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Can cutin and suberin biomarkers be used to trace shoot and root-derived organic matter? A molecular and isotopic approach

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Abstract Cutins of plant shoots and suberins, mostly present in roots could contribute to significant portions of stable soil organic matter. Their biomarker potential, residing in their unique compositions in different plant types, has been used previously to infer sources of organic matter in sediments. These aliphatic plant biopolyesters contain specific biomarkers, which may be used for tracing their fate in soils and sediments, when combined with stable ^{13}C isotope labelling. In order to evaluate the potential use of cutin and suberin biomarkers as indicators of shoot and root contributions from C_3 and C_4 plant origins, the objectives of this study were to 1) identify their constitutive monomers, which are specific for shoots and roots of maize (C_4) and wheat (C_3); 2) evaluate the ^{13}C differences between maize and wheat biomarkers. Mid-chain hydroxy carboxylic acids were mainly found in the aboveground biomass, while α,ω-alkanedioic acids were only present in the roots. The differences in the isotopic composition of the specific monomers between wheat and maize plants (17–18‰ for shoot markers, 19‰ for root markers) were larger than those observed for bulk plant tissues and close to those observed for lignin-derived phenols in other studies. These differences should make it possible to differentiate and quantify the different types and sources of organic matter in sediments and soils. In particular, the molecular and isotopic signatures of cutins and suberins can be used to evaluate the specific dynamics of root vs shoot tissues in soils using C_3/C_4 chronosequences.

Keywords Cutins · Suberins · Soils · Roots · Shoots · Saponification · ^{13}C

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Introduction

Soil organic matter (SOM) influences physical and chemical conditions and its presence is essential to maintain soil quality. Intensive agricultural activities lead to a drastic decrease of SOM contents in cropland soils, where plant residues and microorganisms are the main sources of organic matter (OM) (Kögel-Knabner 2002). A better knowledge of the composition and dynamics of plant and soil OM is necessary to understand the processes of degradation and stabilization of OM in order to favour agricultural practices for a sustainable use of soil.

The stable pool of SOM has been suggested to be enriched in aliphatic structures (Kögel-Knabner et al. 1992; Riederer et al. 1993; Sollins et al. 1996; Augris

et al. 1998), which might be partly attributed to the contribution of cutins and suberins (Kögel-Knabner et al. 1989; Dinel et al. 1990; Nierop et al. 2001; Kögel-Knabner 2002; Otto and Simpson 2006). Cutins are amorphous biopolyesters composed of carboxylic acids, bearing epoxy and alcohol groups, interlinked through ester bonds (Kolattukudy and Walton 1972). Suberins are composed of an aliphatic and an aromatic domain (Bernards 2002). The aliphatic part of suberins is a polyester usually made of carboxylic acids, ω- or α-hydroxylated, of diacids and alcohols. Cutins are embedded within intracuticular waxes and covered with epicuticular waxes to form the plant cuticle that covers the aerial parts of vascular plants (Hunneman and Eglinton 1971; Goñi and Hedges 1990a; Riederer et al. 1993), fruits (Eglinton et al. 1968; Velcheva et al. 1981; Ray et al. 1995), mosses and liverwort (Caldicott and Eglinton 1976; Goñi and Hedges 1990b). Suberins are mostly present in roots, bark and some suberized tissues of plants (Kolattukudy 1981). These biopolyesters act as a protective barrier against chemical, biological and physical attacks and regulate plant water content and gas exchanges (Dean and Kolattukudy 1976; Kolattukudy 1984; Heredia 2003).

Cutins and suberins have often been used as geochemical markers of the vegetation sources in soils and sediments (Cardoso and Eglinton 1975; Cardoso and Eglinton 1983; Goñi and Hedges 1990c; Almendros et al. 1999), but their relative contribution to SOM is poorly known, although they might play a major role in C storage in soils. For the development of management practices, which favour SOM storage, knowledge about the relative contribution and turnover of above and belowground biomass is essential. Such knowledge could be obtained by studying the fate of cutins and suberins (Rasse et al. 2005). Understanding their dynamics in soils requires the use of ^{13}C isotopic tracer at the molecular level.

Following changes in the natural abundance of ^{13}C isotopes is a powerful tool for the study of the fate of plant residues in ecosystems at the global and molecular scale. The bulk ^{13}C isotopic signature of plants is directly related to their photosynthetic pathway and allows for the differentiation between C_3 and C_4 plants (Smith and Epstein 1971). The ^{13}C natural abundance in plants and soils was used to evaluate the turnover of SOM in ecosystems that were initially under C_3 (or C_4) vegetation and changed to a C_4 (or C_3) vegetation. This method was used at the bulk level (Balesdent and Mariotti 1996) and at the molecular level for *n*-alkanes (Wiesenberg et al. 2004a; Quenea et al. 2006), *n*-carboxylic acids (Wiesenberg et al. 2004a), lignin-derived phenols (Bahri et al. 2006) and sugars (Derrien et al. 2006). Despite their importance as potential biomarkers for aboveground and belowground biomass of non-woody plants, the ^{13}C natural abundance of cutin and suberin monomers has never been studied (Amelung et al. 2008). A better knowledge of their isotopic composition in C_3 and C_4 plants would increase their biomarker potential in sediments as well as in soils.

The objective of this study was to evaluate the potential use of biomarkers of cutins and suberins by acquiring information on the quantities, distribution and ^{13}C isotopic composition of cutin and suberin aliphatic monomers in different plant organs. We chose two plants of contrasting photosynthetic pathways, maize and wheat, sampled just before being returned to soil. Leaves, stems and roots of both wheat (*Triticum aestivum* L., C_3 plant) and maize (*Zea mays* L., C_4 plant) were separately analysed. The aliphatic monomers constitutive of the biopolyesters were released by saponification, which was recently shown to release appropriate amounts and diversity of monomers (Mendez-Millan et al., 2010).

Materials and methods

Plant samples

Plant samples were collected just before harvest at the INRA experimental field of "Les Closeaux" in the "Parc du Château de Versailles", France. Leaves, stems and roots of *Zea mays* L. and *Triticum aestivun* L. were separated, air-dried and ground at 100 µm. Allometric relationships between plant organs were taken from Bahri et al. (2006). Leaves, stems and roots represented, respectively, 0.41, 0.39 and 0.20 g g^{-1} plant in maize and 0.37, 0.47, 0.16 g g^{-1} plant in wheat.

In order to remove free lipids before depolymerisation, about 2 g of plant samples were extracted three times with an accelerated solvent extractor Dionex ASE 200 in 30 ml stainless-steel extraction

vessels. The extraction was carried out in three steps (heating time 5 min, static extraction 20 min, pressure 5×10^6 Pa), in dichloromethane/methanol 3:1 (V:V) at 75°C and at 140°C, then in dichloromethane at 140°C (Wiesenberg et al. 2004b).

Analytical procedure: Saponification and derivatisation

Solvent extracted plant samples (100 mg) were refluxed for 18 h in an aqueous solution of potassium hydroxide (KOH, 6%) in methanol (Cardoso and Eglinton 1975; Ray et al. 1995). The solution was filtered and the residue washed with methanol/water (9:1) (V:V). After addition of 150 ml of distilled water, the solution was adjusted to pH 2 with hydrochloric acid (HCl) 6 N in order to isolate the acidic products (Naafs and Van Bergen 2002) and extracted with dichloromethane (50 ml × 3). The volume of the recombined extracts was reduced with a rotative evaporator under low pressure and dried completely under nitrogen. Dried extracts, dissolved in pyridine and BSTFA (N,O-bis (trimethylsilyl)-trifluoroacetamide) containing 1% of trimethylchlorosilane (TMCS) (Altech) were silylated at 70°C for 1 h. Silylation transformed hydroxy and carboxylic acid functions into their trimethylsilyl ether and ester derivatives (TMS ether/TMS ester).

Identification and quantification of the monomers

Silylated saponification products were separated with a gas chromatograph (GC) HP6890 equipped with a SGE BPX-5 column (50 m × 0.25 mm × 0.32 µm), using a He constant flow of 1.5 ml min^{-1}. A 1 µl aliquot was injected in splitless mode, at a temperature of 300°C. The GC oven temperature was programmed at 100°C for 2 min, then from 100 to 150°C at 10°C min^{-1}, from 150 to 200°C at 5°C min^{-1}, and finally at a rate of 2°C min^{-1} from 200 to 350°C and kept 5 min at 350°C. The compounds of interest were chromatographically well resolved, as shown on Fig. 1 for wheat leaves and wheat roots. The same chromatographic conditions were used for their analysis with GC/C-IRMS. Compounds were identified with an Agilent HP5973 Electron Impact (70 eV, scan range *m/z* 40–700, 1.2 scan/s) mass spectrometer (MS) according to their fragmentation pattern supported by comparison with published mass spectra (Eglinton et al. 1968; Hunneman and Eglinton 1971; Holloway and Deas 1973) and with a mass spectra library (Wiley). They were quantified by flame ionisation detection (FID) by using nonadecanoic acid standard, which was added prior to derivatisation and an external calibration with 16-hydroxyhexadecanoic acid. The response factor of the 16-hydroxyhexadecanoic acid relative to nonadecanoic acid was close to 1.

Molecular isotopic analysis

The ^{13}C isotopic signatures of individual compounds were measured in triplicate with a GC HP5890 coupled with an Isochrom III Isotopic mass spectrometer (Micromass-GVI Optima) via a combustion interface (GC/C-IRMS). Samples were injected in the splitless mode. The same column and chromatographic conditions as for the identification and quantification were used. The $\delta^{13}C$ values (expressed in ‰ relative to Vienna PeeDee Belemnite) were determined for the silylated monomers of the molecules of interest. Compounds that were not detected or which concentrations were too low to determine accurately their isotopic signatures, were mentioned as "not analysed" in the result table. We assumed that C-atoms of BSTFA have the same isotopic ratio, as already demonstrated in a previous study on lignin-derived phenols (Dignac et al. 2005). The isotopic values were corrected for the addition of carbon atoms from the trimethylsilyl groups, after measuring the $\delta^{13}C$ values of BSTFA with Elemental Analyser (EA) coupled to IRMS (Micromass NA 1500 NC).

The $\delta^{13}C$ of a compound class (δ_{class}) consisting of several individual compounds with similar chemical structure (such as *n*-carboxylic acids for example) was computed for each tissue by weighting the $\delta^{13}C$ of individual compounds by their concentrations, using the equation:

$$\delta_{class} = \frac{\sum_i \delta_{compi} \times C_{compi}}{\sum_i C_{compi}} \quad (1)$$

where C_{compi} is the concentration of the *i* compound of the class and δ_{compi} is the isotopic ratio of this compound

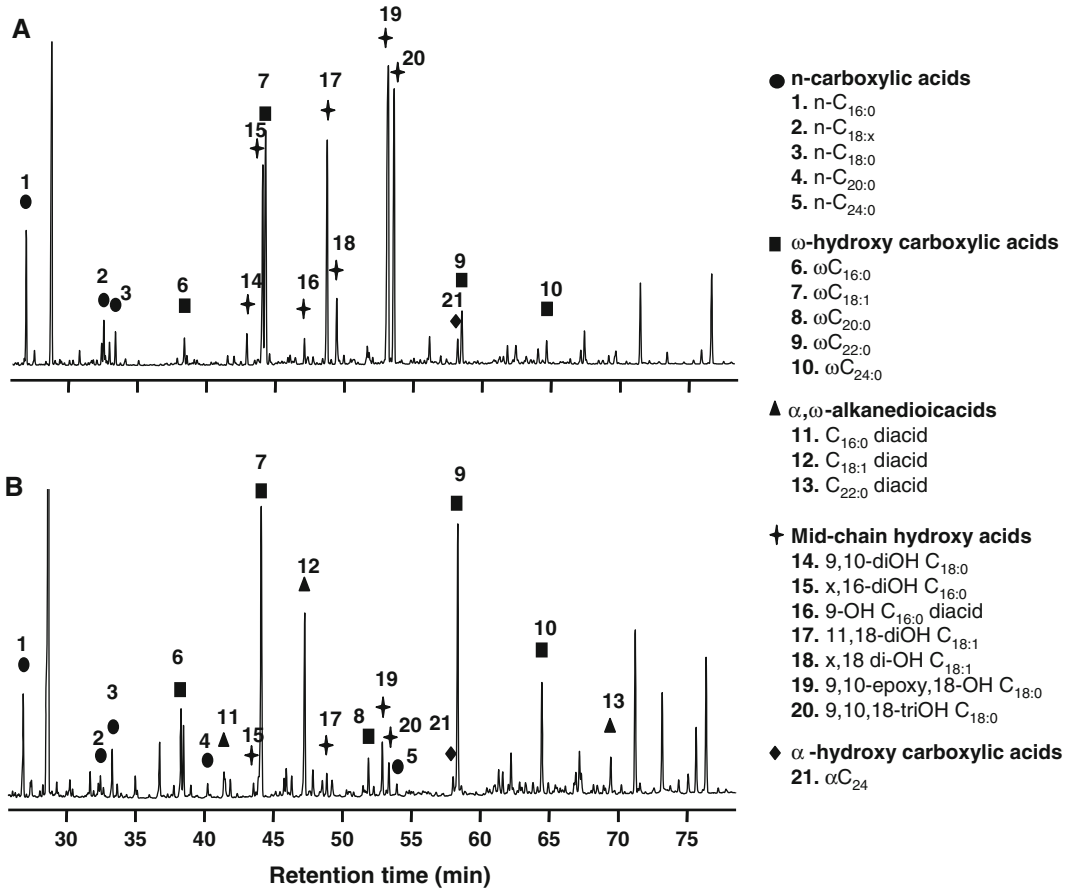

Fig. 1 Chromatographic separation of the aliphatic compounds with GC/MS. Total Ion current traces obtained from the saponification mixtures of wheat leaves (**a**) and wheat roots (**b**)

Results and discussion

Identification and distribution of the aliphatic monomers in plant tissues

Table 1 presents the concentrations of the aliphatic monomers in the different plant organs and their relative contribution to the total concentration of aliphatic monomers released by saponification. The tissues of the wheat plant contained higher concentrations of total identified monomers compared to the corresponding maize tissues (Table 1). The different compounds are presented according to their chemical structures, displayed into six chemical classes.

n-Carboxylic acids and *n*-alcohols

The *n*-carboxylic acids, ranging from *n*-C_{16} to *n*-C_{24} represented 15 to 16% of the total released monomers in wheat tissues, 23 and 25% in maize roots and leaves, respectively, and up to 54% in maize stems. In all the plant tissues, the hexadecanoic acid and the unsaturated octadecanoic acids (*n*-$C_{18:1}$, *n*-$C_{18:2}$ and *n*-$C_{18:3}$ quantified together and labelled *n*-$C_{18:X}$) dominated the *n*-carboxylic acid class. Two *n*-alcohols (octadodecanol and docosanol) were identified in maize roots (Table 1) in very low concentrations (21 and 11 μg g^{-1} representing 2% of the total aliphatic monomers), as already evidenced in suberin of maize primary roots (Zeier et al. 1999).

The *n*-carboxylic acids and *n*-alcohols are found in plant biopolyesters but are also major components of plant waxes. In waxes, carboxylic acids usually range from *n*-C_{12} to *n*-C_{36} (Kolattukudy and Walton 1972; Wiesenberg et al. 2004a; Jandl et al. 2005) and *n*-alcohols are characterized by the predominance of the *n*-C_{26} and *n*-C_{28} compounds (Bull et al. 2000; Kunst and Samuels 2003). Since free-lipids have

Table 1 Concentration (Conc, μg g^{-1} dry sample) of the aliphatic monomers present in the bound lipids of leaves, stems and roots of maize and wheat and relative abundance (RA, in %) of each monomer relative to the total concentration of the aliphatic monomers in the plant tissue

	Maize						Wheat					
	Leaves		Stems		Roots		Leaves		Stems		Roots	
	Conc (μg g^{-1})	RA (%)	Conc (μg g^{-1})	RA (%)	Conc (μg g^{-1})	RA (%)	Conc (μg g^{-1})	RA (%)	Conc (μg g^{-1})	RA (%)	Conc (μg g^{-1})	RA (%)
n-Carboxylic acids												
Hexadecanoic acid (*n*-$C_{16:0}$)	273 ± 3	13.3	127 ± 26	19.6	114 ± 4	8.4	262 ± 4	7.4	105 ± 3	7.9	134 ± 6	7.0
Octadecanoic acid (*n*-$C_{18:0}$)	51 ± 4	2.5	87 ± 13	13.4	67 ± 0	4.9	93 ± 15	2.6	48 ± 8	3.6	51 ± 8	2.7
Octadecenoic acid (*n*-$C_{18:X}$)	150 ± 4	7.3	115 ± 6	17.8	88 ± 3	6.4	186 ± 2	5.3	46 ± 4	3.5	59 ± 2	3.1
Eicosanoic acid (*n*-$C_{20:0}$)	17 ± 0	0.8	6.5 ± 0	1.0	18 ± 0	1.3	nd		nd		25 ± 0	1.3
Tetracosanoic acid (*n*-$C_{24:0}$)	33 ± 3	1.6	15 ± 1	2.3	26 ± 0	1.9	nd		12 ± 2	0.9	23 ± 2	1.2
Total concentration of *n*-carboxylic acids	524 ± 1	25.5	351 ± 32	54.1	313 ± 8	22.9	541 ± 13	15.3	211 ± 17	15.8	292 ± 14	15.3
n-Alcohols												
Octadecanol (Alcohol $C_{18:0}$)	nd		nd		21 ± 0	1.5	nd		nd		nd	
Docosanol (Alcohol $C_{20:0}$)	nd		nd		11 ± 0	0.8	nd		nd		nd	
Total concentration of alcohols					32 ± 0	2.3						
α-Hydroxy carboxylic acids												
2-Hydroxy tetracosanoic acid (α$C_{24:0}$)	95 ± 1	4.6	50 ± 2	7.7	50 ± 2	3.7	80 ± 2	2.3	32 ± 1	2.4	27 ± 1	1.4
ω-Hydroxy carboxylic acids												
16-Hydroxy hexadecanoic acid (ω$C_{16:0}$)	54 ± 4	2.6	52 ± 6	8.0	150 ± 1	10.1	57 ± 1	1.6	nd		134 ± 4	7.0
18-Hydroxy octadecenoic acid (ω$C_{18:1}$)	452 ± 17	22.0	56 ± 4	8.6	234 ± 2	17.1	401 ± 19	11.3	181 ± 5	13.6	349 ± 6	18.2
20-Hydroxy eicosanoic acid (ω$C_{20:0}$)	nd		nd		30 ± 0	2.2	nd		nd		59 ± 2	3.1
22-Hydroxy docosanoic acid (ω$C_{22:0}$)	12 ± 2	0.6	31 ± 1	4.8	68 ± 2	5.0	117 ± 2	3.3	49 ± 1	3.7	354 ± 8	18.5
24-Hydroxy tetracosanoic acid (ω$C_{24:0}$)	70 ± 5	3.4	39 ± 3	6.0	134 ± 4	9.8	67 ± 4	1.9	86 ± 9	6.5	192 ± 20	10.0
26-Hydroxy hexacosanoic acid (ω$C_{26:0}$)	74 ± 1	3.6	27 ± 0	4.2	62 ± 1	4.5	nd		nd		nd	
Total concentration of ω-hydroxy carboxylic acids	662 ± 12	32.3	205 ± 2	31.7	678 ± 7	49.7	642 ± 22	18.2	316 ± 10	23.7	1088 ± 37	56.8
α,ω-Alkanedioic acids												
1,16-Hexadecadioic acid ($C_{16:0}$ diacid)	nd		nd		42 ± 0	3.1	nd		nd		50 ± 4	2.6
1,18-Octadecadioic acid ($C_{18:0}$ diacid)	nd		nd		22 ± 1	1.6	nd		nd		nd	
1,18-Octadecenoic acid ($C_{18:1}$ diacid)	nd		nd		191 ± 3	14.0	nd		nd		289 ± 3	15.1
1,22-Dodecosanoic acid ($C_{22:0}$ diacid)	nd		nd		nd		nd		nd		12 ± 1	0.6
Total concentration of α,ω-alkanedioic acids					255 ± 4	18.7					351 ± 6	18.3

Table 1 continued

	Maize						Wheat					
	Leaves		Stems		Roots		Leaves		Stems		Roots	
	Conc ($\mu g\ g^{-1}$)	RA (%)	Conc ($\mu g\ g^{-1}$)	RA (%)	Conc ($\mu g\ g^{-1}$)	RA (%)	Conc ($\mu g\ g^{-1}$)	RA (%)	Conc ($\mu g\ g^{-1}$)	RA (%)	Conc ($\mu g\ g^{-1}$)	RA (%)
Mid-chain hydroxy acids												
x,16-Dihydroxy hexadecanoic acids ($x = 8$, 9 and 10) (*x*,16-diOH $C_{16:0}$)	166 ± 10	8.1	11 ± 1	1.7	nd		321 ± 17	9.1	90 ± 2	6.8	27 ± 1	1.4
9-Hydroxy hexadecanedioic acid (9-OH $C_{16:0}$ diacid)	32 ± 4	1.6	16 ± 1	2.5	nd		32 ± 1	0.9	130 ± 13	9.8	nd	
9,10-Dihydroxyoctadecanoic acid (9,10-diOH $C_{18:0}$)	nd		nd		nd		54 ± 6	1.5	nd		nd	
9,10,18-Trihydroxyoctadecanoic acid (9,10,18-triOH $C_{18:0}$)	46 ± 4	2.2	6 ± 0	0.9	nd		368 ± 26	10.4	123 ± 8	9.2	30 ± 6	1.6
9,10-Epoxy, 18-hydroxyoctadecanoic acid (9,10-epoxy,18-OH $C_{18:0}$)[a]	118 ± 1	5.8	9 ± 0	1.4	nd		918 ± 51	26.0	262 ± 1	19.7	70 ± 1	3.7
11,18-Dihydroxyoctadecenoic acid (11,18-diOH $C_{18:1}$)	269 ± 4	13.1	nd		5 ± 0	0.4	420 ± 19	11.9	144 ± 3	10.8	29 ± 1	1.5
x,18-dihydroxyoctadecenoic acids ($x = 9$ and 10) (*x*,18 di-OH $C_{18:1}$)	139 ± 2	6.8	nd		nd		160 ± 4	4.5	24 ± 1	1.8	nd	
Total concentration of mid-chain hydroxy acids	770 ± 18	37.5	42 ± 5	6.5	5 ± 0	0.4	2273 ± 117	64.3	773 ± 24	58.0	156 ± 5	8.2
Total concentration of released monomers	2051 ± 5		647.5 ± 37		1365 ± 3		3536 ± 118		1332 ± 43		1914 ± 20	

The standard deviations were calculated from three replicate analyses

nd not detected

[a] Concentration estimated as the concentration of the isomeric mixture of 9-methoxy,10,18-dihydroxyoctadecanoic acid and 9-hydroxy,10-methoxy,18-hydroxyoctadecanoic acids (9-MeOH10,18-diOH $C_{18:0}$ and 10-MeOH 9,18-diOH $C_{18:0}$)

been extracted by ASE prior to depolymerization, we can suppose that most of the waxes have been removed, which is supported by the lack of carboxylic acids with more than 20 carbon atoms and the lack of C_{26} and C_{28} *n*-alcohols. As a result, most of the aforementioned compounds should result from biopolyester depolymerisation.

α-hydroxy carboxylic acid

The $C_{24:0}$ α-hydroxy carboxylic acid was detected, with a relative contribution ranging from 1% of the total monomers in wheat roots to 8% in maize stems. The α-hydroxy $C_{24:0}$ was already observed in plant roots (Zeier et al. 1999; Zimmermann et al. 2000; Franke et al. 2005) but also in leaves (Matzke and Riederer 1990; Franke et al. 2005).

ω-hydroxy carboxylic acids

The ω-hydroxy carboxylic acids (from C_{16} to C_{26}, including a $C_{18:1}$) represented the largest class of aliphatic monomers in maize and wheat roots with more than 50% of the total monomers (Table 1). They were also abundant in leaves (32 and 18% of the total monomers in maize and wheat leaves respectively).

The $\omega C_{20:0}$ was only released from roots and the $\omega C_{26:0}$ only identified in maize tissues. The $\omega C_{18:1}$ was dominating in all the tissues. In roots, apart from the dominant $\omega C_{18:1}$, high contributions of $\omega C_{22:0}$ were observed in wheat, and of $\omega C_{24:0}$ in maize (Table 1). The distribution of ω-hydroxy carboxylic acids in maize roots was close to that obtained by Zeier et al. (1999) in the suberin of cell walls of maize primary roots. The $\omega C_{16:0}$ and $\omega C_{18:1}$ monomers are commonly found in cutins (Holloway 1973; Espelie et al. 1979; Goñi and Hedges 1990a), whereas suberins are generally characterised by monomers with more than 20 carbon atoms with a predominance of the $\omega C_{24:0}$ and $\omega C_{26:0}$ monomers (Matzke and Riederer 1991; Franke et al. 2005). Accordingly, in wheat, the contribution of the ω-hydroxy carboxylic acids with more than 20 carbon atoms to total ω-hydroxy carboxylic acids was lower in leaves and stems than in roots (Table 1). The higher contribution of these long chain compounds to maize aboveground tissues thus reflects the presence of suberized tissues in these organs. Espelie and Kolattukudy (1979) found suberized tissues in the bundle sheaths of *Zea Mays L.* leaves.

α,ω-alkanedioic acids

The α,ω-alkanedioic acids were only released from roots. Their total concentration was similar in wheat and maize (18% of total released monomers, Table 1). The major monomer, the $C_{18:1}$ diacid, represented 15 and 14% of total monomers. It occurred along with very low concentrations of $C_{16:0}$ and $C_{18:0}$ diacids in maize roots and $C_{16:0}$ and $C_{22:0}$ diacids in wheat roots. Zeier et al. (1999) and Zimmerman et al. (2000) already found that the $C_{18:1}$, $C_{16:0}$ and $C_{18:0}$ diacids were the major alkanedioic acids in roots of maize plants at different growth stages.

Mid-chain hydroxy acids

The class named "mid-chain hydroxy acids" comprises seven *n*-carboxylic mono or diacids bearing 2 or 3 hydroxyl groups and 0 or 1 unsaturation. As suggested by Goñi and Hedges (1990a), the 9,10,18-trihydroxyoctadecanoic acid (9,10,18-triOH $C_{18:0}$) can be found as such in the biopolymer or be formed from the conversion of a part of the 9,10-epoxy, 18-hydroxyoctadecanoic acid (9,10-epoxy, 18-OH $C_{18:0}$), since the epoxy function reacts upon basic hydrolysis to be converted into vicinal diols and vicinal methoxy-alcohol groups (Holloway and Deas 1973; Goñi and Hedges 1990a). The isomeric mixture of 9-methoxy,10,18-dihydroxyoctadecanoic acid and 9-hydroxy,10-methoxy,18-hydroxyoctadecanoic acids (9-MeOH 10,18-diOH $C_{18:0}$ and 10-MeOH 9,18-diOH $C_{18:0}$) can derive from the 9,10-epoxy, 18-OH $C_{18:0}$ as well, and was used to quantify the epoxy compound. The quantities of 9,10-epoxy, 18-OH $C_{18:0}$ might thus be underestimated, since the 9,10,18-triOH $C_{18:0}$ acid formed during the depolymerisation could not be distinguished from the preexisting compound and was not taken into account for the estimate of their concentration.

The 9,10-dihydroxyoctadecanoic acid (9,10-diOH $C_{18:0}$) detected in wheat leaves was recognized thanks to the mass spectrum of the corresponding synthetic product published in Eglinton et al. (1968). The 9-hydroxyhexadecan 1,16 dioic acid (9-OH $C_{16:0}$ diacid) present in the stems and leaves of wheat and

maize (Table 1) was previously identified as a major suberin monomer of the *Aspidistra elatior* roots (Zeier and Schreiber 1998).

Two chromatographic peaks were tentatively identified according to their mass spectra (Fig. 2). The *m/z* 530 ion corresponds to M^+ and *m/z* 515 to $[M\text{-}CH_3]^+$. The *m/z* 425 ($[M\text{-}105]^+$) and *m/z* 204 and 217 ions are characteristic for ω-hydroxy carboxylic acids (Eglinton et al. 1968). The major ions, *m/z* 343, 329 and 315 (100%), probably correspond to a cleavage next to the mid-chain hydroxy group ($[TMSiO\text{–}C_{10}H_{17}\text{–}COOTMSi]^+$, $[TMSiO\text{–}C_9H_{15}\text{–}COOTMSi]^+$ and $[TMSiO\text{–}C_8H_{13}\text{–}COOTMSi]^+$, respectively), which leads us to attribute these peaks to the 11,18-dihydroxy octadecenoic acid (11,18-diOH-$C_{18:1}$) and to a mixture of 9,18- and 10,18-dihydroxy octadecenoic acids (*x*,18-diOH-$C_{18:1}$), respectively. Some dihydroxyoctadecenoic acids were previously cited as major monomers of maize cuticle (Espelie and Kolattukudy 1979) and of the leaf cutin of different plant species (Graça et al. 2002).

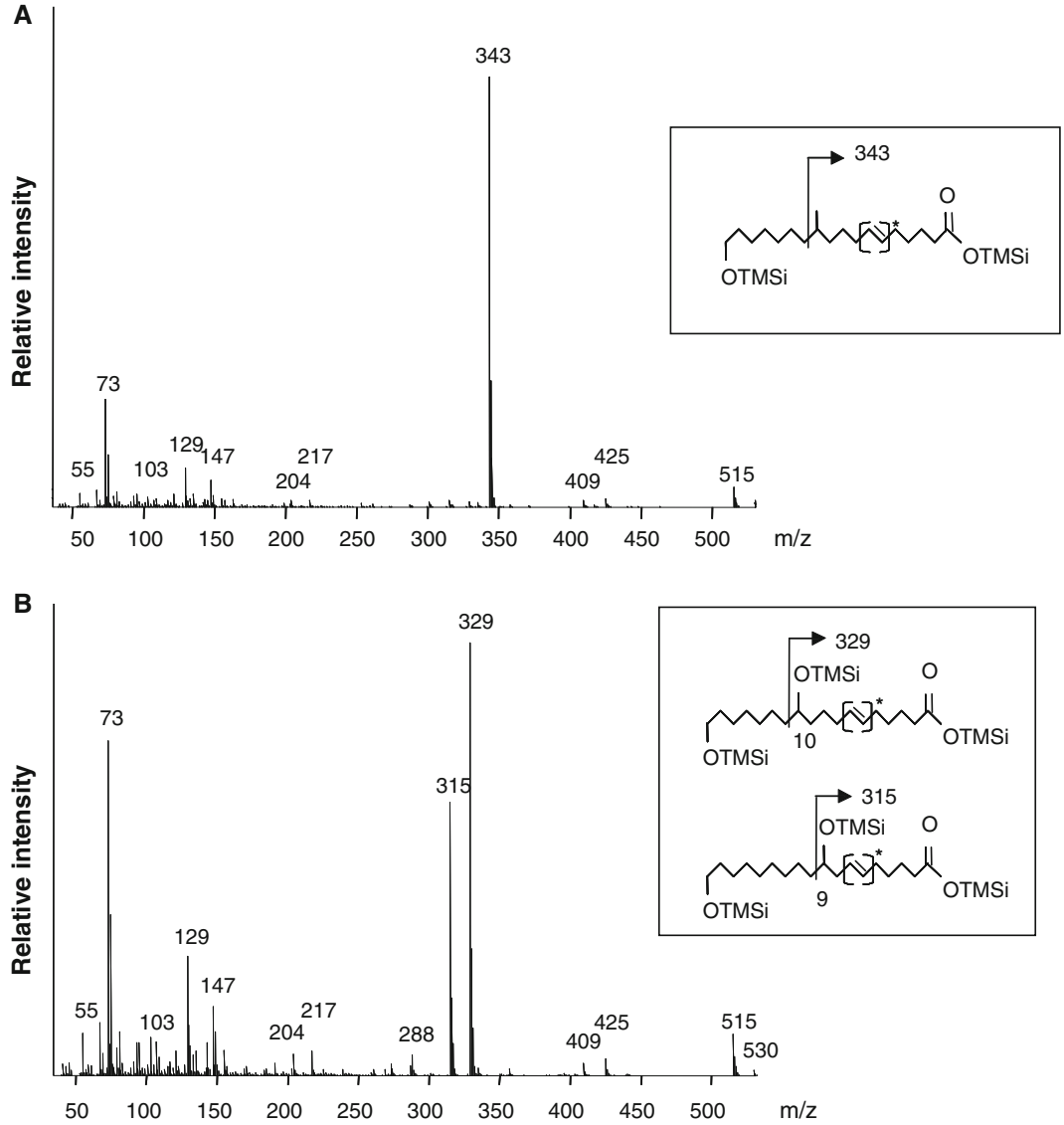

Fig. 2 Mass spectra of (**a**) 11,18-dihydroxyoctadecenoic acid and (**b**) 10,18-dihydroxy octadecenoic acid coeluting with 9,18-dihydroxyoctadecenoic acid; * Position of double bond not determined

The isomeric mixture of (*x*,16)-dihydroxy hexadecanoic acids ($x = 8, 9, 10$) (*x*,16-diOH $C_{16:0}$), the 9,10-epoxy, 18-OH $C_{18:0}$ and the 9,10,18-triOH $C_{18:0}$ were detected in all the samples except maize roots (Table 1) and are the most common monomers identified in cutins (Hunneman and Eglinton 1971; Holloway 1973; Espelie et al. 1979; Espelie et al. 1980; Matzke and Riederer 1991; del Rio and Hatcher 1998).

Leaves were the plant tissues where the mid-chain hydroxy acids were the most abundant, representing 64 and 37% of the total monomers for wheat and maize, respectively (Table 1). Amongst the most abundant compounds of wheat leaves were the 9,10-epoxy,18-OH $C_{18:0}$ (26%) and the 9,10,18-triOH $C_{18:0}$ acid (10%), which accounted only for 6% and 2% of total monomers, respectively in maize leaves. The 11,18-diOH $C_{18:1}$ (13% and 12% in maize and wheat leaves, respectively), and the *x*,16-diOH $C_{16:0}$ (8% and 9% in maize and wheat leaves, respectively) were also major monomers in plant leaves (Table 1). In maize cuticles, Espelie and Kolattukudy (1979) also identified the diOH $C_{18:1}$ acid (with no detail on the location of the hydroxyl groups) and the *x*, 16-diOH $C_{16:0}$ acids as major compounds, while the 9,10,18-triOH $C_{18:0}$ acid was a minor constituent. In wheat cuticles, Matzke and Riederer (1990) only identified the 9,10-epoxy,18-OH $C_{18:0}$ acid and the *x*,16-diOH $C_{16:0}$ acids, accounting for 37% and 21% of the total released monomers, respectively.

Wheat stems contained a high proportion of mid-chain hydroxy acids (58% of the total released monomers, Table 1), while they were minor compounds of maize stems (6.5% of the total released monomers). Their monomeric distribution in wheat stems was close to that of wheat leaves, except for the 9-OH $C_{16:0}$ diacid, which concentration was higher in wheat stems.

The contribution of mid-chain hydroxy acids to root monomers was very low. In maize roots only the 11,18-diOH $C_{18:1}$ could be detected (less than 1% of the total released monomers) (Table 1). In wheat roots, four of the seven mid-chain hydroxy acids could be identified, representing only 8% of the total monomers.

Identification of biomarkers for below and aboveground plant tissues

Although *n*-carboxylic acids constituted a large part of the plant biopolymers (Table 1), they are present in microorganisms as well (Otto et al. 2005) and cannot be used as plant biomarkers in soils. The same is true for the minor *n*-alcohols. The α-hydroxy carboxylic acids might be formed from plant polymers other than cutins and suberins (Molina et al. 2006). Furthermore, in soils, they might also derive from microorganisms and are therefore not specific for plants. These three classes of compounds *a fortiori* cannot be used as cutin or suberin markers and they will be considered together in the following non-specific for plant.

The concentrations of the different classes of compounds in the different plant tissues relative to the total plant were calculated by using the allometric relationships of leaves, stems and roots returned to the soil given by Bahri et al. (2006) (Fig. 3).

The ω-hydroxy carboxylic acids were common to all plant tissues (Table 1). As a result, they cannot be directly related to the above or belowground biomass.

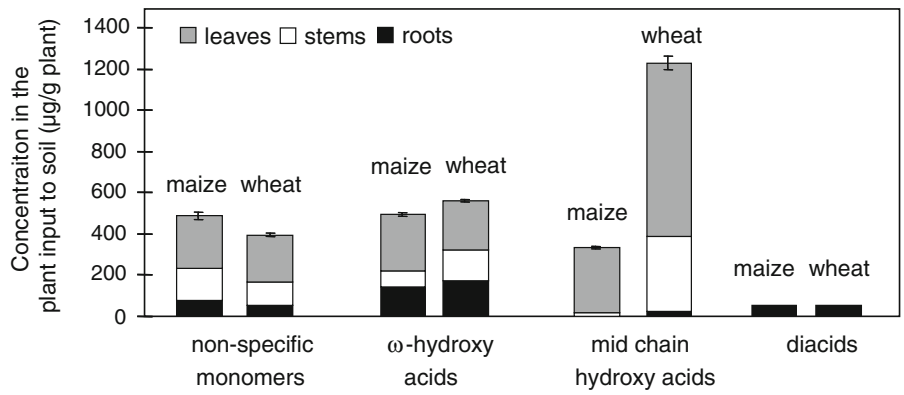

Fig. 3 Concentrations (µg g^{-1} plant) of the non plant specific monomers (*n*-carboxylic acids, *n*-alcohols and α-hydroxy carboxylic acids) and of the plant specific monomers (ω-hydroxy acids, mid-chain hydroxy acids and α,ω-dialkanoic acids) in the plant biomass returned to the soil, according to the plant organ

However, the $\omega C_{20:0}$ was only released from roots (Table 1). Interestingly, ω-hydroxy carboxylic acids with more than 20 carbon atoms ($\omega C_{22:0}$ to $\omega C_{26:0}$) were also found in leaves and stems, whereas in soils they are commonly considered as suberin markers (Nierop 1998; Otto et al. 2005). Given the lack of α,ω-alkanedioic acids in leaves and stems and their presence in roots (Table 1; Fig. 3), these compounds can be regarded as characteristic for belowground plant biomass. By contrast, the contribution of roots to the total concentration of mid-chain hydroxy acids of maize and wheat plants is very low (Fig. 3) and this class of compounds can be considered as specific for aboveground biomass.

In order to evaluate the possible use of the three classes of plant specific compounds (ω-hydroxy carboxylic acids for total plants, diacids for roots and mid-chain hydroxy acids for aboveground tissues) to follow the fate of plant tissues in soils in C_3/C_4 chronosequences, their ^{13}C isotopic signatures were determined and compared.

^{13}C isotopic composition of plant aliphatic compounds in wheat and maize tissues

The $\delta^{13}C$ values of the aliphatic monomers in the different plant tissues are shown in Table 2 for wheat and maize. In agreement with their difference in biosynthetic pathway, maize monomers are ^{13}C enriched when compared to wheat ones. The ^{13}C signatures of the *n*-carboxylic acids varied within a 10‰ range in maize tissues (from −15.6 to −25.6‰ for maize tissues) and a 11.5‰ range in wheat tissues (from −24.4 to −35.9‰ for wheat tissues). The ^{13}C signatures of the *n*-carboxylic acids present in the bound lipids were similar to those of the *n*-carboxylic acids present in the free lipid fraction measured by Wiesenberg et al. (2004a). These authors determined for the free *n*-$C_{24:0}$ a ^{13}C signature ranging from −35.5 to −38.0‰ in wheat and from −20.4 to −27.3‰ in maize tissues. The $C_{18:0}$ *n*-alcohol released from maize roots was ^{13}C-depleted (−29.3‰) compared to the *n*-carboxylic acids of the maize roots. By contrast, the signatures of the $C_{24:0}$ α-hydroxy carboxylic acid were in the same range as those of the C_{24} *n*-carboxylic acids in the different maize and wheat tissues.

The ^{13}C signatures of the ω-hydroxy carboxylic acids ranged from −15.7 to −22.6‰ in maize tissues. In wheat, the ω-hydroxy carboxylic acids had $\delta^{13}C$ ranging from −30.6 to −37.0‰. In maize roots and leaves and in wheat roots, the ω-hydroxy carboxylic acids with more than 20 carbon atoms were ^{13}C-depleted compared to the C_{16} and C_{18} compounds, as already observed for the free *n*-alkanes and *n*-carboxylic acids in wheat and maize (Chikaraishi et al. 2004; Wiesenberg et al. 2004a). This ^{13}C-depletion of compounds with longer chains can be attributed to a depletion along the biosynthesis pathway during the elongation of the carbon chain involving the acetyl-CoA and the malonyl-CoA (Melzer and Schmidt 1987). The high variations in $\delta^{13}C$ can be the result of the many intermediate biomolecules formed during the C chain elongation process (Chikaraishi et al. 2004).

The $\delta^{13}C$ values of the α,ω-alkanedioic acids were in the same range as the other aliphatic compounds, ranging from −15.1 to −20.2‰ in maize roots and from −34.9 to −37.0‰ in wheat roots. The range of ^{13}C signatures of the mid-chain hydroxy acids is less extended in maize tissues (−16.1 to −19.2‰) than in the wheat ones (−26.4 to −38.2‰).

Variation of the $\delta^{13}C$ of compound classes with plant tissues

In each tissue, an average ^{13}C signature was calculated for the ω-hydroxy carboxylic acids (plant markers), diacids (root markers) and mid-chain hydroxy acids (leaves and stems markers, named shoot markers in the following) by weighting the $\delta^{13}C$ values of the individual compounds by their concentrations in the tissue according to Eq. 1 (Fig. 4a). A mean $\delta^{13}C$ value was calculated for *n*-carboxylic acids, α-hydroxy carboxylic acids and *n*-alcohols, labelled as "non-specific compound" class. The values obtained in the present study for the four classes of bound lipid monomers were compared to the data given in the literature for bulk tissues (Dignac et al. 2005) and lignin biomarkers (Bahri et al. 2006) of the same plants (Fig. 4a).

The aliphatic constituents of the biopolyesters were generally depleted in ^{13}C compared to the bulk plant, except for the non-specific compounds in wheat roots that had a similar ^{13}C content as the bulk root tissues. A ^{13}C depletion has already been reported for immobile compounds (such as the plant tissue structural constituents lignins, cutins and

Table 2 ^{13}C signature ($\delta^{13}C$, ‰) of aliphatic monomers in leaves, stems and roots of maize and wheat

	Maize			Wheat		
	Leaves	Stems	Roots	Leaves	Stems	Roots
n-Carboxylic acids						
Hexadecanoic acid (*n*-$C_{16:0}$)	−20.1 ± 0.5	−22.3 ± 0.5	−20.8 ± 0.5	−30.4 ± 0.5	−29.5 ± 0.5	−26.1 ± 0.6
Octadecanoic acid (n-$C_{18:0}$)	−23.0 ± 0.5	−25.6 ± 0.6	−20.8 ± 0.6	−26.8 ± 0.6	−24.4 ± 0.5	−24.9 ± 0.5
Octadecenoic acid (*n*-$C_{18:X}$)	−18.2 ± 0.5	−17.2 ± 0.5	−15.6 ± 1.4	−29.4 ± 0.5	−27.6 ± 0.5	−26.1 ± 0.9
Eicosanoic acid (*n*-$C_{20:0}$)	na[b]	na	−21.3 ± 0.7	na	na	−35.9 ± 1.0
Tetracosanoic acid (*n*-$C_{24:0}$)	−23.2 ± 0.5	−20.7 ± 0.8	−21.3 ± 0.5	na	−33.9 ± 0.5	−35.5 ± 1.0
n-Alcohols						
Octadecanol (Alcohol $C_{18:0}$)	na	na	−29.3 ± 0.5	na	na	na
Docosanol (Alcohol $C_{20:0}$)	na	na	na	na	na	na
α-Hydroxy carboxylic acids						
2-Hydroxy tetracosanoic acid (α$C_{24:0}$)	−22.1 ± 0.5	na	−19.2 ± 0.5	−34.2 ± 0.5	−34.9 ± 0.5	−33.4 ± 0.5
ω-Hydroxy carboxylic acids						
16-Hydroxy hexadecanoic acid (ω$C_{16:0}$)	−16.6 ± 0.5	−18.6 ± 1.0	−15.7 ± 0.5	−34.1 ± 0.7	na	−34.5 ± 0.5
18-Hydroxy octadecenoic acid (ω$C_{18:1}$)	−16.8 ± 0.5	−20.8 ± 0.5	−16.0 ± 0.5	−30.6 ± 0.9	−31.9 ± 0.5	−31.5 ± 0.5
20-Hydroxy eicosanoic acid (ω$C_{20:0}$)	na	na	na	na	na	−37.0 ± 0.6
22-Hydroxy docosanoic acid (ω$C_{22:0}$)	−21.1 ± 0.5	na	−18.0 ± 1.1	−36.5 ± 1.0	−36.9 ± 0.5	−36.9 ± 0.5
24-Hydroxy tetracosanoic acid (ω$C_{24:0}$)	−20.9 ± 0.5	−18.0 ± 0.5	−22.6 ± 1.0	−32.6 ± 1.0	−31.3 ± 0.5	−34.0 ± 0.5
26-Hydroxy hexacosanoic acid (ω$C_{26:0}$)	−18.6 ± 0.7	−18.7 ± 1.0	−21.0 ± 0.7	na	na	na
α,ω-Alkanedioic acids						
1,16-Hexadecadioic acid ($C_{16:0}$ diacid)	na	na	−15.1 ± 0.5	na	na	−36.1 ± 0.9
1,18-Octadecadioic acid ($C_{18:0}$ diacid)	na	na	−20.2 ± 0.5	na	na	na
1,18-Octadecenoic acid ($C_{18:1}$ diacid)	na	na	−16.3 ± 0.7	na	na	−34.9 ± 0.5
1,22-Dodecosanoic acid ($C_{22:0}$ diacid)	na	na	na	na	na	−37.0 ± 0.5
Mid-chain hydroxy acids						
x,16-Dihydroxy hexadecanoic acids (*x* = 8, 9 and 10) (*x*,16-diOH $C_{16:0}$)	−16.4 ± 0.5	na	na	−34.5 ± 0.5	−36.1 ± 0.5	−37.1 ± 0.5
9-Hydroxy hexadecanedioic acid (9-OH $C_{16:0}$ diacid)	−16.1 ± 0.5	na	na	−33.6 ± 2.0	−32.8 ± 0.5	na
9,10-Dihydroxyoctadecanoic acid (9,10-diOH $C_{18:0}$)	na	na	na	−30.7 ± 0.5	na	na

Table 2 continued

	Maize			Wheat		
	Leaves	Stems	Roots	Leaves	Stems	Roots
9,10,18-Trihydroxyoctadecanoic acid (9,10,18-triOH $C_{18:0}$)	−18.1 ± 0.5	−18.9 ± 0.5	na	−34.6 ± 0.7	−37.4 ± 0.5	−26.4 ± 0.9
9,10-Epoxy,18-hydroxyoctadecanoic acid (9,10-epoxy,18-OH $C_{18:0}$)[a]	−17.6 ± 0.5	na	na	−36.1 ± 0.6	−36.8 ± 0.5	na
11,18-Dihydroxyoctadecenoic acid (11,18-diOH $C_{18:1}$)	−17.0 ± 0.5	−19.2 ± 1.3	na	−34.6 ± 0.7	−35.3 ± 0.5	−38.2 ± 0.7
x,18-Dihydroxyoctadecenoic acids (x = 9 and 10) (*x*,18 di-OH $C_{18:1}$)	−17.6 ± 0.5	−17.1 ± 0.6	na	−34.4 ± 0.7	−34.6 ± 0.5	−30.9 ± 1.0

The standard deviations were calculated from three replicate analyses

na not analysed

[a] Estimated as the isomeric mixture of 9-methoxy,10,18-dihydroxyoctadecanoic acid and 9-hydroxy,10-methoxy,18-hydroxyoctadecanoic acids (9-MeOH 10,18-diOH $C_{18:0}$ and 10-MeOH9,18-diOH $C_{18:0}$)

suberins) compared to the mobile ones (sugars) due to a possible fractionation along transport pathways (Hobbie and Werner 2004).

In wheat, the non plant-specific compounds were the most enriched compounds (−28.0 to −30.1‰) when compared to lignin-derived phenols (−31.3 to −33.6‰, Bahri et al. 2006) and to the three plant specific classes (−32.2 to −35.8‰). By contrast in maize, they were the most depleted compounds (−19.9 to −21.4‰) when compared to lignin-derived phenols (−11.2 to −16.2‰, Bahri et al. 2006) and to the three classes of plant specific compounds (−16.4 to −19.2‰). This difference between maize and wheat might be due to the different photosynthetic pathways of organic constituents in C3 and C4 plants (Hobbie and Werner 2004).

In maize, the aliphatic compounds were all depleted compared to lignins, whereas in wheat only the root (α,ω-alkanedioic acids) and shoot (mid-chain hydroxy acids) markers were depleted compared to lignin-derived phenols. The ω-hydroxy acids were also enriched compared to lignins in wheat leaves, while they were depleted in wheat stems and roots. The ^{13}C depletion of the cutin/suberin markers in maize and wheat tissues can be related to the depletion already observed for the free lipid fraction of plants, which was explained by the biosynthetic pathway of lipids in plant cells (Melzer and Schmidt 1987; Rossmann et al. 1991; Hobbie and Werner 2004). However, no clear-cut trend is observed here in the case of wheat tissues.

In maize, the ω-hydroxy acid $\delta^{13}C$ values were similar to those of the cutin (mid-chain hydroxy acids) and the suberin markers (α,ω-alkanedioic acids). By contrast in wheat, a slight ^{13}C enrichment of ω-hydroxy acids in leaves and stems compared to cutin makers is observed. In wheat roots, the ^{13}C content of diacids (−35.1‰) were similar to that of the ω-hydroxy acids.

In both plants, cutin markers (mid-chain hydroxy acids) were ^{13}C-depleted in stems (−18.9‰ in maize, −35.8‰ in wheat) compared to leaves (−17.1‰ in maize, −35.1‰ in wheat) and were both depleted compared to suberin markers (−16.4‰) in maize roots. The $\delta^{13}C$ of diacids in wheat roots (−35.1‰) was similar to that of the cutin markers in leaves. The different isotopic values of the biomarkers in stems, roots and leaves might be explained by a post-photosynthetic ^{13}C fractionation between plant

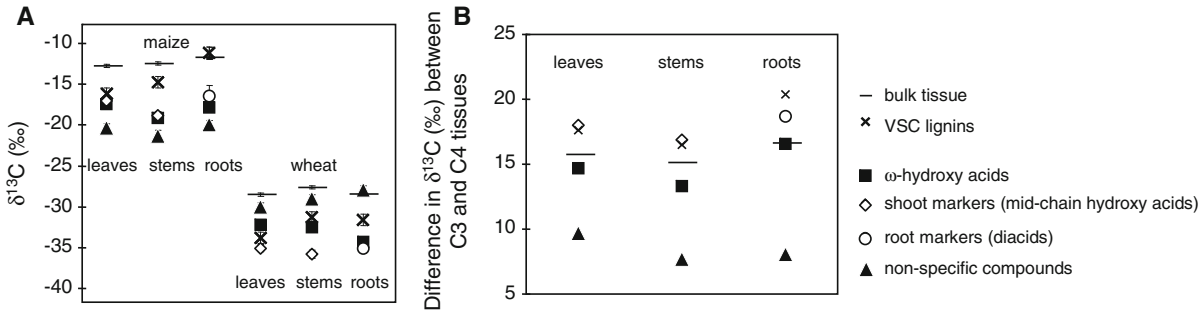

Fig. 4 Comparison of the isotopic signatures in leaves, stems and roots of maize and wheat. **a** Isotopic signatures of the plant aliphatic biopolyesters calculated as the average of the signatures of the monomers weighted by their concentration in the tissue for non plant specific monomers (*n*-carboxylic acids, *n*-alcohols and α-hydroxy carboxylic acids), the ω-hydroxy carboxylic acids, the shoot markers (mid-chain hydroxy acids) and the root markers (diacids). Comparison with published values of the bulk tissues (from Dignac et al. 2005) and of lignins (from Bahri et al. 2006). **b** Difference between the isotopic signatures in wheat (C_4 plant) and maize (C_3 plant)

organs (Hobbie and Werner 2004; Badeck et al. 2005), due to processes such as respiration (Ghashghaie et al. 2003; Tcherkez et al. 2003; Nogués et al. 2004) or transport of metabolites (Hobbie and Werner 2004).

^{13}C isotopic differences of plant aliphatic compounds between wheat and maize

The contrasted variations of the isotopic values of the different compound classes lead to strong differences between C_3 (maize) and C_4 (wheat) values of these classes (Fig. 4b). The highest difference was observed for diacids in roots (18.7‰) and mid-chain hydroxy acids in leaves and stems (18.0 and 16.9‰, respectively). These differences were higher than those observed for ω-hydroxy acids (13.3 and 16.5‰). The non-specific *n*-$C_{20:0}$ and *n*-$C_{24:0}$ also displayed large differences between wheat and maize roots (14.6 and 14.2‰, respectively). The lowest difference was observed for the non specific plant monomers (8.0–9.7‰). Chikaraishi et al. (2004) already observed that the difference in ^{13}C natural abundance of lipids compared to bulk tissues was not the same for C_3 and C_4 plants. These variations might be due to contrasted isotopic fractionation during biosynthesis between C_3 and C_4 plants (Park and Epstein 1961; Tcherkez et al. 2003) and photorespiration, coupled to different allocation patterns (Rossmann et al. 1991; Tcherkez et al. 2003; Hobbie and Werner 2004) and to different environmental stresses during metabolism for C_3 and C_4 plants.

The difference in stable carbon isotope composition of plant biomarkers (mid-chain hydroxy acids specific for shoots, diacids specific for roots) between C_3 and C_4 plants were higher than for bulk tissues (15.1, 15.7, 16.6‰ for stems, leaves and roots, respectively, Dignac et al. 2005) and close to the differences observed for lignins (16.5, 17.6, 20.4‰ in stems, leaves and roots, respectively) by Bahri et al. (2006). In our previous study (Dignac et al. 2005), the difference of isotopic values of lignin-derived phenols in wheat and nine year maize soils was much larger (−32.6‰ under wheat, −25.3‰ after nine year maize) than that observed for bulk soil (−25.9‰ under wheat, −24.4‰ after nine year maize). These differences observed for lignin-derived phenols were high enough compared to the analytical uncertainty to estimate the dynamics of the lignin biomarkers in soils (Bahri et al. 2006).

Conclusions

Cutin and suberin aliphatic constituents are powerful biomarkers commonly used to infer the sources of OM in sediments and soils. In wheat (C_3) and maize (C_4), the ω-hydroxy carboxylic acids were specific for plants but not for a specific plant tissue. By contrast, the α,ω-alkanedioic acids and the mid-chain hydroxy acids were specific for roots and shoots, respectively. Large differences between the C_3 and C_4 species were observed in the isotopic contents of these three classes of biomarkers. Combining

molecular and isotopic information on these biomarkers would be of great value for studying the fate of root/shoot-derived OM in soils using ^{13}C natural abundance labelling in C_3/C_4 chronosequences. The specificity of these monomers for roots and shoots should allow differentiating between the turnover of below and aboveground biomass, which is a major pending question for understanding the contribution of plant OM to C storage in soils.

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