Converting A/I values (ion exchange) to D/L values (reverse phase) for amino acid

2 geochronology

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ABSTRACT

16 Several analytical techniques have been used in amino acid geochronology to measure the
relative abundances of D- and L-enantiomers. During the past two decades, reverse-phase (RP)
18 liquid chromatography has become most common, whereas ion-exchange (IE) liquid
chromatography was widely used prior to the mid-1990s. This study is based on intra-lab paired
20 analyses of RP and IE liquid chromatography to mathematically convert A/I (allo-
isoleucine:isoleucine) values determined with IE to corresponding D/L values for comparison
22 with new RP results. Pooled results of 340 paired IE and RP analyses show that A/I values can

be confidently converted to equivalent D/L values for aspartic acid, glutamic acid, alanine, valine
and isoleucine, with 2σ uncertainties of ± 24 -41% depending on the amino acid. For specific
sample types including gastropod, bivalve, eggshell and whole rock, A/I can readily be converted
to D/L aspartic acid and D/L glutamic acid with 2σ uncertainties of $\pm 20\%$ and 26% ,
respectively, using sample-type-specific relations. A/I is also reliably converted to D/L valine
and D/L isoleucine with 2σ uncertainties of ± 31 and 35% , respectively. Regression equations
are provided to convert A/I to an equivalent D/L value for five amino acids, thereby enabling the
large literature base of AAR results from IE chromatography to be compared and integrated with
AAR results from RP chromatography.

Keywords: Amino acid racemization; isoleucine epimerization; Quaternary geochronology

1. Introduction

Amino acid geochronology is based on the extent to which amino acids preserved in fossils have
interconverted from their initial L-form to their racemized D-enantiomeric form (see Miller et al.
(2013) for a recent review of the method). The ratio of D to L enantiomers serves as the primary
chronometer for amino acid geochronology. Several analytical techniques have been used to
measure D/L values, each with its own advantages, shortcomings, and biases. While several
studies have compared the outcome of various analytical methods (e.g., Bada et al., 1979;
Wehmiller, 1984, 2013; Wehmiller et al., 1988; Kaufman and Manley, 1998; Hearty and
Kaufman, 2009), none have been dedicated to quantifying the difference between the results
from different methods, with the goal of predicting the outcome of one method from another.

The ability to relate D/L values from different analytical methods will enable inter-comparison
46 and integration of amino acid geochronology data, including the growing number of datasets
now archived by NOAA Paleoclimatology (ncdc.noaa.gov/paleo/aar.html; Wehmiller and
48 Pellerito, 2015).

Prior to the turn of the millennium, most amino acid geochronology laboratories used
50 ion-exchange (IE) liquid chromatography to separate amino acids and quantify their abundances.
The procedure yields D/L values for only one amino acid, isoleucine (Ile). More specifically, the
52 method does not separate D and L enantiomers. Instead, separation by IE relies on the more
chemically distinct differences between Ile and its diastereomer, allo-isoleucine (alle). Ile is
54 unusual among amino acids in having two stereocenters and can racemize about either or both
carbon atoms. L-isoleucine racemizes almost exclusively about the alpha carbon to produce D-
56 alle. The reaction by which L-Ile converts to D-alle is known as epimerization, and the extent of
isoleucine epimerization is measured by the ratio of alle to Ile, typically abbreviated “A/I”.

58 In the late 1990s, reverse-phase liquid chromatography (RP) was optimized for amino
acid geochronology (Kaufman and Manley, 1998). It has the advantage of separating D and L
60 enantiomers of several amino acids. Amino acids that racemize at higher rates, such as aspartic
acid (Asp), are optimal for young (Holocene) samples, whereas those that racemize more slowly,
62 such as glutamic acid (Glu) and valine (Val), can be chosen for older samples. A further
advantage of analyzing the extent of racemization in several amino acids is that they can work
64 together as cross checks to identify shells that yield unreliable results. Because the D/L values
among the different amino acids tend to be strongly covariant (e.g., Goodfriend and Meyer,
66 1991; Kosnik et al., 2008, 2013), divergence in these trends might be used to diagnose excessive

sample leaching or contamination, both of which undermine the accuracy of the technique and
68 contribute to high inter-sample variation (Kosnik and Kaufman, 2008). A disadvantage of RP is
that the separation of Ile and aIle, which has been used extensively in amino acid geochronology,
70 can be hampered by interfering compounds in many sample types. Analytical variability of D-
aIle/L-Ile (D/L Ile) obtained by RP among specimens of the same age is therefore typically
72 higher than for other amino acids.

During the past decade, RP has become the most commonly used analytical method in
74 amino acid geochronology, but only a few direct comparisons have been made between RP and
IE datasets (Kaufman and Manley, 1998; Wehmiller, 2013; Powell et al., 2013). Using
76 established inter-laboratory comparison (ILC) samples (bulk-powdered bivalves and eggshells),
Kaufman and Manley (1998) showed that intra-lab A/I values measured using both RP and IE
78 agreed to within 6 to 10%, similar to the result from the one laboratory that used both RP and IE
as reported more recently by Wehmiller (2013). Some differences are expected between
80 subsamples prepared in different vials, in part because the subsample mass is small relative to the
homogeneity of the sample. To eliminate any variability associated with intra-specimen
82 heterogeneities or variability introduced during the hydrolysis step, comparisons between
analytical methods ideally should be based on the same sample solution.

84 Between 1996 and 2010, more than 300 sample solutions were injected onto both IE and
RP HPLCs at the Amino Acid Geochronology Laboratory (AAGL) at Northern Arizona
86 University. They include four sample types: whole rock, gastropod, bivalve, and eggshell from
various study locations (Table 1). The resulting dataset is used in this study to: compare A/I
88 values analyzed by IE to D/L values of several amino acids analyzed by RP; identify which D/L

values might be confidently inferred from A/I values; and to create equations that enable A/I

values to be converted to equivalent D/L values.

2. Methods

2.1. Sample collections and preparation

The dataset is based on collections that were originally analyzed by IE in the AAGL, and that were also injected onto the RP for this study. Results derived from laboratory-heated specimens are not included, however, because the kinetics of racemization are not always strictly analogous to those at ambient temperatures (Tomiak et al., 2013; Demarchi et al., 2013). Most of the IE data, along with one RP dataset, have been previously published in studies that describe the context for the specimens reported here (Table 1). All of the samples were prepared according to standard protocols for each material type, which are outlined in the original studies. All samples were cleaned initially by rinsing with deionized water, sonicating, removing outer layers, or a combination of these procedures. Whole rock samples were disaggregated and dry-sieved prior to cleaning. All samples were then leached with dilute hydrochloric acid (HCl) to varying degree, and hydrolyzed in 7 M HCl for 22 hr at 110°C to obtain total hydrolyzable amino acids.

2.2. Analytical procedures

Samples were analyzed using IE first, followed by RP analysis generally within a month; some were analyzed by RP up to two years later, but were kept frozen between analyses. The IE analyses were performed on either an Agilent 1100/1200 HPLC or an HPLC that was built from components at the AAGL, both fitted with a cation-exchange column, post-column derivatization

with *o*-phthalaldehyde (OPA), and UV-VIS detection. The RP samples were analyzed on an
112 Agilent 1100/1200 HPLC, with a C₁₈ stationary phase, pre-column derivatization with
OPA/IBLC (N-isobutyryl-L-cysteine), and fluorescence detection. D/L values for RP analyses
114 are based on peak areas, which is now common in liquid chromatography because area typically
provides a more accurate measurement of abundance for irregularly shaped peaks. Values for A/I
116 values for IE analyses are based on peak height, which was historically the common practice.
The difference between peak-height and peak-area measurements is discussed in section 3.2. All
118 samples were analyzed in the AAGL except the eggshells were prepared and analyzed using IE
at the INSTAAR laboratory in Boulder, Colorado, then sent to the AAGL for RP analysis. In
120 addition, the ILCs were cleaned and ground at the University of Delaware laboratory, then sent
to the AAGL for hydrolysis and analysis.

122 The D and L amino acid pairs that are regularly quantified by RP at the AAGL are Asp,
Glu, Ser, Ala, Val, and Ile. Phe and Leu are generally poorly resolved and are not considered
124 further. Of the remaining amino acids, Ser is not useful for amino acid geochronology because it
degrades rapidly; therefore, the concentration of Ser is an indication of modern contamination
126 (Miller and Brigham-Grette, 1989). For most samples, an abbreviated run time was used for RP
that provided data only for Asp, Glu, Ser, and Ala. The measurement of D to L ratios in Asp and
128 Glu is complicated by asparagine (Asn) and glutamine (Gln) residues, which are converted to
Asp and Glu, respectively, during hydrolysis. The D/L values for Asp and Glu therefore likely
130 include converted Asn and Gln.

132 2.3. Data analysis

Linear-regression analyses were used to develop equations that relate the extent of epimerization of isoleucine (A/I) to the extent of racemization of various amino acids (D/L). For each amino acid (Asp, Glu, Ala, Val, Ile) three models were fit: (1) a single model with all sample types pooled, (2) a model with a single intercept but with different slopes for each sample type, and (3) a model with different intercepts and slopes for each sample type. Additionally, each model was fit to untransformed and $\log_2$ transformed data, resulting in six models for each of five amino acids.

A number of criteria can be used to select the best model for converting A/I to a D/L values. The coefficient of determination (r^2) quantifies the proportion of variance in the D/L that is predictable by A/I, but comparing different models using r^2 is not statistically straight-forward. Akaike Information Criterion (AICc) quantifies model quality with a penalty for additional model parameters to avoid over parameterizing models. In this analysis our primary objective is to minimize the uncertainty of the predicted D/L values, therefore the model with the most precise predictions is preferred. To that end, rather than traditional model selection criteria such as r^2 or AICc, we use two summary statistics of the predicted uncertainty to identify the best model: the 68th percentile of the predicted uncertainty (equivalent to 1σ uncertainty), and the 95th percentile of the predicted uncertainty (equivalent to the 2σ uncertainty). All three metrics of model quality tend to be in agreement. Notable differences between the 1 and 2σ uncertainty would imply that the model fails to predict well over the full range of observed data. In all cases, prediction uncertainties are determined from the coefficient of variation of raw D/L values. The statistical software package “R” was used to calculate the regressions and the associated uncertainties (version 3.2.3, R core team 2015). Analytical scripts are included as SOM 2.

Only the conversion parameters from the models with the smallest prediction uncertainty
are presented (Table 2). For the pooled regressions, the $\log_2$ transformed model outperformed the
untransformed model for each of the five amino acids. For the sample-type-specific regressions,
the $\log_2$ transformed models with varying slopes and intercepts provided the lowest prediction
uncertainty out of all models tested for each amino acid (although in the case of D/L Ile, the
fixed intercept and variable slope $\log_2$ transformed models gave the same prediction intervals).
The selected models have consistent uncertainties as seen in the similarity of the 1σ and 2σ
values (Table 2), indicating that the models are of consistent quality over the observed range in
D/L values.

3. Results and discussion

3.1. Dataset summary

All laboratory measurements of the same solution analyzed by both IE and RP and used in the
regression analyses are included in the dataset (SOM 1). The number of analyses and the range of
D/L and A/I values analyzed differ among the sample types. A/I values for bivalves and
gastropods range from 0.02-1.16 and 0.02-1.02, respectively. In contrast, A/I values for whole
rocks and eggshells range from 0.03-0.89, and 0.15-1.38, respectively. The maximum D/L value
varies by amino acid, and is often unexpectedly higher than 1.0. This could be due to a
fluorescence bias toward D amino acids (Kaufman and Manley, 1998), or some other unknown
factors.

3.2. Isoleucine analyzed on ion exchange and reverse phase HPLC

176 Depending on the sample type and HPLC column performance, D-alle is often poorly resolved
by RP and is not reliable for amino acid geochronology. Ile D/L values are therefore available
178 for only a subset of 175 specimens, mainly bivalves (Table 2, SOM 1). We compared the extent
to which A/I (from IE) predicts D/L Ile (from RP) using linear regression analysis of the paired
180 values from all sample types pooled (Fig. 1; Table 2). If there is no methodological bias in
measuring A/I (= D/L Ile), we would expect a slope of 1 and an intercept of 0. Log-transformed
182 A/I is a strong predictor of D/L Ile ($r^2 = 0.95$), with a slope of $0.97 (\pm 0.02)$ and an intercept of -
 $0.00 (\pm 0.03)$. The 2σ prediction interval is $\pm 40\%$ for the pooled log-transformed model.

184 The regression models with sample-type-specific slopes and intercepts improved the 2σ
prediction interval to $\pm 35\%$ for the conversion of A/I to D/L Ile (Table 2). The $\log_2$
186 transformed gastropod and eggshell models showed the least methodological bias, with a slope
of 1.01 ± 0.03 and an intercept -0.10 ± 0.08 for gastropods, and 1.01 ± 0.05 and -0.01 ± 0.09 for
188 eggshells. Sample-type-specific models for bivalves and whole rocks yielded slopes of $0.88 \pm$
 0.04 and 0.84 ± 0.07 , and intercepts of -0.01 ± 0.1 and -0.27 ± 0.13 , respectively. The differences
190 in slope and intercept values have several possible causes, including material-specific co-eluting
peaks that obscure D-alle during RP analysis, baseline noise that encroaches on D-alle peak
192 areas (which tend to be small), or possibly the presence of L-alle or D-Ile, or both, which eluted
as separate peaks in RP analysis, but are combined in the alle and Ile peaks analyzed by IE. An
194 additional factor affecting the slope and intercept values may be the limited range of D/L values
for some sample types. For example, the whole-rock dataset includes only 22 analyses, which
196 covers only half of the range of D/L values (0.06 to 0.55).

A final factor that possibly influences the comparison of A/I and D/L Ile values is the conventional reliance on chromatographic peak heights to calculate A/I values versus peak areas to calculate D/L Ile values. The effect of this difference was tested using a subset of 112 analyses from three material types (SOM 1). On average, using peak height in lieu of peak area to calculate D/L Ile values results in minimal differences in the agreement with A/I.

3.3. Relation between A/I and D/L

The relation between A/I and D/L values for Ile (discussed above) plus four additional amino acids (Asp, Glu, Ala, and Val) were explored individually for each of the four sample types and for the pooled dataset. In these equations the slope of the regression line reflects the relative rate of racemization for the two amino acids, so a slope of 1 is not expected. In all cases, the prediction uncertainty was lower for log-transformed regressions, and therefore statistics for the untransformed models are not included in Table 2. The log-transformed model taking into account sample-type-specific slopes and intercepts had the smallest prediction uncertainty intervals for all amino acids. As with Ile, Glu and Val prediction intervals improved substantially for the sample-type-specific parameters (Glu: 41-27%; Val: 38-31%). Asp prediction intervals improved by only ~3% (24-20%), and Ala prediction intervals were only slightly improved with type-specific models (from 33-31%). On the basis of the prediction uncertainty, the amino acids can be ranked from most-to-least skillful for converting A/I to D/L values using type-specific models: Asp > Glu > Val > Ala > Ile. Using the same criteria with the pooled model, the ranking for most-to-least skillful amino acid for the conversion of A/I to D/L is: Asp > Ala > Val > Ile >

Glu. The change in ranking, chiefly the position of Glu, is due to type-specific slope and intercept differences that are apparent in the conversion of A/I to D/L Glu (Figure 2).

3.4. Other factors influencing the comparison of A/I and D/L values

When converting A/I values to D/L values for different specimens, the effect of any difference in pre-treatment procedures must be taken into consideration. The dataset used in this study to derive the equations is based on paired analyses of the same sample solution and therefore does not take into account the effects of different hydrolysis times, or pre-treatments such as bleaching to isolate the intra-crystalline fraction. All of the samples in this study were hydrolyzed for 22 hr; however, shorter hydrolysis times are commonly used for RP analysis. Hydrolysis time is known to affect D/L values, with one study reporting an approximate 10% increase between samples hydrolyzed for 6 hr versus samples hydrolyzed for 20 hr (Kaufman and Manley, 1998). Longer hydrolysis times generally increase D/L values (Canoira et al., 2003), with the difference typically proportionally greater in younger samples, and for amino acids with the higher racemization rates, such as Asp. The influence of pre-treating with bleach to isolate the intra-crystalline fraction is less predictable, with some studies reporting an increase in D/L values (e.g., for *Glycymeris*, Demarchi et al., 2015; ostrich eggshell, Crisp et al., 2013), others a decrease (e.g., for *Patella*, Ortiz et al., 2015), and others no effect (e.g., for ostracodes, Bright and Kaufman, 2011).

Differences in A/I and D/L values among laboratories have been reported in several studies that compared values using ILCs and other reference materials (Wehmiller 1984, 2013; Powell et al., 2013). The A/I and D/L values reported by the AAGL, for example, tend to be

lower than those from other amino acid geochronology laboratories. While ILCs are regularly analyzed at the AAGL to ensure continuity and for reference when reporting laboratory results, no adjustments are made to account for bias. The same is true for the equations developed in this study; they do not resolve issues related to inter-laboratory biases.

Conclusion

A/I values measured using ion-exchange liquid chromatography can be confidently converted to D/L values for five amino acids, with 2σ prediction uncertainty between ± 20 and 35% when considering individual sample types. A/I values converted to D/L values using the pooled sample model yield 2σ prediction uncertainty between ± 24 and 41%, depending on the amino acid. Linear regression slopes and intercepts are provided to use with $\log_2$ A/I values, for both pooled and sample-type-specific models.

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Author contributions

KEW and DSK wrote the manuscript. KEW assembled the dataset and produced the summary statistics. MAK made the figures, completed the regression and error analyses and contributed the R code. PJH provided previously unpublished data.

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Figures

Fig. 1. Relation between A/I measured by ion-exchange chromatography and D/L Ile measured by reverse-phase chromatography. Each point represents a paired analysis of the same sample solution. Both axes are log-scaled. Solid lines are linear regressions for log₂-transformed data; dotted lines are 2σ prediction intervals. The black line is the linear regression of the pooled data (all sample types). Data are listed in supplementary Table SOM1.

Fig. 2. Relation between D/L measured by reverse-phase chromatography (y-axes) and A/I measured by ion-exchange chromatography (x-axes). Each point represents a paired analysis of

the same sample solution. (a) Asp = aspartic acid; (b) Glu = glutamic acid; (c) Ala = alanine; (d) Val = valine. Axes are scaled as log base 2. Solid lines are linear regressions for log₂-transformed data; dotted lines are 2σ prediction intervals. The black line is the linear regression of the pooled data (all sample types). Data are listed in supplementary Table SOM1.

Tables

Table 1. Materials analyzed in this study.

Table 2. Results of linear regression models using log₂ transformed A/I and D/L values, with slopes and intercepts provided for conversions of A/I to D/L. Use Log₂ transformed A/I values in a linear equation, $\text{Log}_2(\text{D/L}) = m(\text{log}_2\text{A/I}) + b$, with the slope and intercept of the appropriate material type. If the data being converted is from a sample type not listed, use the pooled model slope and intercept.

Table 1. Materials analyzed in this study.					
Material	Genus	Location	Reference		
Eggshell	<i>Genyornis</i>	Australia	Miller et al., 1997		
Eggshell	<i>Dromaius</i>	Australia	Miller et al., 1997		
Eggshell ILC-G	<i>Genyornis</i>	Australia	Wehmiller, 2013		
Eggshell ILC-R	<i>Genyornis</i>	Australia	Wehmiller, 2013		
Gastropod	<i>Poecilozonites</i>	Bermuda	Hearty et al., 2004		
Gastropod	<i>Poecilozonites</i>	Bermuda	Hearty et al., 1992		
Gastropod	<i>Lymnaea, Valvata</i>	United States - Idaho	this study ¹		
Gastropod	<i>Cerion</i>	Bahama Islands	Hearty and Kaufman, 2009		
Whole Rock		Australia	this study ²		
Whole Rock		Bahama Islands	Hearty and Kaufman, 2000		
Whole Rock		Australia	this study ³		
Whole Rock		Bermuda	Kindler et al., 2011		
Whole Rock		Australia	this study ⁴		
Bivalve	<i>Macoma, Saxidomas, Mytilus</i>	United States - Texas	McCormack, unpublished ⁵		
Bivalve ILC-A	<i>Saxidomus</i>	United States - Oregon	Wehmiller, 1984		
Bivalve ILC-B	<i>Mercenaria</i>	United States - South Carolina	Wehmiller, 1984		
Bivalve ILC-C	<i>Mercenaria</i>	United States - North Carolina	Wehmiller, 1984		
Bivalve ILC-D	<i>Mercenaria</i>	United States - Virginia	Bakeman, 2006		
Bivalve ILC-Z	<i>Mya</i>	Canada - Northwest Territories	Kaufman and Manley, 1998		
¹ Fish ladder, W of Lifton pumping station, NW Bear Lake North quad (1996)					
² South Australia, Yorke Peninsula - Cape Spencer and Pandolawie Bay (2004)					
³ South Australia, Coorong - various locations (2002-2004)					
⁴ South Australia, Kangaroo Island - various locations (2006)					
⁵ Comal County, Texas - Archaeological site 41CM25 (2003)					

Table 2.												
	Sample type	Slope	±	Intercept	±	r ²	n	AICc	k	cv0.5	cv0.683	cv0.954
<i>Aspartic acid</i>												
	Bivalve	0.43	0.02	-0.2	0.04	0.91	70	-355	9	0.2	0.2	0.2
	Eggshell	0.25	0.03	-0.25	0.04		50					
	Gastropod	0.33	0.01	-0.26	0.03		70					
	Whole Rock	0.38	0.02	-0.35	0.03		150					
	Pooled	0.36	0.008	-0.3	0.02	0.87	340	-239	3	0.23	0.23	0.24
<i>Glutamic acid</i>												
	Bivalve	0.82	0.03	-0.42	0.06	0.95	70	-164	9	0.26	0.26	0.27
	Eggshell	0.97	0.04	-0.53	0.05		50					
	Gastropod	0.66	0.01	-0.3	0.04		70					
	Whole Rock	0.54	0.02	-0.55	0.05		150					
	Pooled	0.64	0.01	-0.5	0.03	0.88	340	126	3	0.41	0.41	0.41
<i>Alanine</i>												
	Bivalve	0.67	0.03	0.27	0.07	0.93	70	-57	9	0.3	0.3	0.31
	Eggshell	0.66	0.04	0.07	0.06		50					
	Gastropod	0.67	0.02	0.36	0.04		70					
	Whole Rock	0.61	0.03	0.26	0.06		113					
	Pooled	0.62	0.01	0.22	0.02	0.92	303	-16	3	0.33	0.33	0.33
<i>Valine</i>												
	Bivalve	0.93	0.04	-0.41	0.09	0.97	70	-37	9	0.3	0.3	0.31
	Eggshell	1.05	0.04	-0.18	0.08		50					
	Gastropod	0.98	0.02	0.12	0.07		34					
	Whole Rock	0.87	0.06	-0.14	0.12		22					
	Pooled	0.94	0.02	-0.22	0.03	0.95	176	43	3	0.38	0.38	0.38
<i>Isoleucine</i>												
	Bivalve	0.875	0.042	-0.011	0.1	0.96	69	11	9	0.34	0.35	0.35
	Eggshell	1.013	0.051	-0.008	0.089		50					
	Gastropod	1.011	0.028	-0.097	0.079		34					
	Whole Rock	0.841	0.07	-0.27	0.133		22					
	Pooled	0.966	0.017	-0.003	0.034	0.95	175	60	3	0.4	0.4	0.4

Reverse Phase Ile D/L

1
0.5
0.2
0.1
0.05
0.02
0.01

0.01

0.02

0.05

0.1

0.2

0.5

1

Ion Exchange A/I

- ◆ Bivalve
- Eggshell
- ▲ Gastropod
- Whole Rock