seems to be a reason for the often observed absence of fish bones in many archaeological sites.

The bone collagen of the bearded dragon, which belongs to the taxonomic class of Sauropodia, displays a very old collagen sequence and is completely different from mammalian bone collagen. It seems that the high amount of

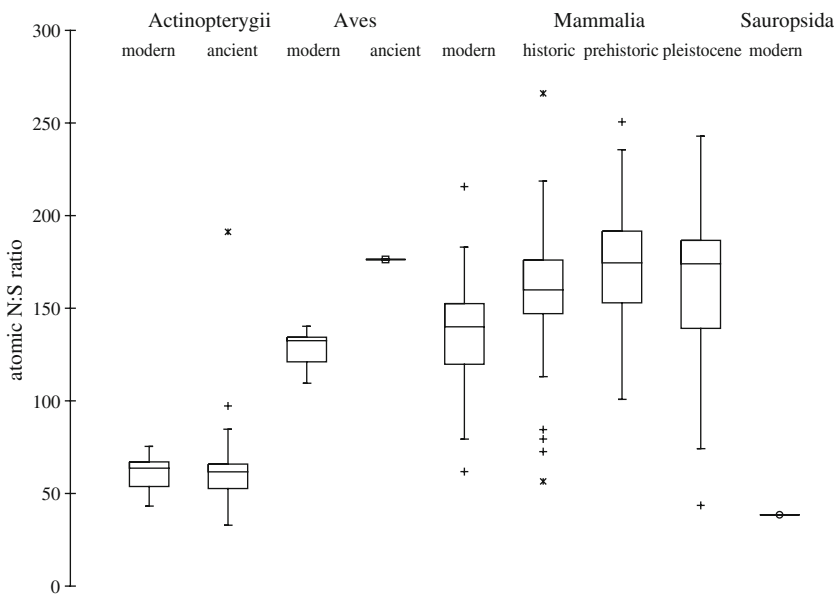

Fig. 6 Box plots of the N:S ratios of samples analysed by mass spectrometry. Results are grouped by class of animals and the time periods they originate from

methionine residues in collagen type I is a very old structural effect. Since there is only limited survival of saurian bones in the archaeological record of the (pre-) historic and Pleistocene period, we do not explore this phenomenon in further detail here.

In Fig. 7, we show the ranges of sulphur isotopic ratios and amounts of sulphur in bone collagen for different classes of animals to demonstrate the power of the method. Marine mammals, like whales and seals from open oceanic waters, and humans and animals living in coastal regions or who consume large amounts of seafood which also have sulphur amounts from 0.15 up to 0.35 wt %, have sulphur isotope ratios ranging from 14 to 19‰ (cluster 1 in Fig. 7). Mammals and birds from terrestrial environments (cluster 2), whose diets are mainly terrestrial, or freshwater fish based with the same range of sulphur amounts (0.15–0.35 wt %), have much lower sulphur isotope values, ranging from −20‰ to 14‰. Therefore, a comparison of values from cluster 1 and cluster 2 helps to identify a marine versus a terrestrial diet or inland-originated food from food from marine or coastal regions. For species with higher amounts of sulphur, ranging from 0.4 to 0.8 wt %, to be found in fish from oceanic salt water environments (cluster 3), have sulphur isotope ratios from 14 up to 19‰ (or more), while freshwater fish have sulphur isotope ratios ranging from −20 to 14‰ (cluster 4). The difference in sulphur isotope ratios between cluster 3 and 4 can be used for studying different feeding patterns in estuarine environments.

Mammalian bone collagen with sulphur amounts below 0.15 and above 0.35 wt % is heavily altered, and therefore does not represent the in vivo structure nor the original sulphur isotope value of the living individual. The most problematic situation for sulphur isotope measurements is the addition of sulphur to the collagen as this must originate from the environment and will have different sulphur isotope values than the bone collagen. The loss of sulphur is less problematic as it will represent only a minor part of the in vivo sulphur containing amino acids in the bone collagen. Fish bone collagen samples with sulphur amounts below 0.4 and above 0.8 wt % need to be excluded from further examinations. Usually, alterations of the sulphur containing amino acids are more obvious for marine specimens because the isotope values drift significantly towards terrestrial isotope values or seawater sulphate values. All results within these ranges should be seen as representative for the in vivo characteristic of the living organisms.

Conclusion

The aim of this study was to establish a robust assessment of quality markers to test the suitability of archaeological bone collagen for sulphur isotope analysis. The theoretical calculation of the weight percent sulphur in bone collagen calculated from DNA and amino acid sequences revealed differences in the amount of sulphur between fish and mammalian collagen type I. The calculated amount of sulphur in fish collagen is 0.4 wt % and for mammalian collagen 0.2 wt %. When we analysed the amount of sulphur in collagen extracted from modern fish and mammalian bone collagen, we determined the sulphur amounts to be 0.63±0.08 and 0.28±0.07 wt %, respectively. Although the measured values for the modern samples are higher in general than the theoretical amounts, we believe that the measured data do represent the accurate in vivo values of the modern animals. It is perhaps possible that the additional sulphur in the modern animals may originate from chondroitin sulphate attached to the collagen type I lysine residues to anchor the fibrils in the mineral

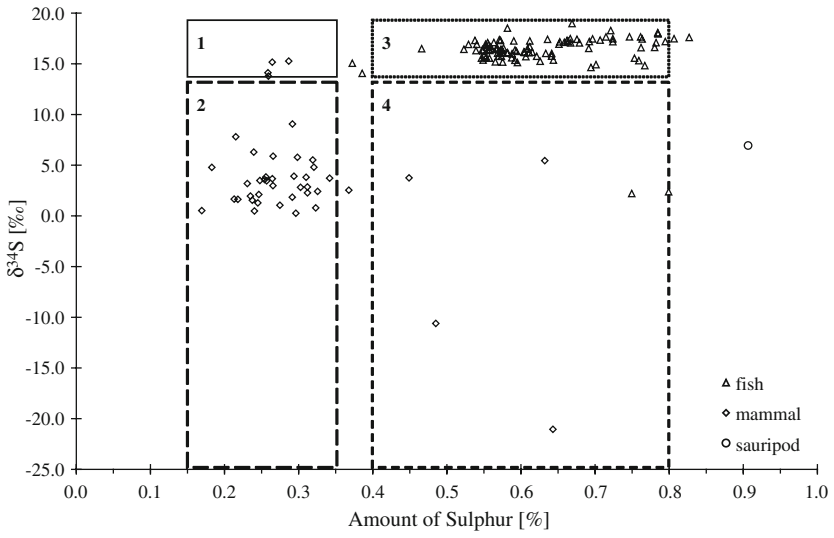

Fig. 7 Scattergram of sulphur isotope ratios versus amount of sulphur of modern samples analysed for this study. Cluster 1 represents sea mammals, cluster 2 terrestrial mammals. Cluster 3 is the range for open sea fish samples and cluster 4 represents freshwater fish values

phase of the bone. Based on these results, we suggest the use of different ranges for the quality control of archaeological samples. Specifically, we suggest that the calculated atomic C:S ratio for mammalian and bird bone collagen should be between 600±300 and for fish bone collagen it should be between 175±50. The supposed atomic N:S ratio for mammals and birds is 200±100, and for fish 60±20.

By applying the calculated marker to archaeological material, we found that it was then possible to exclude obvious outliers and especially to identify samples that contained additional inorganic sulphates. Therefore, we suggest that researchers use the established ranges for sulphur percentages, C:S and N:S ratios with their own data, as a tool to help to identify poorly preserved samples and to then exclude inaccurate sulphur isotope measurements.

Acknowledgements We would like to thank the Max-Planck Society for funding and Christin Ellenberger from the Veterinary Pathology of the University of Leipzig for providing modern animal samples. We would also like to thank Oliver Craig for suggestions to confirm the reliability of our isotopic measurement with conventional methods, and Christine Lehn, who suggested the idea of chondroitin sulphate in the modern bone collagen samples. We would also like to thank the two reviewers for very helpful suggestions and comments on our manuscript.

Appendix

Collagen as a protein is organised as triple helix, containing two α1 chains and one α2 chain of amino acids. The three chains are connected by hydrolysed bonds. The sequences of the amino acids are inherited genetically. Single amino acids are bonded by amide bonds (or peptide bonds). During the reaction of single amino acids to dipeptides water is released. Therefore, the molecular mass of each amino acid within a chain is the amino acid mass subtracted by the molecular mass of water. The number of residues of each amino acid within a chain (for data from DNA sequences) was obtained. For the theoretical calculation of the amount of carbon, nitrogen, and sulphur, the amount of each element in all amino acids was calculated either by molecular mass or as a percentage (see Table 8). We calculated, for each amino acid, the weight percentage of all of the constituent elements and our detailed calculations are shown in Table 9. Published amino acid sequences from species were either presented as a percentage of each amino acid or as the number of residues per 1,000 amino acids (which was then recalculated to percentages). In these cases, the amount of each element was only calculated as a percent of the complete molecule and there is no differentiation between single chains of the collagen molecule. The final percentages therefore represent only a theoretical collagen molecule and post-translational modifications are not taken into account.

The obtained weight percent of all elements of a specimen is further used to calculate atomic ratios by dividing the weight percent multiplied by the quotient of the elemental weights. The resulting ratio represents the atomic ratio of the particular elements.

Table 8 Calculation of amounts of containing chemical elements, by residue and percentage of the amino acid

		Number of residue of element						Percentage of element				
Amino acid		C	N	O	H	S	Molecular weight (g×mol^{-1})	C%	N%	O%	H%	S%
Hydroxyproline	OH-Pro	5	1	3	9	0	131.13	45.8	10.7	36.6	6.9	0.0
Aspartic acid	Asp	4	1	4	5	0	131.09	36.7	10.7	48.8	3.8	0.0
Threonine	Thr	4	1	3	9	0	119.12	40.3	11.8	40.3	7.6	0.0
Serine	Ser	3	1	3	7	0	105.09	34.3	13.3	45.7	6.7	0.0
Glutamic acid	Glu	5	1	4	9	0	147.13	40.8	9.5	43.5	6.2	0.0
Proline	Pro	5	1	2	9	0	115.13	52.2	12.2	27.8	7.9	0.0
Glycine	Gly	2	1	2	5	0	75.07	32.0	18.7	42.6	6.7	0.0
Alanine	Ala	3	1	2	7	0	89.09	40.4	15.7	35.9	7.9	0.0
Cysteine	Cys	3	1	2	7	1	121.16	29.7	11.6	26.4	5.8	26.5
Valine	Val	5	1	2	11	0	117.15	51.3	12.0	27.3	9.5	0.0
Methionine	Met	5	1	2	11	1	149.21	40.2	9.4	21.4	7.4	21.5
Isoleucine	Ile	6	1	2	13	0	131.17	54.9	10.7	24.4	10.0	0.0
Leucine	Leu	6	1	2	13	0	131.17	54.9	10.7	24.4	10.0	0.0
Tyrosine	Tyr	9	1	3	11	0	181.19	59.7	7.7	26.5	6.1	0.0
Phenylalanine	Phe	9	1	2	11	0	165.19	65.4	8.5	19.4	6.7	0.0
Hydroxylysine	OH Lys	6	2	3	14	0	162.19	44.4	17.3	29.6	8.7	0.0
Lysine	Lys	6	2	2	14	0	146.19	49.3	19.2	21.9	9.7	0.0
Histidine	His	6	3	2	9	0	155.16	46.4	27.1	20.6	5.8	0.0
Arginine	Arg	6	4	2	14	0	174.20	41.4	32.2	18.4	8.1	0.0
Asparagine	Asn	4	2	3	8	0	132.12	36.4	21.2	36.3	6.1	0.0
Glutamin	Gln	5	2	3	10	0	146.15	41.1	19.2	32.8	6.9	0.0

Table 9 Calculation of percentages of all elements within the collagen molecule of the DNA sequences for human collagen type I from the database Swiss-prot (P02452, P08123)

ID-number in GenBank			P02452		P08123							
Correlated chain			Chain α1		Chain α2							
AA	Mwt	In Protein ($-H_2O$)	Number of residues	Molecular weight in protein ($g \times mol^{-1}$)	Number of residues	Molecular weight in protein ($g \times mol^{-1}$)	$(2 \times \alpha 1)+\alpha 2$	C wt ($g \times mol^{-1}$)	N wt ($g \times mol^{-1}$)	O wt ($g \times mol^{-1}$)	H wt ($g \times mol^{-1}$)	S wt ($g \times mol^{-1}$)
OH-Pro	131.13	122.12	0	0.00	0	0.00	0.00	0	0	0	0	0
Asp	131.09	122.08	34	4,150.73	21	2,563.69	10,865.16	4,275.92	1,246.60	4,271.84	269.12	0
Thr	119.12	110.11	17	1,871.92	20	2,202.26	5,946.09	2,594.38	756.36	1,727.94	381.00	0
Ser	105.09	96.09	39	3,747.35	31	2,978.66	10,473.36	3,927.60	1,526.73	3,487.87	549.33	0
Glu	147.13	138.12	49	6,768.03	45	6,215.54	19,751.61	8,587.87	2,002.96	6,863.74	1,008.95	0
Pro	115.13	106.12	240	25,469.85	202	21,437.12	72,376.81	40,957.51	9,552.60	10,911.59	4,811.91	0
Gly	75.07	66.06	347	22,922.68	346	22,856.62	68,701.98	24,982.88	14,567.01	16,639.38	3,144.77	0
Ala	89.09	80.09	122	9,770.55	106	8,489.17	28,030.27	12,611.55	4,902.36	5,599.79	1,763.90	0
Cys	121.16	112.15	0	0.00	0	0.00	0.00	0	0	0	0	0
Val	117.15	108.14	20	2,162.80	39	4,217.47	8,543.08	4,744.35	1,106.53	1,263.95	716.65	0
Met	149.21	140.21	7	981.44	5	701.03	2,663.92	1,141.05	266.13	303.99	172.36	609.25
Ile	131.17	122.17	7	855.17	17	2,076.84	3,787.18	2,234.05	434.21	495.98	343.71	0
Leu	131.17	122.17	21	2,565.51	33	4,031.51	9,162.53	5,404.95	1,050.51	1,199.96	831.55	0
Tyr	181.19	172.18	4	688.73	2	344.37	1,721.84	1,080.99	140.07	319.99	90.71	0
Phe	165.19	156.18	15	2,342.76	11	1,718.03	6,403.55	4,432.06	574.28	655.98	371.93	0
OH Lys	162.19	153.18	0	0.00	0	0.00	0.00	0	0	0	0	0
Lys	146.19	137.18	38	5,212.91	31	4,252.64	14,678.45	7,711.06	2,997.44	1,711.94	1,294.19	0
His	155.16	146.15	3	438.45	13	1,899.93	2,776.83	1,369.25	798.38	303.99	134.06	0
Arg	174.20	165.20	53	8,755.35	54	8,920.55	26,431.24	11,530.56	8,964.31	2,559.90	1,935.24	0
Asn	132.12	123.11	11	1,354.23	25	3,077.79	5,786.24	2,258.07	1,316.63	1,503.94	284.24	0
Gln	146.15	137.14	30	4,114.15	22	3,017.05	11,245.35	4,924.51	2,297.11	2,623.90	661.21	0
Total			1,057	10,4172.62	1,023	10,1000.26	309,345.51	144,768.58	54,500.23	62,445.66	18,764.82	609.25
Percentage								46.80%	17.62%	20.19%	6.07%	0.20%

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