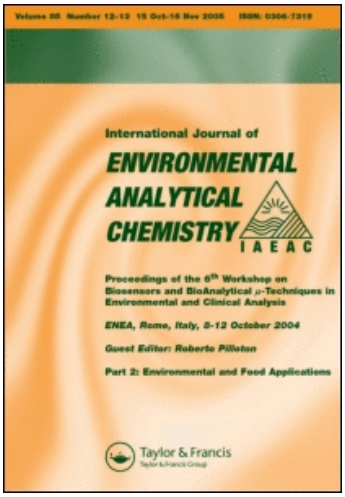

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Unsubstituted and Hydroxy Substituted Fatty Acids in a Recent Lacustrine Sediment

(Lake Léman, Geneva, Switzerland)

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Dedicated to Professor W. Haerdi on the occasion of his 60th birthday

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Unsubstituted as well as α-, β-, ω- and (ω−1)-hydroxy substituted fatty acids were analyzed in a 5 m long sediment core taken from Lake Léman (Switzerland). All these acids occur in three forms: unbound, bound and tightly bound and our results show that there is no conversion from one form to another. The fact that below a burial depth of 30 cm the abundance profiles show no decreasing trend indicates that the diagenetic reactions do not operate below that depth. On the basis of our results, compared with other published data, source correlations are postulated for each of the acid classes. The presence of unsubstituted monounsaturated acids in the C_{20} to C_{32} range probably indicates that long chain fatty acids cannot be considered only as indicators of inputs from higher plants. Finally, C_{27}, C_{29} and C_{31} (ω−1)-hydroxy-acids with unusual structures have also been found in these sediments, as well as 2-methyl nonacosanoic acid; their origin has not yet been established.

KEY WORDS: Sediments, lacustrine, fatty acids, hydroxyacids, biological markers.

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INTRODUCTION

Fatty acids in recent and ancient lacustrine sediments are biological markers which supply information about the paleoenvironmental conditions of the sedimentation basin and the type of organisms living or having lived in a lake and its surroundings.

It has been established that sedimentary organic matter can be divided into an unbound and a bound form, which differ in quality as well as in quantity.[1-3] Recently, another form, called "tightly bound", has been defined.[4,5] It contains mainly fatty acids which are linked to polymeric structures of the bacterial cell walls by amide bonds.[6,7] The unbound acids can be extracted with organic solvents, but the bound ones, which are supposed to be attached to organic polymeric structures by ester bonds, need first a mild hydrolysis. Concerning the tightly bound acids, they are released only after a strong alkaline[5] or acid[6,7] hydrolysis.

We have recently shown that the hydrolysis conditions play an important role on the yields and distributions of the fatty acids recovered from recent sediments and have devised an analytical scheme in order to efficiently extract the bound and the tightly bound acids.[6,7] Using this method, we report here the results of the study of a core from Lake Léman, a post-glacial lake which has been oligotrophic since its formation, some 15000 years ago, but is presently evolving into a mesotrophic status, as is apparent from an examination of the top 15 cm sediments.[8] Our study deals only with sediments deposited before this evolution started. The main objective was to examine the three forms under which the fatty acids occur throughout the sediment core, in order to gain a better understanding of their origins and possible transformations.

EXPERIMENTAL

A 5 m long sediment core was taken from Lake Léman (area: 582 km^2, volume: 89×10^9 m^3, maximum depth: 310 m, altitude: 372 m), under a 25 m water column, using a pneumatic corer, in a zone where the water–sediment interface remains permanently oxic.[8] The core was halved and frozen at -20 °C until analysis. Sections of 10 cm were cut off from one of the halves, the other being laid by.

The centers of the analyzed sections are situated at 0.30, 0.90, 1.80, 2.47, 3.28, 3.62 and 4.42 m depth. The samples were extracted as follows:

The sediment was acidified to pH 3–4, nonadecanoic acid was added as internal standard, and the mixture was filtered. The aqueous phase was then extracted twice with methylene chloride and the remaining sediment was submitted to ultrasonication twice in acetone and twice in methylene chloride. After evaporation of the acetone solutions, containing the interstitial water, the aqueous residues were extracted with methylene chloride. All the methylene chloride extracts were then combined and evaporated *in vacuo* at low temperature. The organic residue was separated on a 15 cm long, 1 cm i.d. column filled with KOH impregnated SiO_2. 3-Hydroxyheneicosanoic acid methyl ester was added as internal standard to the fraction containing the acids before esterification with BF_3/methanol (Fluka AG, Switzerland). The resulting esters were separated by *flash chromatography* on a 20 cm long glass column with an i.d. of 0.6 cm, filled with SiO_2 and using a N_2 pressure of 0.3 to 0.5 bar. The unsubstituted fatty acids were eluted with 50 ml of a 3:1 hexane/methylene chloride mixture, and the hydroxy and dicarboxy acids were eluted with a 9:1 methylene chloride/ethyl acetate mixture. This procedure yields the **unbound** unsubstituted (fraction #1) and hydroxylated (fraction #2) acids. The residual material was submitted to a mild saponification with 5% KOH in methanol during 12 hrs at 100 °C; the internal standards were then added and the sample was extracted as already described. This procedure gives the **bound** fractions #1 and #2.

The remaining sediment was then hydrolysed at 120 °C in 6N HCl during 12 hrs. The pH of the solution was adjusted to 12–13 with KOH and refluxed during 4 hrs. After the addition of the reference compounds, the extraction was performed as described, yielding the **tightly bound** fractions #1 and #2.

The fractions containing the hydroxyacids (#2) were trimethylsilylated with BSTFA (Fluka AG) and analyzed by GC using a Carlo Erba 5300 Mega Series instrument. The abundances of the fraction #1 compounds were measured by comparing the FID areas of the different ester peaks with that of the methyl nonadecanoate peak. Because of the complex chromatographic patterns, the fraction #2 hydroxyacids required a mass fragmentographic analysis. The

GC–MS measurements were performed on a Finnigan 4023 system. All the chromatographic separations were obtained with glass capillary columns (Duran, 0.32 mm × 20 m), coated with OV-73 (Alltech), after splitless injection. The carrier gas (He) pressure was 0.75 bar and the injector and detector temperatures 290 °C. The β-hydroxyacids were quantitated by GC–MS fragmentography of the m/z 175 peaks; the areas were compared to that of the m/z 175 peak of the TMS ether of the β-hydroxyheneicosanoic acid methyl ester used as internal standard. The other hydroxyacids we have studied, i.e. the α-, ω- and (ω − 1)-hydroxyacids, could not be quantitated by the same method because no suitable reference compounds were available. For these acids, we therefore defined "abundance units" in the following way:

α-hydroxyacids: [alpha units]

$$[\text{alpha unit}] = \frac{([\text{M-59}] \times \text{C})}{\text{A}^{175}}$$

where:

[M-59] = area of the $(\text{M-59})^+$ peak of the considered α-OH acid.
C = concentration of the C_{21} β-OH reference compound (μg/g dry sediment).
A^{175} = area of the m/z 175 peak of the C_{21} β-OH reference compound.

The other units, [omega units] and [omega − 1 units], were defined in the same way, using the $(\text{M-47})^+$ and the m/z 117 peak areas respectively. With these abundance units, the importance of the various hydroxyacids in the sediment profiles could be followed for each of the three forms of occurrence.

RESULTS AND DISCUSSION

Unsubstituted acids

The unsubstituted acids were analyzed in the C_{12} to C_{32} range. They include *normal*, branched *iso-* and *anteiso-*, and some mono-unsaturated acids. Polyunsaturated acids, like $C_{18:2}$, were also present in some sections of the core, but only in trace amounts.

Finally, a 2-methyl branched acid was detected in the three forms all along the core. Its molecular weight corresponds to that of a saturated C_{30} compound (ECL = 29.27).

All the distributions show a bimodal pattern, the first mode being centered on C_{16}–C_{18} and the second on C_{24}–C_{26} (Figure 1). The distribution patterns are similar throughout the core in the three fractions, but significant differences are observed in the relative importances of the two modes. Figure 2 shows the change of the abundances of diverse subclasses of unsubstituted fatty acids with the sediment profile in the unbound, the bound, and the tightly bound fractions.

The evolution of the total fatty acid abundances are more or less the same in the three fractions, with a maximum at −3.3 m. The low molecular weight ($C_{13:0}$ to $C_{20:0}$) and the high molecular weight ($C_{22:0}$ to $C_{32:0}$) saturated acids (LFA and HFA) show however differences in their relative abundances in the three fractions. The LFA are more abundant than the HFA in the unbound fractions while the reverse occurs in the bound and tightly bound fractions.

The short chain unsaturated acids ($C_{16:1}+C_{18:1}+C_{18:2}$) are more abundant in the bound fractions, where they show a depth profile similar to that of the LFA. This is not the case for the long chain unsaturated acids ($C_{22:1}$ to $C_{32:1}$) which are present in about the same abundance in the three fractions; their depth profiles resemble those of the HFA. Their relative amounts, compared to the corresponding saturated analogs, show only little variations throughout the core, except for the deepest sections, as illustrated by the ratio HU/HFA in Figure 2.

Iso- and *anteiso-* branched chain fatty acids (BFA) were essentially found in the bound fractions. When expressed in relative amounts versus LFA, their abundances are similar in the bound and tightly bound fractions. The BFA/LFA ratio variations are more or less the same in the three fractions. Finally, the 2-methyl-C_{30} acid shows different abundance profiles in the three fractions; they do not resemble any of those obtained for the above mentioned subclasses.

If, as is generally accepted, the BFA represent bacterial, the HFA higher plant and the LFA mainly autochthonous inputs,[3] our results indicate that the unbound acids essentially originate from plankton. In fact, in this fraction and at any depth, the BFA/LFA and the HFA/LFA ratios are significantly lower than in the two other

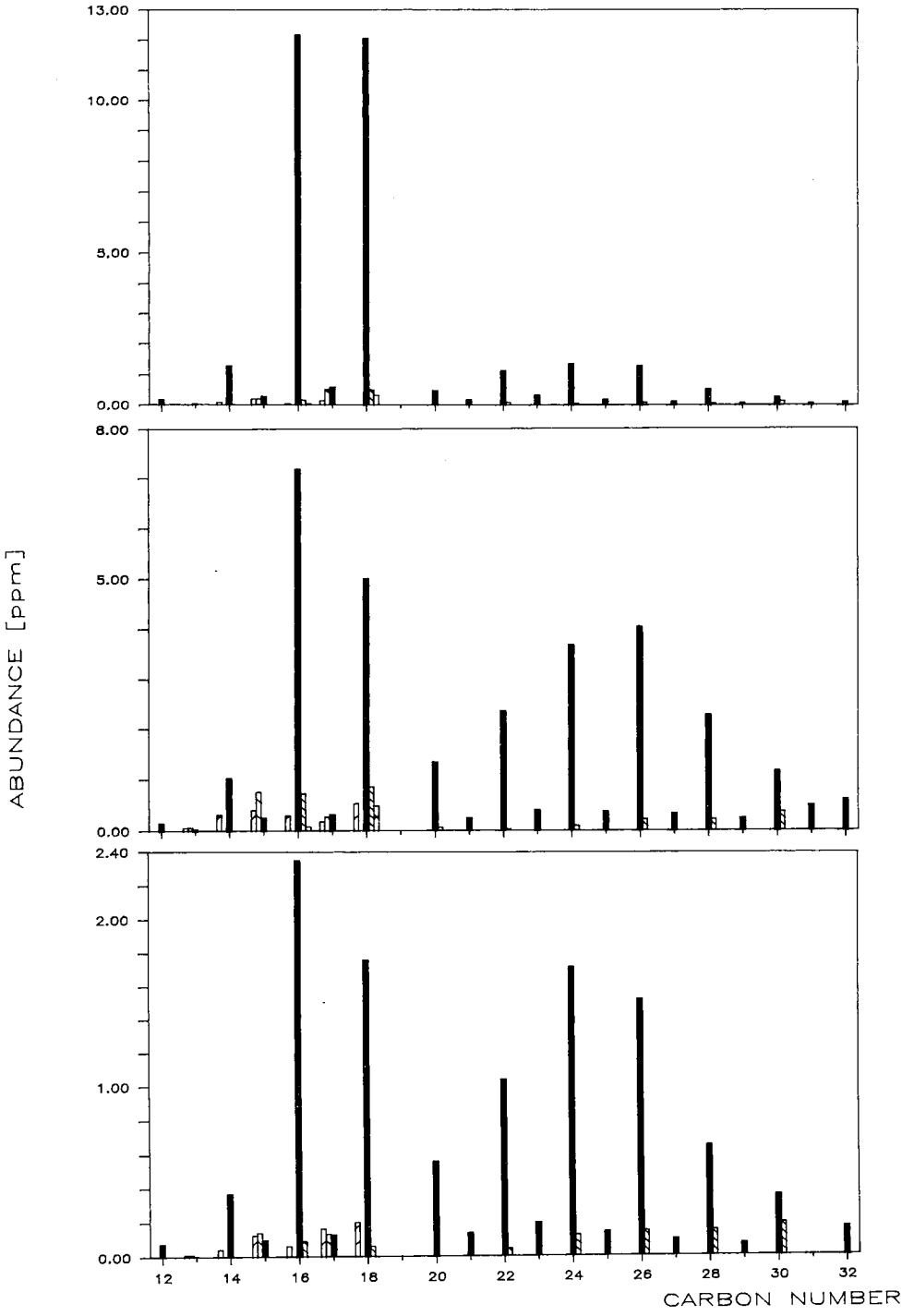

Figure 1 Distributions of the unbound (top), bound (middle) and tightly bound (bottom) unsubstituted fatty acids extracted from Lake Léman sediments at 1.8 m depth. The acids are shown in the following order, from left to right: *iso*, *anteiso*, normal (solid bars), and unsaturated (two unsaturated isomers for C_{18}).

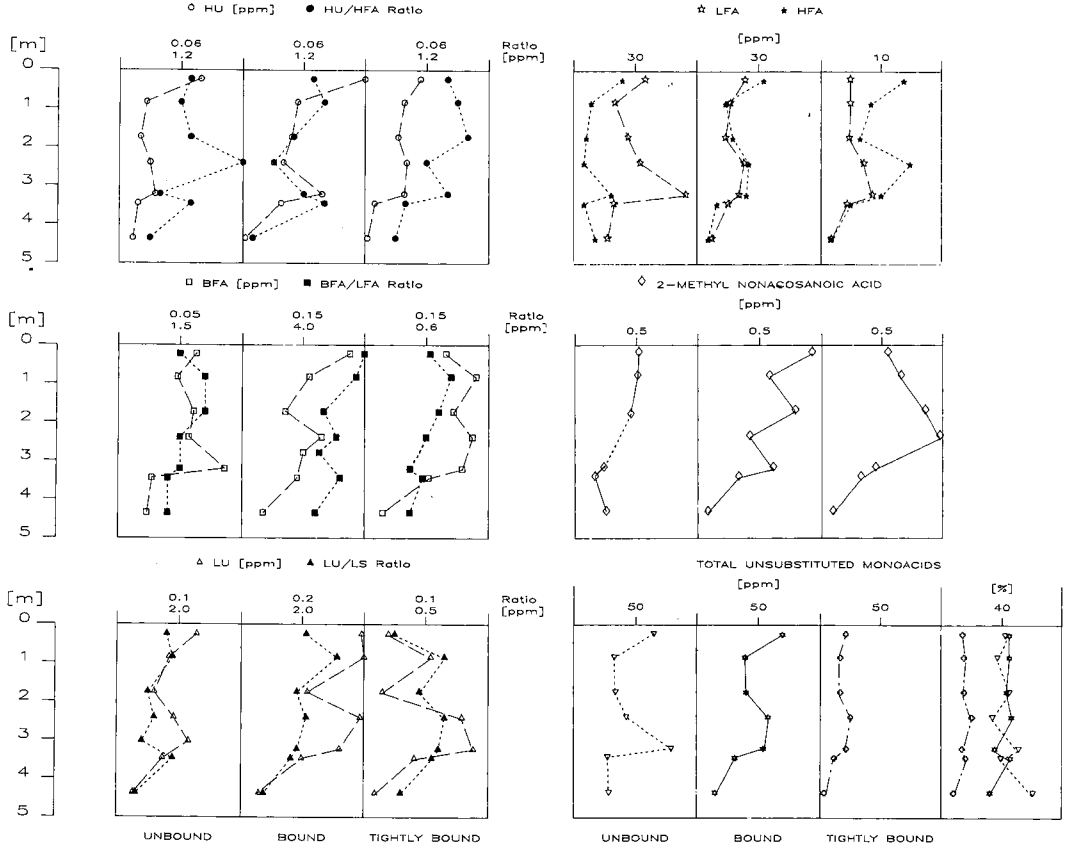

Figure 2 Depth profiles of the unsubstituted acids in Lake Léman sediments. HU, High molecular weight Unsaturated acids ($\sum C_{20:1}$–$C_{32:1}$). LU, Low molecular weight Unsaturated acids ($C_{16:1}+C_{18:1}+C_{18:2}$). LS, Low molecular weight Saturated acids ($C_{16:0}+C_{18:0}$). BFA, Branched Fatty Acids in the C_{12}–C_{18} range. LFA, Low molecular weight normal saturated Fatty Acids C_{12}–C_{18}. HFA, High molecular weight normal saturated Fatty Acids C_{20}–C_{30}. The abundance or ratio scales always begin at 0 (zero).

fractions. The bound and the tightly bound fractions are characterized by higher contributions from bacterial and terrestrial inputs. This is not surprising since the bacterial acids, mainly linked to the membrane lipopolysaccharides by ester and amide bonds, require a saponification or a strong hydrolysis to be released.[9] With regard to the HFA this is however unexpected since in recent sediments the acids in the bound fractions are generally characterized by higher LFA/HFA ratios than those in the unbound ones.[6]

The short-chain monounsaturated acids, mainly C_{16} and C_{18}, are common in nearly all living organisms and only a detailed knowledge of the double bond position and geometry allows them to be used as source indicators.[10-12] In sediments,[13-15] their rapid transformation during early diagenesis, especially under oxic conditions,[8] limits their use as biological markers. They are however better preserved in the bound fractions, where their relative amounts with regard to the corresponding saturated acids are almost four times larger than in the unbound fractions.

The long-chain monounsaturated acids ($C_{22:1}$ to $C_{32:1}$) have also been reported in marine and freshwater sediments, although less frequently than the short-chain ones.[8,10] Their origin is not clear. They have been attributed to land derived organic material,[16] and/or to mycobacteria.[17] Yeasts have also been mentioned as possible sources.[10] The distributions and the depth profiles which we observe for these compounds suggest a source or a diagenetic relationship with their saturated analogs. In fact, in all the organisms which could be potential sources, these acids are generally present in a much lower concentration than the saturated analogs. Thus, if mycobacteria or yeasts were the source of the long chain monounsaturated acids, a large proportion of the corresponding saturated fatty acids should then have the same origin rather than land plant wax esters. Nevertheless, even if this hypothesis is correct, using the HFA as markers for land derived organic matter is still valid, since yeasts as well as mycobacteria are indeed heterotrophic aerobes living mainly on soils or dead plant matter;[18] their fatty acids can thus be considered as terrestrial organic material. On the other hand, it has recently been shown that the algae of the genera *Chlorella* and *Scenedesmus*, common in freshwater lakes, as well as the blue-green alga *Spirulina platensis*, can also synthesize long chain fatty acids, from C_{22} to C_{30}, and their monounsaturated analogs.[19] With the

refinement of the analytical techniques, other aquatic organisms will perhaps also be shown to produce long chain fatty acids. All these considerations indicate that the origin of these acids is still not obvious.

Whatever their origin, the relative amounts of the HFA are usually much lower than those of the LFA, except in the case of some higher plant wax esters. This is also the case in surface sediments where the LFA/HFA ratios are generally maxima at the water–sediment interface but decrease rapidly with depth;[20] this is attributed to a higher stability of the HFA in sediments. The depth profiles we observe from 0.30 to 4.40 m do definitely not show such a decrease. It is thus obvious that the early diagenetic transformations responsible for the decrease of the short chain fatty acids operate essentially in the top 20 to 40 cm of the surface sediment and that the deeper sections conserve the fatty acid fingerprints left after the initial diagenesis. Finally, the relative amounts of the unbound, bound and tightly bound acids do not confirm the hypothesis of a conversion of the unbound and then to the tightly bound forms in the sediment column, as was postulated on the basis of simulation experiments.[21] Our results show that these forms are already present as such at the time of deposition or after early diagenesis.

We have already mentioned that the whole sediment profile corresponds to oligotrophic conditions. The fact that the section at 3.30 m contains significantly more fatty acids than the adjacent sections can therefore not be attributed to changes in the trophic status of the lake. If this increase does not correspond to a higher input, then the organic matter deposited at that time must have been better preserved. A faster sedimentation rate, leading to a rapid burial of the surface sediment[22] and resulting in an early withdrawal of the organic compounds from the initial diagenetic reactions, could explain the higher concentrations found in these sections for the acids in their three forms of occurrence.

Finally, the depth profiles of the 2-methyl C_{30} acid present in the three fractions are quite different from those of the other unsubstituted acids and we do not have any clue for the origin of this acid.

Hydroxyacids

Besides α-, β-, ω-, and $(\omega-1)$-hydroxyacids, we also found in the #2

fractions, a series of α,ω-diacids in the C_9 to C_{30} range. Their abundances were however generally too low to be accurately determined; they will not be considered further in this study.

a) β-hydroxyacids While the bound and tightly bound fractions contain β-hydroxyacids ranging from C_{12} to C_{20} with quite similar distributions, the unbound fractions contain only negligible amounts of them. Abundance–depth profiles are given in Figure 3 and a typical distribution is shown in Figure 4. The *anteiso* C_{15} and C_{17} are more abundant in the tightly bound fraction, while there are more *n*-C_{12}, *n*-C_{14}, $C_{16:1}$, $C_{18:1}$ and $C_{20:1}$ acids in the bound fractions.

Although sedimentary β-hydroxyacids were first attributed to the microbial oxidation of fatty acids,[23] it seems now clear that in the C_{10} to C_{20} range, these acids are synthesized *de novo*, mainly by Gram-negative bacteria.[7] Stereochemical studies of β-hydroxyacids from various organisms have shown that *R* is the natural configuration. As the oxidative degradation leads only to the *S* configuration, it was established[24] that the C_{10} to C_{20} β-hydroxyacids are indeed of bacterial origin, while the homologs with more than 20 carbon atoms might arise from the degradation of long chain fatty acids.

As already mentioned, the distributions of the bound and tightly bound β-hydroxyacids show only slight differences. In fact, as the compositions of the LPS from several Gram-negative bacteria[25,26] show, the ester linked β-hydroxyacids distributions may be somewhat different from the amide linked ones; this could account for the differences we observe in the sediment. It is interesting to note that the even carbon numbered branched β-hydroxyacids found in both the bound and tightly bound forms are almost exclusively *iso*, while the odd carbon numbered acids contain a large proportion of *anteiso* compounds. Although *anteiso* branching is common in unsubstituted bacterial acids, it has never been reported in significant proportions for the β-hydroxyacids. Sedimentary *anteiso* β-hydroxyacids could thus be specific biomarkers for some species of bacteria.

The depth profiles of the β-hydroxyacids (Figure 3) do not show any regular trend and again do not support the hypothesis of a conversion from the bound form to the tightly bound one in the sediment column. These profiles rather reflect the density and the type of the microbial populations existing at the time of deposition.

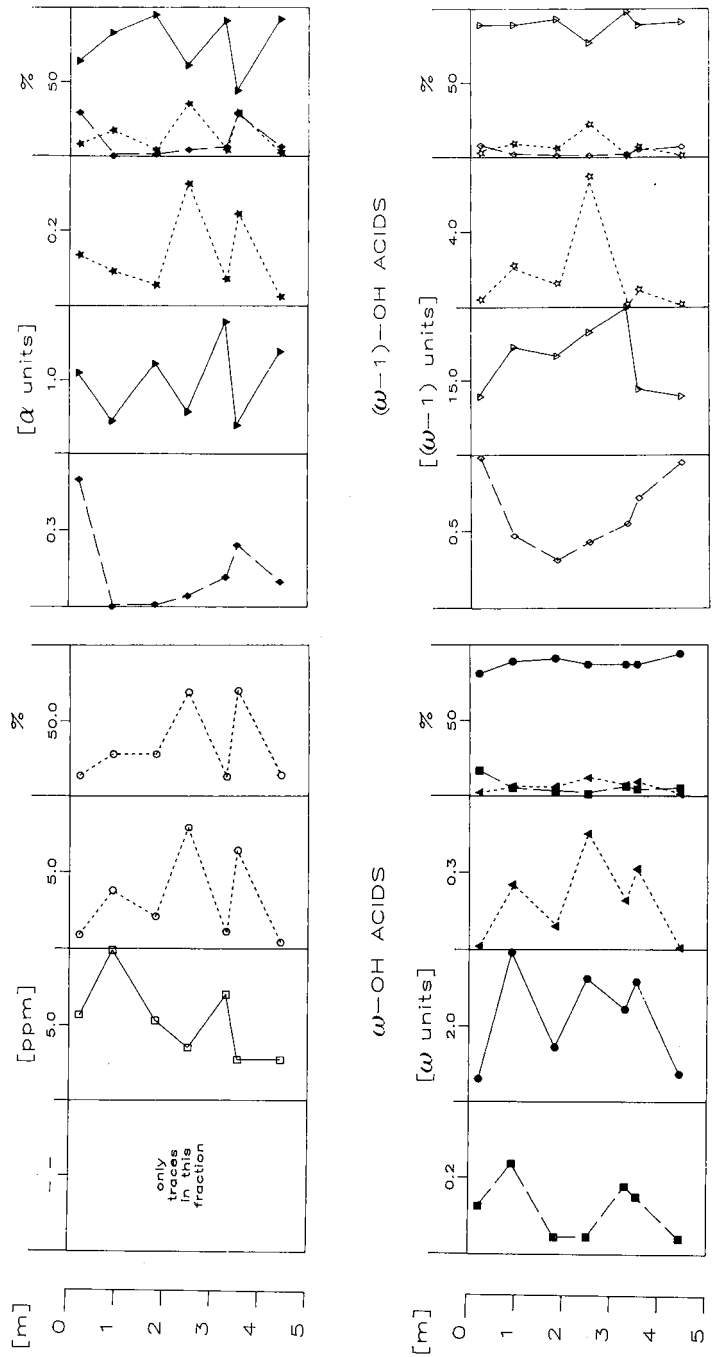

Figure 3 Depth profiles of β-, α-, ω- and (ω–1)-hydroxyacids in Lake Léman sediments. The units are defined in the text (ppm:μg/g dry sediment).

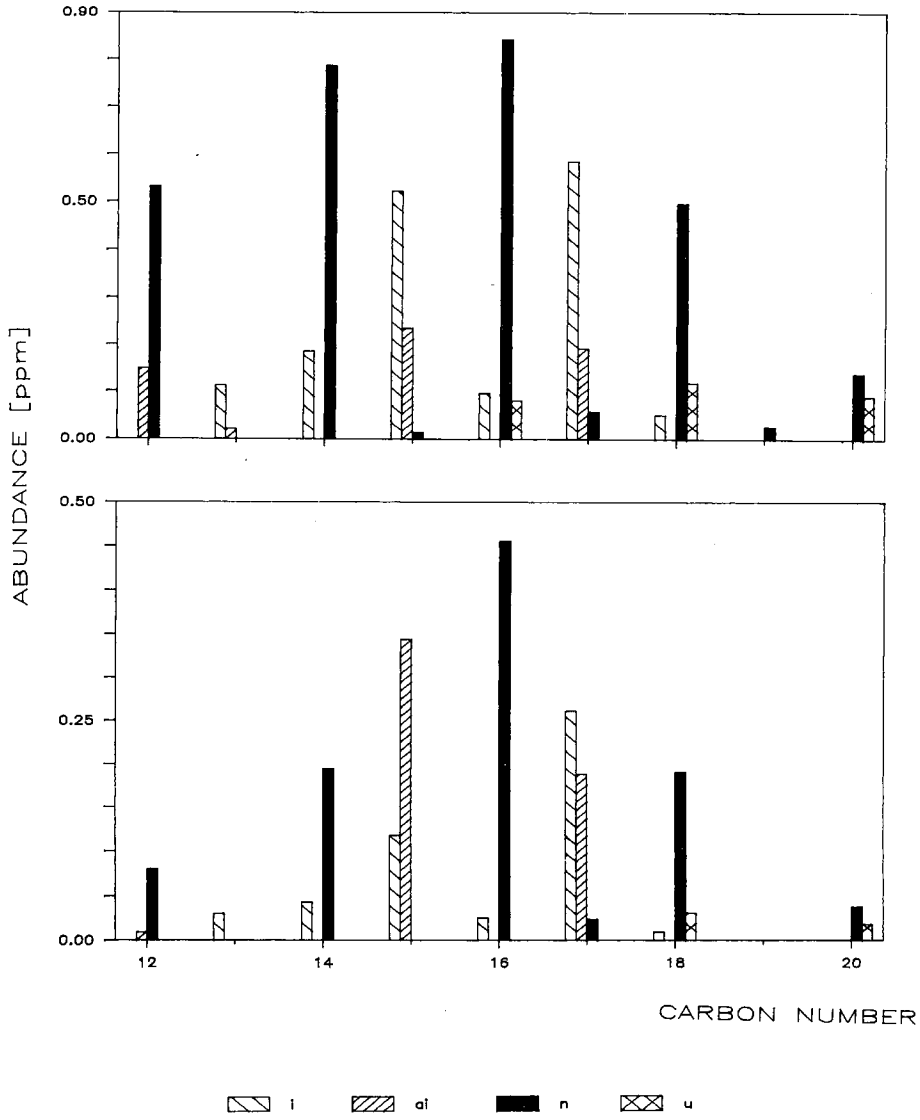

Figure 4 Distributions of bound (top) and tightly bound (bottom) β-hydroxyacids from Lake Léman sediments at 1.8 m depth. i, *iso*; ai, *anteiso*; n, normal; u, unsaturated.

b) α-hydroxyacids The retention times of the TMS ethers of the α- and β-hydroxyacid methyl esters are the same. Since there were only traces of them, we did not investigate quantitatively the α-hydroxyacids shorter than C_{20}. The distributions of these acids throughout the core are roughly the same in the three fractions. Figure 5 shows a typical distribution. The maximum is always at C_{24}, the C_{22} homolog being however often very abundant. Another interesting feature of these distributions is the low carbon preference index (CPI).

α-Hydroxyacids are present in a variety of organisms, including bacteria,[27] yeasts,[28,29] seagrasses,[12] microalgae,[30] animal tissues and plants.[31,32] Bacteria contain essentially short chain acids (C_{10} to C_{18}). In microalgae, the distributions do not resemble those in the sediment we have studied; the CPI values are higher and the short chain (C_{16} to C_{20}) acids generally very abundant. In seagrasses however, the distribution of the C_{20} to C_{28} acids is very similar to that in the Léman sediment, but the CPI is higher. In higher plant leaves, we found small amounts of α-hydroxyacids in the same carbon range and with distributions close to the ones observed in our sediment (Zi Ling Hu, in preparation). These acids could however have belonged to parasitic fungi present on the leaves. Source correlations are therefore difficult to establish.

c) ω-hydroxyacids These acids, detected in the C_{12} to C_{30} range, show throughout the core a bimodal distribution in the three fractions. The first mode is centered on C_{16} and the second, more important, on C_{22}. Typical distributions are shown in Figure 6. The bound fractions contain approximately ten times more ω-hydroxyacids than the unbound and the tightly bound ones.

Bound ω-hydroxyacids are common components of the higher plant cutins and suberins, but the major acids are then C_{16} and C_{18}. Acids in the C_{20} to C_{26} range are uncommon in cutin[33-36] and generally minor components in suberin, although they were found sometimes to be major compounds.[37,38] High proportions of long chain acids with maxima at C_{22}, C_{24}, C_{26} or C_{28} occur in the unbound lipids of diverse seagrasses[12,39] and in the bound lipids of fresh as well as peat forming mosses.[40] Bacteria do not contain ω-hydroxyacids, but various aerobic microorganisms are able to hydroxylate monocarboxylic acids in the ω-position.[41-43]

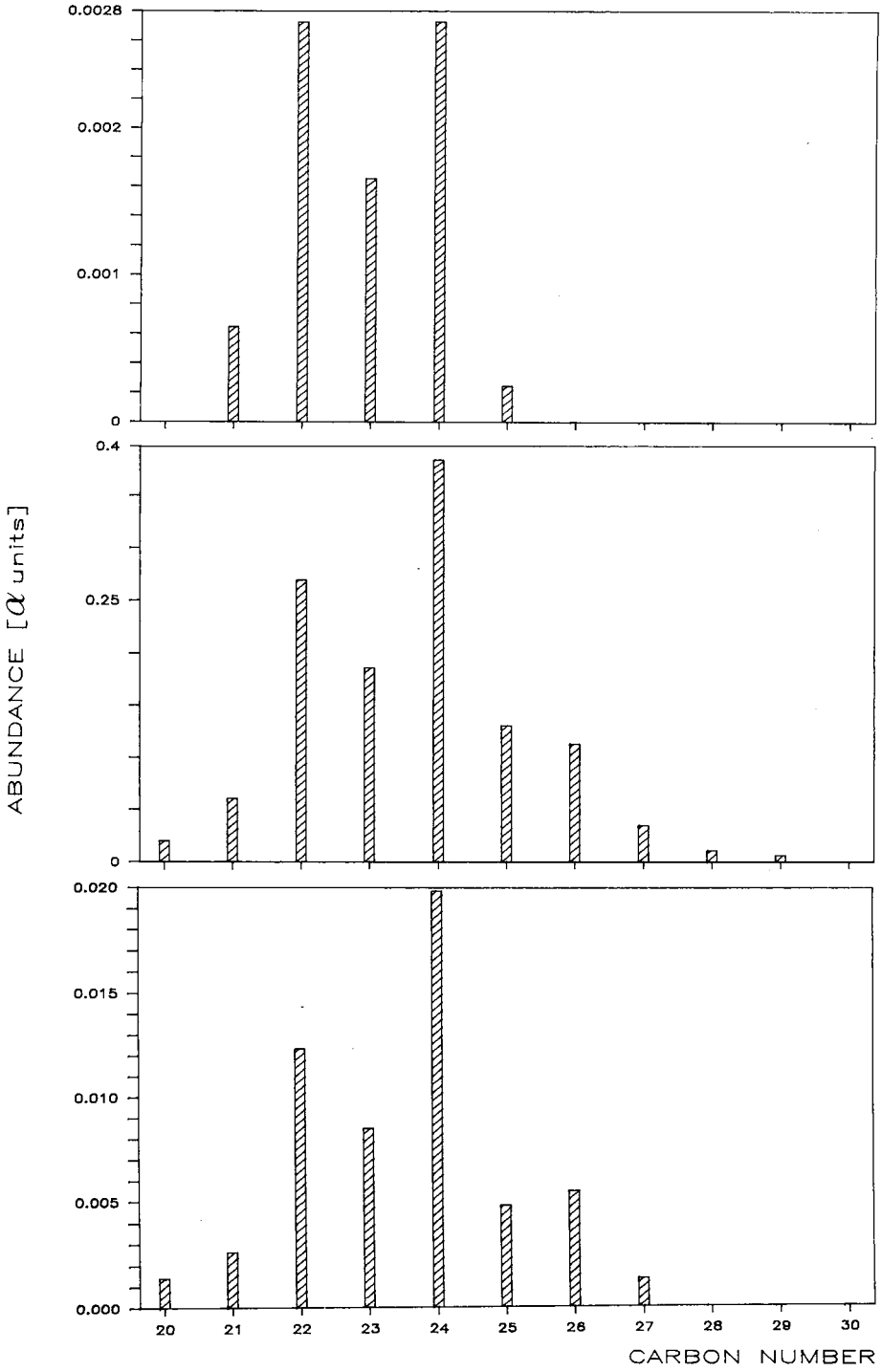

Figure 5 Distributions of unbound (top), bound (middle) and tightly bound (bottom) α-hydroxyacids from Lake Léman sediments at 1.8 m depth.

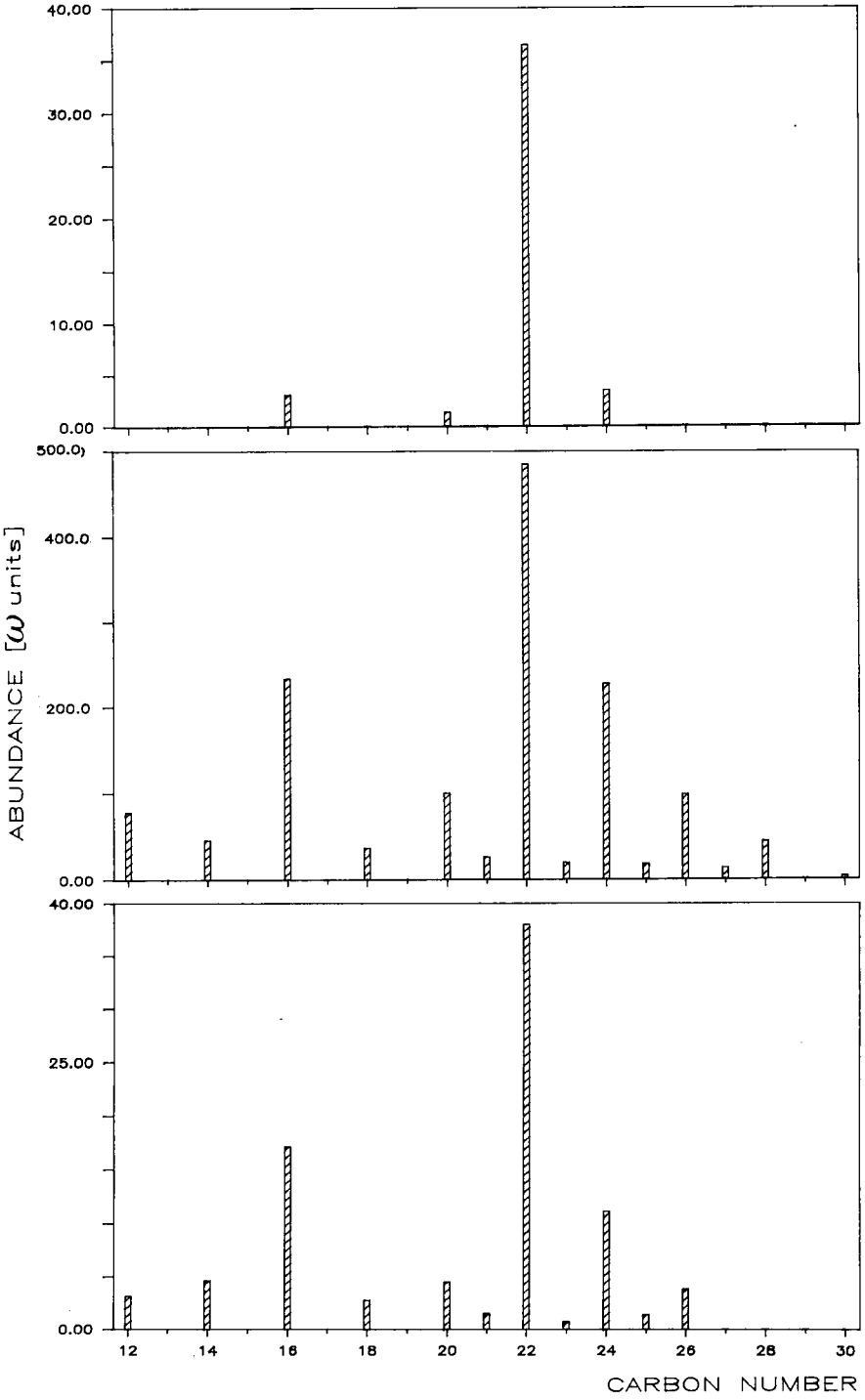

Figure 6 Distributions of unbound (top), bound (middle) and tightly bound (bottom) ω-hydroxyacids from Lake Léman sediments at 1.8 m depth.

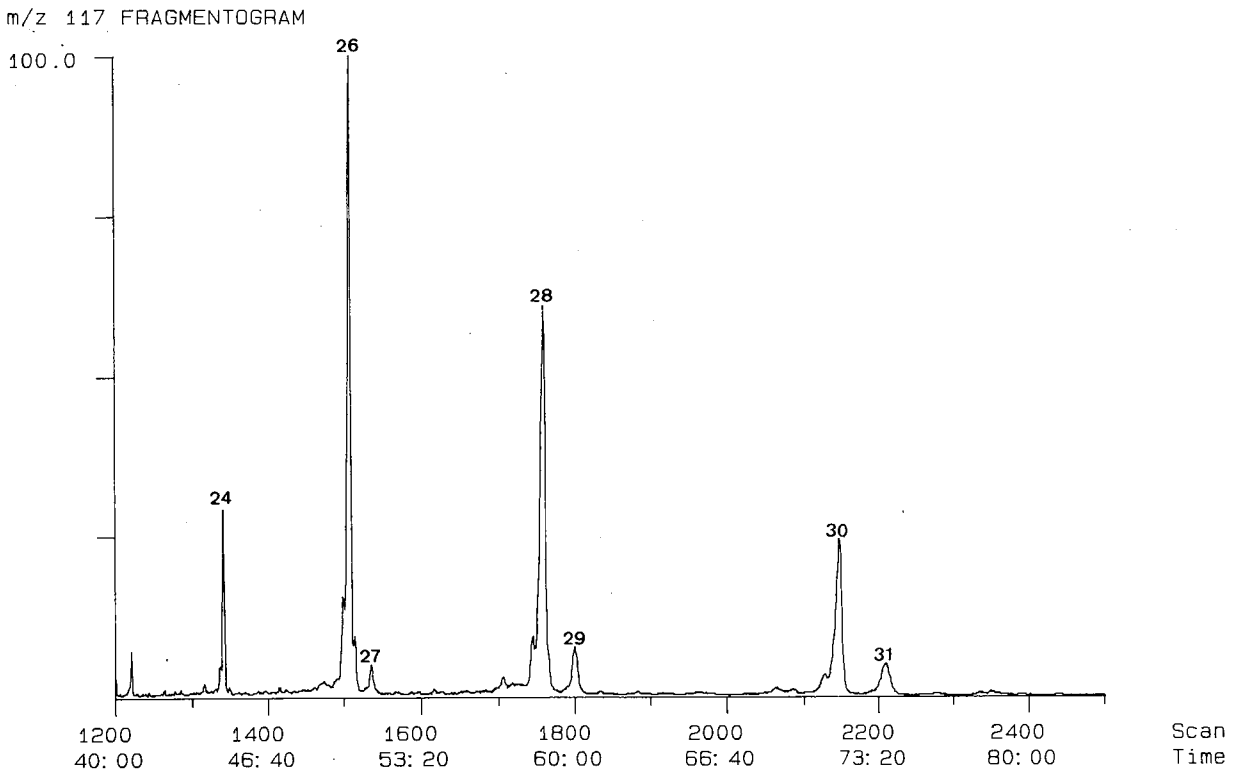

Figure 7 Fragmentogram of the m/z 117 ion, arising from $(\omega-1)$-hydroxyacids, in the bound fraction at 1.8 m depth. The numbers on top of the peaks represent the number of carbon atoms in the molecule.

ω-Hydroxyacids in the same carbon range, with more or less the same distributions as those we have observed, have also been found in various marine and lacustrine sediments; their presence was attributed to land plants,[5,44] to seagrasses[12] and/or to microbial transformations of algal detritus in the oxic water column.[5,31] Land plants and mosses contain these acids in highly resistant biopolymeric structures and could be plausible sources for the sedimentary ω-hydroxyacids since they are mainly present in the bound fraction. The depletion of the short chain acids, which are usually more abundant than the long chain ones, may be due to a preferential degradation by organisms such as fungi and yeasts.[35] The depth profiles for the short chain (C_{12} to C_{18}) and long chain (C_{20} to C_{28}) components are almost the same, enforcing the hypothesis of a unique source. These depth profiles are however surprisingly different from those of the long chain unsubstituted acids and rather similar to the profile of the tightly bound β-hydroxyacids. For the time being we have no explanation for this observation; it just strengthens the doubt we have about sedimentary long chain fatty acids being necessarily indicators of higher plant input.

d) *($\omega-1$)-hydroxyacids* The C_{22} to C_{31} ($\omega-1$)-hydroxyacids show a monomodal distribution centered on C_{28}, except for the unbound and bound acids of the upper levels, which are centered on C_{26}. The tightly bound acids are always centered on C_{28}. In fact the carbon range extends to at least C_{34}, but due to their high retention times and the broadening of the GC peaks, the heavier molecules are difficult to analyze. Interestingly, the odd carbon numbered homologs, only present in the C_{27} to C_{31} range, have GC retention times which are slightly longer than those of the even carbon numbered analogs with one carbon atom less. This suggests that the odd carbon numbered acids are branched, most probably with a methyl group on the ($\omega-2$) position. Branching at position 2 or at any other position can be ruled out on the basis of the mass spectra and GC retention times. Unbranched odd numbered homologs are quasi absent. An example of distribution is given in the mass fragmentogram of Figure 7. Such a strange distribution has already been observed,[31] but its significance has not been discussed. Like the other hydroxyacids mentioned above, the ($\omega-1$)-hydroxyacids are also essentially concentrated in the bound fractions, which contain

more than 90% of them. The depth profiles (Figure 3) are completely different for the three fractions and differ also from those of the other acids.

Reports on the presence of $(\omega-1)$-hydroxyacids in organisms and in sediments are rather scarce. The C_{16} homolog has been found in cutins from bryophytes[45] and some aerobic microorganisms have been shown to hydroxylate alkanes and fatty acids at the $(\omega-1)$ position.[43,46,47] Their presence in a diatomaceous ooze, with a distribution similar to the one we observe, has been attributed to aerobic microorganisms.[31] Seagrasses have also been proposed as a possible source.[48] However, there are too few data available to draw reliable conclusions about the origin of these acids. The depth profiles illustrated in Figure 3 do not show any correlation between the $(\omega-1)$- and ω-hydroxyacids, which would suggest a common source.

CONCLUSIONS

1) The relative amounts of the three forms of acids extracted from a 5 m sediment core of Lake Léman show no evidence of a conversion from the unbound to the bound, or from the bound to the tightly bound forms for any of the fatty acids we have analyzed. Thus, if these conversions do take place, they are operating only in the surficial sediments or, more probably, the acids are already present under these forms at the time of deposition.

2) None of the acid types, whatever their form of occurrence, do show a regular decreasing trend. The acids which survive the early diagenetic processes are preserved in the deeper sections. The depth profiles therefore reflect the changes in the input sources and the effect of the diagenetic reactions which occur before the sediments are buried below the zone in which the microorganisms operate.

3) The tightly bound β-hydroxyacids and the unsubstituted acids shorter than C_{20} show distributions which are significantly different from those of the bound corresponding compounds. Most of them are probably amide linked bacterial acids preserved in the bacterial cell wall remains.

4) The unbound, bound and tightly bound α-hydroxyacids show a similar distribution, which suggests a common origin in the three forms. This is also the case for the ω- as well as for the (ω−1)-hydroxyacids. For each one of these three classes of acids, the amounts present in the tightly bound fractions represent less than 10% of the total amount and remain more or less constant with sediment depth. These acids are definitely not constituents of the bacterial cell wall LPS; the tightly bound acids are thus not only contained in the remains of bacterial membranes but probably also in melanoidine or protokerogen type insoluble organic polymers; part of these acids may also be trapped in mineral matrices.

5) β-Hydroxyacids and short chain ($<C_{20}$) unsubstituted branched acids are specific biomarkers for the bacterial contribution to the sedimentary organic matter. Long chain unsubstituted acids may be used as indicators of terrestrial contribution although not necessarily from a direct input of wax esters of vascular plants; aerobic microorganisms, such as yeasts and mycobacteria, living mainly in terrestrial habitats, could also be responsible for the origin of these long chain fatty acids. The α- and ω-hydroxyacids, present in the sediments of Lake Léman essentially as long chain ($>C_{20}$) compounds, are not specific enough to be useful for input source correlations, although short chain ($<C_{20}$) ω-hydroxyacids can be considered as markers for the terrestrial cutins. Finally, as long as nothing is known on the origins of the (ω−1)-hydroxyacids longer than C_{20} and on that of 2-methyl nonacosanoic acid, they cannot be used as source indicators.

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