The Carbon Isotope Ratio of Alanine in Red Blood Cells Is a New Candidate Biomarker of Sugar-Sweetened Beverage Intake1,2

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Abstract

An objective dietary biomarker would help clarify the contribution of sugar-sweetened beverage (SSB) intake to obesity and chronic disease risk. Previous studies have proposed the carbon isotope ratio ($d^{13}C$) as a biomarker of SSB intake but found associations that were of modest size and confounded by other components of the diet. We investigated whether the $d^{13}C$ values of nonessential amino acids ($d^{13}C_{NEAA}$) in RBCs could provide valid biomarkers that are more specific to SSBs. We assessed the associations of RBC $d^{13}C_{NEAA}$ with SSB intake in a study population of 68 Yup’ik people, using gas chromatography/combustion/isotope ratio mass spectrometry to measure $d^{13}C_{NEAA}$ and four 24-h dietary recalls to assess intake. Among RBC nonessential amino acids, alanine $d^{13}C$ ($d^{13}C_{alanine}$) was strongly correlated with intake of SSBs, added sugar, and total sugar ($r = 0.70, 0.59$, and 0.57, respectively; $P < 0.0001$) but uncorrelated with other dietary sources of elevated $d^{13}C$. We also evaluated whether sweetener intake could be noninvasively assessed using hair $d^{13}C_{alanine}$ in a subset of the study population ($n = 30$). Hair $d^{13}C_{alanine}$ was correlated with RBC $d^{13}C_{alanine}$ ($r = 0.65; P < 0.0001$) and showed similar associations with SSB intake. These results show that $d^{13}C_{alanine}$ in RBCs provides a valid and specific biomarker of SSB intake for the Yup’ik population and suggest RBCs and hair $d^{13}C_{alanine}$ as candidate biomarkers of SSB intake for validation in the general U.S. population. Ultimately, these biomarkers could clarify our understanding of whether and how SSB intake contributes to chronic disease.

Introduction

The consumption of sugar-sweetened beverages (SSBs),6 including sodas, sports drinks, sweetened tea, and other sweetened beverages, has increased over the past 30 y in the United States (1,2). SSB intake has been linked to obesity (3–5) and type 2 diabetes (6), although empirical support for these relationships is mixed (7–9). Associations between self-reported SSB consumption and disease are attenuated by measurement error (10,11). Associations between serum and whole blood $d^{13}C$ of dietary protein sources. Our group recently showed that $d^{13}C$ of dietary protein in RBCs can dramatically improve the validity for sweeteners, it does not dramatically improve the validity for sweeteners, it does not

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3 Abbreviations used: DCM, dichloromethane; NDSR, Nutrition Data System for Research; NEAA, nonessential amino acid; SSB, sugar-sweetened beverage; 24HR, 24-h recall dietary interview; $d^{13}C$, carbon isotope ratio; $d^{13}C_{alanine}$, alanine carbon isotope ratio; $d^{13}C_{NEAA}$, nonessential amino acid carbon isotope ratio.

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control for all dietary confounders, including commercial (corn-fed) meat intake.

Another approach to improving the specificity of δ13C for sweeteners is to measure the δ13C of molecules that favor the incorporation of glucose carbon, thus reducing or eliminating the influence of dietary confounders (26). For example, the nonessential (dispensable) amino acids (NEAAs) in protein have the potential to be synthesized from glucose carbon, whereas the essential (indispensable) amino acids do not (27,28). When an NEAA is synthesized from glucose, its δ13C should reflect the δ13C of recent corn- and cane-based sweetener intake (26). In humans, glucose can significantly contribute to synthesis of NEAAs, particularly alanine (29,30). Here, we propose that measurements of δ13C of individual NEAAs (δ13CNEAA) may provide more specific biomarkers of usual SSB intake than whole-protein δ13C.

The aim of this research is to identify whether δ13CNEAA can provide valid and specific biomarkers of SSB and sugar intake. We evaluate these biomarkers in a Yupik study population from Southwest Alaska, with whom we have an ongoing research relationship focused on diet and chronic disease risk. We evaluated the relationships between δ13CNEAA and self-reported intake of SSBs, added sugar, and total sugar as well as other foods having elevated δ13C: commercial (corn-fed) meats, corn products, and marine foods (fish and marine mammals), which have elevated δ13C from marine bicarbonate (25,31). Intake measures were based on the mean of four 24-h recall dietary interviews (24HRs). As a preliminary assessment of whether hair could provide a less-invasive alternative to RBCs, we also assessed associations among hair δ13CNEAA, RBC δ13CNEAA, and SSB intake in a subset of the study population.

**Participants and Methods**

**Recruitment and procedures.** Data are from the Center for Alaska Native Health Research Nogem Nalluunaitukta (“The Foods’ Marker”) study. This study was approved by the University of Alaska Fairbanks Institutional Review Board and the Yukon-Kuskokwim Health Corporation Human Studies Committee.

In 2008–2009, 68 participants aged 14–79 y were recruited from 2 coastal Yupik communities in Southwest Alaska. All participants completed a demographic questionnaire at enrollment and the first of four 24HRs. Three more dietary interviews were conducted over the next 4 wk, as described elsewhere (25). At ~2 wk after the last dietary interview, fasting blood samples, hair samples, and basic anthropometric measurements were collected. Specimen collection was timed so that the mean age of RBCs (1.5 mo) would coincide with the midpoint of the period over which dietary interviews were conducted (32). Hair samples were collected only from participants with hair length >2 cm and had limited availability following previous analyses. Therefore, the sample size for hair analyses was 30.

**Specimen collection.** Blood was collected into 10-mL EDTA tubes (15% solution, 0.117 mL, 17.55 mg) and centrifuged in the field. Samples were transported to the University of Alaska Fairbanks at −15°C and transferred to long-term storage at −80°C.

A sample of ~50 hairs/participant was clipped close to the scalp with scissors, just inferior to the post-occipital protuberance, and the proximal end of the section was marked with tape. Hair sections were placed into strips of aluminum foil, folded, and stored at room temperature. This study used the section of hair from 2–3 cm from the scalp, as the section from 0–2 cm from the scalp was used for a previous study. Hair was cleaned of tape and other residues with 2 successive 30-min washes with sonication in chloroform/methanol (2:1) and a final 30-min wash with sonication in distilled water (33).

**Amino acid extraction and derivatization.** We extracted amino acids from RBCs and hair samples using protocols described by Popp et al. (34). RBCs (50 μg aliquots) and hair (~1–1.5 mg) were hydrolyzed with 1 mL 6 M HCl (110°C, 20 h). Standard mixtures of 12 amino acids were prepared alongside samples (Sigma-Aldrich Chemie; Fluka Chemie); these underwent all steps including hydrolysis. To eliminate lipids from RBC samples, 2 mL n-hexane/dichloromethane (DCM) (6:5, v:v) was added to the hydrolysates and the lipid phase was removed. The solution was purified by filtration (0.22 μm Millex-GP, Millipore) followed by a rinse with 1 mL 0.01 N HCl (35). Hydrolysates from hair were not lipid-extracted. Norleucine was added to each sample as an internal standard. The hydrolysates were evaporated to dryness at 110°C in a heating block under a gentle stream of N2 for 30 min. After evaporation, samples were stored at 4°C for <2 wk.

Standard amino acid mixtures and isolated RBC and hair amino acids were derivatized to N-trifluoroacetic anhydride isopropyl esters as described elsewhere (36,37). Each sample was propylated in a 0.8-mL solution of acidified 2-propanol (isopropanol:acetyl chloride, 4:1) for 1 h at 110°C, then dried under nitrogen. Trifluoroacetylation was performed by adding 1 mL DCM:N-trifluoroacetic anhydride (1:1, v:v, 10 min at 110°C). Following each reaction, reagents were evaporated under nitrogen and samples underwent successive washes and evaporation of DCM (2 × 250 μL). The samples were transferred to sealed vials in 500 μL DCM.

**Analysis of amino acid δ13C.** δ13CNEAA were analyzed by GC combustion/isotope ratio MS, using a Thermo Trace gas chromatograph, the GC-Isolink combustion interface, and a Delta-V isotope ratio mass spectrometer (Thermo Fisher Scientific) at the Alaska Stable Isotope Facility (Fairbanks, Alaska). The conventional method of expressing δ13C at natural abundance is in permil [‰] abundance of δ13C relative to an international standard (Vienna PeeDee Belemnite,13C/12C = 0.01123), as follows:

\[
\Delta^{13}C = ((^{13}C/^{12}C_{\text{sample}} - ^{13}C/^{12}C_{\text{standard}})) / ^{12}C_{\text{standard}} \times 1000 \%
\]

Thus, a reported difference of 1‰ equals a difference in 13C/12Csample of 1/100,000, requiring isotope ratio MS or similarly precise techniques to measure. Because the standard contains more δ13C than the samples in this study, all δ13C values reported here are negative.

Approximately 2 μg of amino acids (via 1-μL injection) was injected on column in splitless mode at 75°C and separated on a 50-m HP Ultra-1 column (0.32-mm i.d., 0.25-μm film thickness). Amino acids were separated by GC, oxidized completely to CO2, and introduced into the isotope ratio mass spectrometer in a continuous stream of helium. Samples were analyzed in duplicate along with amino acid standards of known isotopic composition and each measured mean amino acid δ13C was corrected relative to the mean amino acid δ13C of standards to account for the exogenous carbon and kinetic fractionation introduced during derivatization (36,37). Analytical errors (SDs) in measuring the derivatized amino acids ranged from 0.1 to 0.9‰, with a mean of ±0.2‰. Errors of corrected δ13C for each amino acid ranged from 0.1 to 1.5‰, with a mean of ±0.5‰.

We obtained δ13C values from 12 amino acids from each RBC sample and 13 amino acids from each hair sample. In this paper, we present data from 6 NEAAs that have the potential to be derived from sugar: alanine, glycine, serine, proline, aspartate/asparagine, and glutamate/glutamine. Asparagine and glutamine are converted to aspartate and glutamate, respectively, during acid hydrolysis; therefore, these amino acids are indistinguishable.

**Assessment of dietary intake.** 24HRs were collected from each participant by certified interviewers using computer-assisted software [Nutrition Data System for Research (NDSR) software 2008; University of Minnesota, Minneapolis, MN]. The majority of interviews were completed in person (93%, n = 261); however, some participants completed either 1 (n = 15) or 2 (n = 2) interviews over the telephone. Participants were asked to recall all food and beverages consumed the day prior to the interview using a multiple pass approach, which included a quick list, forgotten foods probe, detail cycle, and final probe. Interviewers used portion estimation tools, including measuring cups, rulers, bowls, mugs, drinking glasses, and food models. Participants completing phone recalls were given tool packs with a ruler, measuring
in the study, sweeter intake is measured in 3 ways: as SSBs, added sugar, and total sugar. SSB intake was calculated as the sum of servings of sweetened soft drinks and sweetened fruit drinks [servings/d, 237 mL (8 fl oz/serving)]. Added sugar (g/d) is defined as the sum of sugars and syrups added to foods during food preparation or commercial food processing. Total sugar intake (g/d) is defined as the sum of mono- and disaccharides consumed, and included primarily sucrose, fructose, and glucose.

We also present data on intake of other food items having elevated $\delta^{13}$C in this study population, including commercial meats (percent energy), fish and marine mammals (percent energy), and corn products (g/d) (31), because intake of these foods may also affect $\delta^{13}$CNEAA. Commercial meats were defined as meat products purchased from local stores and included poultry, eggs, pork, and beef products. Fish and marine mammals were harvested from the local environment and included both freshwater and marine fish species. Corn products included whole corn and foods made primarily from whole corn, including popcorn, corn cereal, corn chips, and corn tortillas (25).

To describe the diet pattern of the study population more generally, we present total energy intake (kcal), traditional and commercial intake (percent energy), and macronutrient intake (percent energy). Traditional and commercial intake includes all foods harvested from the local environment and purchased from stores, respectively. Access to commercial foods is limited, because the communities participating in this study are off the road system and must be reached by small plane. Each community has 2–3 small stores where limited commercial foods can be purchased, particularly nonperishable or frozen foods, and no restaurants. Traditional foods include fish, marine mammals, moose, caribou, waterfowl, and local greens and berries, with fish and marine mammals contributing $>70\%$ of traditional food energy (39). Major sources of commercial food energy include soda, fruit drinks, commercial meat, crackers, pasta, rice, and vegetable shortening (40).

Statistical analyses. All statistical analyses were performed using JMP version 8 (SAS Institute). The following dietary intake variables were log-transformed for analyses: SSBs (servings/d + 1), added sugar (g/d), and corn products (g/d + 1). Differences in age, sex, and BMI between the complete study sample (total = 68) and the subsample with hair analyses (n = 30) were assessed using Student’s t and $\chi^2$ tests. Associations of dietary intake variables with SSB intake were assessed using Pearson’s product moment correlations, as were associations of SSB intake with $\delta^{13}$CNEAA. Further analyses focused on the $\delta^{13}$C of alanine ($\delta^{13}$Calanine), because only $\delta^{13}$Calanine was strongly associated with SSB, added sugar, and total sugar intake. We used multiple regression models to examine intake of sugar (as SSBs, added sugar, or total sugar), commercial meat, fish and marine mammals, and corn products affected $\delta^{13}$Calanine independently. In these models, $\delta^{13}$Calanine was the dependent variable. We then assessed the ability of $\delta^{13}$Calanine to predict SSB intake with a linear regression model, in which SSB was the dependent variable. To test whether associations of $\delta^{13}$Calanine with SSBs differed by sex or BMI, we fit multiple regression models to examine whether intake of sugar (as SSBs, added sugar, or total sugar) is associated with SSB intake, but not with added or total sugar. There were no associations between the $\delta^{13}$C of glutamate/glutamine, aspartate/asparagine, serine, or glycine and intake of SSBs, added sugar, or total sugar. The associations between $\delta^{13}$Calanine and SSBs, added sugar, and total sugar could be confounded by intake of other foods with elevated $\delta^{13}$C: commercial meat, fish and marine mammals, and corn products (25,31). We tested the specificity of $\delta^{13}$Calanine for SSB intake using a linear model in which the dependent variable was $\delta^{13}$Calanine and the independent variables were SSB, commercial meat, fish and marine mammals, and corn product intake (Table 4). The only significant association was between $\delta^{13}$Calanine and SSB intake. We repeated this analysis using added sugar and total sugar, and again, only the sugar intake variables predicted $\delta^{13}$Calanine (data not shown).

In a linear, predictive model, $\delta^{13}$Calanine explained 49% of the variation in SSB intake (Fig. 1; Table 5). Self-reported sugar intake may be differentially biased by sex and BMI; however, regression coefficients were similar and there were no significant differences across sex and BMI groups (Table 5).

**TABLE 1** Age, sex, and BMI distribution for the complete study sample and the subset of participants with hair samples1,2

<table>
<thead>
<tr>
<th>Sex, % female</th>
<th>Complete sample, n = 68</th>
<th>Hair subset, n = 30</th>
</tr>
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<tbody>
<tr>
<td></td>
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<tr>
<td>Sex, % female</td>
<td></td>
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<tr>
<td>14–19</td>
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<tr>
<td>20–39</td>
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<tr>
<td>40–59</td>
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<tr>
<td>≥60</td>
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<td></td>
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<tr>
<td>Age, y, %</td>
<td></td>
<td></td>
</tr>
<tr>
<td>14–19</td>
<td>49</td>
<td>27.2 ± 6.3</td>
</tr>
<tr>
<td>20–39</td>
<td>32</td>
<td>27.2 ± 6.3</td>
</tr>
<tr>
<td>40–59</td>
<td>40</td>
<td>26.7 ± 6.5</td>
</tr>
<tr>
<td>≥60</td>
<td>12</td>
<td>27.2 ± 6.3</td>
</tr>
<tr>
<td>BMI, kg/m², %</td>
<td>27.2 ± 6.3</td>
<td>27.2 ± 6.5</td>
</tr>
<tr>
<td>&lt;18.5</td>
<td>1</td>
<td>27.2 ± 6.3</td>
</tr>
<tr>
<td>≥18.5 and &lt;25</td>
<td>44</td>
<td>27.2 ± 6.5</td>
</tr>
<tr>
<td>≥25 and &lt;30</td>
<td>24</td>
<td>27.2 ± 6.5</td>
</tr>
<tr>
<td>≥30</td>
<td>31</td>
<td>27.2 ± 6.5</td>
</tr>
</tbody>
</table>

1 Values are means ± SDs or percent of study sample. *Different from the complete sample, $P < 0.05$.
2 Differences in means between the study samples were assessed with t tests and differences in distribution between the study samples were assessed with chi-square tests.

Results

The age, sex, and BMI distribution of the total study population (n = 68) and the subset of the population with hair samples (n = 30) are presented in Table 1. The distribution of age and BMI did not differ between the total study population and the subset with hair samples. A larger proportion of the subset were women, because many men had hair that was either too short to sample or was fully used in prior analyses.

The dietary characteristics of the study population and their associations with SSBs, added sugar, and total sugar intake are presented in Table 2. SSB, added sugar, and total sugar intakes were positively associated with total commercial food intake and percent energy from carbohydrates (Table 2). SSB, added sugar, and total sugar intakes were negatively associated with total traditional intake, percent energy from dietary protein and fat, and fish and marine mammal intake. Intakes of added and total sugar, but not SSBs, were associated with total energy intake and corn product intake. None of the sugar intake variables were associated with percent energy from commercial meats. All sugar intake variables were strongly correlated.

The associations of RBC $\delta^{13}$CNEAA with intake of SSBs, added sugar, and total sugar are presented in Table 3. Only $\delta^{13}$CNEAA was strongly associated with intake of SSBs, added sugar, and total sugar. The $\delta^{13}$C of proline was moderately associated with SSB intake, but not with added or total sugar. There were no associations between the $\delta^{13}$C of glutamate/glutamine, aspartate/asparagine, serine, or glycine and intake of SSBs, added sugar, or total sugar.

The associations between $\delta^{13}$Calanine and SSBs, added sugar, and total sugar could be confounded by intake of other foods with elevated $\delta^{13}$C: commercial meat, fish and marine mammals, and corn products (25,31). We tested the specificity of $\delta^{13}$Calanine for SSB intake using a linear model in which the dependent variable was $\delta^{13}$Calanine and the independent variables were SSB, commercial meat, fish and marine mammals, and corn product intake (Table 4). The only significant association was between $\delta^{13}$Calanine and SSB intake. We repeated this analysis using added sugar and total sugar, and again, only the sugar intake variables predicted $\delta^{13}$Calanine (data not shown).

In a linear, predictive model, $\delta^{13}$Calanine explained 49% of the variation in SSB intake (Fig. 1; Table 5). Self-reported sugar intake may be differentially biased by sex and BMI; however, regression coefficients were similar and there were no significant differences across sex and BMI groups (Table 5).
The correlation between RBC and hair $^{13}$Calanine was $r = 0.65$ ($P < 0.0001$) and the measures generally showed good agreement, with a mean difference of $0.1 \pm 1.5\%$. Agreement was poor for 6 of the samples, with differences from 2.2 to 3.4\%. Figure 2 shows the linear associations between SSB intake and $^{13}$Calanine in RBCs and hair for the subset of participants with both RBC and hair samples ($n = 30$). The slope of the linear relationship with SSB intake did not significantly differ for RBC and hair $^{13}$Calanine; however, the $R^2$ was higher for RBC $^{13}$Calanine (0.50) than for hair $^{13}$Calanine (0.40).

### Discussion

In this Yup'ik study population, we found that $^{13}$Calanine, but not other NEAAs, was strongly correlated with intakes of SSB, added sugar, and total sugar. Furthermore, $^{13}$Calanine was specific to sugar intake, as it was not associated with intake of other foods having elevated $^{13}$C, including corn products, commercial meat, or fish and marine mammals. $^{13}$Calanine predicted 49% of the variation in SSB intake, with each 1% increase in $^{13}$Calanine associated with a 23% increase in SSB intake. The relationship of $^{13}$Calanine with SSBs was consistent across sex and BMI classes, suggesting that SSB intake was not selectively under-reported in these groups. Finally, preliminary data suggested that hair $^{13}$Calanine, a noninvasive measure, had a predictive relationship for SSB that was very similar to RBC $^{13}$Calanine, although the $R^2$ was higher for RBC $^{13}$Calanine. These results show that $^{13}$Calanine is a good biomarker for SSB and sugar intake in Yup'ik people and has promise as a new biomarker of sugar intake for the general population.

This study follows a recent publication from our group presenting a dual-isotope model of sweetener intake based on RBC $^{13}$C and $^{15}$N (25). In both cases, the isotopic measures explained a similar percentage of variation in sweetener intake: 49% of the variation in SSB intake for $^{13}$Calanine and 48% of the variation in total sugar intake for the dual-isotope model (25). However, there were also differences in the performance of the 2 measures: $^{13}$Calanine was not associated with any potential dietary confounders, including commercial meat and fish intake, whereas the dual-isotope model was associated with commercial meat intake as well as sweeteners. Measuring the $^{13}$C of specific amino acids like alanine is significantly more time-consuming and analytically challenging than measuring $^{13}$C and $^{15}$N in whole tissue samples. We expect that $^{13}$Calanine will be most useful as a calibration tool, either for self-report data or more high-throughput biomarkers of sweetener intake. Importantly, both isotopic approaches to measuring sweetener intake require

### Table 2

<table>
<thead>
<tr>
<th>Dietary intake variables</th>
<th>Intake values</th>
<th>SSB (r)</th>
<th>Added sugar (r)</th>
<th>Total sugar (r)</th>
</tr>
</thead>
<tbody>
<tr>
<td>General</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total energy intake, kcal/d</td>
<td>2055 ± 691</td>
<td>0.14</td>
<td>0.36**</td>
<td>0.49***</td>
</tr>
<tr>
<td>Total commercial intake, % energy</td>
<td>78 ± 20</td>
<td>0.54***</td>
<td>0.54***</td>
<td>0.65***</td>
</tr>
<tr>
<td>Total traditional intake, % energy</td>
<td>22 ± 20</td>
<td>-0.54***</td>
<td>-0.54***</td>
<td>-0.65***</td>
</tr>
<tr>
<td>Macronutrients</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protein, % energy</td>
<td>18 ± 6</td>
<td>-0.61***</td>
<td>-0.66***</td>
<td>-0.68***</td>
</tr>
<tr>
<td>Fat, % energy</td>
<td>38 ± 9</td>
<td>-0.56***</td>
<td>-0.48***</td>
<td>-0.49***</td>
</tr>
<tr>
<td>Carbohydrate, % energy</td>
<td>44 ± 14</td>
<td>0.66**</td>
<td>0.64**</td>
<td>0.66**</td>
</tr>
<tr>
<td>Sweeteners</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SSB intake, servings/d</td>
<td>1.4 (1.1, 1.8)</td>
<td>—</td>
<td>0.78***</td>
<td>0.75***</td>
</tr>
<tr>
<td>Added sugars, g/d</td>
<td>74 (62, 87)</td>
<td>0.78***</td>
<td>—</td>
<td>0.92***</td>
</tr>
<tr>
<td>Total sugars, g/d</td>
<td>89 (77, 104)</td>
<td>0.75***</td>
<td>0.92***</td>
<td>—</td>
</tr>
<tr>
<td>Other foods with elevated $^{13}$C</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Commercial meat, % energy</td>
<td>11 ± 8</td>
<td>0.13</td>
<td>0.05</td>
<td>0.23</td>
</tr>
<tr>
<td>Fish and marine mammals, % energy</td>
<td>18 ± 18</td>
<td>-0.48***</td>
<td>-0.44**</td>
<td>-0.56**</td>
</tr>
<tr>
<td>Corn products, g/d</td>
<td>11 (8, 16)</td>
<td>0.22</td>
<td>0.25**</td>
<td>0.36**</td>
</tr>
</tbody>
</table>

1 Values are means ± SDs or geometric mean (95% CI) for log-transformed variables and $r$, assessed with Pearson’s product moment correlation, $n = 68$. *$P < 0.05$, **$P < 0.005$, ***$P < 0.0001$. SSB, sugar-sweetened beverage; $^{13}$C, carbon isotope ratio.

2 Variables were log-transformed for analysis.

### Table 3

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>$^{13}$C</th>
<th>Associations with intake (r)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>SSB</td>
<td>Added sugar</td>
<td>Total sugar</td>
</tr>
<tr>
<td>Alanine</td>
<td>-16.9 ± 1.8 (−20.6, −12.4)</td>
<td>0.70***</td>
<td>0.59***</td>
<td>0.57***</td>
</tr>
<tr>
<td>Aspartate/asparagine</td>
<td>-18.3 ± 2.3 (−25.5, −14.9)</td>
<td>0.23</td>
<td>0.15</td>
<td>0.15</td>
</tr>
<tr>
<td>Glutamate/glutamine</td>
<td>-16.8 ± 2.4 (−23.4, −12.7)</td>
<td>0.17</td>
<td>0.10</td>
<td>0.12</td>
</tr>
<tr>
<td>Glycine</td>
<td>-8.8 ± 1.5 (−12.3, −5.6)</td>
<td>0.05</td>
<td>-0.07</td>
<td>-0.13</td>
</tr>
<tr>
<td>Proline</td>
<td>-16.1 ± 1.4 (−19.0, −13.3)</td>
<td>0.31*</td>
<td>0.14</td>
<td>0.10</td>
</tr>
<tr>
<td>Serine</td>
<td>-8.8 ± 3.4 (−15.1, −2.0)</td>
<td>0.04</td>
<td>0.07</td>
<td>0.09</td>
</tr>
</tbody>
</table>

* Values are means ± SDs (range) and $r$, assessed with Pearson’s product moment correlation, $n = 68$. *$P < 0.05$, **$P < 0.005$, ***$P < 0.0001$. SSB, sugar-sweetened beverage; $^{13}$C, carbon isotope ratio; $^{15}$CNEAA, nonessential amino acid carbon isotope ratio.
TABLE 4  The independent effect of foods with elevated δ13C values on RBC δ13C alanine1,2

<table>
<thead>
<tr>
<th>Dietary variable</th>
<th>β, (95% CI)</th>
<th>R²</th>
</tr>
</thead>
<tbody>
<tr>
<td>SSB, servings/d</td>
<td>0.67 2.24 (1.56, 2.92)***</td>
<td>0.51</td>
</tr>
<tr>
<td>Fish and marine mammals, % energy</td>
<td>-0.00 (-2.41, 2.38)</td>
<td></td>
</tr>
<tr>
<td>Commercial meats, % energy</td>
<td>0.12 (2.25, 7.95)</td>
<td></td>
</tr>
<tr>
<td>Corn products, g/d</td>
<td>0.05 (0.07 (-0.19, 0.34)</td>
<td></td>
</tr>
</tbody>
</table>

1 Values are standardized (β) and unstandardized (β) beta-coefficients and R² for a linear model in which δ13C alanine was the dependent variable and dietary intake measures were the independent variables, n = 68, *** p < 0.0001. SSB, sugar-sweetened beverage; δ13C, carbon isotope ratio; δ13C alanine, alanine carbon isotope ratio.

2 Servings SSB = 237 mL.

Further validation in a more representative U.S. population before they can be used more broadly in nutritional epidemiology. We expect those future studies will further clarify the costs and benefits of these 2 isotopic approaches.

Isotopic biomarkers of sweetener intake in RBC offer some advantages to existing biomarkers of sugar and sweetener intake, in particular, their potential to capture longer term or “usual” intake from a single sample. The time to 85% replacement of human RBCs takes ~3 mo (32), and the amino acids used in RBC synthesis may derive in part from body proteins, which integrate dietary carbon over an even longer period. Thus, isotopic measures in RBCs reflect moderately long-term intake and can be measured in either fasting or nonfasting blood samples. Alternatively, daily extrinsic sugar intake can be measured from 24-h urinary sucrose and fructose (16,17) or from the mean of several spot urines collected across the day (15). However, multiple days of sampling are required to estimate longer term or usual intake, which may present logistical difficulties for some studies. Isotopic measures like RBC δ13C alanine would be especially useful when SSB intake is of particular interest, when a single blood specimen is more feasible to collect, or when only stored, fasted specimens are available.

In the Yup’ik population, δ13C alanine was most strongly associated with SSBs but was also associated with added and total sugar. SSBs are primarily sweetened with corn syrup or associates with SSBs but was also associated with maple syrup, which do not have elevated δ13C values (20). In contrast, added sugar may include beet sugar, honey, and maple syrup, which do not have elevated δ13C values. Total sugar includes added sugar as well as “intrinsically” sugars from milk, fruit, and other sources that do not have high δ13C values (17). For our study population, beet sugar and pure maple syrup were not available in local stores and our dietary assessments found limited use of honey (this study), low intake of dairy (except in youth), and relatively little consumption of fresh fruit (40). Added sugar made up 84% of total sugar intake and δ13C alanine was similarly correlated with both added and total sugar. In a non-Yup’ik population, we expect δ13C alanine would show the strongest associations with SSB intake, followed by added sugar intake, and weaker associations with total sugar intake, as has been found elsewhere (19).

Although alanine is an amino acid, its δ13C was not associated with intake of dietary protein sources having elevated δ13C, namely, commercial meats or fish and marine mammals. Alanine can be synthesized in a single reaction from pyruvate, the end product of glycolysis (42). Early work postulated a “glucose-alanine cycle” in which pyruvate is converted to alanine and recycled back to glucose in the liver (43). In mice that were fed glucose with isotopically labeled carbon, circulating blood alanine was in isotopic equilibrium with blood glucose (44). Results from human studies also support a high level of alanine synthesis from blood glucose (29,30). A recent controlled feeding study in humans found a strong but short-term effect of corn- and cane-based sweetener intake on the δ13C of blood glucose (26). We propose that these short-term effects on blood glucose δ13C are captured in a longer lasting isotopic “record” when glucose is converted to alanine and alanine is incorporated into proteins during protein synthesis. Our data support a substantial level of alanine synthesis from dietary sugar in human RBCs and suggest a lesser contribution of carbon directly from dietary proteins.

Measurements of δ13C alanine in RBCs and hair showed very similar relationships with SSB intake. However, RBC δ13C alanine explained more of the variation in SSB intake than hair δ13C alanine and agreement in δ13C alanine between RBCs and hair was poor for several samples. The study was designed so that the midpoint of the period during which dietary data were being collected matched the mean age of RBCs at the time of specimen collection. Because hair grows at ~1 cm/mo, we estimate that the hair section with the best temporal match to the dietary data would be 1–2 cm from the scalp. Unfortunately, this section was used for another study. Here, we used the section from 2–3 cm, which may have caused a small temporal mismatch between hair and RBC δ13C as well as with the dietary intake data. Nevertheless, these data show that hair δ13C alanine has promise to be
attenuate the relationships observed between independently collected 24HRs, as was done here (10,46). Another may not be representative of usual intake (45). These sources of dietary recall is imperfect, and second, intake on the day of recall study are conservative. 24HRs have 2 sources of error: first, that biomarkers and diet; therefore, the associations presented in this reported dietary intake should attenuate relationships between biomarker of SSB intake has limitations. Error and bias in self-

dcarbon isotope ratio.

\[
\text{SSB intake (in servings/d) = 0.19x + 4.14, R}^2 = 0.50
\]

\[
\text{Alanine } \delta^{13}\text{C} = 0.17x + 3.72, R^2 = 0.40
\]

a noninvasive biomarker of SSB intake and that further investigation of this marker is warranted.

The use of self-reported measures to validate \(\delta^{13}\text{C}_{\text{alanine}}\) as a biomarker of SSB intake has limitations. Error and bias in self-reported dietary intake should attenuate relationships between biomarkers and diet; therefore, the associations presented in this study are conservative. 24HRs have 2 sources of error: first, that dietary recall is imperfect, and second, intake on the day of recall may not be representative of usual intake (45). These sources of error are mitigated by averaging intake across multiple, independently collected 24HRs, as was done here (10,46). Another concern is that energy and sugar intake have been shown to be significantly under-reported (12–14,47,48), which could also attenuate the relationships observed between \(\delta^{13}\text{C}_{\text{alanine}}\) and SSBs (48). In other studies, these biases have been shown to be associated with sex (47,49) and BMI (12,14). However, in this study, we found identical relationships between \(\delta^{13}\text{C}_{\text{alanine}}\) and SSB intake in men and women and in normal weight, overweight, and obese participants.

The design of this study also has important advantages. Validation at the population level is needed to test the effects of dietary background on tissue stable isotope ratios, as multiple foods can affect isotope ratios and both the isotopic distribution of dietary background on tissue stable isotope ratios, as multiple validation at the population level is needed to test the effects of nutritional epidemiology: stable isotope analysis of individual biochemical compounds. We find that the \(\delta^{13}\text{C}\) of RBC alanine performs very well as an objective biomarker of usual SSB and added total sugar intake in the Yup’ik population. We suggest that \(\delta^{13}\text{C}_{\text{alanine}}\) is a very promising candidate biomarker of usual SSB intake for the general U.S. population, because \(\delta^{13}\text{C}_{\text{alanine}}\) appears to be more sensitive to intake of sugar than that of protein sources. However, because the dietary context of the general U.S. population differs significantly from that of the Yup’ik population studied here, validation in a more representative group is needed to more broadly apply this marker of SSB intake. Ultimately, measurement of \(\delta^{13}\text{C}_{\text{alanine}}\) could help refine and clarify our understanding of how SSB intake contributes to obesity and chronic disease.

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6 of 7 Choy et al.


