

Clinical-scale investigation of stable isotopes in human blood: $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ from 406 patients at the Johns Hopkins Medical Institutions

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Objective chemical biomarkers are needed in clinical studies of diet-related diseases to supplement subjective self-reporting methods. We report on several critical experiments for the development of clinically legitimate dietary stable isotope biomarkers within human blood. Our examination of human blood revealed the following: (1) Within blood clot and serum from anonymous individuals (201 males, 205 females) we observed: mean serum $\delta^{13}\text{C} = -19.1 \pm 0.8\text{‰}$ (standard deviation, SD); clot, $-19.3 \pm 0.8\text{‰}$ (SD); range = -15.8‰ to -23.4‰ . Highly statistically significant differences are observed between clot and serum, males and females for both clot and serum. For ^{15}N ($n = 206$), mean serum = $+8.8 \pm 0.5\text{‰}$ (SD); clot $+7.4 \pm 0.4\text{‰}$ (SD); range = $+6.3\text{‰}$ to $+10.5\text{‰}$. Blood serum is enriched in ^{15}N relative to blood clot by $+1.4\text{‰}$ on average, which may reflect differing protein amino acid content. Serum nitrogen is statistically significantly different for males and females, however, clot shows no statistical difference. (2) Relative to clot, capillary blood is marginally different for ^{13}C , but not ^{15}N . Clot ^{13}C is not significantly different from serum; however, it is depleted in ^{15}N by 1.5‰ relative to serum. (3) We assessed the effect of blood additives (sodium fluoride and polymerized acrylamide resin) and laboratory process (autoclaving, freeze drying) commonly used to preserve or prepare venous blood. On average, no alteration in $\delta^{13}\text{C}$ or $\delta^{15}\text{N}$ is detected compared with unadulterated blood from the same individual. (4) Storage of blood with and without the additives described above for a period of up to 115 days exhibits statistically significant differences for ^{13}C and ^{15}N for sodium fluoride. However, storage for unadulterated blood and blood preserved with polymerized acrylamide resin does not change the $\delta^{13}\text{C}$ or $\delta^{15}\text{N}$ isotopic composition of the blood in a significant way. With these experiments, we gain a clinical context for future development of a stable isotope based dietary biomarker. Copyright © 2008 John Wiley & Sons, Ltd.

The leading causes of death in the United States are increasingly linked to poor dietary habits,^{1,2} creating a need for objective chemical biomarkers for use in clinical studies of diet-related diseases. Dietary studies often rely upon self-reported answers to standardized, lengthy questionnaires and other qualitative, subjective techniques of dietary assessment. While providing important subjective information, questionnaires have long been recognized as suboptimal quantitative assessments.^{3,4} With this work, we establish the potential for the use of natural abundance stable isotope mass spectrometry of human blood in the bioassay of dietary intake. Commonly consumed natural and artificial foods exhibit a range of $^{13}\text{C}/^{12}\text{C}$ ratios,^{5–8} it is notable that corn, sugar cane, and derivatives (e.g. high fructose corn syrup (HFCS)) carry conspicuously high $\delta^{13}\text{C}$ signatures.⁹

Food-web studies have long exploited $^{15}\text{N}/^{14}\text{N}$ ratios for their potential to provide insight into trophic level.¹⁰ Newer research has established that short-term diet modifications result in measurable alterations to the natural abundance $^{13}\text{C}/^{12}\text{C}$ and $^{15}\text{N}/^{14}\text{N}$ ratios of a range of biological tissues, spanning muscle, hair, blood, and lipids, from, horses, alpacas, cows, and various other mammals.^{11–18} With respect to human research, labeled-compound stable isotopes are used routinely as tracers in human medical and nutritional research¹⁹ and natural abundance isotopes are often employed in the study of drug metabolism and forensics.^{4,6,15,19–28} Human dietary reconstruction is limited to archaeological specimens,^{14,29–32} a small number of modern anthropological studies of isolated populations,^{28,29} and a small number of studies using hair as a substrate.^{26,31,33}

The stable isotope composition of human tissues has the potential to provide quantitative information about current and past dietary habits. Previous animal studies have demonstrated that mammals acquire the carbon and nitrogen used to build tissues, from diet, and that the isotopic signature of various tissues will reflect the value of

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dominant dietary components^{9,11,13,14,16,18,33–37} and the trophic level.^{10,30,31} Stable isotope geochemists have reliably measured the isotopic signatures of organic substrates accurate to 0.5‰ for more than 50 years.⁵ Wilkinson *et al.*³² compiled the stable isotope composition of blood from 26 individuals to show differences in dietary habits among two population groups of Alaskan natives; we wish to expand in this direction by reporting the variability amongst 406 of individuals of unknown dietary habits. In order to further comment upon the utility of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ in human blood as a potential dietary bioassay, we have produced the largest number of isotopically characterized blood tissue from humans, or for any single species. Here we report on the measurement of the natural abundance stable carbon isotope composition of blood from 406 individuals and the nitrogen isotope signature of blood from 206 anonymous individuals, in order to characterize the isotopic variability seen across an anonymous population. We also explore the ramifications of blood sampling method (capillary vs. venous), and blood storage and preservation methods, on the carbon and nitrogen isotope signatures. We focus on blood as a clinical substrate of long-standing utility and importance, routinely and easily secured, and a well-established bioassay substrate for vital health indices.

EXPERIMENTAL

The data in this paper were collected from several studies that took place from 2006 to 2008. All the blood for these studies was collected within the Johns Hopkins Medical Institutions and given to the Johns Hopkins Krieger School of Arts and Sciences Jahren Laboratory anonymously. All studies contributing data to this paper were performed in accordance with the Helsinki Declaration of 1975 and considered exempt from federal regulation by the Johns Hopkins Homewood Internal Review Board in accordance with 45CFR46.101(b), category (4) or approved by the Johns Hopkins School of Public Health Internal Review Board (00000526, approved August 21, 2008).

Blood from anonymous donors

A total of 406 blood samples were obtained as surplus collected during medical visits to the Johns Hopkins Wyman Park Medical Center. When blood is collected for a laboratory test it is routine for additional blood to be collected in order to facilitate further tests without the nuisance of recall to the patient. We obtained this unused blood for our study. Samples were anonymous, and no information was available to the investigators beyond the date of the blood sampling and the gender of the patient. Equal numbers of male and female blood samples were gained for our analysis. Two hundred samples (from 100 females, 100 males) were collected in 2006 and run exclusively for natural abundance carbon stable isotopes. In 2007 we expanded the study to measure ^{15}N and collected an additional 206 samples (from 105 females, 101 males) to be analyzed for natural abundance carbon and nitrogen stable isotopes. Although compounds are commonly added to blood in order to promote preservation or prevent clotting, the blood for the purpose above was unadulterated (i.e.

without additives). Blood without anticoagulants aided by centrifugation naturally yields fractions of clot and serum. Additives (sodium fluoride, polymerized acrylamide resin) often contain carbon, and they may represent a source of isotopic contamination to the blood sample. Blood samples were transferred into 15 mL polystyrene tubes and centrifuged at approximately 3300–3400 rpm for 14 min in order to separate a cohesive clot from serum.

Blood from recruited subjects

In addition to performing an assessment of the natural variability of isotopic signature within blood from a large human population, we performed two critical tests necessary for any eventual adoption of $\delta^{13}\text{C}$ or $\delta^{15}\text{N}$ analysis of blood as a bioassay. The first test was designed to assess whether a capillary blood sample would provide an isotopic sample of similar quality to venous blood sample. Capillary and venous blood samples were collected from 24 fasting individuals. We used disposable fingerstick lancets, similar to those which form the first step of home blood glucose measurement (i.e., CoaguChek device; Roche Diagnostics, Indianapolis, IN, USA). Our technique involved immediate blotting of capillary blood onto a Whatman spun-glass filter (1 cm in diameter). Prior to the blotting, filters were sterilized in a muffle furnace at 260°C for 2 h. Three drops of blood from one individual (one drop of fluid is approximated as 1 mL) were blotted onto each glass filter; most individuals did not experience discomfort from the fingerstick procedure. Three separate filters were collected for each individual resulting in a total collection of 72 filters. Blotted filters were allowed to dry for 2 h at room temperature, then weighed. Samples weighing between 0.5 and 4.0 mg (not including the filter) were accepted for analysis (the ideal sample weight being 2.0 mg). The filters were enclosed in high-purity tin capsules (each capsule weighing approximately 20 to 40 mg). In order to provide a comparison to the fingerstick method, venous blood samples were collected without additives for each of the 24 individuals. This blood was prepared for isotopic analysis in an identical way to the venous blood described in the previous section (i.e. separation of clot from serum).

It is very common for collected blood to be preserved prior to analysis. The addition of chemical and/or storage at low temperature in order to prevent cellular or biochemical degradation are commonly employed upon collection. In order to assess the isotopic alteration (if any) caused by these techniques, we collected venous blood from 31 individuals and compared the carbon and nitrogen stable isotope compositions of a preserved aliquot with that of the unaltered immediate blood draw. From each of the 31 subjects, three (7 mL) vacutainers of blood were collected: one without additives, one containing sodium fluoride and kaolin (NaF), and one containing polymerized acrylamide resin (PAR). In addition, we tested the effects of autoclaving/freezing (A/FD) and storage against the original isotopic composition of collected blood; if unimportant to isotopic value, both these practices would allow for lower-risk sample handling. Autoclaving was performed using a laboratory sterilizer (Barnstead International, Dubuque, IA, USA) at a temperature of 132°C for 20 min and then

freeze-dried using a FreezeOne 4.5 (Labconco Corp., Kansas City, MO, USA) at a vacuum of $\sim 50 \times 10^{-3}$ mbar and a temperature of -50°C for a minimum of 3 days. Freeze-dried samples were homogenized with a mortar and pestle prior to stable isotope analysis.

Stable isotope analyses

Aliquots (17 μL) of liquid samples (e.g. blood serum) were transferred into high-purity tin capsules and dried for a minimum of 4 h within a sterile chemical fume hood. The amount of sample ultimately introduced into the mass spectrometer ranged from 0.05 to 2.0 mg; the amount of carbon (by mass) in these samples was found to be approximately 41% in serum, and 47% in clot, as determined from the total amount of CO_2 released upon combustion; the amount of nitrogen (by mass) approximately 11% in serum and 14% in clot. Samples were quantitatively combusted to CO_2 in an elemental analyzer (EURO.EA3000; Euro Vector, Milan, Italy) configured with a continuous-flow stable isotope ratio mass spectrometer (Isoprime/Micromass UK Ltd., Manchester, UK). The combustion reactor was maintained at 1020°C and its reagents were replaced approximately every 300 samples. The linear range for the instrument is between 1 and 14 nanoamps (nA), which is equivalent to a linear range of 12 to 140 micrograms (μg) of carbon. Linearity is verified on a daily basis using both variable-sized reference gas injections, and variable-sized standards spanning our sample size range. Each sample was analyzed in triplicate; the values reported represent the mean value of three analyses. The total variability across the three measurements never exceeded 0.3‰. An analytical uncertainty of $\pm 0.1\%$ is associated with each sample measurement. All samples were analyzed in the Jahren Laboratory at the Krieger School of Arts and Sciences, Johns Hopkins University. The natural abundance stable isotope composition ($\delta^{13}\text{C}$ or $\delta^{15}\text{N}$) is reported in units of permil (‰) expressed relative to the international standard for carbon Vienna Pee Dee Belemnite (VPDB) or mean air (AIR) for nitrogen, according to the standard convention [‰]:

$$\delta X = \frac{R_{\text{sample}} - R_{\text{std}}}{R_{\text{std}}} \times 1000$$

Two internal laboratory standards referenced to VPDB were used: powder-form methionine ($\text{C}_5\text{H}_{11}\text{NO}_2\text{S}$) and powder-form peptone, which is a water-soluble protein

derivative of mixed composition. The methionine (-26.8%) and peptone (-14.7%) internal standards were characterized by measuring against the following internationally distributed reference materials supplied by the International Atomic Energy Agency (IAEA): IAEA-CH-6 (-10.45%); IAEA-CH-3 (-24.72%); and IAEA-CH-7 (-32.15%). The internal standards were reported relative to VPDB on a scale normalized by assigning consensus values of -46.6% to L-SVEC lithium carbonate and $+1.95\%$ to NBS 19 calcium carbonate. Natural abundance stable isotope compositions ($\delta^{15}\text{N}$) are reported in units of permil (‰) expressed relative to the nitrogen isotopic composition of air (AIR) according to the standard convention referenced above. Two internal laboratory standards referenced to AIR were used: powder-form peptone, which is a water-soluble protein acid (USGS40 -4.52%); L-glutamic acid (USGS41 $+47.57\%$). The internal standards were reported relative to AIR on a scale normalized by assigning consensus values of $+0.43\%$ to IAEA-N-1 ammonium sulfate and $+180\%$ to USGS32 potassium nitrate derivative of mixed composition and L-histidine ($\text{C}_6\text{H}_9\text{N}_3\text{O}_2$). The internal standards, L-histidine ($\delta^{15}\text{N} -2.84\%$) and peptone ($\delta^{15}\text{N} +5.92\%$), were characterized by measuring against the following internationally distributed reference materials available from the IAEA: L-glutamic. Together, the peptone and L-histidine standards represent the best available two-point calibration of $\delta^{15}\text{N}$ values present in our samples ($+6.3\%$ to $+10.5\%$). Further explanation of this referencing convention can be found in Coplen *et al.*³⁸ Together, the methionine and the peptone represent a two-point calibration that encompassed the range of $\delta^{13}\text{C}$ values present in our samples (-23.4% to -15.8%). Methionine and peptone standards were introduced every 15 samples. Every 12 samples a blank, comprising of an empty tin capsule, was introduced.

RESULTS

Table 1 presents the $\delta^{13}\text{C}$ values of clot and serum collected from 406 anonymous individuals (205 females, 201 males) and the $\delta^{15}\text{N}$ values of 206 individuals (105 females, 101 males), as well as summary statistics. Figures 1(A)–(D) and 2(A)–2(D) represent histograms for carbon and nitrogen isotopes grouped by gender and blood component. Measurement of the carbon isotopes yields the following means: clot

Table 1. $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values of randomly chosen population of 406 individuals, collected during two separate studies, August to November, 2006^a and August to November, 2007. Results shown in Figs. 1–3

	$\delta^{13}\text{C}$						$\delta^{15}\text{N}$					
	Male serum	Female serum	Male clot	Female clot	Total population serum	Total population clot	Male serum	Female serum	Male clot	Female clot	Total population serum	Total population clot
n	201	205	201	205	406	406	101	105	101	105	206	206
Mean	-19.0	-19.3	-19.1	-19.4	-19.1	-19.3	+8.8	+8.9	+7.4	+7.4	+8.8	+7.4
Maximum	-16.3	-15.8	-17.1	-16.4	-15.8	-16.4	+9.8	+10.5	+8.4	+8.7	+10.5	+8.7
Minimum	-21.2	-23.2	-21.3	-23.4	-23.2	-23.4	+7.7	+8.0	+6.3	+6.6	+7.7	+6.3
SD	0.8	0.8	0.8	0.8	0.8	0.8	0.4	0.5	0.4	0.4	0.5	0.4
Range	4.9	7.4	4.3	7.0	7.4	7.0	2.1	2.5	2.2	2.1	2.5	2.5

^aBlood from the study conducted in 2006 was analyzed only for carbon isotopes.

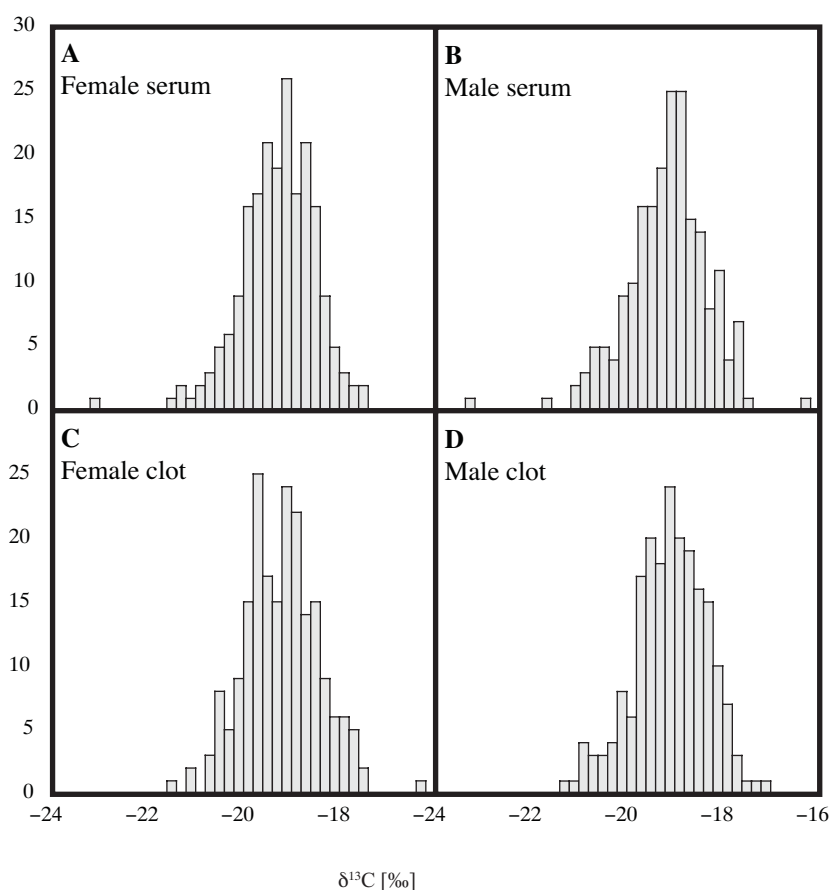


Figure 1. (A–D) Population histograms for $\delta^{13}\text{C}$ values in human blood, separated by gender and blood component (clot and serum). Data were obtained from blood given anonymously by adult females and males (females $n = 205$, males $n = 201$).

(-19.4‰ for females; -19.1‰ for males), serum means (-19.3‰ for females, -19.0‰ for males). Medians compared with the above mean values show a normal for gender and blood media groups; clot medians (-19.4‰ for females; -19.1‰ for males), serum medians (-19.2‰ for females, -19.0‰ for males) and standard deviations for clot (0.8‰ for both females and males) and for serum (0.8‰ for both females and males) were similar for males and females. Based on calculation of p -values, highly statistically significant differences were observed for ^{13}C between males and females ($p < 0.001$), although the difference between the two populations is small (0.3‰); further, a highly statistically significant difference was also observed between the means for serum and clot ($p < 0.001$). The $\delta^{15}\text{N}$ clot means were the same for males and females ($+7.4\text{‰}$); however, a statistically significant difference ($p = 0.012$) was observed between serum means ($+8.9\text{‰}$ for females and $+8.8\text{‰}$ for males). Again, medians compared with the above mean values show a normal for gender and blood media groups; clot medians ($+7.4\text{‰}$ for both males and females), serum medians ($+8.9\text{‰}$ for females and $+8.8\text{‰}$ for males). Standard deviations were similar for gender; clot (0.4‰ for both females and males), and serum standard deviations (0.5‰ for females and 0.4‰ for males). Females had a $\delta^{13}\text{C}$ statistical range of 7.4‰ for serum and 7.0‰ for clot; males had a $\delta^{13}\text{C}$ statistical range of

4.9‰ for serum and 4.3‰ for clot. However, the data that render the range in female values larger than that for males represented less than 2% of the dataset. In contrast, females had a $\delta^{15}\text{N}$ statistical range of 2.5‰ for serum and 2.1‰ for clot; males had a $\delta^{15}\text{N}$ statistical range of 2.1‰ for serum and 2.2‰ for clot.

Figures 3(A)–3(D) plot the isotopic composition of clot versus serum within blood for $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values, grouped by gender. Very small, but highly statistically significant isotopic differences were observed between the $\delta^{13}\text{C}$ value of clot and the $\delta^{13}\text{C}$ value of serum from the same individual. ^{15}N was consistently highly enriched in serum relative to clot from the same individual. For females and males, $\delta^{15}\text{N}_{\text{clot}} + 1.4 = \delta^{15}\text{N}_{\text{serum}}$ (‰) on average.

For a comparison of the isotopic composition of capillary versus venous blood, 24 individuals provided us with both capillary (fingerstick) and venous blood. Capillary blood was analyzed as blood drops on spun-glass filters for comparison with venous blood, separated into clot and serum, from the same individual. Statistical results are shown in Table 2 and Figs. 4(A)–4(D). For this group of 24 individuals, both the $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ maxima and minima comprise a subset of the values gained from the larger population (Table 1). Capillary blood ^{13}C was not found to be statistically significantly different relative to serum and clot (Figs. 4(A) and 4(B))

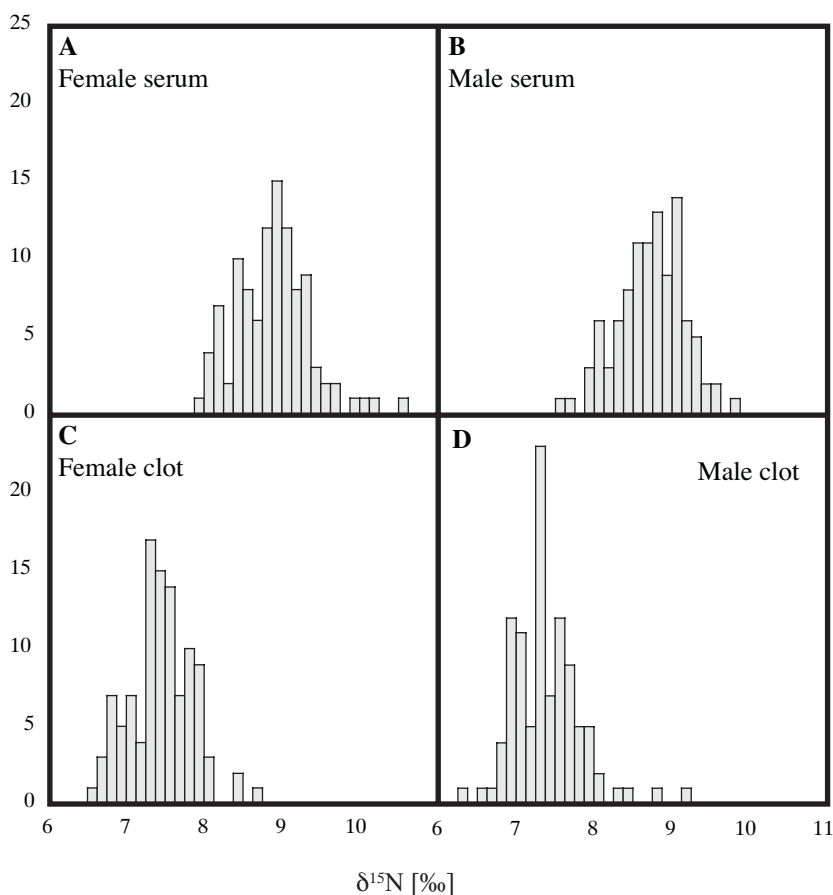


Figure 2. (A–D) Population histograms for $\delta^{15}\text{N}$ values in human blood, separated by gender and blood component (clot and serum). Data were obtained from blood given anonymously by adult females and males (females $n = 105$, males $n = 101$).

separated via venous blood draw. Capillary blood was depleted in ^{15}N (by 1.5‰ on average) relative to serum separated via venous blood draw (Fig. 4(C)). Capillary blood relative to clot separated via venous blood draw (Fig. 4(D)) was also statistically significantly different ($p = 0.014$) for ^{15}N ; however, this represents a relatively smaller difference in blood composition (0.2‰). We note that these relationships are in keeping with the isotopic similarity seen in venous clot and serum with respect to $\delta^{13}\text{C}$ (Figs. 1(A)–1(D) and 2(A)–2(D)), and with the consistent enrichment of venous serum over clot with respect to $\delta^{15}\text{N}$ (Figs. 3(A)–3(D)) discussed earlier.

The effects of common blood additives and storage techniques were investigated using venous blood from 31 individuals; the results are presented in Tables 3(a) and 3(b). As with the capillary blood study, both the $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ maxima and minima comprise a subset of the values gained from the larger population (Table 1). Statistical analysis (Table 4) of $\delta^{13}\text{C}$ values of additive-free blood and autoclaved/freeze-dried blood, blood with sodium fluoride/kaolin (NaF), and blood with polymerized acrylamide resin (PAR) additives for both serum and clot indicate that these methods of blood preservation do not meaningfully alter the $\delta^{13}\text{C}$ or $\delta^{15}\text{N}$ value of a subject's venous blood with the possible exception of nitrogen isotopes for plasma with blood

additive NaF, which shows a difference of marginal significance ($p = 0.063$). After a refrigerated storage period lasting between 58 and 115 days the compositions of blood with PAR additive and unadulterated aged blood are unaffected; however, blood with NaF additive showed statistically significant differences compared with unadulterated blood in serum nitrogen ($p = 0.001$), clot nitrogen ($p = 0.039$), and clot carbon ($p = 0.082$). A full set of p -values calculated for the above discussion is presented in Table 4.

DISCUSSION

Several experiments are needed in order to prove the usefulness of the stable isotope composition of human blood as an indicator of human diet, for the eventual development of an objective bioassay for specific food types. This report represents only one necessary experiment, with some ancillary results meant to assess the practicality of blood sampling and storage. Other necessary experiments, such as feeding studies involving the consumption of known foods and subsequent blood sampling and stable isotope analysis, are underway in cooperation with the 'Test of a Unique Biomarker for the Studies of Obesity and Diabetes' (TUBSOD) project. The primary goal of these experiments was to assess the variability of blood $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values,

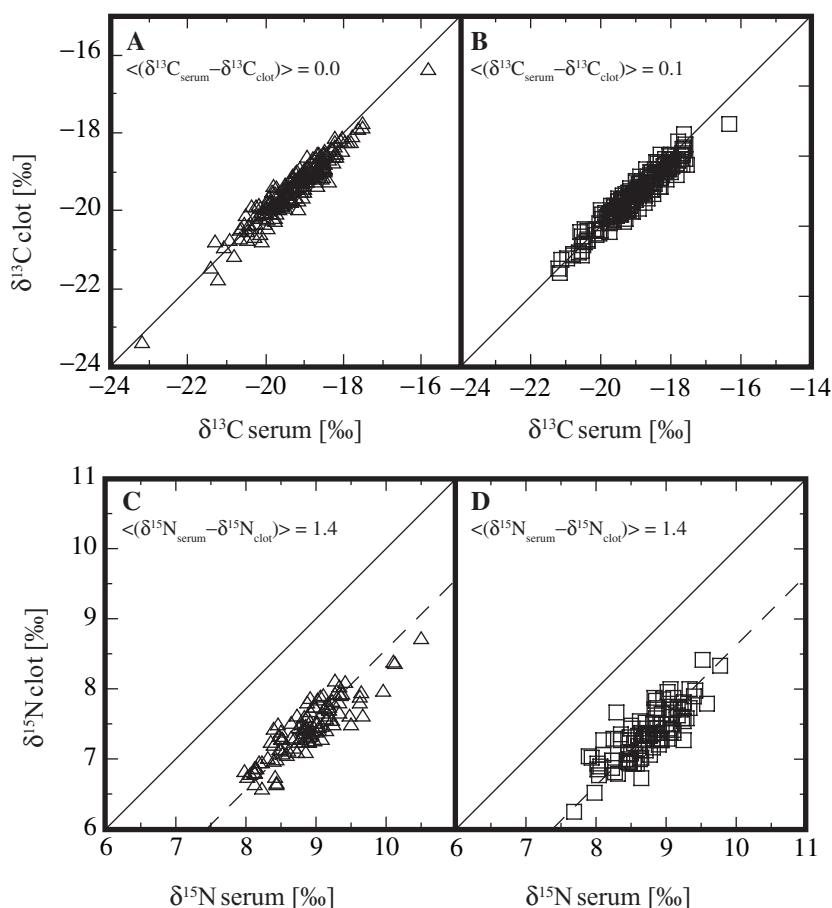


Figure 3. (A–D) $\delta^{13}\text{C}$ ($n = 406$) and $\delta^{15}\text{N}$ ($n = 206$) values of clot versus serum, as separated from venous blood; females designated by \triangle , males designated by \square . R^2 values for each regression are as follows: 0.91 (female $\delta^{13}\text{C}$); 0.92 (male $\delta^{13}\text{C}$); 0.77 (female $\delta^{15}\text{N}$); 0.65 (male $\delta^{15}\text{N}$). 1:1 lines are shown; dashed lines represent the mean offset from 1:1 line for each dataset.

Table 2. $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ samples from capillary and venous samples from 24 randomly chosen, fasting, individuals, collected between October and December, 2007. Results shown in Fig. 4

	$\delta^{13}\text{C}$			$\delta^{15}\text{N}$		
	Venous			Venous		
	Capillary	Serum	Clot	Capillary	Serum	Clot
n	24	24	24	24	24	24
Mean	-20.0	-19.7	-19.8	+7.3	+8.8	+7.1
Maximum	-18.2	-18.1	-18.2	+8.2	+9.8	+8.0
Minimum	-21.8	-21.5	-21.6	+6.6	+8.0	+6.6
SD	0.8	0.8	0.8	0.4	0.4	0.3
Range	3.6	3.4	3.4	1.6	1.9	1.4

and to evaluate whether the variability seen was wide enough to encompass a range of hypothesized diets. If such variability existed in an anonymous population with a presumably wide range in dietary habits, it would be one good sign for the possible use of blood $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values as a reflection of dietary habits.

The first step in such an analysis is to ascertain the range in $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values of foods important to the modern diet. We have pursued this using previously published compilations, as well augmenting our own report of food $\delta^{13}\text{C}$ values⁶ with $\delta^{15}\text{N}$ determinations (for a compilation of the data used, see Table S1 in the Supporting Information). Jahren *et al.*⁶ reported $\delta^{13}\text{C}$ values for approximately 100 plant-derived foods obtained from local grocery stores and highlighted that corn and cane sweeteners exhibited $\delta^{13}\text{C}$ signatures significantly higher than the $\delta^{13}\text{C}$ values associated with other vegetables, and speculated on the utility of a ^{13}C -based biomarker of sweetener consumption. Of the foods analyzed by Jahren *et al.*,⁶ 59 contained sufficient nitrogen for $\delta^{15}\text{N}$ analysis. We note that products such as pure high-fructose corn syrup (HFCS) contain no nitrogen, and so were not analyzed for $\delta^{15}\text{N}$ value; however, processed foods (e.g. cake mix) did contain sufficient nitrogen and carbon for isotopic analysis. Figure 5 shows the grouping of commonly consumed dietary items in $\delta^{15}\text{N}$ versus $\delta^{13}\text{C}$ space. Data was gained from diverse publications that reported values for commonly consumed meats,^{30,39,40} dairy products,^{30,39,40} edible plants,^{6,22,26,30} corn and corn derivatives,⁶ processed foods,^{6,39} and other animals (ferret, cat, dog,

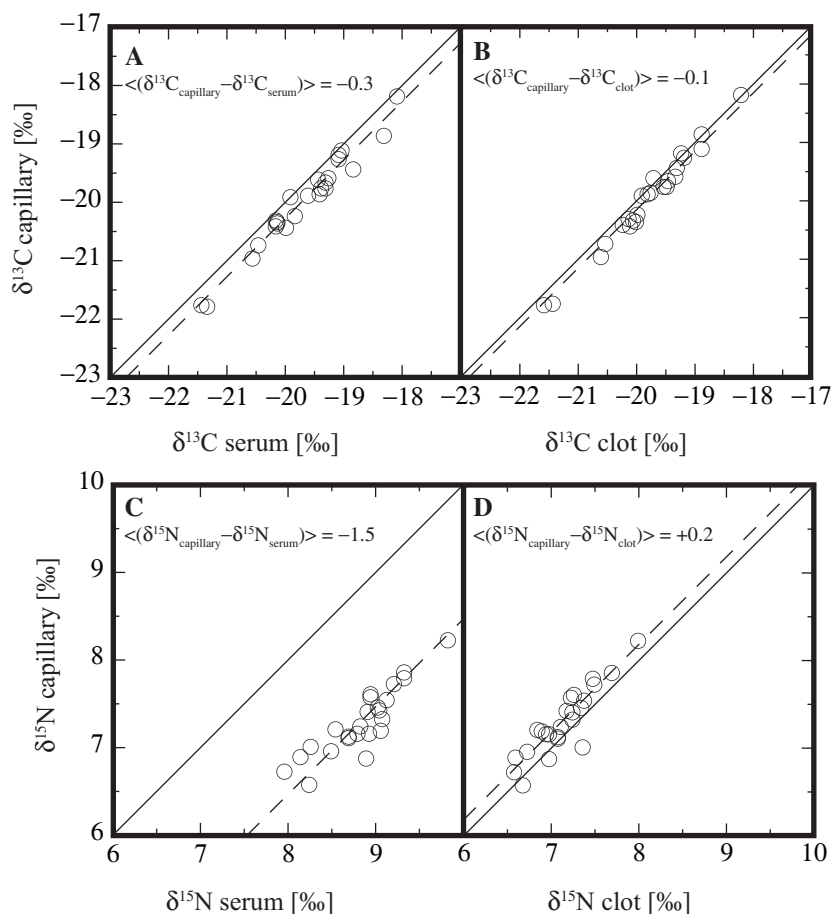


Figure 4. (A–D) $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values of capillary blood obtained via fingerstick versus clot and serum from the venous blood of the same individual ($n = 24$). R^2 values for each regression are as follows: 0.96 (serum $\delta^{13}\text{C}$); 0.98 (clot $\delta^{13}\text{C}$); 0.79 (serum $\delta^{15}\text{N}$); 0.81 (clot $\delta^{15}\text{N}$). 1:1 lines are shown; dashed lines represent the mean offset from 1:1 line for each dataset.

Table 3a. $\delta^{13}\text{C}$ values of venous blood clot and serum from 31 randomly chosen, fasting, individuals, collected between October and December, 2007

	Non additive		NaF		PAR		A/FD	
	Serum	Clot	Plasma	RBC	Serum	Clot	Serum	Clot
n	31	31	31	31	31	31	31	31
Mean	-20.1	-20.2	-20.1	-20.2	-20.1	-20.2	-20.1	-20.1
Maximum	-18.7	-18.7	-18.7	-18.8	-18.8	-18.7	-18.6	-18.6
Minimum	-21.6	-21.9	-21.7	-21.9	-21.7	-22.0	-21.6	-21.9
Standard Deviation	0.7	0.7	0.7	0.7	0.7	0.7	0.7	0.7
Range	2.9	3.2	3.0	3.0	2.9	3.3	3.0	3.3

Table 3b. $\delta^{15}\text{N}$ values of venous blood clot and serum from 31 randomly chosen, fasting, individuals, collected between October and December, 2007

	Non additive		NaF		PAR		A/FD	
	Serum	Clot	Plasma	RBC	Serum	Clot	Serum	Clot
n	31	31	31	31	31	31	31	31
Mean	+8.8	+7.2	+8.7	+7.2	+8.7	+7.2	+8.8	+7.2
Maximum	+9.3	+7.8	+9.2	+7.8	+9.2	+7.7	+9.2	+7.7
Minimum	+7.8	+5.7	+7.7	+5.9	+7.8	+5.9	+7.7	+5.8
Standard Deviation	0.3	0.4	0.3	0.4	0.3	0.4	0.3	0.4
Range	1.5	2.2	1.5	1.9	1.5	1.9	1.5	1.8

Table 4. Correlation between stable isotope values of (NaF = sodium fluoride, PAR = polymerized acrylamide resin) and laboratory process (autoclave/freeze-dry = A/FD) compared with unadulterated blood

	$\delta^{13}\text{C}$				$\delta^{15}\text{N}$			
	Day 0		Aged		Day 0		Aged	
	Serum	Clot	Serum	Clot	Serum	Clot	Serum	Clot
NaF	EQ ^a	EQ	EQ	0.082 ^c	0.063	EQ	0.001	0.039
PAR	EQ	EQ	EQ	0.134	0.101	EQ	EQ	EQ
A/FD	EQ	0.435	n.d. ^b	n.d.	EQ	EQ	n.d.	n.d.
Non-additive	n.d.	n.d.	EQ	0.465	n.d.	n.d.	0.112	EQ

^aEQ = equivalent when compared with unadulterated blood within 0.05‰, therefore no *p*-value is calculated.

^bn.d. = not determined. A/FD blood was not measured after aging as this process is assumed to halt biological processes. Non-additive blood was not measured on day 0; it represents a split of unadulterated blood and therefore is assumed to be of the same composition. ^cNumbers in the table represent a calculation of the *p*-value for comparison with unadulterated blood.

cow, goat, horse, rabbit, hare, mouse).^{22,39,40} Note that we necessarily limited Fig. 5 to contain only items for which one study reports both $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values. A great number of additional studies exist which report only $\delta^{13}\text{C}$ values,^{8,11,13,14,18,33,34,40,41} the range of $\delta^{13}\text{C}$ values reported in such studies is in keeping with the data depicted in Fig. 5.

Each food represented in Fig. 5 is detailed within Table S1 (see Supporting Information), which also lists carbon and nitrogen isotope values. In total, values from 141 foods/animal tissues were compiled, and several trends emerged upon examination of the $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values. With respect to carbon, the majority of edible plants fell between $\delta^{13}\text{C} = -28\text{‰}$ and -24‰ . Corn and corn derivatives formed an exception to this, exhibiting much higher values: $\delta^{13}\text{C} = -11\text{‰}$ to -14‰ . Meat and animal products (e.g. dairy) spanned this range ($\delta^{13}\text{C} = -27\text{‰}$ to -10‰), reflecting the known variation in plant-type diet among herbivores (e.g. alfalfa, grass, corn feed).⁴² Processed foods followed similar patterns to the edible plants, falling within two ranges: $\delta^{13}\text{C} = -24\text{‰}$ to -20‰ (wheat-dominated isotopic signature) and $\delta^{13}\text{C} = -11\text{‰}$ (corn-dominated isotopic signature). With respect to nitrogen, edible plants exhibited lower $\delta^{15}\text{N}$ values (-3‰ to $+3\text{‰}$) than meat and animal products ($+3\text{‰}$ to $+13\text{‰}$) in agreement with previous observations of ^{15}N enrichment with elevated position in the food chain.⁴³ As expected, given their wide range of ingredients (e.g. flour from different grains, dairy products, carrageenan, gelatin, etc.), the $\delta^{15}\text{N}$ values of processed foods ranged from -1‰ to $+6\text{‰}$. Based on the data compiled here, the mean \pm standard deviation isotopic values of edible plants, meat and animal products, corn and processed foods, occupy distinct ranges in $\delta^{15}\text{N}$ versus $\delta^{13}\text{C}$ space (Fig. 5).

Many researchers have contributed data to the study of the relationship between the stable isotope composition of human tissue and the diet of the individual sampled.^{20–23,30–32,39,44,45} This technique has been applied within the context of

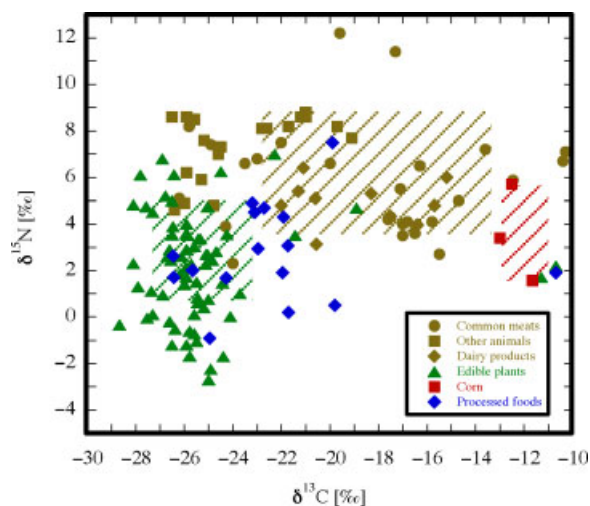


Figure 5. A compilation of $\delta^{15}\text{N}$ versus $\delta^{13}\text{C}$ values for commonly consumed meats,^{30,39,40} dairy,^{30,39,40} other animals,^{22,39,40} edible plants,^{6,22,26,30} corn and corn derivatives,⁶ and processed foods.^{6,39} Shaded rectangle portions represent mean values \pm standard deviations for each group of food represented on the graph.

archaeological studies, to assess diet and contextualize social status.^{23,44,46–48} Because archaeological specimens primarily consist of bone (including collagen) and occasionally hair, these human tissues have been considered premium substrates for isotopic work. We have chosen to focus upon the body's reservoir of blood as a potential substrate for modern isotopic dietary biomarkers that could serve multiple assays in tandem. However, other researchers have stressed the simplicity, storage potential, and essentially non-invasive sampling of hair^{20,21,23,30,31,39,40,49} and fingernail^{22,49} (for a compilation of modern tissue from humans and other mammals, see Table S2 in the Supporting Information). Petzke^{30,31} presented data from 99 individuals self-reported as omnivores (individuals consuming meat,

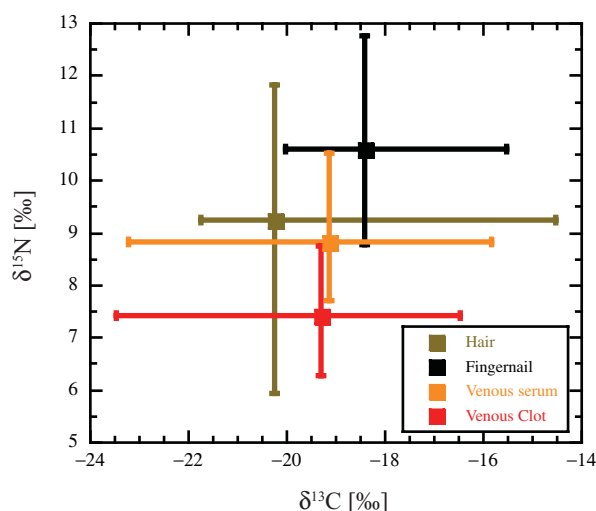


Figure 6. A compilation of mean and total range in $\delta^{15}\text{N}$ versus $\delta^{13}\text{C}$ values for human hair,^{20,21,23,30,31,39,40} fingernail,²² blood serum and clot.³² Only data from modern tissues is included.

animal products, and edible plants), 15 ovo-lacto vegetarians (individuals consuming dairy products and edible plants), and 6 vegans (individuals consuming edible plants). According to the observations of self-reported intake of Petzke *et al.*, hair from humans with self-reported omnivorous diets on average occupy an isotopic space of $\delta^{13}\text{C} = -19.6 \pm 0.4\text{‰}$ and $\delta^{15}\text{N} = +9.9 \pm 0.6\text{‰}$; ovo-lacto vegetarians occupy an isotopic space of $\delta^{13}\text{C} = -20.2 \pm 0.3\text{‰}$ and $\delta^{15}\text{N} = +7.7 \pm 0.5\text{‰}$; and vegans occupy an isotopic space of $\delta^{13}\text{C} = -20.9 \pm 0.3\text{‰}$ and $\delta^{15}\text{N} = +6.2 \pm 0.4\text{‰}$.

In order to examine the isotopic composition of the tissues mentioned above (i.e. hair, blood components, and fingernail) in relation to blood, our preferred substrate, we compiled reported data for the carbon and nitrogen isotopic signatures for hair,^{20,21,23,30,31,39,40} fingernails,²² blood clot,³² and serum (Fig. 6). Although isotopic data for bone collagen⁴⁴ also exists, we chose to limit the tissues in the figure to modern human specimens. We wish to stress that these data do not comprise different tissues taken from the same individual, but rather a comparison of ranges found for each tissue, sampled in the context of several different studies. The total spread of values encompassed by all the data is large: $\delta^{13}\text{C}$ ranges from -23‰ to -15‰ and $\delta^{15}\text{N}$ ranges from $+6\text{‰}$ to $+13\text{‰}$. Note also that the mean and range for hair data most closely overlap with those for blood serum in Fig. 6; therefore, we compare the ranges observed by Petzke *et al.*³⁰ of dietary habit with our dataset of blood serum $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values in Fig. 7.

The observations of Petzke *et al.*³⁰ provide a useful context because they compare self-reporting of dietary habits directly with measured isotopic ranges in humans, and assigned ranges in isotopic value with dietary regime. As detailed above, we have no information beyond gender for the 406 individuals who donated blood serum for the analyses shown in Fig. 7. We presume that these donors visited the Johns Hopkins Medical Institutions for a diverse set of medical complaints, and had a concomitantly diverse set of dietary habits. If we compare the $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values of the blood serum from these donors (Table 1) with the quantitative ranges set forth by Petzke *et al.*³⁰ for the identification of dietary habit, we see that the $\delta^{15}\text{N}$ values of serum span the range of ovo-lacto vegetarians to omnivores. This distribution is similar to the distribution of dietary habits among self-reporting US adults; approximately 0.5% of the population is vegan and approximately 1% of the population is vegetarian, leaving approximately 98.5% of the population as omnivorous.⁵⁰ Because we do not have dietary information for any of these donors, we can only offer conjecture about the relatively heavy carbon isotope values shown in the anonymous population sampled. Earlier we emphasized the uniquely high $\delta^{13}\text{C}$ value of corn (-10‰ to -14‰ ; Fig. 5), which is enriched in ^{13}C relative to other plants via the isotopic selectivity inherent to C4 photosynthesis, a relatively recent evolutionary innovation. Until further studies, including controlled feeding in combination with blood draw, are performed that quantitatively link the $\delta^{13}\text{C}$ value of diet to the $\delta^{13}\text{C}$ value of human blood, our suggestion that corn and corn derivatives are evident in the isotopic signature of blood serum shown in Fig. 7 remains a speculation.

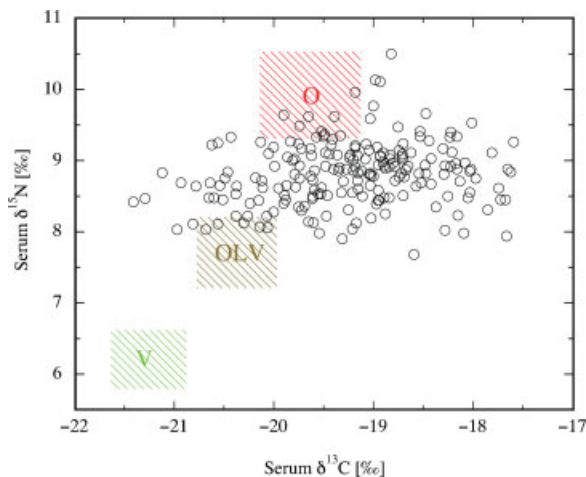


Figure 7. $\delta^{15}\text{N}$ versus $\delta^{13}\text{C}$ for blood serum from an anonymous population of 206 individuals (Table 1). Dietary ranges observed for omnivores (O), ovo-lacto vegetarians (OLV), and vegans (V) after Petzke *et al.*³⁰ A small number of data points are excluded because they fall outside of the range of this graph. This figure is available in colour online at www.interscience.wiley.com/journal/rcm.

In the event that further experiments prove that stable isotope signatures in blood provide a useful bioassay for diet, we have answered several practical questions relevant to the implementation of routine $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ analysis of blood. First, our work with venous blood showed small, but statistically significant carbon and nitrogen isotopic differences between males and females. If an isotopic bioassay is applied to unpreserved blood, we have revealed important considerations regarding the use of clotted versus unclotted substrate (i.e. serum) for analysis. Our carbon isotope analyses showed small, but statistically significant differences between serum and clot components (Figs. 1 and 3); in contrast, the $\delta^{15}\text{N}$ value of serum is enriched by a mean value of 1.4‰ compared with clot from the same blood sample (Figs. 3(C) and 3(D)). This difference may arise from the difference in rates of reconstitution in different blood components, and it is possible that serum would reflect shorter-term dietary inputs. We suggest that different blood components may be useful in tracking different dietary components; for example, blood clot may better reflect dietary protein intake, while serum may better reflect dietary carbohydrate intake.

Based on the results of our capillary blood study (Fig. 4), we see that the $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values of capillary blood most closely reflect those of blood clot. In the event that blood serum is used as the preferred isotopic substrate, it may be possible to assume the composition of patient serum, by applying a $+1.5\text{‰}$ correction to a capillary blood sample $\delta^{15}\text{N}$ value (Fig. 4(C)). Our work to assess the possibility of isotopic contamination by common preservatives yielded positive results: the addition of PAR was shown not to affect the $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ composition of the blood to which it has been added. This remains true over several months of storage. In contrast, the addition of NaF, showed a small, but marginally statistically difference on Day 0, and statistically

significant differences after several months of storage. This is particularly important because it suggests that archived blood, or blood drawn for other purposes, can be a useful isotopic substrate, in spite of age, storage, and addition of PAR. However, blood with NaF additive may not represent the most ideal substrate for isotopic work, particularly if it has been aged. Finally, we found no isotopic difference between autoclaved/freeze-dried blood and fresh blood, which allows us to freely sterilize blood via the autoclaving process, therefore making it safer to handle. Freeze drying has the potential to extend the storage of the blood and preservation of the isotopic composition indefinitely.

SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article.

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