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Stable Isotope Ratios of Marijuana. I. Carbon and Nitrogen Stable Isotopes Describe Growth Conditions*

ABSTRACT: There remains significant uncertainty in illicit marijuana cultivation. We analyzed the $\delta^{13}C$ and $\delta^{15}N$ of 508 domestic samples from known U.S.A. counties, 31 seized from a single location, 5 samples grown in Mexico and Colombia, and 10 northwest border seizures. For a subset, inflorescences and leaves were analyzed separately. These data revealed a strong correspondence, with inflorescences having slightly higher $\delta^{13}C$ and $\delta^{15}N$ values than leaves. A framework for interpreting these results is introduced and evaluated. Samples identified as outdoor-grown by $\delta^{13}C$ were generally recorded as such by the Drug Enforcement Administration (DEA). DEA-classified indoor-grown samples had the most negative $\delta^{13}C$ values, consistent with indoor cultivation, although many were also in the outdoor-grown domain. $\delta^{15}N$ indicated a wide range of fertilizers across the dataset. Samples seized at the single location suggested multiple sources. Northwest border $\delta^{13}C$ values suggested indoor growth, whereas for the Mexican and Colombian samples they indicated outdoor growth.

KEYWORDS: forensic science, stable isotope ratio, drug intelligence, *Cannabis sativa*

Marijuana (*Cannabis sativa* L.) is the most widely used illicit drug in the United States (1–4) and is associated with a range of public health concerns (e.g., 5, 6–12). In spite of this, there remains significant uncertainty in predicting its cultivation and distribution. Effectively addressing the illicit trade of marijuana is aided by an understanding of its cultivation practices and distribution routes, thereby contributing to useful forensic drug intelligence (13). Stable isotope, chemical composition, and genetic approaches have been applied to the identification of the geographic sources of plants, different genetic strains, and cultivation methods (14–21). As stable isotope ratios record aspects of a plant's growth environment (22), they have a unique potential to yield critical information on geographic origin and cultivation of marijuana.

Previous work has shown the potential utility of stable isotope ratio data for marijuana in a forensic context (19–21,23,24). Stable isotope ratio data have the potential to provide links between seized samples, information on the variety of sources supplying individual areas, as well as information on temporal changes in distribution and cultivation practices based solely on the analysis of seized samples. However, no general framework for interpretation exists and, to the authors' knowledge, there has not yet been an effort to routinely monitor the isotopic composition of marijuana sold across the U.S.A.

We report here the results of a large survey of the stable isotope ratios of marijuana cultivated within the borders of the United States and suggest a framework for interpreting these results, with the goal of advancing the utility of stable isotope ratios in forensic investigations and intelligence gathering on marijuana trafficking in the U.S.A. The emphasis here focuses on the analysis of carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) isotope ratios of marijuana.

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Plant carbon isotope ratios are controlled by the isotopic composition of the carbon source (atmospheric CO_2) and, in C_3 plants like marijuana, the supply and demand of CO_2 in photosynthesis (25). Background atmospheric CO_2 has a $\delta^{13}C$ value of approximately −8‰, and is generally spatially invariant at large scales (*c.* ±0.2‰; 26). Diffusion of CO_2 to the sites of photosynthesis and the enzymatic fixation of CO_2 discriminates against the heavier isotope of carbon (^{13}C) resulting in plant tissues that are depleted relative to the atmospheric source. The supply rate of CO_2 to photosynthesis has a dominant effect on plant $\delta^{13}C$ and is itself controlled by the degree of stomatal opening on leaf surfaces. Stomates respond to several stimuli, but in general leaf conductance to CO_2 declines as plants are water limited (25). This decline in stomatal "openness" reduces the supply of CO_2 and increases the relative amount of $^{13}CO_2$ in the intercellular spaces, resulting in an increased $\delta^{13}C$ of the fixed carbon when plants are water stressed. Increased irradiation functions similarly, increasing photosynthesis relative to the supply of CO_2 and increasing plant $\delta^{13}C$. As stomates open (e.g., in shaded conditions) intercellular CO_2 increases and the $\delta^{13}C$ of fixed carbon is increasingly influenced by the photosynthetic discrimination against $^{13}CO_2$; so shaded plants tend to have more negative $\delta^{13}C$ values. The CO_2 produced from plant and animal respiration or from the burning of fossil fuels is strongly depleted in ^{13}C because the origin of that CO_2 is ultimately ^{13}C-depleted plant material (27). As such, the $\delta^{13}C$ of plants grown in an environment with a significant amount of respiration not mixing with the atmosphere, or fossil fuel-sourced CO_2 (such as bottled carbon dioxide) will exhibit $\delta^{13}C$ values much lower than those grown in the typical background atmosphere.

Although the processes that control the $\delta^{15}N$ of plants and soils are more complicated and less well understood than that of plant $\delta^{13}C$, the nitrogen isotope ratios of plants are also primarily controlled by the isotopic composition of the plant's nitrogen source and internal transformations (28–30). As such, plant $\delta^{15}N$ values can provide information on the types of fertilizers used, especially

when comparing inorganic (e.g., NH_4NO_3) to organic (e.g., manure) fertilizers. The distinction between inorganic and organic fertilizers is observable primarily because the $\delta^{15}N$ of inorganic fertilizers are generally much more depleted than those of organic fertilizers (31).

Based on these theoretical and empirical expectations from the literature and previous work on marijuana carbon and nitrogen isotope ratios, we introduce an isotope-ratio-based framework for interpretation and evaluate its utility for marijuana grown in known United States counties. We further analyze a sample set seized from a location in San Diego, CA, as well as a small number of samples grown outside the United States, as case studies in the application of the framework.

Methods

We analyzed 508 domestic marijuana samples from 30 states and 218 counties across the U.S.A. (see Fig. 1; average sample size 20 g), seized by the Drug Enforcement Administration (DEA) between 2003 and 2006. In addition to the 508 samples from known counties of origin, we obtained and analyzed 31 domestic seizures of unknown origin from San Diego marijuana dispensaries. We also acquired and analyzed 10 northwestern U.S.A. border samples, 3 authentic Colombian samples, and 2 authentic Mexican samples all seized in 2005. In total, we analyzed 554 individual marijuana samples.

Of the 508 domestic samples of known origin, the DEA identified 382 of them as having grown outdoors and 59 as having been cultivated indoors (67 were of unknown growth environment, perhaps having been harvested or dried prior to seizure). In addition to information on geographic origin and indoor versus outdoor growth, we were generally provided other information as determined by law enforcement personnel, including seizure date, whether plants were classified as marijuana, sinsemilla (unfertilized/sterile female buds), or ditchweed (plants occurring as weeds, not purposefully cultivated), maturity of the crop, height of the crop, diameter of the crop canopy, and whether or not seeds were present. All domestic samples of known origin were provided to us via the University of Mississippi.

For all samples from which it was possible to isolate leaf material, we analyzed leaf-only fractions. We also analyzed inflorescence-only fractions from 228 samples. In cases where leaf or inflorescence fractions were not obtainable, we analyzed a mixture of leaf and inflorescence material. Generally, we pulverized ~200 mg of dried samples with mortar and pestle, filtering and regrinding residual large particles by passing ground material through 250 μm stainless steel sieves until the complete sample was ground and homogenized.

For analysis of carbon and nitrogen isotopes, we loaded 1.8 ± 0.18 mg of sample (from the original ~200 mg homogenized subsample) into tin capsules (3.5 × 5 mm; Costech, Valencia, CA), and then analyzed the marijuana samples in duplicate on an elemental analyzer coupled to an isotope ratio mass spectrometer (Delta Plus EA-IRMS; Thermo Scientific, Waltham, MA). Samples were corrected to an internal reference material (yeast) calibrated to the PDB standard (belemnite carbonate standard from the PeeDee Formation, SC) and atmospheric N_2. Overall precision based on multiple analyses of the yeast reference was ±0.09‰ for both $\delta^{13}C$ and $\delta^{15}N$. We reanalyzed marijuana samples with replicate $\delta^{13}C$ standard deviations of greater than 0.4‰. We also reanalyzed samples with replicate $\delta^{15}N$ standard deviations of greater than 0.7‰ (thresholds identify duplicates with standard deviations greater than approximately the 99th percentile of all standard deviations of duplicate runs). We reanalyzed the samples with low nitrogen content (<2%) at twice the routine mass (3.6 ± 0.36 mg) to equalize peak areas. For all samples analyzed more than twice, values greater than one standard deviation from the mean of all replicates were omitted from the reported mean sample value. Across the database, the average standard deviations for replicate analysis of the same subsample were 0.08‰ for $\delta^{13}C$ and 0.13‰ for $\delta^{15}N$.

To evaluate within-sample heterogeneity, we measured carbon and nitrogen isotope ratios on two independently obtained and ground leaf subsamples from ten outdoor grown samples. The first subsample was ~200 mg (same as for all other samples discussed here) and the second was ~1000 mg. These included samples from a wide geographic range and all three plant types (marijuana, sinsemilla, and ditchweed). The results of this comparison revealed a high correspondence between the two subsamples ($R = 0.991$ for

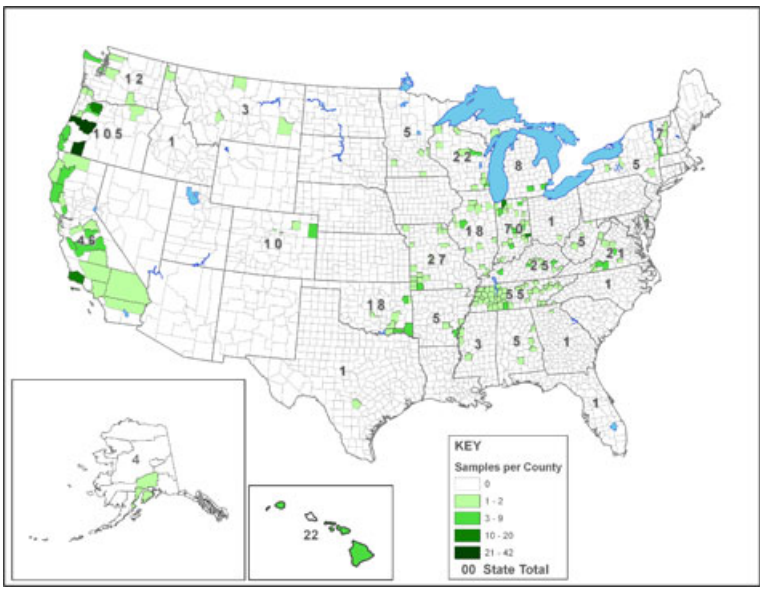

FIG. 1—*Geographic distribution of marijuana eradication samples. Numbers shown are the number of specimens obtained for each state.*

$\delta^{13}C$ and 0.997 for $\delta^{15}N$), although there was a statistically significant difference (paired *t*-test $p. > |t| = 0.02$, mean difference = 0.4‰) for $\delta^{15}N$ and a nonsignificant difference (paired *t*-test $p > |t| = 0.07$, mean difference = 0.1‰) for $\delta^{13}C$ between the two groups. For $\delta^{15}N$ the mean difference was small relative to the observed differences between samples, but larger than measurement precision. Upon closer examination it was clear that those samples exhibiting the greatest deviations (for both $\delta^{15}N$ and $\delta^{13}C$) were those samples that contained a high proportion of inflorescence material and were also "heavily manicured," meaning they contained an abundance of small pieces relative to larger, distinct leaves and inflorescences. Those samples with abundant leaf material and that were less manicured ($n = 6$) did not show significant differences between the first and second subsampling. Our subsampling regime thus should have faithfully represented the source sample, with the caveat that "contamination" by inflorescence material can affect the measured $\delta^{13}C$ and $\delta^{15}N$ values (see also results below), introducing a potential small positive bias in heavily manicured samples.

All data were imported into a relational database that also included DEA and University of Mississippi data. Orthogonal regression analyses were conducted to evaluate relationships between components (JMP 7.0; SAS Institute Inc., Cary, NC). Analyses of variance and paired *t*-tests were also conducted to evaluate whether different fractions or identified growth environments differed significantly.

Results and Discussion

A large range of $\delta^{13}C$ values was observed for the samples of known origin in the U.S.A., ranging from −51.8‰ to −20.3‰ (see Fig. 2). A similarly large range of $\delta^{15}N$ values were also observed from −7.9‰ to 29.5‰. As expected, across all sample types, samples identified as having been grown indoors had significantly lower mean $\delta^{13}C$ values (±1 SD) than did those identified as having been grown outdoors (−33.1‰ [6.5‰] and −28.2‰ [1.7‰], respectively; $F_{1,641} = 224.5$, $p < 0.0001$). No significant differences were observed between growth environments for $\delta^{15}N$.

Paired *t*-tests revealed highly significant differences between components for both $\delta^{13}C$ and $\delta^{15}N$ ($p < 0.0001$) for samples from which leaves and inflorescences were obtainable, with mean differences of 0.6‰ and 1.1‰ for $\delta^{13}C$ and $\delta^{15}N$, respectively (inflorescences greater than leaves for both). Although there were clear differences in mean values, strong, positive relationships were observed between components (see Fig. 3). Orthogonal regressions fitted to these paired data yielded slopes of 1.0 for both $\delta^{13}C$ and $\delta^{15}N$ and *y*-intercept values of 2.2‰ for C and 1.0‰ for N (inflorescences regressed against leaves). Leaf and inflorescence C and N isotopes were strongly coupled, with inflorescence isotope ratios consistently greater than leaf values from the same samples by ~1‰ for both. These results agree with previous reports (21) that C and N isotope ratios provide a significant potential to match samples, either to each other or of a sample to a crop.

Although previous reports suggested that $\delta^{13}C$ and $\delta^{15}N$ can yield geographic information (20,23), there were no clear U.S.A.-regional differences in either the carbon or nitrogen isotope ratios in our broad dataset, even when comparing plants grown in counties known to have quite different local climates. This lack of a regional carbon or nitrogen isotope signal may be due to cultivation practices of growers across the U.S.A., such as supplemental irrigation or shading, as well as significant variations in cultivation practices and growth environments within given climates that might have masked the expected local climate effects. In any case, there was no evidence from these 508 samples that $\delta^{13}C$ or $\delta^{15}N$ provides geographic information on marijuana cultivation in the U.S.A.

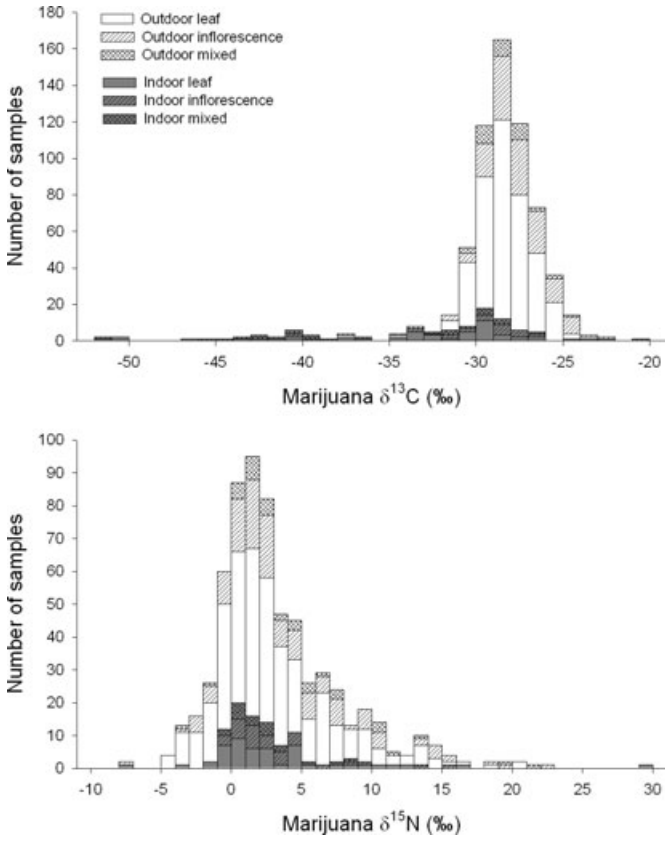

FIG. 2—*Variations in the carbon and nitrogen isotope ratios of leaf organic matter among individual marijuana samples. Carbon isotope values below −32‰ indicate indoor growth and above −29‰ outdoor growth. Nitrogen isotope values below 0‰ indicate use of ammonia-based fertilizers, between 0‰ and +5‰ nitrate-based fertilizers, and above +5‰ organic fertilizers such as manure.*

We suggest that marijuana $\delta^{13}C$ and $\delta^{15}N$ are most appropriately applied to determining and tracking cultivation methods or to applications where sample matching is of interest and provide a framework for interpreting these isotope ratios. The suggested framework consists of the identification of thresholds for categorizing samples (based on theoretical models or empirical observations for leaf C and N isotope ratios) and a simple bivariate plot of sample results relative to these thresholds. Within this framework then, the categories for carbon isotope ratios are indoor-grown, shade- or indoor-grown, and outdoor-grown. For nitrogen they are inorganic fertilizer (or no fertilizer) and organic fertilizer use. The threshold for identifying indoor-grown plants was based on theoretical expectations from a mechanistic model of carbon isotope fractionation in plants (25). Based on this model, we estimated that the minimum theoretical $\delta^{13}C$ for outdoor-grown marijuana leaves was −32‰, a value consistent with the lower threshold of values reported in the literature for C_3 plants (32,33). The threshold is conservative in that conditions for this minimum are likely to be rare. Thus, there is a high degree of confidence that tissues with $\delta^{13}C$ values more negative than −32‰ must be supplied with CO_2 with $\delta^{13}C$ values more negative than that of the average atmospheric CO_2 in an enclosed environment. Values greater than −32‰ but less than −29‰ are identified as shade- or indoor-grown based on the same model and literature. Those with $\delta^{13}C$ values greater than −29‰ are identified

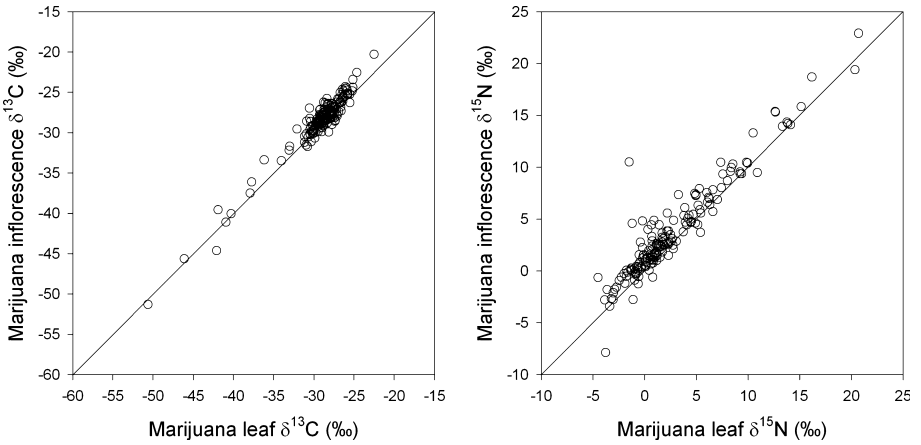

FIG. 3—*Stable isotope ratios of marijuana leaf and inflorescence material are positively correlated, with carbon and nitrogen isotope ratios exhibiting nearly 1:1 relationships. Orthogonal linear regression showed strong, nearly 1:1 relationships with relatively small positive offsets for flowers versus leaves (see text for regression statistics; 1:1 line shown).*

as outdoor-grown. It is important to note that the "outdoor-grown" classification could encompass indoor-grown plants, depending primarily on how well-ventilated the indoor environment was. As such the "outdoor-grown" classification primarily indicates open-grown plants, not specifically whether grown inside or outside a structure.

Although there are large isotopic fractionations in soils and plants that remain somewhat poorly understood for nitrogen (28,30), marijuana nitrogen isotope ratios are strongly associated with the isotopic composition of the nitrogen fertilizers used (e.g., 21). As atmospheric N_2 is the source of N for inorganic fertilizers, their isotopic compositions are near 0‰, resulting in marijuana tissues that are also near 0‰ (21,31,34). Organic fertilizers, on the other hand, are derived primarily from animal waste and therefore generally have significantly enriched $\delta^{15}N$ values, thereby increasing the $\delta^{15}N$ values of soil N (31). Marijuana plants fertilized with organic fertilizers therefore exhibit higher $\delta^{15}N$ values than those fertilized with inorganic fertilizers. Based on the results of Denton et al. (21), we assigned a threshold of 7‰ for leaves from marijuana plants fertilized with inorganic versus organic fertilizers. Clearly some variation is expected around this value, with the magnitude of that variation somewhat uncertain but again based on previous empirical observations, it should not be more than 2–3‰. It is also important to retain some additional caveats for nitrogen, primarily related to actual cultivation practices of growers. It is possible that a mixture of fertilizers would be used, thus contributing to multiple nitrogen source isotopic compositions and resulting in relatively ambiguous $\delta^{15}N$ values. Also, the amount of fertilizer added could contribute to variability in plant N source $\delta^{15}N$. Finally, plants fertilized with inorganic N fertilizers will have $\delta^{15}N$ values similar to plants that have received no fertilization. With these caveats in mind, however, we believe that this framework provides a useful approach to interpreting variability across datasets and especially among different locations or periods of time in order to identify potential changes in sources or cultivation practices.

The graphical framework is shown in Fig. 4. Several domains are identified for interpreting marijuana leaf $\delta^{13}C$ and $\delta^{15}N$, with the values dividing the domains based on the above described expectations. Plotting $\delta^{13}C$ and $\delta^{15}N$ results in this bivariate space allows one to visualize the distributions of samples and compare plants identified as indoor and outdoor grown (results for leaf data shown in Fig. 5). With two individual exceptions, all of the U.S.A.

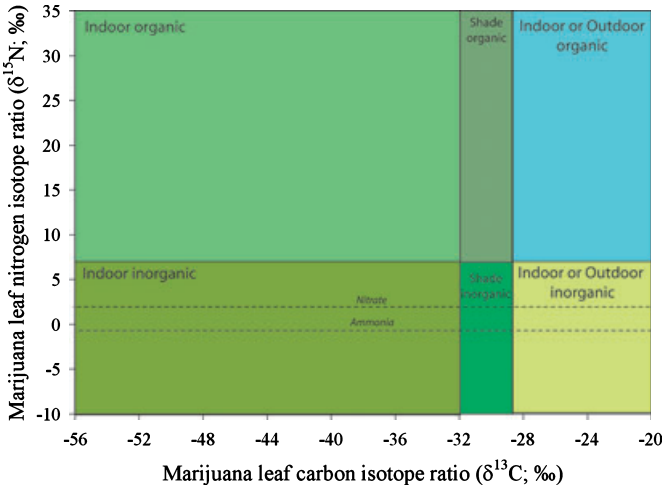

FIG. 4—*Diagrammatic representation of domains in carbon and nitrogen isotope ratio space consistent with different growth environments. The carbon and nitrogen isotopic composition of the sample indicates indoor versus outdoor growth, as well as type of fertilizer used. Carbon isotope ratios of less than −32‰ indicate indoor growth and those less than −29‰ indicate either outdoor growth in shade or indoor growth. Nitrogen isotope ratios less than 7‰ indicate the use of inorganic fertilizers and above organic (e.g., manure; 21). The median values for nitrate and ammonia fertilizers are shown based on a previous survey for reference (34).*

leaf samples of known origin and identified as outdoor-grown fell within the outdoor-grown domain. In addition, the outdoor-grown plants showed a wide range of $\delta^{15}N$ values indicating a diversity of nitrogen fertilizers. A significant number of the samples identified as indoor-grown yielded $\delta^{13}C$ values that fell within the indoor-grown domain, but also exhibit a large range of $\delta^{13}C$ values, with approximately half of the values falling outside the indoor-grown domain. These samples also exhibited a somewhat more narrow range of $\delta^{15}N$ values, but included individual values that spanned nearly the entire range of the outdoor-grown samples.

As expected, these $\delta^{13}C$ and $\delta^{15}N$ results for marijuana grown in the United States and compared with the framework expectations indicated a wide range of growing conditions. There was also clear evidence of indoor-grown plants being supplemented with CO_2, based on the low $\delta^{13}C$ values observed for some samples (up to 20‰ lower than previous reports; 21, 35). Also not surprisingly, the $\delta^{15}N$ results strongly suggested that plants grown in the U.S.A.

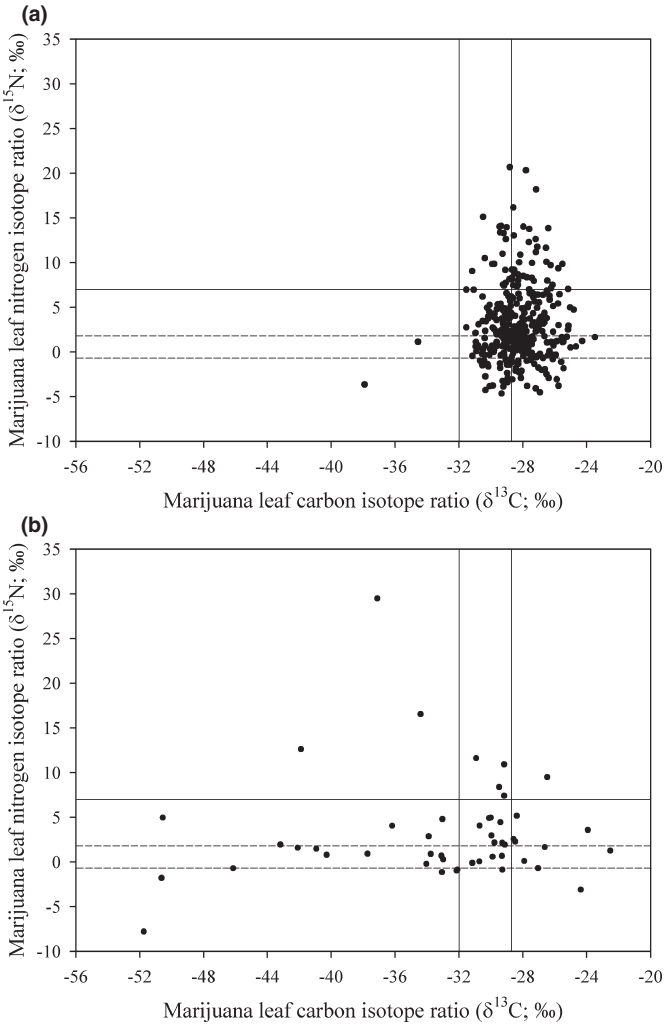

FIG. 5—*Nitrogen versus carbon isotope ratios of marijuana leaf material for domestic eradication samples from plants identified as having been grown outdoors (a) or indoors (b). Samples with $\delta^{15}N$ values over 7‰ are likely fertilized with organic fertilizers (e.g., manure). Samples with $\delta^{13}C$ values less than −32‰ are likely indoor grown, between −32‰ and −29‰ either indoor or shade grown.*

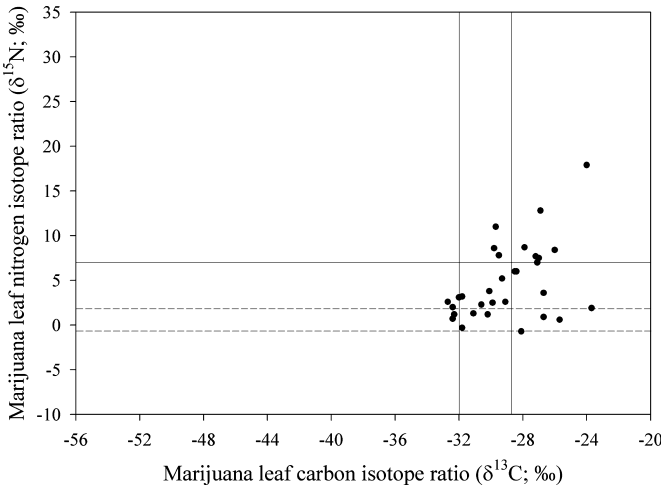

FIG. 6—*Marijuana leaf $\delta^{15}N$ versus $\delta^{13}C$ of samples obtained from seizures in San Diego, with unknown sources. These results suggest a wide range of growth environments and fertilizers, including both organic and inorganic N fertilizers and some indoor cultivation.*

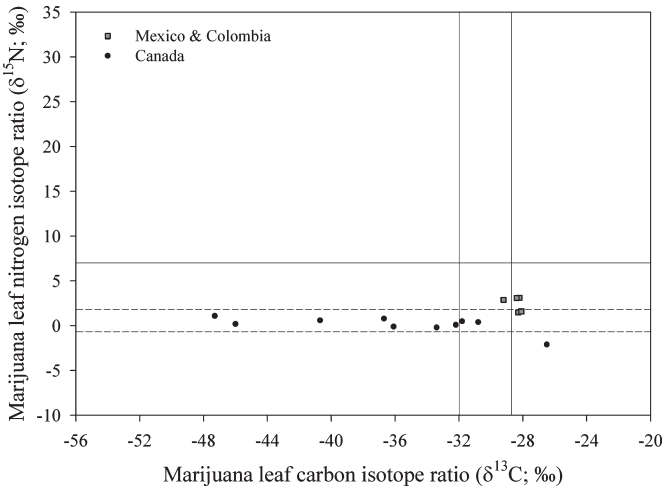

FIG. 7—*Marijuana leaf $\delta^{15}N$ versus $\delta^{13}C$ of samples cultivated outside the borders of the United States (note two Colombian samples are mixed tissue samples). These results show a very narrow range of $\delta^{15}N$ values, consistent with inorganic fertilizer use for all non-U.S. samples and, not surprisingly, a predominance of indoor growth for the northwest border seizures (Canada).*

were fertilized with a wide range of nitrogen fertilizers, dominated by inorganic fertilizers, or perhaps no fertilizer application, but also including many derived from organic sources. These results provided strong evidence that C and N isotope ratios record important aspects of marijuana cultivation, yielding a record with a rich potential to aid forensic investigations and drug intelligence.

The results from the samples seized at the San Diego, CA dispensaries are shown in Fig. 6 and are interpreted based on this framework. These results suggested that these plants were grown predominately outdoors, although there were some individual samples that were likely grown indoors. In addition, there was a wide range of nitrogen isotope ratios observed, including inorganic and organic fertilizer use. These results strongly suggested that this location obtained marijuana from a wide variety of suppliers, growing under a wide variety of conditions.

The results from non-U.S.A. seizures are shown in Fig. 7. A clear difference between the northwest border seizures and the Mexican and Colombian samples was observed. As might be expected, the marijuana seized at the northwest border and presumably grown in Canada or Alaska had carbon isotope ratios consistent with indoor growth. All non-U.S.A. samples showed a narrow range of $\delta^{15}N$ values clustered around the values consistent with inorganic fertilizers. Although the samples known to be grown in Mexico and Colombia also had $\delta^{15}N$ values consistent with inorganic fertilizer use, their carbon isotopic composition indicated outdoor growth, again as might be expected given the climatic differences.

Although more detail on nitrogen isotopic fractionations in particular, as well as greater understanding of the ranges of carbon isotope values observed for indoor-grown marijuana plants is warranted, we believe that the introduced framework for interpreting $\delta^{13}C$ and $\delta^{15}N$ is a productive approach. Monitoring marijuana $\delta^{13}C$ and $\delta^{15}N$ from locations of interest over time could allow the detection of changes in cultivation practices at the retail level. In addition, changes in the variability over time within a given location could detect changes in diversity of origins by inferring diversity of origins from diversity of growing conditions evidenced in

the isotope ratios. In addition, we believe that sample matching or exclusion of potential matches through the measurement and comparison of sample $\delta^{13}C$ and $\delta^{15}N$ is possible based on the strong correspondence between components of the marijuana plant we observed. Finally, although we did not observe strong geographic differences based on sample $\delta^{13}C$ and $\delta^{15}N$, it should be possible to evaluate claims made about the origin(s) of seized specimens using C and N isotope ratios, if source samples are also available for comparison.

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