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Nicolas Bossard, Jérémy Jacob, Claude Le Milbeau, Elisabeth Lallier-Vergès, Valery T. Terwilliger, et al.. Variation in  $\delta D$  values of a single, species-specific molecular biomarker: a study of miliacin throughout a field of broomcorn millet (*Panicum miliaceum* L.). *Rapid Communications in Mass Spectrometry*, 2011, 25, pp.1-9. <10.1002/rcm.5092>. <insu-00602348>

**HAL Id: insu-00602348**

**<https://insu.hal.science/insu-00602348v1>**

Submitted on 22 Jun 2011

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**Variation in  $\delta D$  values of a single, species-specific molecular biomarker: a study of miliacin throughout a field of broomcorn millet (*Panicum miliaceum* L.)**

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## **Abstract**

Compound-specific  $\delta D$  analyses of land plant derived biomarkers preserved in lake sediments are gaining increasing interest in paleoclimatic studies because of their potential to record essential information on the climatic conditions that prevailed at the time of their synthesis. The accuracy of inferences about climate would be better constrained if the variability in  $\delta D$  values of possible inputs were better studied at catchment scales.

We measured the  $\delta D$  values of miliacin (olean-18-en-3 $\beta$ -ol methyl ether) extracted from millet seeds of plants collected in 21 stands spatially distributed in a field with visually heterogeneous soil organic matter contents. The use of a single molecular biomarker extracted from a single plant species eliminates the possibility of variability caused by differences in plant type.

$\delta D$  values differed between plants by as much as 50‰, and average  $\delta D$  values per stand differed from one another by a maximum of 30‰. Differences in  $\delta D$  values within stands could be as high as between stands, suggesting that  $\delta D$  values are not related to macroscale heterogeneities in soil organic matter content. In addition,  $\delta D$  values were unrelated to factors indicative of differences in environment such as plant height, seed weight and miliacin concentration. The average miliacin  $\delta D$  value was representative of the area sampled however since it was normally distributed ( $p < 0.05$ ). Thus, the possibility is good that the  $\delta D$  value in a sediment sample represents the central tendency of its sources in a catchment, and any climatic information that they record.

**Keywords:** Compound-specific isotope analysis, hydrogen isotopes, spatial variability, miliacin, millet, pentacyclic triterpenes.

## 1. Introduction

Compound-specific stable hydrogen isotope analyses are increasingly receiving attention for their potential to record paleoclimatic conditions. The rationale for using this technique is that the hydrogen isotopic composition ( $\delta D$ ) of individual molecules records information on the climatic conditions at the time of their biosynthesis.<sup>[1],[2],[3],[4]</sup> Once released in the environment, molecules synthesised by a large array of producers in the catchment can then be transported to lake sediments where, if conditions remain favourable, they can be preserved for million years.<sup>[5]</sup> The  $\delta D$  of molecules that accumulated through time in sediments can thus provide integrative inferences about past climate changes. Nonetheless, the accuracy of these inferences requires understanding the quantitative relationships between compound-specific  $\delta D$  values and the controlling environmental factors.

An effective means of quantifying relationships between  $\delta D$  of individual molecules and environmental factors is by calibration over natural or controlled environmental gradients. Calibration studies have demonstrated that  $\delta D$  values of lipids from higher plants are primarily controlled by the  $\delta D$  values of the sources of the hydrogen used for their synthesis, normally meteoric waters, which, in turn, are climatically controlled.<sup>[6]</sup> Nonetheless, other factors can also affect the  $\delta D$  values of lipid compounds from higher plants. Unlike submerged, aquatic photosynthesizing organisms; the hydrogen in water that land plants use to synthesize organic compounds will have been exposed to fractionations from evaporation in the soil and from transpiration. Although the precise effects of evaporation and transpiration on the  $\delta D$  values of the source water for lipid synthesis in higher plants is presently a contentious issue, their associated fractionations may lead to a 30‰ enrichment in deuterium in higher plant lipids over those of phytoplankton.<sup>[7],[8],[9]</sup> Soil properties affect the vertical distribution of the soil water  $\delta D$  values and thus the  $\delta D$  values of water absorbed by plants depending on their rooting system.<sup>[10]</sup> The type of vegetation (trees/herbs for example) not only controls the depth of root penetration but also the intensity of soil water evaporation and leaf water transpiration.<sup>[11]</sup> Depending on biosynthetic (isoprenoids/acetogenic lipids) and possibly photosynthetic pathways ( $C_3/C_4/CAM$ ), hydrogen will be subjected to different fractionation during enzymatic processes that, in turn, will lead to varying  $\delta D$  of biochemicals.<sup>[1],[2],[12],[13]</sup> Finally, water use efficiency as well as potential variations of water source  $\delta D$  with time may also have impacts on the  $\delta D$  of higher plant lipids.<sup>[14],[15]</sup>

The aforementioned potential sources of variability could lead to a considerable range of  $\delta D$  values contributing to the pool of plant-derived lipids preserved in lake sediments. Part of this variability could be due to environmental differences (soil properties for example) among the source areas of plants. Another source of variability could arise from differences in  $\delta D$  values of

the same lipid from different plant types within the same source area. The few studies of possible plant sources to lake sediments within a catchment suggest that the differences in  $\delta D$  values of *n*-alkyl lipids can be large among different plant types.<sup>[11],[16],[17],[18]</sup> The potential of sediments to accumulate *n*-alkyl lipids with a large spectrum of  $\delta D$  values raises the question of the confidence level of paleoclimatic records based on compound-specific  $\delta D$ .

A way to discriminate between environmental and biological factors affecting the  $\delta D$  of plant lipids and to avoid the bias induced by plant types is to analyze molecules that are specific to certain plant taxa. Most of the  $\delta D$  work on land plant derived lipids has been restricted to *n*-acids, *n*-alkanes and sterols that are common across most taxa. We have detected, in tropical lake sediments, pentacyclic triterpene methyl ethers (PTME) derived mainly from Gramineae.<sup>[19]</sup> One of these PTMEs, miliacin (olean-18-en-3 $\beta$ -ol methyl ether; Figure 1), was also detected in the sediments of Lake le Bourget where, at least within the French Alps, it has a unique source plant: millet (*Panicum miliaceum*).<sup>[20],[21]</sup>

The objective of the research reported herein is to examine the variability in  $\delta D$  values of miliacin extracted from millet seeds collected randomly in a field, which constitutes an intermediate scale between a single plant and an entire lake catchment. We address the specific question of variability in  $\delta D$  with environmental change by restricting the study to a single compound from a single species. This allows us to exclusively examine the impacts of local environmental parameters. To the best of our knowledge, this is the first paper to evaluate environmental variation in  $\delta D$  of a land plant derived lipid that, if extracted from sediments, would not be confounded by variation in plant type. Our findings will be readily applicable to the use of miliacin  $\delta D$  in sediments for paleoclimate studies but are also thought to be extendable to other specific compounds.

## **2. Site, materials and methods**

### **2.1. Sample collection**

Samples of *P. miliaceum* (var. Sunrise) were collected during August 2009 at harvest time from a field in Mézières-lez-Cléry, near Orléans, France (47°50'15", 1°48'20"E and ca. 100m above sea level). Mean  $\delta D$  of precipitation was  $-41.1 \pm 7.2\text{‰}$  during the 1996-2005 time period at the closest Global Network for Isotopes in Precipitation (GNIP) monitoring station (Orléans-La-Source). This field was chosen considering the variability in soil properties as illustrated by different shades of grey on aerial picture (Figure 2) that indicate contents of organic matter. The heights of plants of *P. miliaceum* growing in the field were measured from the first node up to the top of the main panicle. Plant heights range from ca. 30 cm up to 120 cm, with higher plants

developed on more organic soils, as determined by field measurements. 26 stands representative (numbered from 183 to 210) of the various contexts (heights of the millet plants and soil color) were selected in the field. From 1 to 5 plants (3 in most cases for a total of 61) were taken at each stand at less than 30cm apart. The whole plants were sampled and dried in an oven at 35°C for 48 h. The weight of the main panicle was determined after drying.

## **2.2. Miliacin extraction and purification**

For each plant sample, twenty seeds of the main panicle were ground to powder and weighed. These twenty seeds provide a sufficient amount of miliacin for determination of  $\delta D$  values. We analyzed two sets of twenty seeds from a single panicle of each plant and we observed no significant differences either in millet seed weight, miliacin concentration or miliacin  $\delta D$  values between sets. Lipids were ultrasonically extracted three times with organic solvents (DCM:isopropanol 2:1), and the lipids extracts were then combined. The total extract was then separated into neutral, acidic and polar fractions by ion exchange column on aminopropyl-bonded silica. The neutral lipid fraction was eluted with DCM:isopropanol 2:1 and then dried under nitrogen. Miliacin (Figure 1) was purified from the neutral fraction by flash chromatography on activated silica by using the following solvents of increasing polarity. After the elution of aliphatic and aromatic hydrocarbons, miliacin was collected in fractions eluted with 2mL hexane:toluene (1:1) and 2mL hexane:ethyl acetate (19:1). Miliacin-containing fractions were then combined, dried under nitrogen and stored at -4°C until analysis by Gas Chromatography-Mass Spectrometry (GC-MS) and Gas Chromatography-isotope ratio Mass Spectrometry (GC-irMS). 5 $\alpha$ -cholestane was added prior to analysis by GC-MS for quantification purposes.

## **2.3. GC-MS analyses**

Miliacin contents and purity were assessed by GC-MS on a TRACE gas chromatograph coupled to a Polaris GCQ mass spectrometer (ThermoFinnigan, Bremen) according to previously described methods.<sup>[19]</sup> The gas chromatograph was fitted with an Rtx-5 MS capillary column (5m column guard, 30 m, 0.25 mm i.d., 0.25  $\mu$ m film thickness). The gas chromatograph operating conditions were: temperature held at 40°C for 1 min, then increased from 40 to 300°C at 20°C.min<sup>-1</sup>, with final isothermal hold at 300°C over 30min. The sample was dissolved in toluene and injected splitless in a 2 $\mu$ L volume, with the injector temperature set at 280°C. The carrier gas was Helium and the flow was set at 1.4mL.min<sup>-1</sup>. The mass spectrometer was operated in the electron ionisation (EI) mode at 70eV ionization energy and scanned from 50 to 650 Da.

## **2.4. GC-irMS analyses**

Miliacin  $\delta D$  values were determined by using a Trace gas chromatograph equipped with a Triplus autosampler coupled to a DeltaV Advantage isotope ratio mass spectrometer through a GC-Isolink pyrolysis interface and a ConFlo IV dilution system (ThermoScientific, Bremen). Chromatographic conditions were the same as those used in GC-MS, except that the GC column (J&W DB5, 30m, 0.25 mm i.d., 0.25  $\mu\text{m}$  film thickness) was slightly different. 1  $\mu\text{L}$  of miliacin dissolved in toluene was co-injected with an internal standard constituted by 1  $\mu\text{L}$  of a mixture of *n*-alkanes (*n*-C<sub>16</sub> to *n*-C<sub>30</sub>) with  $\delta D$  values ranging from -46.3 to -242.6‰ determined offline (Arndt Schimmelmann, Indiana University, Bloomington, USA). Due to high miliacin purity, no significant coelution of other compounds with *n*-alkanes was observed (Figure 3). Data were then normalized to the V-SMOW isotopic scale by using the  $\delta D$  of the *n*-C<sub>25</sub> and *n*-C<sub>27</sub> alkanes as reference.<sup>[1]</sup> All miliacin  $\delta D$  values are reported in ‰ units relative to the V-SMOW scale. The 13 remaining *n*-alkanes were used for accuracy assessment, with the exception of the *n*-C<sub>29</sub> alkane that co-eluted with 5 $\alpha$ -cholestane (Figure 3). Each sample was randomly (i.e. not one after the other and not in logical order in the sequence of injection) injected at least 3 times. When necessary, supplementary injections were performed and aberrant values were not taken into account by using a Dixon test ( $p < 0.05$ ). The mean precision of miliacin  $\delta D$  values was 5.9‰ (from 0.3 to 12.0‰), i.e. within common precision ranges reported for natural samples.<sup>[15],[22],[23]</sup> The *n*-alkanes mixture was analysed every nine injections, as external standard. The overall precision for the *n*-alkane standard (injected with and without sample) is around 3‰ (1 $\sigma$ ,  $n=361$ ), with no notable difference between internal and external standards, which suggests no significant coelution.<sup>[1]</sup> Measured *n*-alkanes  $\delta D$  values are in good agreement with those measured offline, except for a significant drift at elevated  $\delta D$  values. The best match between offline and online  $\delta D$  values for *n*-alkanes is between -240 and -70‰. The expected miliacin  $\delta D$  values (-80 to -150‰) fall within this range. The H<sub>3</sub><sup>+</sup> factor was determined daily prior to analysis and ranged from 3.7 to 3.8 in the course of analysis.

### 3. Results

#### 3.1. Miliacin $\delta D$ values means and standard deviations

Miliacin  $\delta D$  values range from -144.5‰ to -98.1‰; i.e. with a difference of almost 50‰ (Table 1, Fig. 4a). Standard deviations of the repeated analysis of each sample range from 0.3 to 12‰ with a mean standard deviation of 5.9‰. Miliacin  $\delta D$  values of individual plants were normally distributed (Figure 4b) about an average of -121.1‰ ( $\pm 9.1$  sd) (Kolmogorov-Smirnov,  $p < 0.05$ ). When  $\delta D$  values average of each plant are again averaged by stand, they range from -132.8 ‰ to -102.8‰ (Figure 4c); i.e. a difference almost reaching 30‰. In this case, standard

deviation of  $\delta D$  values in each stand ranges from 2.1 to 19.3‰, with a mean standard deviation of 6.9‰. Miliacin  $\delta D$  values per stand show a normal distribution (Figure 4d) with an average of -120.7‰ ( $\pm 6.5$  sd) (Kolmogorov-Smirnov,  $p < 0.05$ ).

### 3.2. Comparison of miliacin $\delta D$ values with other parameters

To examine whether miliacin  $\delta D$  values varied systematically with environmental condition, we tested the values for correlation with the following environmentally influenced parameters: standard deviation of miliacin  $\delta D$  values, plant height, panicle weight, 20 seeds weight and miliacin concentration. Plant heights range from 20 to 120cm, panicle weight from 0.1 to 8.8g, 20 seeds weight from 25 to 160mg and miliacin concentrations from 0.08 to 4.8  $\mu\text{g}\cdot\text{g}^{-1}$  seed (Table 1). No linear relation could be established between miliacin  $\delta D$  values and other parameters ( $r^2 < 0.15$ ).

## 4. Discussion

### 4.1. $\delta D$ of pentacyclic triterpenes

Very few studies have reported  $\delta D$  values for pentacyclic triterpenes, probably because these compounds are secondary metabolites and constitute minor components in biological and geological systems, when compared to *n*-alkyl lipids. Average miliacin  $\delta D$  values (-121‰ V-SMOW) are in agreement with some of the few other values that have been reported for pentacyclic triterpenes.  $\delta D$  values of -139 and -171‰ have been reported for lupenol, -142‰ for lupenone, -144‰ for an unidentified pentacyclic triterpenone and -151 and -165‰ for two unidentified pentacyclic triterpenols, all extracted from *Spartina alterniflora*.<sup>[1],[15]</sup> For taraxerol,  $\alpha$ -amyrin and an unidentified pentacyclic triterpene extracted from the surface sediments of a pond in Massachusetts,  $\delta D$  values were around -170/-180‰.<sup>[2]</sup> Various pentacyclic triterpenes extracted from *Daucus carota* were highly depleted, with -252‰ for  $\delta$ - and  $\beta$ -amyryns, and values ranging from -226 to -239‰ for unidentified pentacyclic triterpenols.<sup>[1]</sup>

In contrast to *n*-alkyl lipids (acetogenic lipids), which are produced from acetyl-CoA in the plastid, pentacyclic triterpenes are produced in the cytosol, also from acetyl-CoA, via the mevalonic pathway.<sup>[1],[24]</sup> This explains a systematic 50 to 100‰ offset between *n*-alkyl lipids and sterols or triterpenols, directly from plants or from soils and sediments.<sup>[1],[12],[22],[25],[26],[27],[28]</sup> The origin of this difference is still unclear and could be related either to the source of hydrogen (directly from H<sub>2</sub>O or *via* NADPH), distinct pools of NADPH within the cell or it could result from kinetic isotope effects associated to hydrogenation.<sup>[1],[25],[26]</sup>

Differences in  $\delta D$  among pentacyclic triterpenes with various structures (such as lupane, oleanane, ursane, taraxerane) could arise from fractionation intervening during the ultimate steps of

their biosynthesis that involves the cyclisation of 2,3-oxidosqualene and subsequent rearrangements through methyl group and double bond migrations, and ring expansion/contraction.<sup>[29]</sup> In fact, very little is known on the fractionation of hydrogen isotopes associated to the synthesis of pentacyclic triterpenes. The  $\delta D$  of various pentacyclic triterpenes produced by a single plant could therefore provide key information on hydrogen isotopic effects associated to their synthesis, as it was remarkably as was performed for phytol.<sup>[26]</sup>

#### 4.2. Compound-specific $\delta D$ variations over a small area

The almost 50‰ range in miliacin  $\delta D$  values within the area of a single field is within the range of compound-specific  $\delta D$  variations of less plant specific compounds in larger-scale paleoenvironmental studies (see section 4.4.). The importance of the 50‰ range can be put into perspective by comparing it with the ranges of compound-specific  $\delta D$  values recorded in the few other studies of plants over small areas, either under natural or controlled conditions.

Studies in natural systems of homogeneous climate (such as our study site) reveal large differences in  $\delta D$  values of plant lipids. In a study of 48 land and aquatic plant species grouped by life form growing in the catchment of a small pond,  $\delta D$  values of *n*-C<sub>30</sub> fatty acids varied by 100 ‰ between all species, 70 ‰ among tree species, and 40 ‰ among grasses.<sup>[11]</sup> Within trees developed in the same area, the difference in *n*-alkane  $\delta D$  values can reach 30‰ for deciduous trees (*Betula* spp. and *Quercus* spp.) and up to 35‰ for evergreen trees (*Pinus strobus* and *Tsuga canadensis*)<sup>[14]</sup> Similarly, a difference of ca. 60‰ was noted between  $\delta D$  values of *n*-alkanes produced by trees growing in woody grassland (*Atalaya* sp.) and trees growing in woodland (*Acacia* sp.) at few tens of meters of distance.<sup>[16]</sup> When grasses are considered, the differences in  $\delta D$  values of *n*-alkanes extracted from different species growing in individual sites of the US Great Plains was as large as 50‰.<sup>[13]</sup> Thus, the range of miliacin  $\delta D$  values we observed falls within the ranges observed for *n*-alkyl lipids extracted from various plants in natural conditions over a small area.

In controlled environment chambers, environmental variations that could affect the  $\delta D$  of biochemicals are reduced, when compared to natural systems. The range of  $\delta D$  values is thus expected to be narrower. Nevertheless,  $\delta D$  values of *n*-alkyl lipids from plants have been found to differ by as much as 90‰ between tree and grass species growing in a controlled environment chamber.<sup>[18]</sup>  $\delta D$  values of *n*-alkanes and *n*-alkanoic acids each differed by a maximum of 60‰ between several grass species.<sup>[13],[30]</sup> When  $\delta D$  values of *n*-alkanes were compared among species of a single photosynthetic pathway (C<sub>3</sub> or C<sub>4</sub>), however, this difference was reduced to 30‰.<sup>[13]</sup> Within tree species, a maximum of 30‰ difference in  $\delta D$  values of a single alkanolic acid was

found among individuals of a single oak species.<sup>[30]</sup> None of these studies (either under natural or controlled conditions) had more than four replicates per species, however, thereby limiting conclusions on intraspecies variability in  $\delta D$  values.

These large ranges in compound-specific  $\delta D$  values reported in both controlled and natural conditions have been attributed to several major causes that are, in most cases, interconnected.

Studies indicate that by taking up isotopically distinct water and through different transpiration dynamics, the  $\delta D$  values of the water used for lipid biosynthesis may vary systematically between plant life forms (e.g. trees, shrubs and grass). The  $\delta D$  values of soil water can decrease with depth due to a decreasing loss of D-depleted water as vapour during evaporation. Differences in depths of rooting systems among plant life forms can thus lead to uptake of waters with different  $\delta D$  values.<sup>[8],[13],[16],[31]</sup> Before its use in lipid biosynthesis, the water is exposed to further D-enrichment during transpiration and the magnitude of that enrichment may also vary by life form. The two fractionations; evaporation of soil water and transpiration may minimize or maximise differences between plant life forms in  $\delta D$  values of water used in lipid biosynthesis. For example, grasses tend to take up most of their water at the surface where it is most D-enriched whereas deeper soils are a more important source of water for trees. . Tree leaves may have higher transpiration rates than grasses because their leaves are exposed to direct sunlight and wind exposure, and do not benefit from canopy effect and direct soil moisture.<sup>[11]</sup> The D-enrichment of water taken up by grasses relative to water taken up by trees may thus be offset by lower water losses via transpiration in grasses than in trees.

Differences in leaf waxes  $\delta D$  values in distinct plants species also arise from differences in plant physiology and photosynthetic pathways. The impact of leaf morphology and architecture is still debated.<sup>[13]</sup> Differential fractionation of hydrogen isotopes between C<sub>3</sub>, C<sub>4</sub> and CAM plants has also been proposed but there is still no consensus on whether this arises from isotope effects related to metabolisms or to physiological and morphological differences.<sup>[8],[13],[18],[25],[26],[32]</sup> The last parameter to be taken into account for explaining the variability of biochemical  $\delta D$  values relates to the strategies developed by plants for their water use.<sup>[11],[13],[33]</sup> The negative correlation between  $\delta^{13}C$  and  $\delta D$  values of plant lipids suggests that variable water use efficiency influences inter-species differences in leaf wax  $\delta D$  values.<sup>[8],[14],[18]</sup> None of these explanations can be invoked in our case that concerns a single biochemical produced by a single species developed under relatively homogeneous conditions.

### **4.3. Origin of variability in miliacin $\delta D$ values**

Our data bring new information about the spatial variability of compound-specific  $\delta D$  values. By focusing our study on a single compound specific to one plant, we reduce interspecies offsets but also reduce potential variability related to micro-environmental conditions, biosynthetic and photosynthetic pathways and physiological factors (including leaf structure and rooting system). Despite these precautions, a large range of miliacin  $\delta D$  values (50‰) is found in millet seeds collected from plants developed on a ca. 1000 m<sup>2</sup> area. However, this range is not necessarily attributable to differences in environmental conditions.

The normal distribution of miliacin  $\delta D$  values per plant implies a single dominant controlling parameter. The lack of correlation of miliacin  $\delta D$  values with the standard deviation of these values and miliacin concentrations excludes any analytical bias. No correlation could be found between miliacin  $\delta D$  values and parameters related to plant yields (plant height, panicle weight and weight of 20 seeds). Because plant heights were visually correlated with soil properties in the field, this suggests that there is no relationship between miliacin  $\delta D$  values and soil properties. This is confirmed when intra- and inter-stand variability is compared. If soil properties were a major factor, one could expect larger differences between stands (at decimetric scale) than within a single stand, which shows similar soil properties at decimetric scale. The similarity of intra-stand (mean of standard deviation of miliacin  $\delta D$  values per stand; 6.9) and inter-stand (standard deviation of mean  $\delta D$  values per stand; 6.5) miliacin  $\delta D$  values variability thus allows us excluding decimetric-scale differences in soil properties as a dominant control parameter. Conversely, the Gaussian distribution of miliacin  $\delta D$  values suggests that precipitation  $\delta D$  and mean hydrological regime that prevail at the field scale constitute the dominant control parameters.<sup>[30]</sup>

Because our study was focused on a single plant, several potential parameters (plant form, photosynthetic and biosynthetic pathways, plant physiology) that have been proposed to explain compound-specific  $\delta D$  differences in spatial studies (see 4.2.) can be discarded. Large-scale differences in soil properties cannot either be invoked. Although this remains to be tested, the dispersion of miliacin  $\delta D$  values could result from micro-scale heterogeneities in soil properties and/or in relative humidity. For example, the millet field was heterogeneously but densely covered by the weed *Cynodon dactylon* (Bermuda grass) at harvest time. The competition for water uptake between these two plants could partially explain local differences in miliacin  $\delta D$  values. Alternatively, these differences could also be related to intrinsic (phenotypic and genotypic) variability within *P. miliaceum*.<sup>[34]</sup>

#### **4.4. Consequences on the climatic significance of compound-specific $\delta D$**

The maximum difference of miliacin  $\delta D$  values between plants analysed from the Mézières-lez-Cléry millet field is 50‰. This range must be compared to known ranges of compound-specific  $\delta D$  values found in both actual and ancient natural systems in order to estimate the confidence level of paleoclimatic interpretations based on the variations of  $\delta D$  values of lipids extracted from sediments archives. Throughout a latitudinal transect (from 70°N to 40°N) in Europe, leaf wax C<sub>27</sub> *n*-alkane extracted from various deciduous trees had  $\delta D$  values ranging from ca. -140 to -210‰ (i.e. a 70‰ range) whereas sedimentary leaf wax C<sub>27</sub> *n*-alkane from the same transect showed  $\delta D$  values from -130 to -220‰ (i.e. a 90‰ range).<sup>[7],[33]</sup> However, it is not clear whether these ranges of  $\delta D$  values purely reflect a climatic gradient or also attests to vegetation changes through latitude because the *n*-alkanes studied are common to a wide range of higher plant taxa. In this study, a wide variety of deciduous trees were analysed, with potentially large interspecies differences in compound-specific  $\delta D$  values (see section 4.2.). Nevertheless, such calibration studies permit inferences about shifts in compound-specific  $\delta D$  values with environmental and climatic variations. In sedimentary archives, a 80‰ shift in *n*-alkanes or *n*-acids  $\delta D$  were interpreted as the transition between a savannah and tropical forest due to different hydrological conditions.<sup>[35],[36],[37]</sup> Thus, up to 80 ‰ range of compound-specific  $\delta D$  values in recent and ancient systems is indicative of highly contrasted environmental conditions. These ranges are in the same order as that found for miliacin  $\delta D$  values, at a single location. The overlap between the range of local variability under the same environmental regime and the range of drastic environmental changes could, if only the range is considered, induce serious doubt on the confidence level to which the  $\delta D$  of sedimentary lipids can be interpreted in paleoclimatology.

The number of samples of a single species analysed in this study permits a more thorough examination of the significance of the 50‰ miliacin range in  $\delta D$  values. Miliacin  $\delta D$  values follow a normal distribution (average = -121.1‰ of all plants and -120.7‰ of plants averaged by stand; Figure 4). In the meantime, the range of  $\delta D$  values diminishes from ca. 50‰ (all plants) to 30‰ (average by stands). 90% of miliacin  $\delta D$  values per plants are comprised between -112 and -130‰ V-SMOW, i.e. a 20‰ range (Figure 4b). This range is evidently reduced when miliacin  $\delta D$  values are averaged by stand (from -114 to -126‰; i.e. a range of 12‰; Figure 4d). Consequently, the probability for obtaining miliacin  $\delta D$  values that are representative of the whole millet field from remotely sampling a single plant appears rather high.

Sedimentary lipids constitute an integrative signal of all lipids produced in the catchment. If variation in  $\delta D$  values of species specific molecular biomarkers was high within a small,

climatically homogeneous area, the resolution of a climatic interpretation from an entire catchment would be low. Our findings suggest that, although noteworthy variation in miliacin  $\delta D$  values under such conditions is possible, variation is more commonly small. Research efforts now need to concentrate on how well the original lipid  $\delta D$  value is archived in sediments. Following the incorporation of their source plants into sediments, the  $\delta D$  values of lipids may be affected by (i) selective degradation during diagenesis; (ii) temporal offsets due to differences in transportation times of plant sources to sediments; and (iii) uneven representation of environmental heterogeneities within the catchment. Significant D depletion in soil relative to overlying plant n-alkyl lipids but little difference in  $\delta D$  values of isoprenoids in soil and overlying plants suggested larger diagenetic effects in the former than the latter category of compounds.<sup>[28]</sup> D depletion of surficial sedimentary lipid compounds relative to surrounding plants has been attributed to offsets between the time lipids were produced and their accumulation in sediments.<sup>[33]</sup>

If we exclude these temporal and diagenetic offsets, the  $\delta D$  value of sedimentary miliacin would record the weighted mean miliacin  $\delta D$  value of all millet biomass produced in the catchment; i.e. the variability is restrained to the mean value, with no distortion. This hypothesis should be tested by examining the propagation of variability from the source to the sedimentary archive, i.e. the representativeness of sedimentary lipid  $\delta D$  compared to catchment-scale lipid  $\delta D$ .<sup>[34]</sup>

## 5. Conclusion

This study contributes to constraining the climatic inferences that can be made from  $\delta D$  analyses of molecular biomarkers preserved in lake sediments that may have originated under a variety of environmental conditions throughout a catchment. The analysis of the spatial distribution of  $\delta D$  values measured on a single molecule extracted from a single plant species grown in a field with heterogeneous soil properties shows differences as large as 50%. This large difference is not necessarily related to environmental conditions. Despite the large range between maximum and minimum values, however, most miliacin  $\delta D$  values were tightly clustered in a normal distribution about the mean and thus varied little within the study area. Similarly, the  $\delta D$  values of miliacin in lake sediments may average miliacin  $\delta D$  values over their source areas.

## Acknowledgments

This work was undertaken within the PalHydroMil project, supported by Agence Nationale de la Recherche grant (ANR JCJC, 2011-2013) and NSF project n° BCS0551668. Mr Sévin and Mr Beaulieu are acknowledged for giving access to the millet field. N. Bossard beneficiates from a PhD grant provided by the Ministère de la Recherche et de l'Enseignement Supérieur (Action

Thématique Prioritaire, 2009-2012). V.J. Terwilliger is hosted at ISTO under the auspices of Le Studium® (Institute for Advanced Studies). The authors wish to thank J.R. Disnar and S. Gogo for useful discussions and reviewers who greatly improved a previous version of the manuscript.

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## Table captions

Table 1: Inventory of millet plant samples by stand together with plant height, panicle weight, weight of 20 seeds, miliacin concentration in seeds and miliacin  $\delta D$  values and standard deviations of miliacin extracted from seeds. Average and standard deviation of miliacin  $\delta D$  values by stand are calculated as the arithmetic average and standard deviation of miliacin  $\delta D$  values of plants developed on the same stand. n represents the number of replicates per sample in GC-irMS.

Sample n°	Stand	Plant height (cm)	Panicle weight (g)	Weight of 20 seeds (mg)	Miliacin concentration (ng.g <sup>-1</sup> seeds)	Miliacin $\delta D$ value (‰V-SMOW)	Standard deviation	n	
183-33	183	33	0.35	74	240	-112.8	7.7	9	
183-35		35	0.4	59	139	-136.0	1.7	16.5	
184-65		65	2.04	84	4775	-110.3	6.5	3	
184-70	184	70	1.51	141	1292	-122.3	-116.4	6.6	6.0
184-73		73	3.11	136	1813	-116.5	6.5	3	
185-79	185	79	1.59	123	438	-124.9	0.3	3.3	2
185-81		81	2.09	125	2755	-120.2	1.5	3	
186-73		73	1.76	123	350	-127.9	4.6	5.1	3
186-80	186	80	1.68	123	702	-120.6	5.2	3	
187-61		61	1.2	127	387	-125.9	11.1	7.5	3
187-66	187	66	1.71	102	1026	-115.3	5.3	3	
188-43		43	1.11	89	145	-132.4	4.1	-	4
189-69		69	1.14	135	451	-106.4	2.0	3	
189-71	189	71	3.3	129	481	-144.5	-123.5	6.4	19.3
189-74		74	1.1	93	566	-119.7	1.0	3	
190-36		36	0.25	76	328	-118.7	4.0	2	
190-39	190	39	0.36	73	351	-120.4	-121.0	10.3	2.6
190-41		41	0.4	88	296	-123.8	5.8	3	
191-63	191	63	2.79	76	82	-125.4	-125.4	8.6	-
192-61		61	1.84	99	317	-102.8	-102.8	3.5	-
193-78		78	5.08	127	468	-117.2	-115.2	9.6	2.9
193-87	193	87	1.96	130	303	-113.1	2.5	3	
194-79		79	3.8	130	572	-127.0	6.7	3	
194-82	194	82	2.75	158	558	-119.5	-124.1	3.7	4.0
194-86		86	3.64	137	557	-125.8	5.4	3	
196-87		87	8.78	135	629	-134.7	0.0	1	
196-88	196	88	7.81	142	94	-130.9	-132.8	3.1	2.7
197-82		82	3.53	142	758	-134.8	5.3	3	
197-88		88	8.59	143	767	-125.0	-123.7	5.1	11.8
197-93	197	93	2.94	148	261	-111.3	4.3	3	
198-87		87	2.72	117	348	-124.3	11.1	6.5	6
198-90		90	3.31	105	265	-115.2	-119.7	10.0	8
199-74	199	74	3.06	140	250	-114.9	9.8	3	
199-85		85	2.75	111	356	-116.6	-121.3	4.7	9.7
199-86		86	3.33	145	224	-132.5	6.7	3	
200-26	200	26	0.13	25	156	-121.0	-121.0	10.4	-
201-36		36	0.38	123	295	-108.1	-106.7	8.1	2.1
201-41	201	41	0.74	121	323	-105.2	5.4	3	
202-21		21	0.14	51	441	-98.1	11.3	2	
202-54	202	54	1.93	117	344	-106.3	-105.8	6.9	7.5
202-60		60	2.71	125	353	-113.0	9.9	6	
203-75		75	2.34	134	335	-109.6	9.9	3	
203-81	203	81	8.06	144	511	-127.6	-122.1	2.8	10.8
203-82		82	3.9	147	707	-129.0	1.8	3	
204-105	204	78	1.1	91	247	-117.1	10.3	6	
204-78		90	3.07	123	230	-124.5	-121.0	6.6	3.8
204-90		105	1.69	132	359	-121.3	7.3	6	
205-73	205	73	2.01	142	316	-122.7	5.8	3	
205-75		75	2.13	127	339	-125.2	-122.0	7.1	3.6
205-84		84	3.21	133	331	-118.0	4.5	3	
207-67	207	67	5.59	158	3370	-122.5	4.9	3	
207-72		72	2.11	136	1028	-124.7	-125.5	4.3	3.5
207-90		90	7.01	146	693	-129.3	1.5	3	
208-87	208	87	3.7	140	339	-110.4	-115.4	12.0	7.0
208-89		89	2.2	133	299	-120.3	6.1	2	
209-55		55	0.82	82	2001	-126.8	-126.8	2.1	-
210-43	210	43	1.38	83	310	-120.9	3.6	3	
210-50		50	4.28	151	89	-139.8	7.8	4	
210-70		70	4.71	143	410	-122.3	-127.9	4.9	8.4
210-71		72	3.79	128	485	-133.7	4.8	4	
210-72		74	3.63	136	383	-122.9	5.7	5	

## Figure captions

Figure 1: Structure of miliacin (olean-18-en-3 $\beta$ -ol methyl ether).

Figure 2: Location of *P. miliaceum* field, heterogeneity of soil properties on aerial picture (grey scale) and location of samples.

Figure 3: Chromatogram obtained by GC-irMS illustrating the distribution of miliacin and of a series of *n*-alkane standards ( $nC_{16}$ - $nC_{30}$ ) with  $\delta D$  values measured offline (acquired from Arndt Schimmelmann, Indiana University, Bloomington, USA) used for normalising miliacin  $\delta D$  values. \* indicates the 5 $\alpha$ -cholestane, used as quantitation standard.

Figure 4: a- Distribution of  $\delta D$  values of miliacin extracted from the seeds of 61 plants collected in the Mézières-lez-Cléry field sorted by increasing plant height, which was considered pertinent due to the visual correlation between plant height and soil properties in the field. Maximum and minimum miliacin  $\delta D$  values and the corresponding range (-98‰ to -144‰ V-SMOW; range of ~50‰ V-SMOW) are reported.; b- Frequency histogram of miliacin  $\delta D$  values of all plants with calculated mean ( $\mu$ ) and standard deviation ( $\sigma$ ); c-  $\delta D$  values and standard deviation of miliacin  $\delta D$  values averaged per stand, sorted by increasing plant height. Maximum and minimum of averaged miliacin  $\delta D$  values (from -105 to -135‰ V-SMOW; range of 30‰) are reported; d- Frequency histogram of miliacin  $\delta D$  values averaged per stand with calculated mean ( $\mu$ ) and standard deviation ( $\sigma$ ).

Figure 1

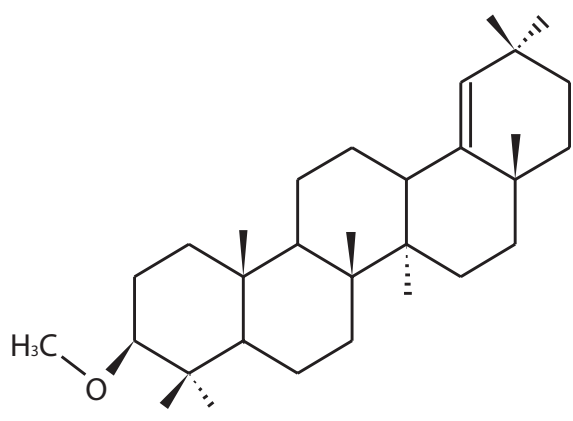


Figure 2

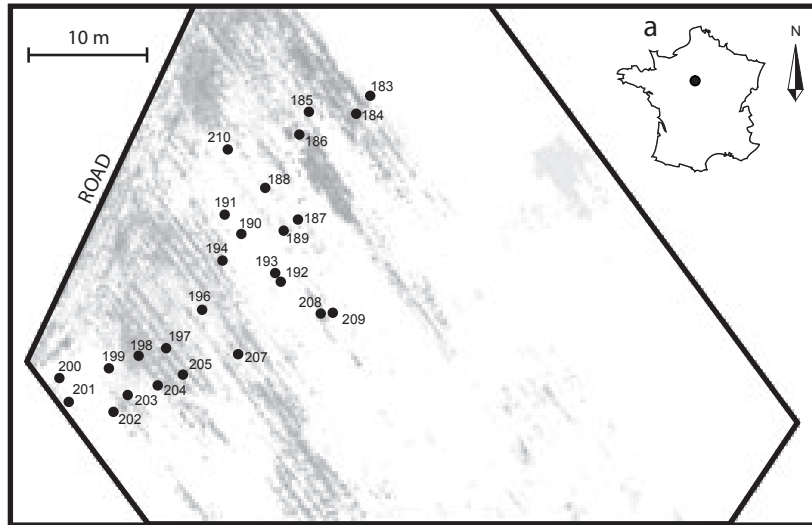


Figure 3

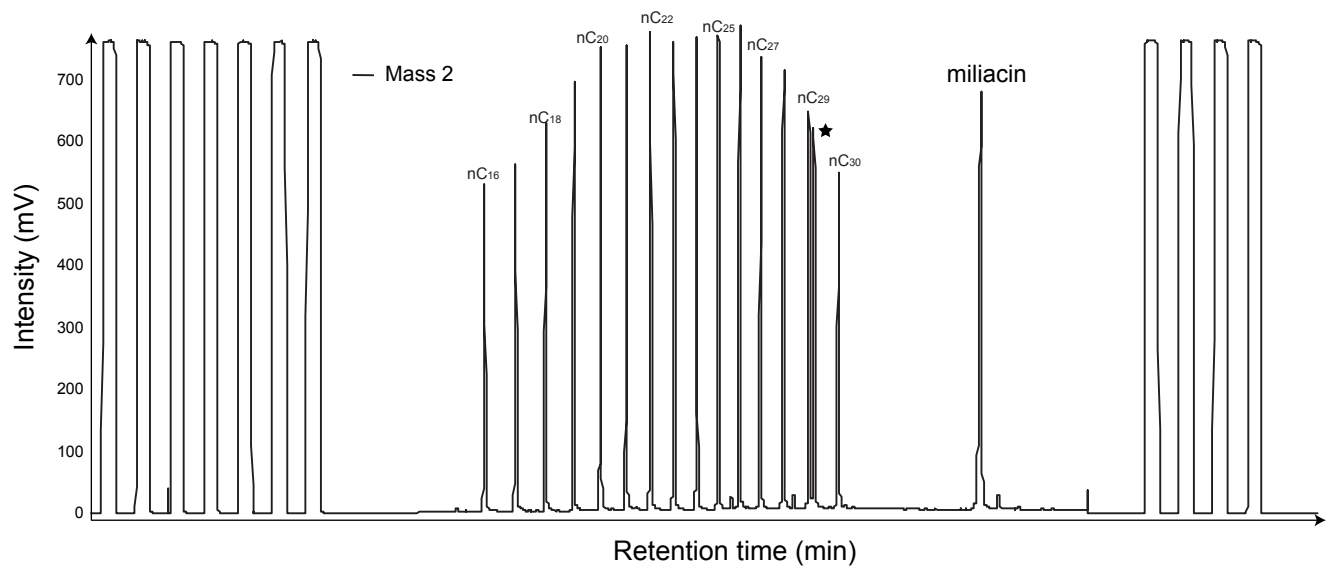


Figure 4

