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► **To cite this version:**

Eric Lichtfouse, Gersende Berthier, Sabine Houot, Enrique Barriuso, Valerie Bergheaud, et al.. Stable carbon isotope evidence for the microbial origin of C14-C18 n-alkanoic acids in soils. *Organic Geochemistry*, 1995, 23 (9), pp.849-852. 10.1016/0146-6380(95)80006-D . hal-00192937

**HAL Id: hal-00192937**

**<https://hal.science/hal-00192937>**

Submitted on 29 Nov 2007

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Revised version

Organic Geochemistry 23, 849-852, 1995.

doi:10.1016/0146-6380(95)80006-D

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## Stable carbon isotope evidence for the microbial origin of C<sub>14</sub>-C<sub>18</sub> *n*-alkanoic acids in soils

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**Abstract-** In order to delineate the origin of soil fatty acids, crop soil samples have been incubated for 21 days *in vitro* either with unlabelled or <sup>13</sup>C-labelled glucose. Analyses of C<sub>14</sub>-C<sub>32</sub> *n*-alkanoic acids from monocarboxylic acid fractions, as methyl esters, by gas chromatography-combustion-isotope ratio monitoring mass spectrometry (GC-C-IRMS) show that C<sub>14</sub>, C<sub>16</sub> and C<sub>18</sub> *n*-alkanoic acids are <sup>13</sup>C-labelled, thus demonstrating their derivation from soil microorganisms, e.g. fungi or bacteria, growing during the experiment. Higher *n*-alkanoic acids, C<sub>16</sub>-C<sub>33</sub> *n*-alkanes, and C<sub>22</sub>-C<sub>30</sub> *n*-alkanols have not been significantly labelled, thus suggesting their derivation from other sources, e.g. higher plants. This short-term tracer experiment using stable carbon isotopes represents a novel and fruitful approach to study organic matter transformations in soils and other systems such as sediments.

*Key words* - carbon-13, soil fatty lipids, *n*-alkanes in soils, *n*-alkanols in soils, *n*-alkanoic acids in soils.

### INTRODUCTION

Free or bound fatty acids widely occur in almost all living organism and also where organic matter is being decomposed, such as soils and sediments (Parker, 1969, Morrisson, 1969, Albrecht and Ourisson, 1971, Douglas *et al.*, 1971, Schnitzer and Neyroud, 1975, Seifert, 1977, Schnitzer and Khan, 1978, Perry *et al.*, 1979, Moucawi *et al.*, 1981, Mackenzie *et al.*, 1983, Gillan and Sandstrom, 1985, Grimalt and Saiz-Jimenez, 1989, Grimalt *et al.*, 1989, Dinel *et al.*, 1990, Amblès *et al.*, 1991, De Leeuw and Largeau, 1993, and refs. therein). The precise biological origin and the chemical processes leading to the accumulation of *n*-fatty acids within degraded organic matter are still speculative because 1) these substances may derive from a wide variety of biological precursors, some of which have undoubtedly not been identified yet, 2) the molecular structure of these substances is poorly informative, and 3) little is known about the degradation processes of organic matter. For instance, soil *n*-alkanoic acids may arise by direct input from plants or soil organisms, or by oxydation of soil aliphatic lipids such as *n*-alkanes, *n*-alkanols and *n*-alkanoic acids.

After the pioneering work by Parker (1961-62) on δ<sup>13</sup>C values of individual fatty acids from algae and marine grass, some reports suggest that such isotopic studies

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at the molecular level might bring some information on the origin and fate of fatty acids (Vogler and Hayes, 1980, Lichtfouse and Collister, 1992, Abrajano *et al.*, 1994). In soils and sediments, fatty acids are believed to derive from a wide variety of biological sources, such as plants, animals, algae, microbes and their degradation products (Perry *et al.*, 1979, Gillan and Sandstrom, 1985, Grimalt and Saiz-Jimenez, 1989, Grimalt *et al.*, 1989, Dinel *et al.*, 1990, Zelles *et al.*, 1992, Amblès *et al.*, 1994, and refs. therein). On the other hand, the distribution of fatty acids is currently used to quantitate the microbial biomass and even to subgroup individual bacterial species (Perry *et al.*, 1979, Gillan and Sandstrom, 1985, Zelles *et al.*, 1992, Thompson *et al.*, 1993). However, the applicability of such methods relies highly on the biological origin and transformation processes leading to the accumulation of fatty acids in soils and sediments. Also, in order to evaluate the possible microbial origin of soil fatty acids, versus other biological precursors such as higher plants, we wish to report here a simple short-term tracer experiment with  $^{13}\text{C}$ -labelled glucose.

### EXPERIMENTAL

Soil samples (120 g dry weight) from a crop field (Grignon, France) were sterilized with  $\gamma$  rays, then incubated for 21 days in the dark at pF 2 and 28°C with soil water from the same field and either distilled water (blank) or D-glucose "Glu" ( $\delta^{13}\text{C} = -10.60\text{‰}$ ) or D-glucose " $^{13}\text{C}$ -Glu" ( $\delta^{13}\text{C} \sim +500\text{‰}$ ). Soils were dried, finely ground then extracted with  $\text{CHCl}_3$ -MeOH (3/1 v/v). Alkane, alcohol and acid fractions were obtained from the extracts as described elsewhere (Lichtfouse *et al.*, 1994a). The alcohol fraction was acetylated with a large excess of acetic anhydride in pyridine (1/1, v/v) 1 h. at 50°C. After the addition of water and  $\text{CH}_2\text{Cl}_2$  (3x), the organic phases were mixed then washed with water (3x), dried overnight over  $\text{CaCl}_2$ , concentrated under reduced pressure and fractionated by thin layer chromatography on silica gel eluting with  $\text{CH}_2\text{Cl}_2$  (using cholesterol acetate as reference), to give a mono-acetate fraction ( $R_F \sim 0.7$ ). Acid fractions were treated overnight with an excess of diazomethane in diethyl ether. After concentration (ventilated hood), the residue was fractionated by thin layer chromatography on silica gel eluting with ethyl acetate-*n*-hexane (5/95 v/v; using octacosanoic acid methyl ester as reference), to give a mono-ester fraction ( $R_F \sim 0.5$ ). These derivatization reactions, and subsequent thin layer chromatographic steps, were found to be essential for the required chromatographic resolution needed for gas chromatography-combustion-isotope ratio-mass spectrometry (GC-C-IRMS) analysis (Lichtfouse *et al.*, 1991).

Relative abundances of alkanes, alkanols (as mono-acetates), and alkanolic acids (as monomethyl esters), were measured by gas chromatography. They were found to be similar for the incubation of distilled water (blank) and glucose. Carbon isotope analyses are described elsewhere (Lichtfouse *et al.*, 1994b). Isotopic compositions are summarized in Table 1 and are expressed in per mil. relative to the PDB standard:  $\delta^{13}\text{C} = [({}^{13}\text{C}/{}^{12}\text{C}_{\text{sample}}/{}^{13}\text{C}/{}^{12}\text{C}_{\text{std}}) - 1] \times 10^3$ , where  ${}^{13}\text{C}/{}^{12}\text{C}_{\text{std}} = 0.0112372$ .  $\delta^{13}\text{C}$  values of *n*-alkanes, *n*-alkanols and *n*-alkanoic acids from the incubation with distilled water (blank) and from the incubation with unlabelled glucose ("Glu") were found to be similar.  $\delta^{13}\text{C}$  values of alcohols and monocarboxylic acids, and their respective errors, have been corrected for the contribution of the added methyl group ( $\delta = -79.7 \pm 3.9\text{‰}$ ) and acetyl group ( $\delta = -50.1 \pm 1.8\text{‰}$ ), respectively, using pure isotopic standards (palmitic acid, arachidic acid, *n*-hexadecanol, cholesterol, 5 $\alpha$ -cholestan-3 $\beta$ -ol,

ergosterol). The  $\delta^{13}\text{C}$  value of the added acetyl group ( $-50.1\text{‰}$ ) is significantly different from that of the derivatization reagent, acetic anhydride ( $-29.3 \pm 0.1\text{‰}$ ). This important  $^{13}\text{C}$ -depletion, which is due to isotopic fractionation of the reagent, shows that derivatization of both the isotopic standards and the samples must be done under the same conditions (temperature, etc.) and preferably at the same time, to allow a correct calculation to be made of the  $\delta^{13}\text{C}$  value of the added carbon (see Rieley, 1994). The GC-C-IRMS analysis of *n*-alkanols and sterols as their trimethylsilyl ether derivatives has been previously reported by Jones *et al.* (1991).

Table 1.  $\delta^{13}\text{C}$  values for soil aliphatic lipids, in ‰ versus PDB.

Carbon Number	<i>n</i> -Alkanes			<i>n</i> -Alkanols			<i>n</i> -Alkanoic acids		
	Glu	$^{13}\text{C}$ -Glu	$\Delta\delta^*$	Glu	$^{13}\text{C}$ -Glu	$\Delta\delta^*$	Glu	$^{13}\text{C}$ -Glu	$\Delta\delta^*$
14							-28.4	-20.0	$\pm 0.9$
16	-27.1	-28.4	$\pm 0.6$				-25.0	-9.9	$\pm 0.9$
17	-29.4	-29.9	$\pm 0.6$						
18	-27.7	-28.1	$\pm 0.6$				-24.8	-16.9	$\pm 0.9$
20							-29.5	-27.5	$\pm 0.8$
21							-29.9	-30.3	$\pm 0.8$
22				-31.4	-31.1	$\pm 0.8$	-30.7	-30.1	$\pm 0.8$
23							-31.5	-32.0	$\pm 0.8$
24				-32.3	-32.2	$\pm 0.8$	-30.0	-30.3	$\pm 0.8$
25	-34.4	-33.0	$\pm 0.6$				-32.9	-32.1	$\pm 0.8$
26				-34.7	-34.3	$\pm 0.8$	-32.5	-32.8	$\pm 0.8$
27	-33.3	-32.6	$\pm 0.6$				-34.5	-34.2	$\pm 0.8$
28				-34.3	-34.2	$\pm 0.8$	-33.9	-34.1	$\pm 0.8$
29	-34.7	-34.9	$\pm 0.5$				-35.3	-34.9	$\pm 0.8$
30				-34.2	-34.5	$\pm 0.8$	-34.5	-35.1	$\pm 0.8$
31	-34.5	-34.9	$\pm 0.5$				-35.1	-35.0	$\pm 0.7$
32							-33.6	-34.7	$\pm 0.7$
33	-34.2	-34.6	$\pm 0.5$						

\*Experimental errors. For *n*-alkanols and *n*-alkanoic acids, these errors have been corrected for the contribution of the added carbon, in a similar way as that described by Rieley (1994). Noteworthy, the errors for acids decrease from  $\pm 0.9\text{‰}$  to  $\pm 0.7\text{‰}$  with increasing C number, highlighting the higher precision that should theoretically be gained when the acid carbon number increases.

## RESULTS AND DISCUSSION

The distribution of *n*-alkanes, *n*-alkanols and *n*-alkanoic acids from soils incubated 21 days either with distilled water (blank) or glucose are similar, showing the absence of alteration of soil biomarker profiles by the glucose experiments. The substances analysed after the glucose experiments are therefore good representatives of those from the initial soil. The isotopic composition of the *n*-alkanes, *n*-alkanols and

*n*-alkanoic acids from soils incubated either with glucose, or with  $^{13}\text{C}$ -labelled glucose, are reported in Table 1.  $\text{C}_{14}$ ,  $\text{C}_{16}$  and  $\text{C}_{18}$  *n*-alkanoic acids have incorporated  $^{13}\text{C}$ -enriched carbon (Figure 1), the highest enrichment being observed for the  $\text{C}_{16}$  acid (+ 8.4‰). A portion of these acids must therefore have been synthesized by soil microorganisms growing during the experiment. This finding is in good agreement with the occurrence of  $\text{C}_{14}$ - $\text{C}_{18}$  *n*-fatty acids in various microorganisms (Kolattukudy, 1976). An algal origin can be ruled out in our experiment because the glucose incubations were performed in the dark. Also the most probable biological precursors for  $\text{C}_{14}$ - $\text{C}_{18}$  *n*-fatty acids are bacteria, fungi and actinomycetes because these organisms are, with algae, the main soil microorganisms.

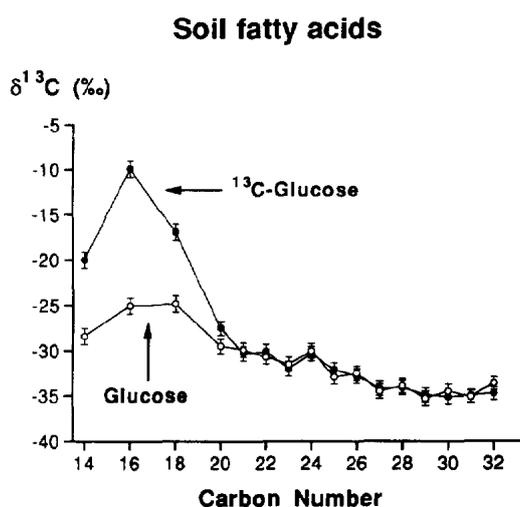


Figure 1. Isotopic composition of *n*-alkanoic acids from soils incubated either with glucose or  $^{13}\text{C}$ -glucose. Note the  $^{13}\text{C}$ -enrichment of  $\text{C}_{14}$ ,  $\text{C}_{16}$  and  $\text{C}_{18}$  *n*-alkanoic acids.

On the other hand, higher *n*-alkanoic acids, *n*-alkanes and *n*-alkanols have not been significantly labelled during the glucose incubation. Firstly, it is suggested that soil microorganisms are not their biological precursors, at least under the conditions of our experiment. Indeed, these substances are probably derived from organisms which were not grown during the experiment, as suggested by their occurrence, or the occurrence of their immediate precursors, in animals, algae and higher plants (Kolattukudy, 1976).

Secondly, special attention is called to the absence of labelling of the  $\text{C}_{16}$  and  $\text{C}_{18}$  *n*-alkanes. Indeed, these substances being the close biosynthetic equivalents of *n*-alkanoic acids, it can be inferred that the microorganisms synthesizing the  $\text{C}_{16}$  and  $\text{C}_{18}$  labelled acids during the glucose experiment did not produce their reduced counterparts in notable amounts. Secondly, the absence of isotopic labelling in the short-chain *n*-alkanes also suggests that their synthesis through abiotic reduction of their acid precursors has not taken place in our experiment, although this might not be true for longer time periods.

In addition, it should be noted that a significant  $^{13}\text{C}$ -enrichment of short-chain versus long-chain fatty acids occur also in the blank experiment (see the "Glucose" curve on Figure 1). For instance, the  $\text{C}_{16}$  and  $\text{C}_{18}$  acids show an enrichment of about +

10‰ relative to the C<sub>31</sub> acid. Since the results of the labelled experiment demonstrate a microbial origin for these compounds, this enrichment is apparently associated with their synthesis in the soil. It is therefore suggested that their microbiological precursors are consuming <sup>13</sup>C-enriched nutrients. The occurrence of carbon sources like sugars and amino acids fits particularly well with this first hypothesis because these nutrients are highly biodegradable, thus preferentially consumed, and are usually <sup>13</sup>C-enriched relative to the bulk carbon (Deines, 1980). Alternatively, isotope effects associated with assimilation, metabolism and biosynthesis are also possible, as discussed by Hayes (1993). Whatever mechanisms cause the <sup>13</sup>C-enrichment of short-chain versus long-chain acids in the blank sample, the labelled experiment has clearly disclosed that these compounds are of microbial origin.

### CONCLUSION

It has been demonstrated, using isotope tracers, that C<sub>14</sub>, C<sub>16</sub> and C<sub>18</sub> soil *n*-alkanoic acids derive, at least partly, from soil microorganisms. Incubations of soils, or sediments, with <sup>13</sup>C-labelled substrates, followed by <sup>13</sup>C isotope analysis at the molecular level, represent a novel and promising way to study the origin and fate of biological markers.

*Acknowledgements*- We thank J.-N. Rampon and G. Bardoux for technical assistance. This work was supported by the *Institut National de la Recherche Agronomique (Action Intégrée Prioritaire "Écodynamique des substances à caractère polluant")*.

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