Using Kinetic Proteomics and Linear Algebra to Discover Changes in Amino Acid Metabolism

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**Audience Analysis:**

My primary audience is scientists and researchers in proteomics and bioinformatics. The journal I chose is Bioinformatics published by Oxford Academic, and I followed their style guide (Oxford SciMed). They have a selection of general topics they accept, and my research would fall under the Gene Expression section which accepts work related to proteomics and mass spectrometry. Because this is a bioinformatics journal, I need to focus on explaining my computational methods and how they apply to the proteomics data I am using. One of my major references was published in this journal, so it gives me a reliable source to compare my manuscript with.

My secondary audience are people in the BYU Life Sciences department. People in this department will have a general knowledge of biological concepts, but I still need to be careful about cutting jargon and explaining my methods clearly. Although they might not be interested in every aspect of my research, I hope to adequately describe the background, gap in the literature, and impact of my project.

**Abstract**

Maintaining proper protein synthesis, maintenance, and degradation is necessary to stay healthy. Aging, cancer, disease, diet, and other factors are known to change this delicate balance of protein homeostasis. Low calorie, low protein diets extend the lifespan of mice but the exact mechanism for why this happens is still unknown. While well-developed proteomic methods are commonly used to study homeostasis changes at the protein level, methods to measure amino acid homeostasis are lacking. This method would help us understand how the body changes how it makes molecules with different circumstances, and why those changes are related to age, disease, and cancer. Using data from a kinetic mouse diet experiment, we developed a method to take proteomic data and deconvolute amino acid metabolic markers known as n-values. N-values are the number of hydrogens replaced by heavy hydrogen in an isotopic labeling experiment. While changes in amino acid metabolism are noticeable in the results, our experiment importantly uncovered two areas where our computational methods can be improved.

**Introduction**

Ageing, cancer, disease, and diet can all change protein homeostasis, or proteostasis (Bartke *et al.* 2001, Fontana and Partridge 2015, Chen *et al.* 2023, Kasturi 2024). These changes can be detected in complex samples by using isotopic labeling to measuring protein synthesis, degradation, and turnover over a period, also referred to as kinetic proteomics (Doherty *et al.* 2009, Price *et al.* 2010, Karunadharma *et al.* 2015). Kinetic proteomics is useful because it allows you to see dynamic changes over time, instead of seeing single proteomic snapshot.

These proteomic changes can have remarkable effects on the individual. For example, methionine restriction, calorie restriction, and low-protein diets increase longevity and overall health in mice (Bartke *et al.* 2001, Fontana and Partridge 2015). The mechanisms for how these diets increase longevity are unclear, but repeated studies using mice and kinetic proteomics could yield interesting results.

We ran a kinetic mouse diet experiment to see if we could measure shifts in protein metabolism. Our in-house DeuteRater software is designed to analyze kinetic data from tissue metabolically labeled with heavy water (D2O). This includes calculating n-values for proteins, or the number of hydrogens replaced by heavy hydrogens (deuterium or 2H) (Naylor *et al.* 2017). Proteomic level data has many uses, but we also want to know if dietary influences cause molecules to be assembled differently. This is especially important for amino acids, the building blocks of proteins. If proteostasis is changing, this must mean amino acid homeostasis is changing as well.

DeuteRater previously calculated protein n-values by summing up the literature-based n-values of all the amino acids in its sequence (Naylor *et al.* 2017). These literature n-values are the average labeling sites for mammalian tissue and have usually been found to be stable in mice (Naylor *et al.* 2017). This is a convenient and computationally efficient way to calculate n-values for proteins, but it does not account for any possible changes in amino acid metabolism. When adapting DeuteRater to work for kinetic lipid data, a new method of n-value calculations was developed to calculate empirical n-values, or n-values from the experimental data (Carson *et al.* 2017). We used this method for proteins to compare the literature and empirical n-values of each protein.

As mentioned, DeuteRater only calculates n-values for proteins, not amino acids. So, to accomplish our goal of discovering changes in amino acid metabolism, we developed a method to estimate amino acid n-values using linear algebra and the kinetic proteomic data from our mouse diet experiment. After running the data through DeuteRater and calculating protein n-values, we used a least-squares matrix algorithm to estimate the amino acid n-values for each diet.

Our underlying assumption is that the literature and empirical n-values for each protein will be similar. Any changes at the amino acid level will likely be washed out and be less noticeable for a large protein or peptide. Therefore, a scatterplot of empirical n-values plotted over their literature n-values should generally follow a y=x linear trend. We hypothesized that essential amino acid n-values would also be like their literature n-values. The main variable of the mouse experiment was diet, and since essential amino acids can be made by the organism, different diets would have a smaller influence on their metabolism. We expected non-essential amino acids to show a larger change in empirical n-value compared to their literature n-values.

**Materials and Methods**

**Mouse Diet Experimental Design**

56 mice were labeled with D2O and randomly assigned to one of four diets (figure 1): low-protein ad libitum (LP AL), low-protein calorie restricted (LP CR), high-protein ad libitum (HP AL), and high-protein calorie restricted (HP CR). Diets LP AL and LP CR each had nine mice, diet HP AL had 18 mice, and diet HP CR had 20 mice. The number of mice between the low-protein and high-protein diets is different because they were done independently.

Each mouse was given an initial shot of 10% D2O to get labeled material into their system quickly. To keep the mice at ~5% D2O enrichment, we provided 8% D2O enriched water to offset the unlabeled water in their food. Each mouse was kept in a separate cage to prevent the mice from eating other diets.

Mouse blood serum and brain samples were collected across a range of timepoints, with at least two mice being taken down for each. Timepoints ranged from zero (after the initial injection) up to 32 days. Blood serum was analyzed using an Off-Axis Integrated Cavity Output Spectroscopy machine (OA-ICOS) to figure out the percent D2O enrichment. This is recorded, along with when the sample was taken. Brain samples are prepared and run through liquid-chromatography and mass spectrometry pipeline (LC-MS). This provides intensity or abundance information for the proteins in the sample. Abundance, retention time (how long it took for the molecule to pass through the LC-MS), mass-to-charge ratio (m/z), and other identifying information are saved in a .tsv file. DeuteRater uses the time and enrichment data from the blood serum, LC-MS data from the brain samples, and an ID file of lipids or proteins to look for. DeuteRater then calculates the number of hydrogens replaced by deuterium for each molecule.

**DeuteRater and N-Value Calculations**

DeuteRater is the name of our software designed to calculate and graph kinetic curves for lipids and proteins labeled with D2O (Naylor *et al.* 2017). This process includes calculating n-values for individual molecules, or the number of hydrogens replaced by heavy hydrogens (2H or deuterium). Previously, n-values for proteins and peptides were assumed to be stable and always calculated by summing together literature established amino acid n-values (Naylor *et al.* 2017). Lipids do not have established n-values making this method unusable for lipids and their constituents, so we created a method to calculate n-values from our kinetic experiment data.

From a kinetic experiment, we know the chemical formula, m/z, retention time, and intensity for each lipid measured. The measured intensity of each molecule will show up as a spectrum, with m/z on the x-axis and intensity on the y-axis (Figure 2Bii). As more D2O is incorporated, the spectrum will shift in a predictable way (Naylor *et al.* 2017). For any given molecule at any time, it will be completely unlabeled (0% deuterium, 100% hydrogen), fully labeled (100% deuterium, 0% hydrogen), or somewhere in between. We generate these theoretical unlabeled and labeled spectra using emass (Rockwood 2006). Emass needs the chemical formula, the number of deuterium, and the percent enrichment (5% with our mice), and returns the theoretical spectrum.

The theoretical spectra are calculated for each combination of hydrogen and deuterium so we can calculate the fraction new (Figure 2Bi and 2Biii). The fraction new is calculated for each collection of corresponding peaks using Equation 1 and is the portion of the population that is new.

For example, if a molecule had 20 hydrogen total, 20 theoretical fully labeled spectra would be generated: 20 hydrogen and 0 deuterium, 19 hydrogen and 1 deuterium, …, 0 hydrogen and 20 deuterium. A standard deviation is calculated from the fraction new values of each peak, and whichever combination of hydrogen and deuterium has the lowest standard deviation, that number of deuterium is selected as the n-value (Figure 2Bv).

**Amino Acid N-Value Calculations**

N-values for proteins were calculated with the n-value calculation module in DeuteRater, but calculating amino acid n-values required more steps. Since we knew the sequence and n-value for each protein, we could use a least-squares matrix equation to solve for amino acid n-values. We created a matrix where each row is a protein sequence, and each column is the number of each amino acid found in each sequence (Table 1). A corresponding list of the calculated protein n-values was also provided for the equation.

Like Figure 1A, each amino acid has a specific number of possible locations where deuterium can replace hydrogen. Knowing this, we implemented a lower and upper bound to the least-squares solution with zero being the lower bound (an n-value cannot be negative) and the max number of labeling sites as the upper bound (see Max Labeling Sites column of Table 2). An open-source Python library called SciPy has a module available to solve a linear least-squares regression problem with upper and lower bounds (Virtanen *et al.* 2020). To evaluate this algorithm, we first created our protein and amino acid count matrix based on the literature protein n-values. The least-squares model accurately solved for the literature-based amino acid n-values (figure 3).

**N-Value Quality Control Filters**

The least-squares solution used for amino acid calculations is sensitive to noise (Wedin 1972). A few filters were enforced to ensure only the highest quality n-values were used. First, only standard deviations less than or equal to 0.05 were included. Second, low-intensity proteins tend to be noisier compared to high-intensity proteins (Wells *et al.* 2023). To avoid this potential source of noise, only the top 75% of the most abundant proteins were used.

**Results**

**Literature vs. Empirical Protein N-Values**

One of our underlying assumptions was that protein n-values should be close to their literature n-values. This means if we plot the empirical or experimental-based n-values over the literature n-values, we should see a general linear y=x trend. This did not seem to be the case with the data from the four diets (figure 4). Instead, the linear trend line for each of the diets after filtering by standard deviation (< 0.05) and intensity (top 75%) did not match the y=x trend. This seemed to be caused by extreme outliers with low standard deviations, but much higher empirical n-values compared to their literature n-values. Applying a least-squares model to this data would likely not produce correct results, so another attempt at removing noisy data was made.

**Max Labeling Site Filter**

When investigating the extreme outliers from Figure 4, we noticed their n-values were suspiciously high. It was decided that most of these outliers could justifiably be removed if we added a filter for the max number of labeling sites for each protein. Figure 2A shows each protein will have a specific number of possible labeling sites. Consequently, this number is also the maximum number of deuterium that could be theoretically attached to the protein. Any protein assigned to an n-value greater than the maximum number of labeling sites should therefore be excluded, since it is not possible to have a n-value that high.

Applying this max labeling site filter improved the quality of the data (Figure 5). Except for the LP AL diet, the filtered data points showed linear trends much closer to the y=x trend we were expecting. Importantly, most of the extreme outliers seen previously with high standard deviations and high n-values were now excluded.

**N-Value Calculation Improvements**

Another interesting observation was made when looking at graphs from the n-value calculation process (Figure 6). Graph A is an example of a good n-value with a low standard deviation. The n-value calculation algorithm found a local minimum close to the literature n-value. Graph B shows a poor example where the standard deviation loosely follows a 1/x trend, or an exponential decay function. The n-value in this case is not believable because it selects the maximum number of deuteriums as the supposed n-value. This method could be improved if we allow for fractional n-values or multiply the standard deviations by x (the number of deuterium).

**Amino Acid N-Values**

Table 2 displays the results of the least-squares solution for each diet. Essential amino acid n-values changes were higher on average than non-essential amino acids. This was surprising since we hypothesized non-essential amino acids would have a larger change. For each diet, the empirical n-values were close to the literature n-values for tryptophan, which is what we expected. Conversely, methionine and phenylalanine are also essential amino acids, and their empirical n-values ranged from to their max number of possible labeling sites. For non-essential amino acids, glutamine and alanine had interesting results. For the low-protein diets, glutamine showed n-values different than the literature n-value. Alanine was the opposite, where the high protein diets showed a change in n-value compared to the literature n-value. Although these results are interesting, it is important that we first address the issues with noise and n-value calculations before we make any claims.

**Discussion**

We tried to find diet-dependent changes in amino acid metabolism by calculating empirical n-values and using a least-squares matrix method to estimate amino acid n-values. Noise heavily influenced the results, and even after applying filters for standard deviation and intensity, the data was not dependable. Although we cannot confidently show diet-dependent n-value changes in amino acids, our experimental method revealed two ways we can improve our n-value calculations.

Figure 6 points out an underlying problem with the current n-value calculation method. Perhaps there are local minimums with a more exact n-value, but we are skipping over them. The way n-values are calculated does not allow for non-integer n-values, but theoretically an n-value is not constrained to integers. Interpolating fractional n-values could improve the accuracy of the n-value calculations and reduce noise that is skewing the amino acid n-value results.

Taking advantage of the exponential decay trend could also prove to be useful. If we see molecules with curves resembling exponential decay, we could multiply the standard deviations by the amount of deuterium. This would penalize the high n-values while amplifying local minimums hidden by the exponential decay function.

**Conclusion**

Although our kinetic proteomics and linear algebra method needs improvement, we still see interesting differences in metabolism between essential vs non-essential amino acids and between diets. The method is sensitive enough to reveal metabolic differences at the amino acid level, and we are confident with better n-value calculation methods we can uncover important amino acid metabolic mechanisms that affect health and longevity.

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A screenshot of a video game

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**Figure 1. The kinetic mouse diet experiment workflow.** Mice were randomly assigned to one of four diets: low-protein ad libitum (LP AL), low-protein calorie restricted (LP CR), high-protein ad libitum (HP AL), and high-protein calorie restricted (HP CR). The mice kept at a consistent 5% D2O enrichment. At least two mice were taken down at each timepoint for reproducibility. D2O enrichment was found from the blood serum using an LGR-ICOS machine. Brain samples were run through a liquid-chromatography mass spectrometry workflow. DeuteRater uses the time and enrichment data, LC-MS data, and an ID file of molecules to look for. DeuteRater then calculates the number of hydrogens replaced by deuterium for each molecule.

A diagram of a graph

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**Figure 2. The process of calculating n-values, or the number of hydrogens replaced by deuterium.** (A) A molecule will have a specific number of possible locations (in this case, ten) where hydrogen can be replaced by deuterium (2H). Highly exchangeable locations are not included, because the deuterium would inevitably be replaced and not show up in mass spectrometry (Wells *et al.* 2023). (B) For each combination of hydrogen and deuterium, a theoretical unlabeled (i) and fully labeled spectrum (iii) is generated and used with the measured or empirical spectra (ii) to calculate a fraction new for each peak (iv). See Equation 1 for how fraction new is calculated. A standard deviation is calculated for each combination of hydrogen and deuterium, and whichever combination has the lowest standard deviation, the number of deuterium is selected as the n-value for that molecule (v) (Naylor et al. 2017).

**Equation 1.** Fraction new is the portion of the population is new (see Figure 2 for a visual representation). It is calculated using the empirical spectra, theoretical fully labeled spectra, and the theoretical unlabeled spectra in the above equation. Fraction new is calculated as part of the n-value calculation process (see Figure 2).

**A =** Protein Sequence (rows), Amino Acid Sequence Counts (columns)

**b =** Protein Empirical N-Values= [34, 48, 10, 32, 28, 17, … etc.]

**x =** amino acid n-values

**minimize 0.5 \* ||A x - b||\*\*2**

subject to lower bound (0) <= x <= upper bound (max amino acid labeling sites)

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
|  | **Alanine** | **Cysteine** | **Glycine** | **Lysine** | **…** |
| **Protein 1** | 3 | 2 | 5 | 3 | … |
| **Protein 2** | 6 | 7 | 9 | 4 | … |
| **Protein 3** | 8 | 1 | 6 | 12 | … |
| **Protein 4** | 5 | 5 | 8 | 17 | … |
| **Protein 5** | 4 | 2 | 7 | 5 | … |
| **Protein 6** | 2 | 9 | 11 | 2 | … |
| **…** | … | … | … | … | … |

**Table 1 and Equation 2. A theoretical example of the matrix used to solve amino acid n-values.** This least-squares method minimizes the equation 0.5 \* ||A x - b||\*\*2, where A is a matrix with protein sequences as rows and amino acid counts as columns, b is a corresponding list of protein n-values (the number of hydrogens replaced by deuterium), and x is a list of the amino acid n-values needed to be solved. The lsq\_linear function in the scipy.optimize library does these calculations and can accept lower and upper bounds (Virtanen *et al.* 2020). We set zero as the lower bound and the max number of amino acid labeling sites as the upper bound.

A group of graphs with different colored dots

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**Figure 3. Evaluating the SciPy least-squares model by plotting the solved amino acid n-values (y-axis) over the literature amino acid n-values (x-axis).** Using a matrix with protein sequences as rows and amino acid counts as columns, we provided a corresponding list of literature-based protein n-values, the number of hydrogens replaced by deuterium, calculated by summing together amino acid n-values for each sequence. If the least-squares method is correct, we should see a linear y=x trend when plotting solved amino acid n-values over literature-based amino acid n-values. For each diet, the least-squares model produced amino acid n-values matching the literature (Naylor *et al.* 2017).

A screenshot of a computer screen

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**Figure 4. The correlation between the literature n-value and empirical n-value for each protein.** These scatterplots were made to test whether empirical n-values for proteins will be close to their literature n-values. The grey dots are all proteins measured along with a gray linear fit line. The colored dots are proteins with a standard deviation of less than or equal to 0.05 and were in the top 75% most abundant for each diet. A colored linear fit line was applied to those data points. The black dashed line is a linear y=x trendline we hypothesized the data points would follow.

A screenshot of a computer screen

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**Figure 5. The correlation between the literature n-value and empirical n-value for each protein after applying the max labeling sites filter.** The grey dots are all proteins measured along with a gray linear fit line. The colored dots are proteins with an n-value less than or equal to the maximum number of possible labeling sites, an n-value standard deviation of less than or equal to 0.05 and were in the top 75% most abundant for each diet. A colored linear fit line was applied to those data points. The black dashed line is a linear y=x trendline we hypothesized the data points would follow.

A graph of a graph of a number of deuterium

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**Figure 6. A good and poor example of n-value calculations for different proteins.** The fraction new standard deviation (y-axis) is plotted over the number of deuteriums (x-axis) during the n-value calculation process. Ideally, the lowest standard deviation is a good estimate of the n-value for each molecule (graph A). Sometimes the lowest standard deviation does not accurately represent a good n-value estimate if no strong local minimums are present (graph B).

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Amino Acid** | **LP AL N-Values** | **LP CR N-Values** | **HP AL N-Values** | **HP CR N-Values** | **Literature N-Values** | **Max Labeling Sites** |
| **Phenylalanine** | **0.94** | **0.98** | **7.82** | **0** | **0.47** | **8** |
| Histidine | 4.41 | 0.24 | 0 | 0.82 | 2.15 | 5 |
| Isoleucine | 5.48 | 4.96 | 0 | 2.35 | 0.8 | 10 |
| Lysine | 1.15 | 1.99 | 0 | 5.6 | 0.3 | 9 |
| Leucine | 3.93 | 2.98 | 1.98 | 4.24 | 0.7 | 10 |
| **Methionine** | **3.75** | **0.55** | **0** | **8** | **1.02** | **8** |
| Threonine | 2.98 | 5 | 2.98 | 3.83 | 0.21 | 5 |
| Valine | 1.94 | 0 | 0 | 3.82 | 0.79 | 8 |
| **Tryptophan** | **0** | **0** | **0.27** | **0.46** | **0.06** | **8** |
| **Average Essential AA Change** | **2.01** | **1.13** | **0.73** | **2.51** |  |  |
|  |  |  |  |  |  |  |
| **Alanine** | **4** | **4** | **2.95** | **0.89** | **3.95** | **4** |
| Cysteine | 0 | 3 | 0 | 0 | 1.67 | 3 |
| Aspartic Acid | 3 | 0 | 2.66 | 1.44 | 2.5 | 3 |
| Glutamic Acid | 4.16 | 4 | 5 | 2.76 | 4.18 | 5 |
| **Glycine** | **2** | **1.6** | **0.9** | **1.98** | **1.84** | **2** |
| Asparagine | 0 | 3 | 2.62 | 0 | 1.15 | 3 |
| Proline | 0 | 0 | 4.52 | 0 | 1.57 | 7 |
| **Glutamine** | **1.78** | **5** | **3.27** | **3.33** | **3.62** | **5** |
| Arginine | 0 | 2.41 | 0 | 7 | 3.4 | 7 |
| Serine | 3 | 0 | 1.62 | 3 | 1.96 | 3 |
| Tyrosine | 5.64 | 0 | 2.45 | 1.18 | 0.5 | 7 |
| **Average Non-Essential AA Change** | **-0.25** | **-0.3** | **-0.03** | **-0.43** |  |  |
| **Combined Average AA Change** | **0.77** | **0.34** | **0.31** | **0.89** |  |  |

**Table 2. Amino acid n-values estimated from a least-squares matrix algorithm.** LP and HP are low and high protein. AL and CR are ad libitum and calorie restricted. Average n-value changes were calculated for essential and non-essential amino acids for each diet. The literature n-values column represents the average literature n-value for mammalian tissue (Naylor *et al.* 2017). The maximum labeling sites column is the number of possible labeling sites for each amino acid. Ideally, essential amino acid n-values would be close to their literature n-values. Alternatively, we expected there to be more change in non-essential amino acid n-values. This was sometimes true (such as tryptophan, alanine, and glutamine), but not true in other cases (phenylalanine and glycine).