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The influences of cultivation setting on inflorescence lipid distributions, concentrations, and carbon isotope ratios of *Cannabis sp.*

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ABSTRACT

While much is known about how the growth environment influences many aspects of floral morphology and physiology, little is known about how the growth setting influences floral lipid composition. We explored variations in paraffin wax composition in *Cannabis sp.*, a cash crop grown both indoors and outdoors across the United States today. Given an increased focus on regulation of this crop, there are additional incentives to certify the setting of *Cannabis* cultivation. To understand the impacts of the growth environment, we studied distributions, concentrations, and carbon isotope ratios of *n*-alkanes isolated from *Cannabis sp.* inflorescences to assess if variations within these lipid parameters were related to known growth settings of specimens seized by federal agents. We found that *Cannabis* plants cultivated under open-field settings had increased inflorescence paraffin wax abundances and greater production of lower molecular weight *n*-alkanes relative to plants grown in enclosed environments. Further, the carbon isotope ratios of *n*-C_{29} from *Cannabis* plants grown in enclosed environments had relatively lower carbon isotope ($\delta^{13}C$) values compared to plants from open-field environments. While this set of observations on seized plant specimens cannot address the particular driver behind these observations, we posit that (a) variations in irradiance and/or photoperiod may influence the distribution and concentration of inflorescence lipids, and (b) the $\delta^{13}C$ value of source CO_2 and lipid concentration regulates the $\delta^{13}C$ values of inflorescence *n*-C_{29} and bulk *Cannabis* plant materials. Nonetheless, by using a cultivation model based on $\delta^{13}C$ values of *n*-C_{29}, the model correctly identified the growth environment 90% of time. We suggest that these lipid markers may be used to trace cultivation methods of *Cannabis sp.* now and become a more powerful marker in the future, once the mechanism(s) behind these patterns is uncovered.

1. Introduction

The policies surrounding the use and distribution of marijuana (*Cannabis sp.*) are controversial within the United States. While possession, cultivation, and sales of marijuana remain illegal under the Federal Controlled Substance Act, the District of Columbia and the States of Washington, Colorado, Oregon, and Alaska have recently legalized marijuana for personal use and additional States have current ballot measures. In response, the Department of Justice released a series of enforcement priorities seeking to avert the public health consequences of marijuana usage; curb trafficking and violence associated with illegal marijuana distribution and sales by criminal enterprises; and limit transport of marijuana between jurisdictions with differing marijuana laws. These District and State jurisdictions are now working to develop regulatory mechanisms for the production and sales of marijuana and other marijuana-derived products; however, given the nascent state of the legislation, there remain numerous ambiguities within these regulations. In particular, within jurisdictions where *Cannabis* production and sale are legal, the growth environments of *Cannabis* cultivation are highly regulated by the local government. As an example, the State of Colorado requires the physical locations of *Cannabis* cultivation—such as individual fields or specific glass/hothouses—as well as the

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site of production facilities to be certified and all crops and products must be inventoried. Thus, there is a need for product traceability during plant cultivation, harvest, shipment, and following the manufacture of *Cannabis* products.

Stable isotope analysis of marijuana has demonstrated its potential to improve the forensic and law enforcement communities' understanding of marijuana production methods, growth environments, and trafficking networks [1–3]. In this respect, carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) isotope values have proven moderately useful. In a series of papers, Shibuya and colleagues demonstrated the potential to differentiate three of the five major production regions of marijuana cultivation in Brazil based on observed differences in the bulk $\delta^{13}C$ and $\delta^{15}N$ isotope values of seized marijuana samples [4,5]. West and others followed with a study of eradicated and seized material from the U.S., but could not distinguish region-of-origin based on bulk $\delta^{13}C$ and $\delta^{15}N$ values alone [6]. While cultivation location could not be assigned in this dataset, the growth environment could be identified using $\delta^{13}C$ values as plants grown outdoors had unique values compared to plants grown in a greenhouse system [6].

In plants, stable carbon isotope ($\delta^{13}C$) values reflect the additive influences of the $\delta^{13}C$ value of atmospheric CO_2 and isotopic fractionations associated with diffusion and carbon fixation [7,8]. These fractionation events depend on the ratio of the concentrations of atmospheric CO_2 inside (c_i) and outside (c_a) of the leaf. Given that the $\delta^{13}C$ value of atmospheric CO_2 and plant fractionation factors are relatively fixed, c_i/c_a is responsible for the majority of isotopic variability for a given species living in natural and managed environments [7,8]. Factors influencing a plant's $\delta^{13}C$ value through variation in c_i/c_a are broadly related to plant-water relations and irradiance [7]. The c_i/c_a is responsive to changes in the stomatal conductance, with important influences on conductance being ambient water vapor deficit, soil moisture, and leaf temperature [9–17]. Both field and laboratory studies have provided extensive evidence for the impact of plant-water relations and irradiance on $\delta^{13}C$ values in a variety of plant tissues [18–22]. However, there may be cases where bulk plant tissues are not available, particularly with drug compounds derived from plants, and there have been very few experiments carried out under semi-controlled conditions to understand how these processes effect the distributions of and $\delta^{13}C$ values of specific plant molecules [23–25].

Analysis of non-refractory *Cannabis sp.* compounds, particularly the cannabinoids and other terpenoids, has been an area of significant scientific research [26–28]. The distribution of cannabinoids has been used to discriminate between *Cannabis* strains and geographic origin of marijuana strains [29,30]. Recently, compound-specific isotope analysis (CSIA) of the carbon isotope values of cannabinol (CBN), cannabidiol (CBD) and THC has been demonstrated as feasible [31]. However, it has been well documented that the distribution of cannabinoids can vary markedly within a single plant, through a plant's life cycle, as plant material ages, and within a single seizure collection [32–35]. These variations complicate the standardized usage of cannabinoid distributions and isotope ratios of these compounds as regulatory tools and illustrate the need for the development of a method using refractory, unchanging compounds to monitor and source *Cannabis* compounds.

High molecular weight straight-chain alkanes (*n*-alkanes) are ubiquitous in higher plants including *Cannabis sp.* [36]. Furthermore, *n*-alkanes are highly refractory and are not altered by isotopic exchange at normal surface temperatures and pressures [37]. These characteristics make *n*-alkanes a possible tool for the regulation and certification of *Cannabis*-derived products.

Here, we present chain-length distributions, concentrations, and stable carbon isotope compositions of *n*-alkanes extracted from *Cannabis* inflorescences seized by the U.S. Drug Enforcement Agency (DEA) from clandestine growing operations employing either enclosed, greenhouse systems or open-field farming methods. This experimental design allows us to investigate the impacts of cultivation method on plant waxes and we hypothesized that cultivation method is recorded in *Cannabis* n-C_{29} carbon isotope ratios, similar to the information recorded by bulk *Cannabis* materials [6,38]. To test this hypothesis, we analyzed 84 *Cannabis* inflorescences of U.S. origin from known cultivation settings (i.e., enclosed system vs. open-field environments) and explored the association between growth settings and the distributions, concentrations, and $\delta^{13}C$ values of n-C_{29}.

2. Methods

2.1. Sample localities and materials collected

We analyzed inflorescences from 84 fully mature domestic marijuana samples of known origin from 53 counties within 18 states (Table 1). Samples analyzed here are a subset of materials used in studies by West et al. [6,39] and Hurley et al. [38,40]. In this study on compound specific isotope analyses, samples were selected from 9 states where possession and usage of marijuana is illegal (AR, FL, IN, KY, MO, TN, TX, WI, and WV) and from 9 additional states with various state-level statutes ranging from legal medical usage (HI, IL, and MT), medical and possession decriminalization (CA, NY, and VT), and legalization (AK, OR, WA). *Cannabis* inflorescence, leaf material, stems, and in some cases roots and seeds were collected between 2003 and 2006 through the U.S. Drug Enforcement Administration's (DEA) eradication efforts. Notes were provided reporting the growth setting (i.e., enclosed, open-field) employed at the clandestine growing operation for all specimens. In addition, information regarding number of plants seized, approximate canopy-cover, and plant height was reported for some, but not all samples. No information regarding the species or specific cultivar of *Cannabis* was provided. Materials used in this study were collected from archived material that was desiccated and stored in 4-ml glass vials at the University of Utah since initial sample intake. Of the 84 samples, 62 of them were noted by the DEA as having been grown in open-field environments and 22 as having been cultivated within enclosed environments.

2.2. Lipid extraction, identification, and quantification

Samples (50–400 mg) of inflorescences were isolated and pulverized with a mortar and pestle, filtering and regrinding residual large particles by passing ground material through a 250-μm stainless steel sieve until all material was ground and homogenized. Lipids were extracted from 100 to 300 mg of powdered inflorescences with 2:1 dichloromethane (DCM)/methanol by ultra-sonication (30 min × 2). The resulting total lipid extracts were concentrated under a stream of purified nitrogen using a FlexiVap Work Station (Glas-Col, Terre Haute, IN, USA), transferred to 4-ml glass vials, and further evaporated under a gentle stream of N_2 gas. Extracts were then separated into compound classes by column chromatography using 1 g deactivated silica gel (70–230 mesh) in an ashed Pasteur pipette, and eluted with 2 ml hexane to obtain the saturated hydrocarbons following Tipple and Pagani [41].

Compounds were identified and their abundances were quantified using a Thermo Ultra gas chromatograph (GC) fitted with a programmable-temperature vaporization (PTV) injector and flame ionization detector. Hydrocarbons were introduced to the PTV injector at 40 °C, followed by a 50 °C/s ramp to 320 °C. The GC oven temperature program utilized was 60–320 °C at 15 °C/min