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^{13}C -dating, the first method to calculate the relative age of molecular substance homologues in soil

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Abstract

This article reports the design of ^{13}C -dating, the first method to calculate the relative age of molecular substance homologues occurring in fractions from the same soil sample. Soil is a major carbon pool impacting modern climate by CO_2 release and uptake. Molecular substances that sequester carbon in soils are poorly known due to the absence of methods to study molecular-level C dynamics over agricultural time scales, e.g. 0-200 years. Here I design a method to calculate the relative age of the plant-derived C_{31n} -alkane occurring in 6 fractions from a soil sample naturally ^{13}C -labelled by maize cropping during 23 years. Soil fractions are the bulk soil extract, two humin-encapsulated fractions and three particle-size fractions. Results show that C_{31n} -alkane homologues have relative ages ranging from -6.7 years for the humin-encapsulated homologue to +25.1 years for the 200-2000 μm fraction homologue. Such a wide variation of 31.8 years evidences temporal pools of molecular substances in soil. This finding also reveals that physical encapsulation can strikingly change the dynamics of a single molecular substance. ^{13}C -dating thus allows to assess the carbon storage potential of molecular substances from crop soils. Such knowledge will help to identify molecular compounds, associated soil pools and agricultural practices that favour carbon sequestration. ^{13}C -dating is further applicable to any environmental sample containing organic matter subjected to a ^{13}C isotope shift with time. ^{13}C -dating will also help to study the sequestration and delayed release of chemicals in various disciplines, such as pollutants in environmental sciences, pharmaceuticals in medicine, and nutrients in food science.

Keywords Soil organic matter; Humin; ^{13}C ; C sequestration; Maize wax n-alkane; Agriculture; Dating method; Physical sequestration; Encapsulation; Pollutants; Pharmaceuticals; Food science

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Introduction

Molecular issues

Soil is a major carbon pool impacting climate change by CO₂ release and uptake (Lal, 2010, Stavi and Lal, 2011, Lichtfouse et al. 2009, 2010, 2011). Agricultural practices can either sequester or deplete soil carbon in the long run (Benbi and Brar, 2009, Hazarika et al. 2009, Doumbia et al. 2009, Halberg et al. 2010, Novak and Fiorelli, 2010, Pati et al., 2010, Lichtfouse et al., 2011). Geoengineering using for instance biochar addition to soil is a potential technique to sequester soil carbon and in turn decrease atmospheric CO₂ levels (Sohi et al. 2010). However mechanisms that control carbon sequestration are not fully understood because knowledge of the dynamics of molecular substances occurring in dead organic matter is scarce (Marschner et al., 2008). Dead matter indeed contain very complex mixtures of organic substances inherited from the decay of living matter over short to long time periods (Schnitzer 1991, Hedges et al., 2000, Ohkouchia and Eglinton, 2008). In soils, although ¹⁴C-dating of bulk carbon (Wan et al., 1996) suggests the existence of organic substances aged up to several thousand years, dynamics at the molecular level are poorly known (Lichtfouse, 1995, 1997, 1999, Lichtfouse et al., 1998a,b, Hedges and Oades, 1997, Bull et al. 2000, Gleixner et al., 2002, Wiesenberg et al., 2004, Freier et al., 2010, Rubino et al., 2010). Such knowledge would help to define agricultural and land management practices that will favour carbon sequestration and, in turn, offset global warming.

Sequestration in temporal pools

Sequestration of an individual substance in different pools of a same sample is a poorly known phenomenon despite major potential implications for pollutant bioavailability and plant nutrient release (Figure 1) (Kubicki and Apitz, 1999, Sutton and Sposito, 2005). Given the extreme complexity of the soil matrix, individual substance homologues may indeed occur in the same soil sample within different physical and temporal pools, and thus can have various ages and turnovers (Lichtfouse, 1999). For instance a free substance should be younger than the bound homologue encapsulated in humin, the macromolecular organic network of soils. A such hypothesis stems from reports showing for example the unexpected preservation of proteins in sediments (Knicker and Hatcher, 1997). The existence of temporal pools of molecular substances is of fundamental importance to understand the mechanisms of carbon cycling at the molecular level. The existence of temporal pools is also of applied importance to identify which individual substances and associated pools contribute to carbon sequestration. A suitable method is therefore necessary to assess the relative age of individual substances.

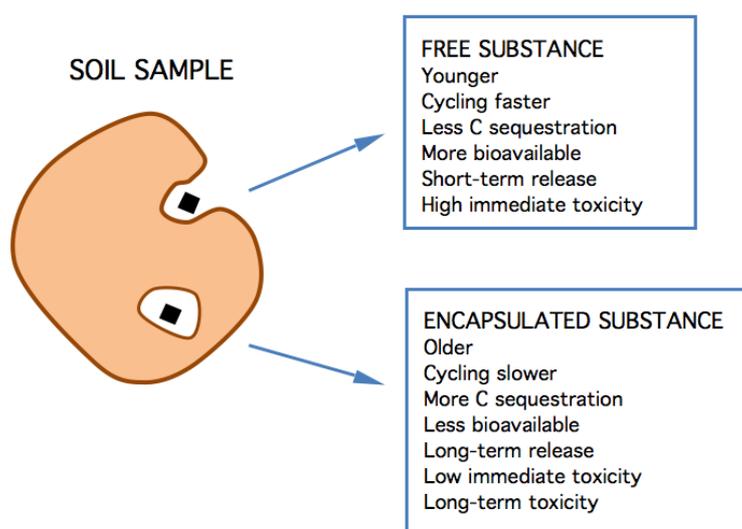
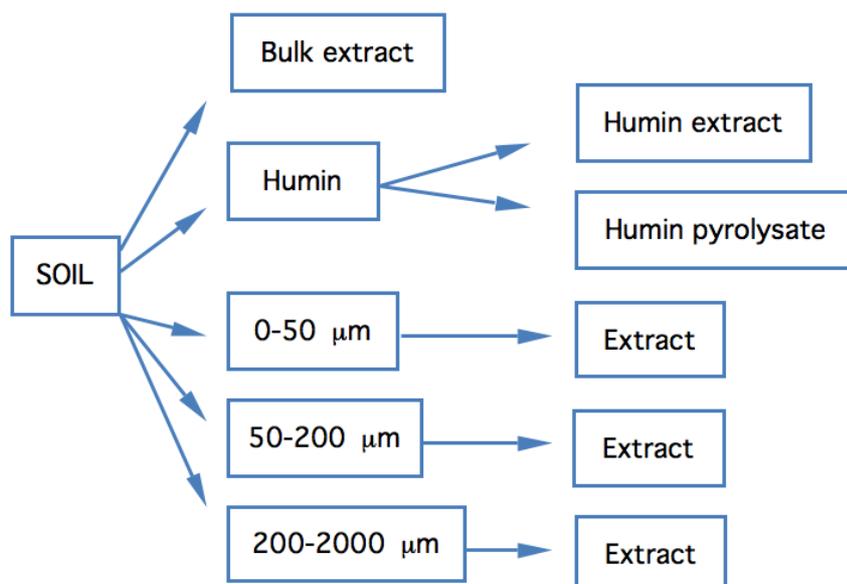


Figure 1. Encapsulation of molecular organic substances in soil. This scheme shows the consequences of physical encapsulation on the behaviour of organic substance homologues. Such consequences are relevant for carbon sequestration, plant nutrient storage and long-term pollutant release in waters, for example. The concept of encapsulation is also relevant for the delayed release of encapsulated pharmaceuticals and food substances (Mishra et al., 2010, Fang and Bhandari 2010).



Fractions	$\delta^{13}\text{C}$ (‰)	Maize-C (%)	t_1 (years)	Relative age (years)
Bulk soil extraction	-28.1 ^c	50.33	23	0
Humin-encapsulated ^a	-29.7 ^c	39.74	16.3	- 6.7
Humin-encapsulated ^b	-29.7 ^c	39.74	16.3	- 6.7
0-50 μm fraction	-27.3 ^c	55.63	26.2	+ 3.2
50-200 μm fraction	-24.3 ^c	74.50	44.1	+ 21.1
200-2000 μm fraction	-24.0 ^c	77.48	48.1	+ 25.1

a) $\text{C}_{31}n$ -alkane homologue from the humin extract, b) $\text{C}_{31}n$ -alkane homologue from the humin pyrolysate, c) data from Lichtfouse 1995, 1997, 1999, Lichtfouse et al. 1998b, Cayet and Lichtfouse, 2001. A possible pyrolytic carbon contribution to the humin-encapsulated $\text{C}_{31}n$ -alkane released by pyrolysis (b) can be excluded as explained previously (Lichtfouse et al. 1998a,b). Indeed gas chromatography analysis of the pyrolysate alkane/alkene fraction show that C_{27} - $\text{C}_{33}n$ -alkanes have a strong odd-number predominance, typical of plant waxes, without n -alk-1-ene homologues typical of pyrolytic products (Lichtfouse et al. 1998a). Whereas C_{11} - C_{26} show no odd/even predominance and have n -alk-1-ene homologues typical of pyrolytic products. In other words, pyrolysis is a means to release compounds that are physically encapsulated, that is without strong chemical bonds. Here the trick is to find a means to distinguish pyrolytic versus encapsulated homologues.

Figure 2. Top: fractionation of a soil sample into 6 fractions containing plant-derived n -alkanes. Bottom: relative age of soil $\text{C}_{31}n$ -alkane homologues

Dating at the molecular level

^{14}C -dating can date organic matter aged from hundreds to thousands of years, and has been successfully applied at the molecular level in sediments (Pearson and Eglinton, 2000, Pearson et al., 2001, Ohkouchia and Eglinton, 2008). Nonetheless ^{14}C -dating is less suitable to study the turnover of organic matter over short time periods relevant to agriculture and climate change, e.g. from 0 to 200 years. This issue is due both to the global ^{14}C contamination of modern samples by atomic bomb testing around 1950 (Harkness et al., 1986) and to the higher standard deviation of ^{14}C -dating versus the rapid turnover of recent organic material in soils (Trumbore, 2000). Alternatively, ^{14}C -spiking of tagged compounds is another technique used for instance to study pesticide biodegradation (Barriuso et al., 2008). However ^{14}C -spiking is less representative of native compounds because spiking in itself does not represent the natural behaviour of native compounds such as plant-derived substances found in crop soils. Therefore a method is needed to assess the dynamics of individual soil organic substances over short time periods. Here I report a novel method, named ^{13}C -dating, designed by conceptualising previous findings (Lichtfouse 1995, 1997, 1999, Lichtfouse et al. 1998b, Cayet and Lichtfouse, 2001). ^{13}C -dating allows to measure the relative age of organic substances from dead organic matter that has been ^{13}C -labelled at natural abundance.

Experimental

Detailed experimental procedures and data are given elsewhere (Lichtfouse 1995, 1997, 1999, Lichtfouse et al. 1998b, Cayet and Lichtfouse, 2001). Briefly, soils were sampled in an experimental maize crop field in France. Cropping maize, a naturally ^{13}C -enriched plant, is a means to follow the fate of plant carbon into soil organic matter. In this article I discuss data from a soil sample cultivated 23 years with maize. The C_{31} soil *n*-alkane, a compound derived from plant waxes, was analysed in 6 fractions from the same soil sample. Fractions are the bulk soil extract; the humin-encapsulated *n*-alkane fraction released either by extraction or pyrolysis; and *n*-alkanes in 0-50 μm , 50-100 μm , and 200-2000 μm particle-size fractions (Figure 2).

The bulk soil extract was obtained by CHCl_3 -MeOH 3/1 (v/v) extraction, then was fractionated by several steps of silica gel chromatography to yield an alkane fraction suitable for ^{13}C analysis (Lichtfouse, 1997). Humin was obtained by treatment of the CHCl_3 -MeOH pre-extracted soil sample by HF/HCl to remove minerals (Lichtfouse et al., 1998b). Two pools of encapsulated alkanes were isolated from humin: a pool of alkanes occurring in the CHCl_3 -MeOH humin extract, and a pool released by pyrolysis of the CHCl_3 -MeOH pre-extracted humin. Particle-size fractions were isolated from the soil sample by sieving in water followed by density separation (Cayet and Lichtfouse, 2001). Alkane fractions were obtained from particle-size fractions by CHCl_3 -MeOH extraction followed by several steps of silica gel chromatography. Alkane fractions were then analysed for $\delta^{13}\text{C}$ values by gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS). I reviewed the main applications of compound specific isotope analysis (Lichtfouse, 2000).

Results and discussion

Turnover of soil molecular substances

The principle of ^{13}C -dating at the molecular level is built on previous data from ^{13}C -labelling cultivation experiments (Lichtfouse 1995, 1997, 1999, Lichtfouse et al. 1998b, Cayet and Lichtfouse, 2001). Specifically, cropping maize, a naturally ^{13}C -enriched C_4 plant, on soils previously cultivated with naturally ^{13}C -depleted C_3 plants, e.g. wheat, slowly incorporates ^{13}C -enriched plant matter in soil (Figure 3). As a consequence the C_{31} maize leaf wax *n*-alkane of $\delta^{13}\text{C}$ -20.6‰ is progressively introduced into the soil and mix with the ^{13}C -depleted C_{31} soil *n*-alkane of $\delta^{13}\text{C}$ -35.7‰. The $\delta^{13}\text{C}$ value of soil C_{31} *n*-alkane thus increases from -35.7‰ to -28.1‰ after 23 years of maize cropping (Lichtfouse 1995). A such isotope shift allows to calculate the percentage of maize-derived carbon within the soil C_{31} *n*-alkane:

$$\text{Equation 1} \quad M = 100 \cdot (\delta - \delta_0) / (\delta_m - \delta_0)$$

Where M is the percentage of maize-derived C in the soil substance, δ denotes the $\delta^{13}\text{C}$ value of the soil substance at a given time of maize cultivation, δ_0 depicts the $\delta^{13}\text{C}$ value of the soil substance before maize cultivation (-35.7‰), and δ_m refers to the $\delta^{13}\text{C}$ value of the maize substance (-20.6‰). This calculation shows that the soil $\text{C}_{31}n$ -alkane contains 50.3% of maize-derived carbon after 23 years of calculation. Therefore about half of the soil $\text{C}_{31}n$ -alkane is renewed after 23 years. The dynamics of the soil $\text{C}_{31}n$ -alkane can be further modelled using a first-order kinetic law (Lichtfouse 1997):

$$\text{Equation 2} \quad M = 100 \times (1 - \exp(-0.031t_1))$$

Where M refers to the percentage of maize-derived C in the soil substance and t_1 denotes the time of maize cultivation in years. Accordingly the complete renewal of 99% of the soil $\text{C}_{31}n$ -alkane will last 148 years. This finding represented the first determination of the turnover of soil carbon at the molecular level (Lichtfouse 1997).

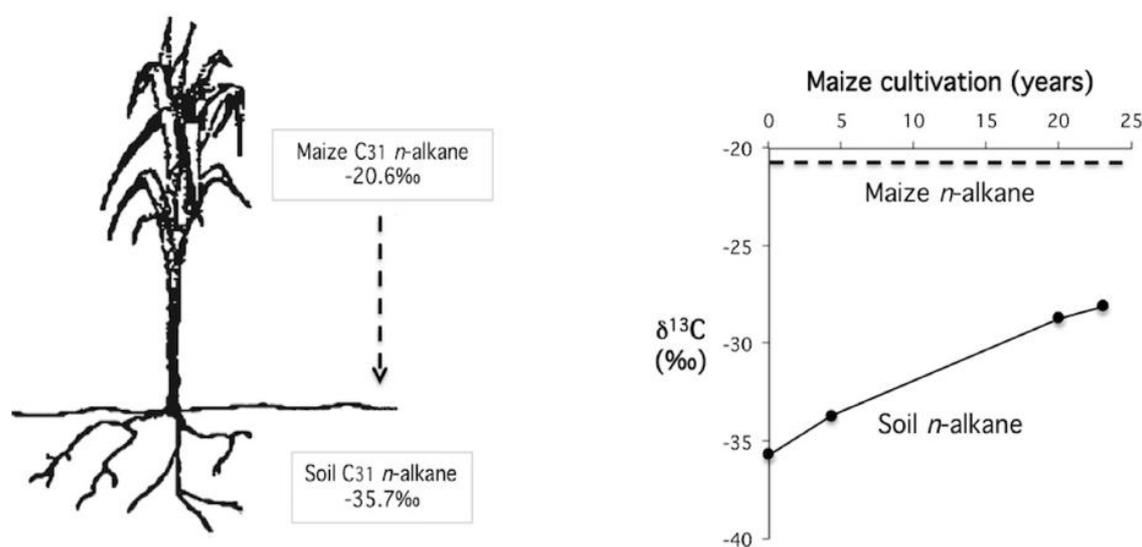


Figure 3. Labelling individual soil organic compounds at natural abundance. Maize (*Zea mays*), a naturally ^{13}C -enriched plant, is cultivated on a soil which was previously cultivated with ^{13}C -depleted plants. The C_{31} leaf wax n -alkane of $\delta^{13}\text{C}$ -20.6‰ , is progressively introduced into the soil and mix with the ^{13}C -depleted C_{31} soil n -alkane ($\delta^{13}\text{C}$ -35.7‰). The isotope value of the soil n -alkane therefore increases over years of maize cultivation (Lichtfouse, 1995).

Physical encapsulation of substance homologues

Access to the turnover further allows to measure the relative age of substance homologues occurring in 6 fractions from the same soil sample (Figure 2). The free $\text{C}_{31}n$ -alkane is isolated from the bulk soil by solvent extraction (Lichtfouse 1995, 1997). The encapsulated $\text{C}_{31}n$ -alkane homologue is isolated from humin either by extraction or pyrolysis (Lichtfouse et al. 1998b, Lichtfouse 1999). $\text{C}_{31}n$ -alkane homologues are also extracted from soil particle-size fractions (Cayet and Lichtfouse, 2001). Previous occurrence of substance homologues in compartments of soils and modern sediments were suspected but not proven due to the lack of method allowing to distinguish compounds of identical molecular structure. This issue is solved here by measuring different ^{13}C isotope ratios for $\text{C}_{31}n$ -alkane homologues occurring in 6 fractions from a soil cultivated 23 years with maize (Figure 2).

Results show a wide range of $\text{C}_{31}n$ -alkane $\delta^{13}\text{C}$ values from -29.7‰ for the humin encapsulated homologue to -24.0‰ for the homologue in the 200-2000 μm particle-size fraction. As a consequence the oldest $\text{C}_{31}n$ -alkane homologue is encapsulated into humin and contains only 39.74% of maize C, whereas the youngest $\text{C}_{31}n$ -alkane homologue occurs in the 200-2000 μm particle-size fraction and contains 77.48% of maize C. Such a large difference demonstrate unambiguously for the first time that the turnover of an individual substance vary widely depending on its physical localisation within the soil matrix. This finding implies also that the stability and degradation of substances in soils cannot be explained solely by their molecular

structure, but should take physical protection into account. Physical encapsulation of organic molecules was previously hypothesised but definitive proof was lacking. Here the lower maize-C content of *n*-alkane homologues evidence the mechanism of physical encapsulation at the molecular level.

A possible effect of biodegradation on the ^{13}C -dating method should be considered. Indeed, in theory, free compounds should be more accessible to microbes and should thus be more biodegraded than bound compounds. If biodegradation occurs, then the $\delta^{13}\text{C}$ values may be changed if biodegradation induces $^{13}\text{C}/^{12}\text{C}$ isotope fractionation. However petroleum biodegradation experiments using microbial communities show the absence of $\delta^{13}\text{C}$ shift of alkanes (Mazeas et al. 2002). A further comparison of petroleum samples of increasing degree of biodegradation also shows the absence of ^{13}C isotope shifts of *n*-alkanes (Sun et al., 2005). In addition a 23-year decomposition experiment of *Calluna vulgaris* shoots using litter-bags buried in a peat bog shows the absence of $\delta^{13}\text{C}$ shifts of *n*-alkanes despite 90% weight loss of the plant shoot biomass (Huang et al. 1997). To conclude, a possible effect of biodegradation on the ^{13}C -dating method due to a ^{13}C shift can be reasonably excluded for the results of this article.

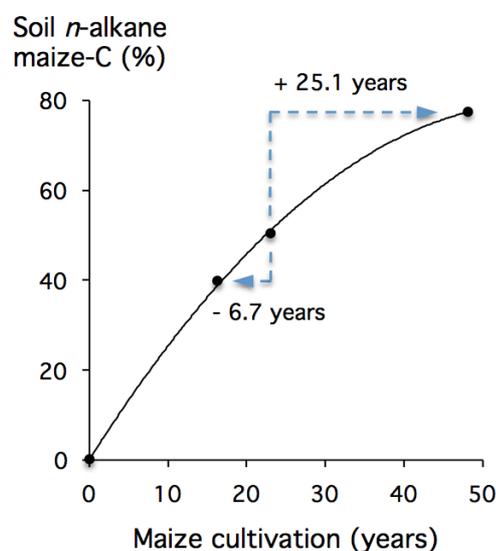
Relative age

The relative age of C_{31}n -alkane homologues occurring in different fractions from the same soil can be calculated according to:

$$\text{Equation 3} \quad \text{Relative age} = t_1 - t_2$$

Where t_1 refers to the time of maize cultivation calculated using kinetics of maize C content in the free C_{31}n -alkane from the bulk soil (Equation 2). t_2 denotes the time of maize cultivation of the soil sample, of 23 years in our example (Figure 2). In other words t_1 represents the cultivation time at which the C_{31}n -alkane from soil fractions, e.g. humin-encapsulated and particle sizes, would have the same maize C content as the free C_{31}n -alkane from the bulk soil extraction (see arrows in Figure 4). For instance the humin-encapsulated C_{31}n -alkane from the soil cultivated 23 years with maize has a maize C content equal to free homologue from the soil cultivated 16.3 years, thus yielding a relative age of -6.7 years. Similarly the C_{31}n -alkane from the 200-2000 μm particle-size fraction of the soil cultivated 23 years with maize has a maize C content equal to free homologue from the soil cultivated 48.1 years, thus yielding a relative age of +25.1 years. Those findings bring unambiguous evidence for temporal pools of individual substance homologues in soil (Lichtfouse 1999). The wide age difference of 31.8 years between the two *n*-alkane fractions reveals that physical encapsulation can strikingly change the dynamics of a molecular substance. To conclude, ^{13}C -dating is a novel method to calculate the relative age of organic substance homologues in soils.

Figure 4. Principle of ^{13}C -dating calculation of the relative ages of C_{31}n -alkane homologues in two soil compartments, the humin-encapsulated and the 200-2000 μm particle-size fraction, from a soil cultivated 23 years with maize. The kinetic curve refers to equation 2 (Lichtfouse, 1997). Note that the C_{31}n -alkane encapsulated in humin is 6.7 years younger than the C_{31}n -alkane in the bulk soil extract. Note also that the C_{31}n -alkane from the 200-2000 μm particle-size fraction is 25.1 years younger than the C_{31}n -alkane in the bulk soil extract. Those findings demonstrate both physical encapsulation and the occurrence of temporal pools at the molecular level. The wide age difference of 31.8 years between the two *n*-alkane fractions reveals that physical encapsulation can strikingly change the dynamics of a molecular substance.



Implications

^{13}C -dating is a method requiring the occurrence of an isotope shift with time, either at natural abundance or by ^{13}C -labelling. This method is particularly useful over short-time periods of 0-200 years where ^{14}C -dating is less suitable. ^{13}C dating is applicable to study individual substances from complex media such as soils, palaeosols, sediments, sewage sludge's, and organic matter from waters and airborne particulates. Noteworthy, ^{13}C dating should not be used to measure absolute ages because ^{13}C dating is based on kinetic modelling. Concerning climate change ^{13}C -dating should be particularly useful to identify molecular compounds and associated fractions that sequester carbon over decades to centuries. A such knowledge would indeed help to define sustainable agricultural systems that are sequestering carbon. ^{13}C -dating can also be applied to study the mechanisms of pollutant trapping in complex environmental samples (Lichtfouse et al., 1998b). Such knowledge would be useful to understand for example why herbicides are still released in waters years after the herbicide ban (Barth et al. 2009). Understanding pollutant trapping at the molecular level is also needed to define agricultural practices that reduce the use of pesticides (Alletto et al., 2010). ^{13}C dating can also be used to study the turnover of substances in biological samples (Lichtfouse, 2000). For instance this non-radioactive technique would allow to investigate the metabolism of naturally ^{13}C -enriched maize-derived food substances or ^{13}C -tagged pharmaceuticals and food substances in the human body and other living organisms. Indeed the concept of encapsulation is highly relevant to medicine and food science (Mishra et al., 2010, Fang and Bhandari 2010).

Conclusion

^{13}C -dating allows to calculate the relative age of substance homologues occurring in various pools from the same sample. Results show large variations from -6.7 to +25.1 years of the relative age of the C_{31n} -alkane in 6 fractions from the same soil sample. Such wide age difference of 31.8 years reveals that physical encapsulation can strikingly change the dynamics of a molecular substance. ^{13}C -dating will thus be useful to identify substances and associated fractions that sequester carbon in soils. ^{13}C -dating is potentially applicable to study the dynamics of organic substances occurring in complex media subjected to an isotope shift over time, such as sediments, water, sludge's, airborne particulates and biological samples. ^{13}C -dating is also potentially applicable to study the delayed release of molecular substances in medicine and food science.

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