Evaluation of two lipid removal methods for stable carbon and nitrogen isotope analysis in whale tissue

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Abstract

RATIONALE

The presence of lipids in animal tissues can influence the interpretation of stable isotope data, particularly in lipid-rich tissues such as the skin and muscle of marine mammals. The traditionally employed chloroform:methanol delipidation protocol has the potential to alter $\delta^{15}N$ values in proteinaceous tissues. Our objective was to determine whether cyclohexane is an alternative extraction method, effectively removing lipids without altering $\delta^{15}N$ values.

METHODS

Kidney, liver, muscle, and skin samples were collected from beach-cast Sowerby's beaked whales (*Mesoplodon bidens*). Control subsamples were processed without delipidation extraction, and duplicate subsamples were extracted with either chloroform:methanol or cyclohexane. $\delta^{13}C$, $\delta^{15}N$, and C:N values were determined by continuous-flow elemental analysis

isotope ratio mass spectrometry. Paired Wilcoxon tests were used to evaluate the change in isotope values after extraction, and unpaired Wilcoxon tests were used to evaluate difference in isotope values between extractions.

RESULTS

Cyclohexane is an effective delipidation technique for tissues with low and moderate lipid content. Chemical delipidation influenced $\delta^{15}N$ values; extracted samples generally showed an increase in $\delta^{15}N$ values which varied 0.0‰ to 1.7‰. Chloroform:methanol extraction resulted in alterations to $\delta^{15}N$ values greater than analytical precision for all analyzed tissues. Changes to $\delta^{15}N$ values after cyclohexane extraction were at or near analytical precision in liver and muscle but greater than analytical precision for kidney and skin.

CONCLUSIONS

We recommend processing duplicate subsamples for stable isotope analysis, one with and one without extraction in order to obtain accurate values for each isotope. Prolonged chemical extractions are not necessary to effectively remove lipids. When samples are limited, we suggest using cyclohexane for tissues with low or moderate lipid content, and chloroform:methanol for higher lipid-rich tissues.

Introduction

Stable isotope analysis (SIA) of animal tissues is a rapidly expanding tool applied to a variety of environmental, ecological, anthropological, and forensic problems; however, interpretation of stable isotope data can be confounded by a suite of variables related to sample design, collection, preparation, and analysis[1,2]. Animal tissues are comprised of multiple compound classes (e.g., proteins) and compounds (e.g., amino acids), each with potentially different isotopic compositions[3]. The isotopic composition of bulk (whole) tissue is an average of

the isotopic composition of the constituent molecules weighted by their relative proportion[4,5]. If the relative proportion of isotopically distinct tissue components varies among bulk samples, then tissue composition will contribute to measured population stable isotope means and distributions. Wildlife and anthropological studies addressing questions of spatial origin, movement behavior, or diet commonly focus on largely proteinaceous tissues such as muscle, feather, hair keratin, or bone collagen for isotopic analyses[6,7]. Such tissues commonly also contain lipids, potentially influencing $\delta^{13}C$ values and C:N ratios[8-10]. On average, synthesized body lipids tend to be depleted in ^{13}C compared to synthesized proteins, so that the presence of lipids within protein samples tends to reduce bulk tissue $\delta^{13}C$ values. The degree of isotopic differentiation can vary depending on lipid and protein composition, nutritional status, and other physiological effects[8,11,12]. Soft tissues such as muscle, liver, and subcutaneous connective tissues frequently act as physiological lipid stores. Lipid contents in these tissues may be high and markedly variable among individuals[13]. Failure to consider lipid content when conducting tissue-based studies can therefore bias data interpretation and lead to erroneous conclusions about diet or movement patterns[9,14,15]. Two approaches have been proposed to address the problem of lipid content in mixed tissue isotope analyses: statistical isotopic correction models and chemical removal of lipids.

Statistical isotopic correction models aim to account for the influence of ^{13}C depleted lipids retrospectively using C:N ratios as predictors of lipid content and mass balance approaches to correct measured values[16]. These models typically are established by statistical regression between measured $\delta^{13}C$ values and C:N ratios and may also utilize measured or estimated end member values for pure lipid, pure protein, or expected protein:lipid offsets. The coefficients associated with statistical lipid correction models are likely to vary according to tissue type,

physiology, and metabolic status. Therefore, while a variety of models are available, they do not generate consistent results between and within species and tissue types[13,17-20]. Thus, lipid correction models must be parameterized for each study and may still yield inconsistent results[21-24].

Chemical lipid extraction provides a rapid and consistent means of ensuring lipid removal. The most common method for lipid extraction is a polar solvent solution of chloroform:methanol. This technique, in use for more than 60 years, is effective at removing lipids. However, the process is relatively aggressive, potentially also influencing the relative proportions of amino acids present because of the higher solubility of the polar amino acids in polar solutes[9,25]. As $\delta^{13}C$ and $\delta^{15}N$ values vary among individual amino acids, altering the relative proportions of amino acids present in a protein following chloroform:methanol extractions can alter the isotopic compositions of both carbon and nitrogen in bulk protein analyses. Non-polar solvents, such as hexane and diethyl ether, provide an alternative means of lipid removal. All amino acids are relatively insoluble in non-polar solvents, so the use of non-polar solvents for lipid extraction carries less risk of unintentional alteration of amino acid and bulk protein isotopic compositions [11,14]. Despite years of study and the rise in the use of stable isotope analyses of animal tissues, the relative performance of different chemical extraction approaches as applied to specific tissues of different species is still not well characterized. As a result, there is a conflicting body of evidence about the effects of lipid extraction on $\delta^{13}C$ and $\delta^{15}N$ values and a lack of consistency in extraction methods employed across studies. In addition to avoiding the potential effects of chemical extraction on target protein isotopic compositions, it may be beneficial to avoid chemical extraction for simple time and cost considerations.

For any given species, tissue, and study there is often uncertainty regarding: (1) whether tissue lipid extraction is a necessary step prior to stable isotope analyses; and if so, (2) the magnitude of undesirable isotopic alteration that should be expected associated with different chemical extraction methods. This is especially problematic in the case of poorly studied species, tissues with few case studies in the literature, and tissues with high and variable lipid contents.

In this study we evaluated two methods of lipid removal, chloroform:methanol and cyclohexane, and their effects on $\delta^{13}C$, $\delta^{15}N$, and C:N values in four tissue types collected from Sowerby's beaked whales (*Mesoplodon bidens*), a rare and elusive species. Cyclohexane is a nonpolar solvent frequently used to extract lipids for lipid research studies but has only occasionally been used in stable isotope analyses[26-31]. Whale tissue, especially skin, is lipid-rich and has proven particularly challenging to evaluate with statistical isotopic correction models [13,17,23]. Thus, it is often assumed to be necessary to use a chemical extraction method when processing whale tissue. Here, we assessed the necessity of using a chemical lipid extraction method in tissue for this whale species, the degree to which each method altered isotope ratios, and how any changes to isotope values may influence interpretation of these values.

Materials and Methods

Sampling, sample preparation, and stable isotope analysis

We obtained samples of kidney (n = 18), liver (n = 17), muscle (n = 18), and skin (n = 24) from 26 stranded *M. bidens* (n = 77 total tissue samples). Samples were opportunistically collected from beach-cast carcasses from various locations along the Scottish coastline by the Scottish Marine Animal Stranding Scheme and stored at -20 ℃. We collected ~0.5 g subsamples of frozen tissues and preserved them in 95% ethanol for <1 week for transport. Ethanol is a commonly used preservative for soft tissues that can contribute to lipid removal and increase

120 $\delta^{13}C$ values in the tissues of some species, but typically has small and insignificant effects on
121 $\delta^{15}N$ values[32-35]. Prior to analyses we removed excess ethanol, subsampled each tissue sample,
122 freeze dried the samples individually for 16 hours, and ground dried tissues with mortar and
123 pestle. We subsampled 10 samples from each tissue type to serve as an unextracted control; these
124 samples were submitted for stable isotope analysis without lipid extraction. For each of the 77
125 tissue samples, we extracted one subsample with 2:1 chloroform:methanol for 30 minutes,
126 manually agitating samples every 5 minutes. We repeated this process with a duplicate sample
127 for cyclohexane extraction. Lipid extraction timelines vary among studies from minutes to days;
128 we employed a single 30-minute extraction to keep extraction methods consistent between our
129 two protocols. Longer extraction times, particularly for chloroform:methanol, are often
130 employed on tissues[8,11,14]. However, it is unclear if prolonged extraction is necessary to
131 effectively remove lipids, especially on finely ground materials. Lipid extracted samples were
132 dried at 60 ℃ for 16 hours post extraction. Between 0.5 and 0.8 mg of each sample was loaded in
133 3x5mm tin capsules and submitted for C and N stable isotope analysis.
134 Stable isotope analysis was completed at the Smithsonian Institution Museum
135 Conservation Institute Stable Isotope Mass Spectrometry Laboratory using a Thermo Delta V
136 Advantage mass spectrometer in continuous flow mode coupled to an Elementar vario ISOTOPE
137 Cube Elemental Analyzer via a Thermo Conflo IV (ThermoFisher Scientific, 168 Third Avenue
138 Waltham, MA USA 02451). We used V-PDB and Air to calibrate $\delta^{13}C$ and $\delta^{15}N$, respectively.
139 Two standards, an in-house Costech Acetanilide (Costech Analytical, 26074 Avenue Hall, Suite
140 14 Valencia, CA USA 91355) and Urea-UIN3, calibrated to USGS40 and USGS41 (L-glutamic
141 acid), were included between every 10 samples to ensure accuracy and precision, with an

analytical precision of +/-0.2‰ (1σ). Weight percent carbon and nitrogen values were calibrated to the in-house acetanilide standard with an analytical precision of +/- 0.5%.

Data analysis

Our data analyses addressed four questions: (1) are both lipid removal techniques effective; (2) how much variance is there between chloroform:methanol and cyclohexane extracted samples; (3) does delipidation extraction meaningfully change $\delta^{13}C$, $\delta^{15}N$, and C:N values; and (4) do extraction methods change isotope values in similar ways? To answer question (1), we evaluated the C:N ratios post extraction for all samples (n = 77) because he C:N ratio often is used to evaluate the presence of lipids in tissue samples, and previous studies have identified a significant relationship between larger C:N ratios, higher lipid proportions, and lower $\delta^{13}C$ values in some animal tissues[8]. We used these same 77 samples to address question (2), employing paired Wilcoxon tests to compare $\delta^{13}C$, $\delta^{15}N$, and C:N values between each subsample of chloroform:methanol and cyclohexane extracted tissue. We then used a subset of these samples (n = 40; 10 of each tissue type) to address questions 3 and 4, comparing $\delta^{13}C$, $\delta^{15}N$, and C:N values of the unextracted control samples to those same tissues post extraction. We selected these tissues because there was enough of each sample for pre- and post-extraction analysis and duplicate analysis, if needed. To address question (3), we used paired Wilcoxon tests to evaluate differences in pre- and post-extraction values for each extraction method to explore how extraction method changed isotope values ($\delta^{13}C$ and $\delta^{15}N$) and their relationship to each other (C:N ratios). For question (4), we used unpaired Wilcoxon tests to compare the degree and direction of change in values between the same tissue subsamples extracted with chloroform:methanol and cyclohexane. We considered p-values ≤ 0.05 significant, and statistical analyses were performed using R[36] with RStudio[37].

We use two delta notations to express our results. The first is the standard delta notation δ, which is the parts per thousand difference between the sample and international standards, expressed as $\delta^{y}X = [(R_{sample} - R_{standard})/(R_{standard})]$, where X is the element, y is the atomic mass of the stable isotope, and R is the ratio of heavy to light isotopes. The second is Δ notation, used to represent the difference between two δ values. In this paper we use it to represent the difference between extracted and unextracted values (e.g. $\Delta^{13}C = \delta^{13}C_{extracted} - \delta^{13}C_{unextracted}$).

Results and Discussion

For question (1), we found both extraction methods effectively removed lipids from tissues with relatively lower initial lipid content. A 30-minute chloroform:methanol extraction effectively delipidated lipid-rich tissues , and a 30-minute cyclohexane extraction was moderately effective at delipidating lipid-rich tissues . C:N ratios were reduced to < 5 in all 77 chloroform:methanol extracted samples, and in all but 1 cyclohexane extracted skin sample (Figure 1). There is currently no consensus regarding "correct" marine mammal C:N ratios following delipidation; some sources suggest tissues with C:N (by mass) values > 3.5 contain sufficient lipid to significantly complicate tissue $\delta^{13}C$ interpretations, while others consider values between 4 and 5 acceptable[8,13,38]. Our chloroform:methanol extracted samples had a mean C:N ratio of 3.4 (range: 3.0 – 4.7), and the cyclohexane extracted mean was 3.6 (range: 3.0 – 6.4). Thus, chloroform:methanol C:N ratios in this study fell within multiple definitions of acceptable C:N ratios, demonstrating that prolonged extraction times, especially on ground tissue, are not necessary. Likewise, cyclohexane C:N ratios for most tissues also fell within acceptable C:N ratios, and longer extractions with this method may only be required on lipid-rich tissues, such as skin.

For both extraction methods, mean skin C:N values were greater than total sample mean (chloroform:methanol = 3.8; cyclohexane = 4.1), and muscle, liver, and kidney C:N mean were less than total sample mean (chloroform:methanol = 3.2, 3.2, and 3.2 respectively; cyclohexane = 3.3, 3.4, and 3.3 respectively) (Figure 1). The observed relationship between $\delta^{13}C$ values and C:N ratios post extraction begins to level out when C:N ratios exceed 4, and extrapolation of the relationship to infinite C:N ratios suggests that the $\delta^{13}C$ value of pure lipid in Sowerby's beaked whale tissues is between -20‰ and -25‰. Based on the observed relationship between $\delta^{13}C$ values and C:N ratios (Figure 1), together with the assumed C:N ratio of pure protein[8], we suggest that beaked whale tissue samples with C:N ratios around 3.5 do not require chemical extraction or statistical correction.

Paired Wilcoxon tests for question (2), variance between chloroform:methanol and cyclohexane extracted samples (n = 77), demonstrated that $\delta^{13}C$ values of kidney, liver, and skin subsamples extracted with chloroform:methanol were significantly different than subsamples of those same tissues extracted with cyclohexane, and the difference in muscle tissue values approached significance (Table 1). For $\delta^{15}N$ values, only kidney subsamples were significantly different between the two extraction methods. The mean differences in $\delta^{15}N$ values in kidney, liver, muscle, and skin tissues were 0.5‰, 0.3‰, 0.4‰, and 0.3‰ respectively, and differences in $\delta^{15}N$ between extracted subsamples ranged from 0.0‰ to 1.7‰. C:N values were significantly different in kidney,liver, and skin subsamples (Table 1).

Finally, we addressed questions (3) and (4), evaluating the effect of lipid extraction on isotope values and variation in values between differently extracted subsamples of the same tissue sample. Below we summarize the treatment effects and recommendations for each tissue type:

Kidney

Unextracted C:N ratios ranged between 3.2 and 3.7 with a mean of 3.3 and low variation among individuals (Table 2). Chloroform:methanol extraction reduced C:N ratios and decreased mean $\delta^{13}C$ values. Both extraction methods increased variation among individuals in $\delta^{13}C$ and $\delta^{15}N$ values. Chloroform:methanol extraction resulted in greater variation among individuals for $\Delta^{13}C$ values, and both extraction methods had similar variation among individuals in $\Delta^{15}N$ and $\Delta C:N$ values (Table 3, Figure 2). Due to the low C:N ratios in unextracted samples and inconsistent changes to among variation among individuals in $\delta^{13}C$ and $\delta^{15}N$ values, we recommend avoiding lipid extraction in whale kidney samples.

Liver

Unextracted C:N ratios ranged between 3.2 and 4.0 with a mean of 3.4 and a small variation among individuals (Table 2). Chloroform:methanol extraction reduced C:N ratios and decreased mean $\delta^{13}C$ values and variation among individuals in $\delta^{13}C$ values. $\delta^{15}N$ values and variation among individuals remained largely unchanged after both extraction methods. Both extraction methods had similar variation among individuals in $\Delta^{13}C$, $\Delta^{15}N$, and $\Delta C:N$ values; however, mean $\Delta C:N$ between extraction methods was significantly different (Table 3, Figure 2). Due to low C:N ratios in unextracted tissues, we recommend avoiding lipid extraction in whale liver samples. However, due to the reduction in variation among individuals in $\delta^{13}C$ values and relatively low effect on $\delta^{15}N$ and $\Delta^{15}N$ values post extraction, a short extraction with chloroform:methanol may be useful in some studies.

Muscle

Unextracted C:N ratios ranged between 3.1 and 6.8 with a mean of 3.7 and a large variation among individuals (Table 2). Both extraction methods effectively reduced mean C:N

ratios below 3.5 and reduced among individual variability in $\delta^{13}C$ values. Both extraction methods increased mean $\delta^{15}N$ values to a similar extent, but chloroform:methanol resulted in greater variation among individuals. Chloroform:methanol extraction resulted in greater variation among individuals in $\Delta^{13}C$, $\Delta^{15}N$, and $\Delta C:N$ values (Table 3, Figure 2). We therefore recommend cyclohexane extraction for whale muscle samples.

Skin

Unextracted C:N ratios ranged between 3.3 and 11.7 with a mean of 6.4 and a large variation among individuals (Table 2). Both extraction methods significantly reduced mean C:N ratios and reduced variation among individuals in $\delta^{13}C$ values, though variation among individuals post cyclohexane extraction was greater than post chloroform:methanol extraction. Both extraction methods increased mean $\delta^{15}N$ values to a similar extent, but chloroform:methanol extraction resulted in increased variation among individuals. Chloroform:methanol extraction resulted in greater variation among individuals for both in $\Delta^{13}C$ and $\Delta C:N$ values, whereas cyclohexane extraction resulted in greater variation among individuals in $\Delta^{15}N$ values (Table 3, Figure 2). We therefore recommend subsampling whale skin samples and submitting one samples for stable isotope analysis without lipid extraction to obtain an accurate $\delta^{15}N$ value, and one after extraction with chloroform:methanol for an accurate $\delta^{13}C$ value.

Conclusions and Recommendations

Our results indicate that cyclohexane is an effective delipidation technique for tissues with low and moderate lipid content, but not as effective as chloroform:methanol with lipid-rich tissues, such as whale skin. In the sampled Sowerby's beaked whale tissues, the $\delta^{13}C$ value of lipids is between -20‰ and -25‰, and tissues with lower C:N ratios, such as kidney and liver,

256 do not require delipidation (Table 2). Samples extracted with cyclohexane resulted in generally
257 lesser changes to $\delta^{15}N$ compared to chloroform-methanol extraction, with some differences being
258 at or near analytical precision, suggesting that this extraction method is less likely to alter the
259 abundance of amino acids in the sample.

260 It is possible to aggressively delipidate tissues multiple times to obtain a desired C:N
261 ratio, but increasingly aggressive extractions dramatically increase the risk of altering amino acid
262 compositions and associated bulk protein $\delta^{13}C$ and $\delta^{15}N$ values. We found that a single 30-
263 minute extraction effectively removed lipids in most tissue samples, suggesting that prolonged
264 lipid extraction of hours or days may be unnecessary, especially for ground tissues. Thus, we
265 recommend avoiding aggressive delipidation when possible except in lipid-rich tissues such as
266 whale skin. For these Sowerby's beaked whale tissues, C:N values < 5 indicate lipids have been
267 removed while preserving the relative abundance of amino acids; we anticipate repeating this
268 analysis on the same tissue types from other whale species would yield comparable results.

269 Lipid content in tissue samples and how the presence of lipids effects $\delta^{13}C$ is an
270 important consideration when designing animal studies. Our work provides insight into selecting
271 the appropriate delipidation technique, if applicable, for a variety of tissues with varying levels
272 of lipid content. When ample tissue is available and funding permits, we recommend reporting
273 isotope values from both unextracted and chloroform:methanol extracted samples. Researchers
274 would then consider $\delta^{15}N$ values from the unextracted sample and $\delta^{13}C$ from the extracted
275 sample in studies. However, for rare or scarce tissues, or when funding limits processing to one
276 sample, we recommend using cyclohexane for tissues with low or moderate lipid content, and
277 chloroform:methanol for lipid-rich tissues.
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Table 1.

Mean (± SD) $\delta^{13}C$, $\delta^{15}N$, and C:N values of chloroform:methanol and cyclohexane delipidated Sowerby's beaked whale tissues. *P* values are for paired Wilcoxon tests to evaluate difference in values post extraction method in subsamples of the same tissue sample.

			Chloroform:methanol		Cyclohexane		
	Tissue	*n*	Mean	SD	Mean	SD	*P*
$\delta^{13}C$	Kidney	18	-17.7	0.76	-18.0	0.82	0.014
	Liver	17	-17.8	0.62	-18.2	0.84	0.001
	Muscle	18	-18.1	1.08	-18.3	0.90	0.081
	Skin	24	-19.1	0.93	-19.5	1.15	0.007
$\delta^{15}N$	Kidney	18	13.3	0.80	13.1	0.72	0.012
	Liver	17	13.2	0.88	13.2	0.85	0.712
	Muscle	18	12.6	0.82	12.7	0.96	0.865
	Skin	24	12.7	0.94	12.6	0.91	0.331
C:N	Kidney	18	3.2	0.10	3.3	0.15	0.002
	Liver	17	3.2	0.10	3.4	0.20	<0.001
	Muscle	18	3.2	0.22	3.3	0.32	0.899
	Skin	24	3.8	0.45	4.1	0.69	0.014

Table 2.

Mean (± SD) $\delta^{13}C$, $\delta^{15}N$, and C:N values for unextracted, chloroform:methanol lipid extracted, and cyclohexane lipid extracted Sowerby's beaked whale tissues. *P* values pertain to paired Wilcoxon tests comparing mean values pre and post extraction to evaluate the magnitude of change each extraction method has on values.

			Unextracted		Chloroform:methanol			Cyclohexane		
	Tissue	*n*	Mean	SD	Mean	SD	*P*	Mean	SD	*P*
$\delta^{13}C$	Kidney	10	-18.0	0.70	-17.7	0.92	0.084	-18.0	0.96	0.492
	Liver	10	-18.1	0.98	-17.7	0.65	0.037	-18.0	0.96	0.375
	Muscle	10	-18.9	1.38	-18.4	0.75	0.048	-18.5	0.92	0.193
	Skin	10	-21.1	2.03	-18.9	0.89	0.002	-19.5	1.09	0.004
$\delta^{15}N$	Kidney	10	13.1	0.83	13.2	0.90	0.375	12.9	0.87	0.275
	Liver	10	13.3	0.86	13.2	0.82	0.557	13.3	0.83	0.492
	Muscle	10	12.4	0.75	12.4	0.77	0.769	12.5	0.61	0.375
	Skin	10	12.2	0.73	12.4	0.86	0.106	12.3	0.80	0.232
C:N	Kidney	10	3.3	0.16	3.2	0.11	0.004	3.3	0.17	0.625
	Liver	10	3.4	0.25	3.2	0.09	0.006	3.4	0.23	0.232
	Muscle	10	3.7	1.12	3.3	0.28	0.009	3.3	0.39	0.027
	Skin	10	6.4	2.35	3.7	0.41	0.002	4.1	0.53	0.004

401 *Table 3.*

402 Mean (± SD) $\Delta^{13}C$, $\Delta^{15}N$, and $\Delta C:N$ values between delipidated and unextracted Sowerby's beaked whale tissues (extracted value – unextracted
403 value). *P* values pertain to unpaired Wilcoxon tests to evaluate difference in the change to isotope values by delipidation method.

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			Chloroform:methanol		Cyclohexane		
	Tissue	*n*	Mean	SD	Mean	SD	*P*
$\Delta^{13}C$	Kidney	10	0.7	0.53	0.0	0.39	0.123
	Liver	10	0.4	0.48	0.1	0.45	0.143
	Muscle	10	0.5	0.85	0.3	0.63	0.529
	Skin	10	2.2	1.39	1.6	1.16	0.248
$\Delta\delta^{15}N$	Kidney	10	0.2	0.48	-0.2	0.49	0.315
	Liver	10	-0.1	0.36	-0.1	0.31	1.000
	Muscle	10	0.1	0.41	0.1	0.20	0.853
	Skin	10	0.2	0.26	0.1	0.32	1.000
$\Delta C:N$	Kidney	10	-0.1	0.11	0.0	0.10	0.075
	Liver	10	-0.3	0.19	-0.1	0.14	0.015
	Muscle	10	-0.4	0.94	-0.4	0.74	0.739
	Skin	10	-2.7	2.26	-2.3	2.17	0.529

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