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Effect of Season and Variety on the Differentiation of Geographic Growing Origin of Pistachios by Stable Isotope Profiling

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The objectives of this study were to demonstrate if seasonal or variety differences affected the feasibility of stable isotope profiling methods to differentiate the geographical growing regions of pistachios (*Pistachia vera*). Bulk carbon and nitrogen isotope analyses of ∼150 pistachios samples were performed. Isotope ratios were determined using a stable isotope mass spectrometer. The pistachio samples analyzed were from the three major pistachio-growing regions: Turkey, Iran, and the United States (California). Geographic regions were well separated on the basis of isotope ratios. Seasonal effects were found to affect some isotopes for some regions. Pistachio varieties within specified geographic regions were not found to affect the discriminating power of stable isotopes, for the varieties tested. This paper reports the development of a simple chemical profiling method using bulk stable isotope ratios that may be widely applied to the determination of the geographic origin of foods.

KEYWORDS: Geographic authenticity; stable isotopes; *Pistachia vera*; geographic origin; geographic indications; bulk isotope ratio; pistachios; chemical profiling

INTRODUCTION

Geographic indications are increasingly serving as a marketing tool that can add economic value to agricultural products by conveying a cultural identity through a region of origin. Recognizing the value of specific human skills and natural resources in the productive process creates a unique identity for food products (*1*). The WTO Agreement on Trade-Related Aspects of Intellectual Property Rights (TRIPS), signed in 1995, was established to protect names of particular food products associated with certain geographic locations (Food Geographic Indications) (*2*). However, financial incentives motivate dishonest retailers and resellers to misidentify the geographic origin of commodities (*3*) and food products (*4*−*8*). Most publications in the area of geographic classification have focused primarily on processed foods, particularly wines (*4*, *9*) and juices and, to a lesser extent, cocoa (*7*, *8*) and olive oil (*10*). The development of analytical chemistry methods that can confirm food labels identifying geographic indications in food commodities is therefore opportune (*12*). Bioterrorism and public health security preparedness include protecting a nation's food supply. As an economic and public safety tool, isotope ratio analysis of foods provides a scientific foundation to geolocate foods on the basis of their isotopic composition.

Plants and animals reflect characteristics of their environment and physiology through the stable isotope ratios of elements (e.g., $^{13}C/^{12}C$, $^{15}N/^{14}N$, $^{18}O/^{16}O$, and $^{2}H/^{1}H$) that form compounds in the organisms. Isotope ratios have been used in a chemical profiling method to determine geographic origin of biota (*13*, *14*). Chemical, physical, and biological processes can have significant isotope fractionations. The principle of employing stable carbon isotope methods is that the distributions of isotopes in organic matter are a function of photosynthetic fixation, temperature, plant type (e.g., C3 vs C4 plants) (*15*), and/or the environment (e.g., latitude) (*16*). For example, the $^{13}C/^{12}C$ ratios vary with geography and climate. Depending on the plant type (e.g.. C3 or C4), each photosynthetic pathway discriminates differently the heavier carbon isotope present in atmospheric CO_2. In addition, plants in humid environments, for instance, take in more CO_2; they develop a lower ratio of ^{13}C to ^{12}C than plants in drier environments. Many chemical processes affect nitrogen isotopic composition such as denitrification and mineralization. Climate and ecosystem variations such as soil types and annual temperatures and precipitation have been reported to affect nitrogen isotope ratios (*17*). Some geographical spatial variability in foliar nitrogen isotope ratios has been observed (*17*). Nitrogen isotope ratios varied from 3 to 15‰ relative to a small geographic region (*17*). The range of nitrogen isotopic ratios was reported to reflect the spatial variability in atmospheric versus soil bioavailable nitrogen (*18*).

Stable isotopes have been used to classify the geographic origins of olive oil (*19*), milk/cheese (*20*, *21*), wine (*22*), whiskey (*23*), flavors (*24*, *25*), wheat (*26*), and orange juice (*27*). Various degrees of success have been reported. Overall, many authenticity studies were surveyed in nature (<30

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samples), and therefore conclusions concerning the effectiveness of these techniques should be made prudently.

Pistachio variation in quality, food safety (e.g., aflatoxins), import/export fees, legal implications, and financial concerns makes determining the country of origin for pistachios important, especially because the world pistachio export market is valued at over U.S. $600 million. In 1997, the European Union (EU) banned Iranian pistachios because their shipments exceeded allowed levels of aflatoxins. The ban lasted only 3 months; however, aggregate imports from other countries (e.g., the United States) dropped 40%. Lack of specific geographic origin information may have contributed to the overall reduction of pistachio consumption in 1997 (*28*). Each country's applied tariff rates and national laws on commodities vary dramatically. For example, Israeli law prohibits importation of goods from Iran. In 1997 evidence was presented that $10 million worth of Iranian pistachios had been purchased by Israeli importers (*29*). The pistachios were sold below Israeli market value, undermining the world price. Methods to determine geographic origin would be another tool available for such disputes. Pistachio producers and traders are motivated to discover objective chemical techniques for determining the geographic origin of pistachios. Only a few varieties are produced in most countries, and California produces only one variety (Kerman). Over 85% of the pistachios are grown in Iran (~50%), the United States (California) (~25%), and Turkey (~10%).

In our preliminary paper (*30*) we showed that isotope ratios were different among the geographic study sites and may be able to provide information about the geographic origin of samples. These preliminary results encouraged us to extend the measurements to a greater number of pistachio samples from different countries, subregions, seasons, and varieties to verify the possibility of their use for the characterization of geographic origins of pistachios.

The purpose of this study was to determine the effect of season and variety on the feasibility of differentiating raw pistachios grown in the three different major regions of the world, Iran, Turkey, and the United States, using isotope ratios. In addition, the feasibility of further separation of these subregions was investigated. Here we present data from three different sites representing a >450 chemical parameter data set.

MATERIALS AND METHODS

Apparatus. Nitrogen (δ^{15}N‰) and carbon (δ^{13}C‰) stable isotopes and bulk C/N ratios were measured on a stable isotope mass spectrometer (MS) (Finnigan MAT-251, ThermoFinnigan, Waltham, MA). Isotopic data use the standard isotopic delta notation (δ), in per mil (‰), relative to the Pee Dee Belemnite (PDB) scale for carbon isotopes and relative to air (^{15}N) for nitrogen. By convention, the following equation for δ was used for carbon (and an analogous equation for nitrogen):

$$\delta^{13}\text{C‰} = [(^{13}\text{C}/^{12}\text{C}_{\text{sample}}) - (^{13}\text{C}/^{12}\text{C}_{\text{std}})/(^{13}\text{C}/^{12}\text{C}_{\text{std}})] \times 1000$$

Enrichment of heavy isotopes, relative to the standard, gives positive values, whereas enrichment of light isotopes, relative to the standard, gives negative values. Calibration to PDB was done through the NBS-19 and NBS-20 standards of the National Institute of Standards and Technology (Gaithersberg, MD).

Sampling, Preparation, and Analysis. Turkish and Iranian samples were collected on-site and shipped directly to our laboratory. Chain-of-custody was maintained for all samples. U.S. samples were provided by the California Pistachio Commission. Specific subregions/cities, variety, and season information was known for all samples analyzed. Each pistachio sample was analyzed as the whole nut (no shell). Samples for isotope analysis were dried overnight at 60 °C, ground to a fine powder using a small coffee grinder (2 oz, Toastmaster, Boonville, MO), and loaded in capsules for MS analysis. The chemical analytical technique is well suited to the analysis of modest to small samples; a minimum of 2.0 ± 0.5 mg can be used. A total of 146 pistachio samples were analyzed from the United States, Iran, and Turkey (n = 47, 63, and 36, respectively). Samples from two growing seasons were analyzed from two regions: Iran in 2000 (n = 23) and 2001 (n = 40) and the United States in 2000 (n = 18) and 2001 (n = 29). Two pistachio varieties were analyzed from two regions: Iran, Fandoghi (n = 36) and Kaleh Ghochi (n = 27); and Turkey, Sliirt (n = 27) and Keten Gomlegi (n = 9). To minimize any potential for day-to-day bias, samples from any designated group were typically analyzed in three different batches. Samples in each batch were resampled, ground, loaded in capsules, and analyzed on different days.

Quality Control and Statistical Analysis. Each sample was analyzed in triplicate. NIST 8542 and NIST 8548 samples were also analyzed in each batch. External precision estimates of δ^{15}N‰ and δ^{13}C‰, based on replicate analysis of acetanilide and oxalic acid standards, were ±0.12 and ±0.11‰, respectively. Graphical presentations and t test used SigmaPlot 2003 for Windows, version 8.0, SPSS, U.K., Ltd. Model results used S-Plus 2000, Lucent Technologies, Inc. The use of statistical and multivariate analysis for characterizing the geographic growing origin has been previously described (*30*).

RESULTS AND DISCUSSION

Regional Isotope Ratio Analysis. Bulk nitrogen and carbon isotopes were determined and reported as δ^{15}N‰, measured as N_2 , and δ^{13}C‰, measured as CO_2, and total bulk carbon/nitrogen ratios were calculated for the three regions tested (n = 146). The mean values of the C/N ratios, δ^{15}N‰, and δ^{13}C‰, were determined for pistachio samples grown in three different countries; the United States and Iran were statistically different for all three parameters (C/N, δ^{15}N‰, and δ^{13}C‰, p value $\ll$ 0.0001). U.S. and Turkish pistachios were also statistically different for all three parameters (p value $\ll 0.0001$, unpaired t test). Iranian and Turkish pistachios were statistically different for C/N and δ^{15}N‰ (p value $\ll 0.0001$). However, the δ^{13}C‰ was not statistically different (p value = 0.577, unpaired t test) between Iranian and Turkish pistachios.

Unlike many other chemical profiling techniques used to differentiate geographic origin, where pattern recognition methods are required to make group separations (*8*, *9*), here a simple plot of bulk C/N versus δ^{15}N‰ provides excellent group separations of the three countries (**Figure 1A**). The separation by country is all the more notable because the data set included two growing seasons and several pistachio varieties. Tree-based models provide an alternative method for classification problems. A simple hierarchical tree of decision rules is shown in **Figure 1B**, which is useful for the prediction/classification of pistachios in this data set. Restricting the tree model to three terminal nodes as shown results in a good prediction of the data set, with a misclassification error rate of <5%. Adding two additional nodes provides nearly prefect prediction of the data set (data not shown). As might be expected, principal component analysis performed as previously described (*30*) provides good separation of the data. PC 1 and PC 2 account for 65 and 31% proportions of the variance, respectively, a cumulative proportion of 96% (**Figure 1C**).

The δ^{15}N‰ values for pistachio samples from Iran, Turkey, and the United States showed greater variability than the δ^{13}C‰ values and ranged from −3 to 10. Higher δ^{15}N has been attributed to greater plant uptake of soil-dissolved inorganic nitrogen, whereas lower δ^{15}N has been attributed to greater plant uptake of the low-δ^{15}N atmospheric nitrogen (ammonium) (*17*). The three geographic regions (Iran, Turkey, and the United States) were each statistically different from the others. Whereas

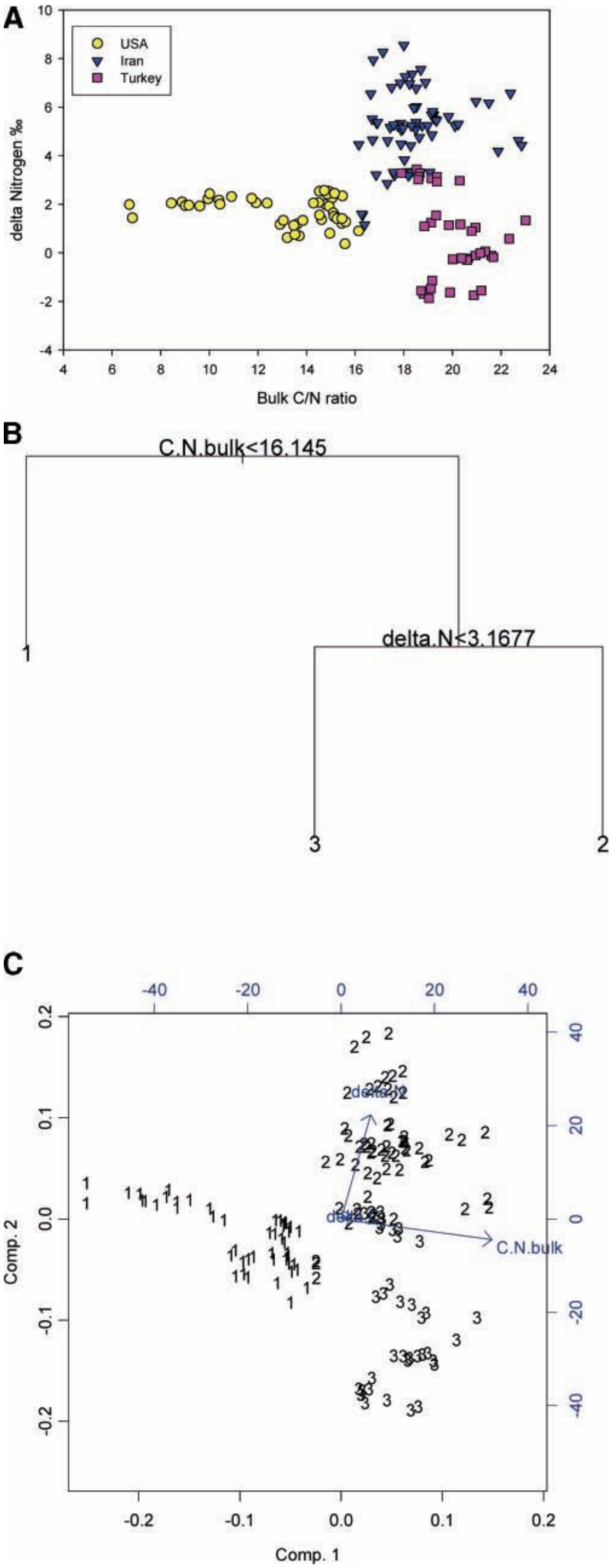

Figure 1. All panels show three geographic growing regions, several pistachio varieties within each region, and two growing seasons (n = 146). (**A**) stable isotope (δN‰) and bulk C/N ratio versus three geographic growing origins; (**B**) tree-based model results in a simplified hierarchical tree of decision rules useful for classification of pistachios (use of the decision rules from the tree model results in <5% misclassification error rate); (**C**) plot of the first two PCs for pistachios from three different regions. In panels **B** and **C**, 1 indicates the United States; 2, Iran; and 3, Turkey.

Table 1. Seasonal Bulk Stable Isotope Values (± 1 Standard Deviation) of Dry Weight for 2000 and 2001 Iranian and U.S. Pistachios

country/season	n	bulk C/N av	δN‰ av	δC‰ av
Iran/2000	23	17.85 ± 1.13	5.13 ± 1.81	−26.89 ± 0.348
Iran/2001	40	18.78 ± 1.62	5.20 ± 1.41	−26.86 ± 0.98
U.S./2000	18	10.64 ± 2.52	2.09 ± 0.22	−27.22 ± 0.18
U.S./2001	29	14.60 ± 0.84	1.53 ± 0.64	−28.01 ± 0.56

Turkish δ^{15}N‰ pistachio values were typically from −2 to 3.0, U.S. δ^{15}N‰ values were from 0 to 2.5, and Iranian δ^{15}N‰ values were typically from 1 to 9 (**Figure 1A**).

Similar to δ^{15}N‰, the bulk C/N ratios in pistachio samples displayed a fair amount of variation, and values ranged from 13 to 23. Whereas Turkish C/N ratios typically were 18−23, U.S. C/N ratios were 6−16, and Iranian C/N ratios were typically 16−23. The bulk C/N ratio and δ^{15}N‰ could be used to predict geographical origin for this two-season, multivariety, three-country data set (**Figure 1B**).

Conversely, δ^{13}C‰ values for pistachio samples from Iran, Turkey, and the United States showed modest variability and ranged between −28.5 and −24.5. U.S. and Turkish samples tended to have δ^{13}C‰ values between −29 and −27, whereas Iranian pistachio sample value typically were −27.5 to −25. This range in δ^{13}C‰ values is typical of other commodities, such as olive fruit (*31*, *32*). The modest range in δ^{13}C‰ in olive fruit was attributed to the strict discrimination of the Calvin biosynthetic process (*31*). Even though there is only a modest range in δ^{13}C‰, there is a statistically significant difference between U.S. and Iranian/Turkish pistachios, probably, because in addition to the plant discrimination process, there are environmental contributions to isotope discrimination (*16*, *17*). Values of δ^{13}C‰ have been used to examine environmental variation, including water, latitude, and elevation effects (*16*). Guy and Holowachuk (*16*) found that δ^{13}C‰ values decreased (were more negative) with increasing rainfall. The U.S. pistachios were statistically different and more negative than pistachios from Iran or Turkey. This may indicate that the U.S. samples experienced more moisture (more rainfall/irrigation water) during the study period. Both Iranian and Turkish pistachios are grown in high elevation plains, where there may be less available moisture. In addition to rainfall, values of δ^{13}C‰ have also been correlated with latitude (*16*); however, there is little latitude difference among the three countries in this study, and the δ^{13}C‰ values were not associated with the small latitude differences for the subregional sites within the study.

Seasonal Isotopic Ratio Analysis: Seasonal variability was guardedly investigated here because samples for each season were not always from the same farms, although general subregions were resampled. Two seasons were collected from Iran (n = 63) and the United States (n = 47) (**Table 1**). The C/N ratio, δ^{15}N‰, and δ^{13}C‰ were not statistically different for the two seasons in Iran (p value = 0.02, 0.8, and 0.9, respectively) (**Figure 2**). There were, however, seasonal differences in U.S. pistachio samples (**Figure 2**). The U.S. C/N ratio, δ^{15}N‰, and δ^{13}C‰ were statistically different (p value ≪ 0.0001, 0.0001, and 0.0001, respectively). The U.S. pistachio δ^{13}C‰ values were more negative for the 2001 season. The average 2001 annual rainfall for this region was ∼10% higher than that in 2000, consistent with the isotope trend (*33*). However, there is little literature to indicate that the magnitude of variation of δ^{13}C‰ was based on seasonal moisture differences (*26*, *32*). The modest difference in rainfall coupled with

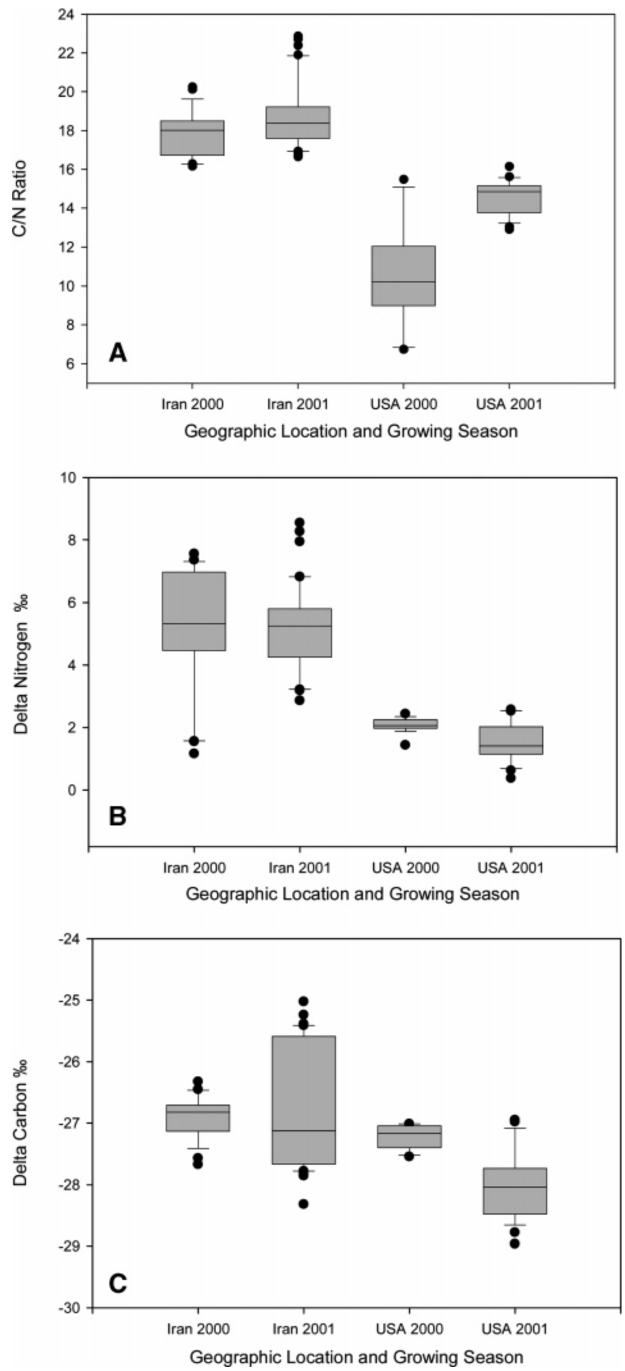

Figure 2. Seasonal variation of C/N ratio, δN, and δC of pistachios from Iran and the United States. The boundary of the box indicates the 25th and 75th (top and bottom) percentiles. The line within the box marks the median. The whiskers above and below the box indicate the 90th and 10th percentiles. All box plot outliers are designated a ●.

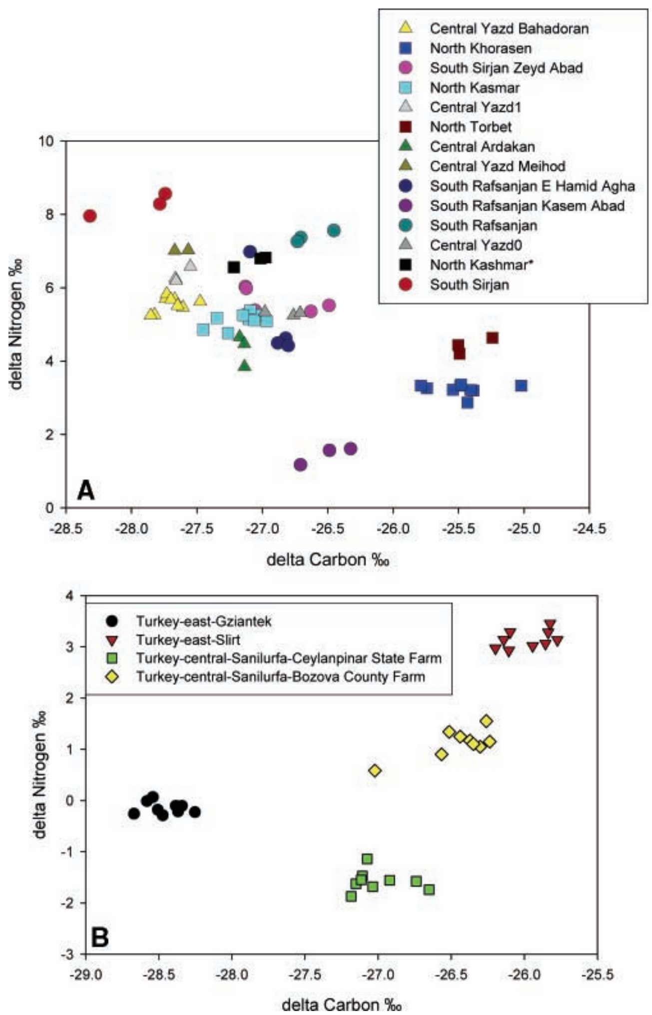

Figure 3. (**A**) Subregional geographic locations from Iran: north (■), central (▲), and south (●) and sublocation geographic designations (see legend) versus carbon and nitrogen isotopic ratios. (**B**) Turkish pistachios, subregional and sublocation geographic designations (see legend) versus carbon and nitrogen isotopic ratios.

irrigation would seem to be a tenuous association. Also, because all of the ratios are statistically different, rainfall/irrigation alone is unlikely to account for the observed differences, although general climatic environmental differences do influence isotope ratios. Alternatively, U.S. pistachio results could indicate that there are subregional differences and that an even larger database would need to be developed to confirm these results. Although all U.S. samples were from California, the same farms/trees were not systematically sampled, and the differences seen may be due to seasonal environmental effects or subregional differences. It appears from this data set that to confirm the effects or lack thereof of seasonal impact on geographic isotopic chemical profiling methods, additional seasonal samples should be analyzed. Overall, however, the magnitude of the seasonal difference in U.S. isotopic values is small compared with the other geographic regions tested. Therefore, it does not adversely affect the isotopic geochemical profiling method. For example, the 2001 U.S. pistachio samples ($n = 29$) were predicted with 100% success when using a tree model generated from the other samples (Iran, Turkey, and 2000 U.S., $n = 117$).

Subregional Isotopic Ratio Analysis. Regional pistachios were subdivided first into subregional units and then further subdivided into sublocations. Three subregional growing areas were recognized for Iranian pistachios: north, central, and south. Iranian and Turkish subregional units were found to have some modest clustering character based on isotopic ratios (**Figure 3**). Iranian pistachio samples from each sublocation, however, were strongly clustered on the basis of the isotopic ratios (**Figure 3A**). Nearly all of the pistachios were resampled and analyzed so clusters are representative of the pistachio isotopic values and they are not an artifact of the analysis or analytical bias. Although sublocations cluster, the general subregional growing areas do not exclusively cluster by subregion (i.e., north, central, south). This is also true for the Turkish subregional units, central and east, which are not as strongly clustered in their small sublocation units (**Figure 3B**). Therefore, one could not a priori

Table 2. Variety Differences in Bulk Stable Isotope Ratios (± 1 Standard Deviation) by Dry Weight

country	variety	*n*	bulk C/N av	δN‰ av	δC‰ av
Iran	Fandoghi	36	18.18 ± 0.90	4.93 ± 1.20	−26.89 ± 0.87
Iran	Kaleh Ghochi	27	18.78 ± 2.05	5.49 ± 1.91	−26.84 ± 0.71
Turkey	Sliirt	27	19.79 ± 1.11	0.47 ± 2.02	−27.14 ± 1.05
Turkey	Keten Gomlegi	9	20.51 ± 1.43	1.12 ± 0.27	−26.45 ± 0.24

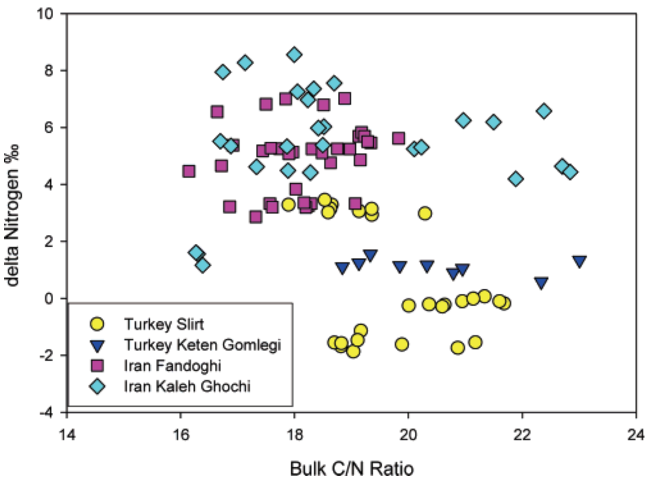

Figure 4. Variety differences in Turkish and Iranian pistachio bulk C/N ratio versus δN‰.

predict the isotopic ratios on the basis of subregional designations. It appears to be an important caveat of authenticity research that, without an adequate fully representative database, predictions should be made prudently.

Development of tree model classifications while withholding specific subregional samples still results in excellent geolocating success. For example, a tree model developed with U.S., Turkish, and Iranian samples ($n = 124$) minus all samples from the northern Iran region had a >98% success rate of the training data set. This tree model was then used to classify the northern Iran samples ($n = 25$), and it had a 100% success rate. Similarly, success rates were achieved with various combinations of training/predicting data sets of subregional pistachio isotope samples. Therefore, within this data set, although there are some subregional differences relative to the overall isotopic differences among the three regions, the subregional differences are small and do not adversely affect geo-locating success.

Pistachio Variety Isotopic Ratio Analysis. The differences in pistachio variety were also investigated. Two varieties from Iran were analyzed, Fandoghi and Kaleh Ghochi ($n = 63$), and two varieties from Turkey were analyzed, Sliirt and Keten Gomlegi ($n = 36$) (**Table 2**). The C/N, δ^{15}N‰, and δ^{13}C‰ values for the two Iranian varieties were not statistically different (p value = 0.12, 0.16, and 0.81, respectively, Student's unpaired *t* test). The C/N, δ^{15}N‰, and δ^{13}C‰ values for the two Turkish pistachio varieties were not statistically different (p value = 0.13, 0.35, and 0.06, respectively, Student's unpaired *t* test). The pistachio varieties do not separate readily, as seen in **Figure 4**, as a function of variety only, although embedded in such an analysis is variation of growing area because we do not have different varieties from adjacent pistachio trees. As compared to geographic differences, variety does not appear to affect the isotopic differences seen within this data set.

Models developed without a specific variety were still able to successfully classify the geographic origin, as might be expected because there is no statistical difference between varieties within a geographic region. For example, a tree model developed with U.S., Iranian, and only Keten Gomlegi Turkish pistachios ($n = 119$) was able to successfully (100%) classify Turkish Sliirt samples ($n = 27$).

Conclusions. A continuing challenge for a study of this nature is to ensure that sample data used to develop the predictions or models do in fact adequately represent all of the underlying variability of the entire population, including seasonal variability and variety. Although for some geographic regions there were seasonal differences, this did not affect classification success. Of the varieties tested, variety did not adversely affect the isotopic profiling success for geolocating pistachios. Use of multiple profiling approaches, however, provides converging lines of evidence that geolocating pistachios with isotopic ratios combined with other chemical profiling techniques would afford even more confidence in the results. Within the boundaries of this study, bulk C/N and stable isotope analysis to determine geographic growing origins of pistachios from multiple seasons and multiple varieties appears to be feasible.

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