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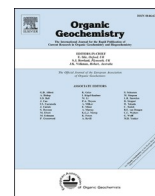
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Comparison of Curie-point pyrolysis with tetramethylammonium hydroxide (TMAH) and base catalyzed methanolysis in leaf and root biomarker decomposition studies

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ABSTRACT

Cutin and suberin have frequently been used as plant-part specific biomarkers in sediments and soils. The two most commonly used analytical methods described in the literature to study cutin and suberin are pyrolysis with tetramethylammonium hydroxide (TMAH) and base-catalyzed methanolysis with potassium hydroxide (KOH). Both methods are usually combined with gas chromatography (GC) to analyze and identify the resulting compounds. However, until now, a critical assessment of the compatibility of the results obtained by both methods was missing.

Here, we compared the two techniques on Scots pine (*Pinus sylvestris*) and Norway spruce (*Picea abies*) roots and needles that decomposed for three years in litterbags. KOH methanolysis released a broader suite of long-chain alcohols, diols and mid-chain hydroxy-fatty acids, identifying 19 tissue-specific biomarkers, whereas pyrolysis with TMAH yielded 10 diagnostic compounds; only seven markers overlapped. The concentrations of analogous molecules decreased in different ways over time, preventing any generalizable conclusions from being drawn about the stability of the original biopolymers. Thus, the methods are complementary rather than interchangeable. Pyrolysis yields fewer, more temporally stable signals and requires substantially less sample preparation per analysis, making it well suited to quantitative source apportionment; by contrast, methanolysis involves multi-step sample preparation and provides richer structural detail that facilitates biomarker discovery across taxa and matrices.

1. Introduction

In recent decades, there has been substantial interest in the chemical structure and functional roles of the plant biopolymers cutin and suberin. Aliphatic plant polyesters, such as cutin and suberin, are crucial functional membranes in plants, controlling water, gas, and solute transportation processes, and providing protection against microbial infections (Franke and Schreiber, 2007; González-Valenzuela et al., 2023). Ester-bound lipids derived from cutin and suberin have been considered as potential precursors for stable soil organic matter and thus possible carbon sinks in the global carbon cycle (Lorenz et al., 2007; Berg and McLaugherty, 2020). Additionally, specific cutin- and suberin-derived ester-bound substituted fatty acids have been used as biomarkers to estimate the relative input of leaves and roots to the soil

(e.g., Crow et al., 2009; Spielvogel et al., 2014; Burger et al., 2024).

Although both polymers act as hydrophobic diffusion barriers, they occur in distinct tissues and interfaces within the plant. Cutin is present on the outer surfaces of leaf, fruit, and primary-shoot epidermal cell walls, while suberin serves as an apoplastic interface, forming a barrier between living cells and the external environment, as well as separating different tissues within the plant (Franke and Schreiber, 2007; Fich et al., 2016). Suberin is particularly abundant in cork cells, which are prevalent in bark, roots, and healed plant wounds. Both cutin and suberin predominantly consist of *n*-fatty acids (FAs) and substituted FAs, such as mono-, di-, and tri-hydroxy FAs, alkane dioic acids, and epoxy FAs, linked by ester bonds to form an aliphatic plant polymer (Kolattukudy, 1981; Graça, 2015). Cutin and suberin are chemically related yet distinct polymers, differing in composition and in their spatial patterns

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of accumulation within the apoplast (Philippe et al., 2020). Released ester-bound lipids typically span chain lengths of C12–C24, with leaf- and needle-derived fractions commonly dominated by C16 and C18 homologues (Altmann et al., 2021a; Altmann et al. 2021b). α,ω -Dihydroxyhexadecanoic acid is well represented in leaves and needles; however, it has not yet been reported from roots (Kolattukudy, 1981).

In recent decades, various analytical methods for studying cutin and suberin have been developed (Fig. 1). Spectroscopic methods such as Fourier-Transform Infrared Spectrophotometry (FTIR) and Nuclear Magnetic Resonance Spectroscopy (NMR) are widely used to analyze the chemical bonds of the intact polymer without prior degradative steps, which might alter the properties of the samples (Villena et al., 2000; Fang et al., 2001). However, these chemical bonds are regarded as less source specific as biomarker-based methods (Amelung et al., 2008).

The detection of specific macromolecules such as cutin and suberin is typically achieved based on their monomer constituents which can be resolved using gas chromatography (Del Rio and Hatcher, 1998). Such analyses require prior degradation of the samples to cleave the links between the monomers. The necessary depolymerization can be achieved chemo-thermally through pyrolysis, biologically using enzymes, or chemically via methods such as solvolysis, reductive, or oxidative techniques (Goñi and Hedges, 1990; Zeier and Schreiber, 1998; Naafs et al., 2005).

Base-catalyzed methanolysis with KOH proceeds in methanolic solution, where KOH generates methoxide (MeO^-) in situ ($\text{KOH} + \text{MeOH} \rightleftharpoons \text{MeO}^- \text{K}^+ + \text{H}_2\text{O}$). Methoxide effects nucleophilic acyl substitution at polyester ester linkages (addition–elimination), releasing the acyl components predominantly as methyl esters; pre-existing hydroxyl groups on released monomers remain as $-\text{OH}$ and are typically derivatized (e.g., silylated) prior to GC/MS (Carey & Sundberg, 2007; Järvinen et al., 2009). In the literature, both “methanolysis” and “hydrolysis” are used depending on solvent composition: “methanolysis” refers to anhydrous ($\approx 100\%$) MeOH, whereas “hydrolysis” denotes MeOH/ H_2O mixtures, which modulate the relative roles of methoxide and hydroxide in acyl cleavage (Carey & Sundberg, 2007).

Analytical pyrolysis encompasses elimination, rearrangement, substitution, addition, and redox processes under rapid heating (Moldoveanu, 1998). For saccharides and hydroxy fatty acids, a commonly used approach is modified pyrolysis with tetramethylammonium hydroxide (TMAH): TMAH promotes thermally assisted

hydrolysis and in-situ methylation, converting carboxyl groups to methyl esters and hydroxyl groups to methyl ethers during depolymerization (de Leeuw & Baas, 1993; Challinor, 2001). Although only one of several possible analytical approaches, pyrolysis with TMAH is widely applied in soil science and yields reproducible, interpretable datasets (Kögel-Knabner, 2000; Nikolaus et al., 2023; Charamba et al., 2024). So far, it has been shown that pyrolysis with TMAH and base-catalyzed methanolysis with KOH are both suitable for releasing ester-bound lipids. Several studies have directly compared the molecular profiles yielded from SOM macromolecules under different analytical methods (Nierop et al., 2006; Crow et al., 2009; Mendez-Millan et al., 2010). Previous studies have shown that different chemical degradation methods can influence the composition and concentrations of analytical products derived from cutin and suberin (Graça and Pereira, 2000; Järvinen et al., 2009). To our knowledge, the comparability of pyrolysis with TMAH and base-catalyzed methanolysis with KOH to study needle and root derived biomarkers, i.e. cutin and suberin, has not been validated in soil-like environments.

To address these concerns, the objective of the present study was to compare the applicability of base-catalyzed methanolysis with KOH and pyrolysis with TMAH for the analysis of leaf- and root-derived aliphatic polyesters that are commonly used as representative for cutin and suberin.

Specifically, we aimed to:

- (i) Test whether the qualitative and quantitative information of root- and needle-specific biomarkers is similar for both analytical methods.
- (ii) Analyze whether the temporal trends of relative root- and needle-specific biomarker abundance during litter decomposition are similar for both methods.

For this purpose, we used samples of Norway spruce (*Picea abies*) and Scots pine (*Pinus sylvestris*) from a three-year litter bag decomposition experiment conducted in eastern Finland.

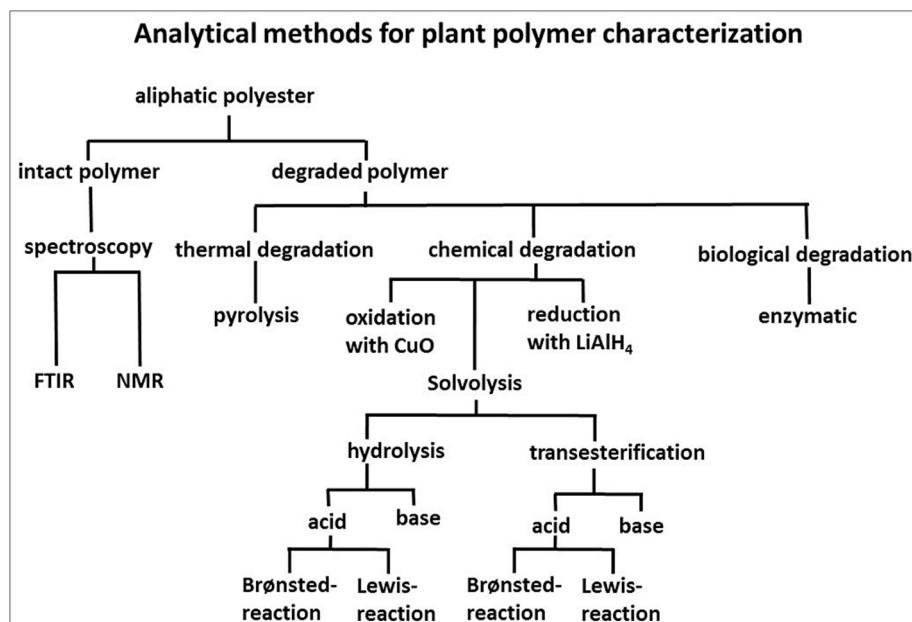


Fig. 1. Overview of analytical methods to analyze aliphatic biopolymers.

2. Material and methods

2.1. Samples

The samples were obtained from a litter bag experiment conducted in eastern Finland (63°51' N, 28°58' E, 220 m a.s.l.). The average air temperature during the experiment was 1.3 °C, and the average annual precipitation was 450 mm (Palviainen et al., 2004). Roots and needles were exposed in litter bags in the field for three years and were sampled annually. The soil, derived from sandy till, is classified as a Haplic Podzol (IUSS, 2022) with a 3 cm thick organic layer (mor humus). The bags containing foliage and branches were placed on top of the organic layer while those containing roots were placed under the organic layer (Palviainen et al., 2004). The observed mass loss of the plant litter material is shown in Table 1.

2.2. Extraction and quantification of root and leaf-derived biomarkers

2.2.1. Free lipid extraction

The contents of the bags were ground using an A 10 IKA analytical mill, and subsamples were oven-dried at 105 °C for 24 h (Palviainen et al., 2004). The field samples were combined into a composite sample, from which duplicates were prepared for subsequent analyses. Since the focus of this study was on macromolecules, free lipids were extracted prior to further processing. For this, 0.2 g of each sample was extracted using accelerated solvent extraction (ASE) with a Dionex 200 ASE extractor. The extraction was performed using 11 mL extraction cells and a dichloromethane/methanol (DCM/MeOH) (93:7 v/v) solvent mixture (Jansen et al., 2006). Extraction conditions included a temperature of 75 °C and a pressure of 17·10⁶ Pa, with a heating phase of 5 min and a static extraction time of 20 min (Jansen et al., 2006). The free lipids were not considered for this study, as they were not expected to contain individual root- or needle-specific compounds (Amelung et al., 2008).

2.2.2. Ester-bound lipid extraction and derivatization (base catalyzed methanolysis)

After extraction, the samples underwent base-catalyzed methanolysis to release ester-bound lipids (Hunneman and Eglinton, 1972). The extraction residues were refluxed for 3 h in 20 mL of 1 M KOH/MeOH. After cooling, the suspension was centrifuged for 20 min at 2500 rpm. Each soil residue was then extracted via sonication for 15 min in 30 mL of a DCM/MeOH (1:1 v/v) mixture. The suspension was centrifuged again, and the supernatant was combined with that from the first

Table 1

Total mass loss, C and N content of litter materials during decomposition (from Palviainen et al., 2004).

| | <i>Picea</i> | | | | <i>Pinus</i> | | | |
|---------------------|--------------|------|------|------|--------------|------|------|------|
| | Needles | | | | Needles | | | |
| Year of incubation | 0 | 1 | 2 | 3 | 0 | 1 | 2 | 3 |
| Total mass loss (%) | 0 | 27.4 | 46.6 | 62.6 | 0 | 32.7 | 54.3 | 68.8 |
| C content (%) | 52 | 49.9 | 49.1 | 50 | 52 | 54.2 | 54.5 | 54.1 |
| N content (%) | 0.9 | 1.2 | 1.6 | 1.8 | 1.1 | 2.0 | 2.4 | 2.6 |
| C/N ratio | 59 | 41 | 31 | 28 | 48 | 28 | 29 | 28 |
| | <i>Picea</i> | | | | <i>Pinus</i> | | | |
| | Roots | | | | Roots | | | |
| Year of incubation | 0 | 1 | 2 | 3 | 0 | 1 | 2 | 3 |
| Total mass loss (%) | 0 | 19.4 | 29.1 | 33.9 | 0 | 32.0 | 39.9 | 49.4 |
| C content (%) | 49.1 | 50.2 | 51.1 | 49.9 | 46.2 | 50.1 | 51 | 50.2 |
| N content (%) | 0.6 | 0.7 | 0.4 | 0.3 | 0.7 | 1.1 | 1.0 | 1.2 |
| C/N ratio | 77 | 25 | 26 | 25 | 62 | 26 | 26 | 26 |

centrifugation step.

The combined extracts were acidified to pH 1 by adding 6 M HCl. Solvent was removed by evaporation, and the samples were sequentially redissolved in 50 mL of ultrapure water and DCM before being transferred to separation funnels. Methanolates were recovered via liquid-liquid extraction in the separation funnels. Anhydrous Na₂SO₄ was added to the combined DCM phase to remove residual water. The DCM extracts were then concentrated by evaporation under N₂, transferred to 2 mL glass vials, and dried. Deuterated hexadecanoic acid was added as an internal standard for relative quantification.

Aliquots of the total extracts, following solvent extraction and base-catalyzed methanolysis, were converted to trimethylsilyl (TMS) derivatives by reacting with *N,O*-bis-(trimethylsilyl) trifluoroacetamide (BSTFA) for 1 h at 70 °C (Jansen et al., 2006). After derivatization, the solutions were dried under N₂ to remove excess BSTFA and re-dissolved in 200 µL of cyclohexane. The depolymerization products obtained via base-catalyzed methanolysis were identified as methyl ester trimethylsilyl ethers.

2.2.3. Thermally assisted hydrolysis and methylation (THM, pyrolysis with TMAH)

Thermally assisted hydrolysis and methylation (THM) analyses were performed by adding a droplet of tetramethylammonium hydroxide (TMAH) in water (25%) to a few milligrams of solvent-extracted residue, which was pressed onto a ferromagnetic wire with a Curie temperature of 600 °C. The wet sample was dried using a 100 W halogen lamp. This procedure was repeated three times to ensure complete methylation. The wire with the sample was then manually inserted into a Horizon Instruments Curie-Point pyrolyser. This analytical procedure is hereafter referred to as pyrolysis with TMAH. Samples were heated for 5 s at 600 °C. The depolymerization products generated by pyrolysis with TMAH were identified as methyl ester methyl ethers.

2.2.4. Gas chromatography–mass spectrometry (GC/MS)

The GC/MS analysis for both the methanolates and the pyrolysed samples was performed with a ThermoQuest Trace GC 2000 gas chromatograph connected to a Finnigan Trace MS quadrupole mass spectrometer. The methanolates were separated via cold on-column injection of 1 µL of the extract onto an HP-5MS fused-silica column (30 m × 0.25 mm id, 0.25 µm film thickness) at 45 °C.

The GC oven was temperature-programmed from 65 °C (2 min), to 120 °C (held 5 min) at 70 °C/min, then to 325 °C (held 15 min) at 5 °C/min.

Pyrolysed samples were analysed in an online procedure where the pyrolyser was directly coupled to the GC/MS apparatus. Separation took place on a DB-5 fused silica column (J and W 30 m × 0.32 mm i.d., 0.25 µm film thickness). The GC oven conditions were: 40 °C (1 min) to 320 °C (held 15 min) at 7 °C/min.

He was the carrier gas at 1.4 ml/min. MS detection was in full scan mode over a mass to charge ratio (*m/z*) of 50–650, cycle time 0.65 s, after electron ionization at 70 eV. Ester-bound lipids were assigned from mass spectra and retention times. For semi-quantitative interpretation, diagnostic ratios (hereafter referred to as proxies) were calculated following Christensen et al. (2005). An isotopically labeled internal standard was tested but discarded because homogeneous distribution in solid samples could not be ensured, which would have biased quantification. For consistency and comparability among depolymerization methods, we therefore omitted an internal standard and used native hexadecanoic acid (FA16) as a proxy indicator of degradation, which was consistently present in all analytes.

3. Results and discussion

3.1. General ester-bound lipid composition

The methanolates derived from base-catalyzed methanolysis were

identified as fatty acids (FAs), ω -hydroxy alcanoic acids (ω FAs), α,ω -alkandioic acids (diacids), and mid-chain hydroxy alcanoic acids (Table 2), consistent with previous reports on cutin- and suberin-derived products (Mendez-Millan et al., 2010; Angst et al., 2016). However, not all of these molecular types were detected by pyrolysis with TMAH (Table 2). Specifically, long-chain alcohols and long-chain diols detected by methanolysis were typically absent from the pyrograms. These findings are consistent with prior pyrolysis studies on needles/leaves, in which these compounds have not been reported (cf. Nierop, 2001).

Notably, only two mid-chain hydroxy fatty acids were detected by pyrolysis: 9,16-dihydroxyhexadecanoic acid (9,16-diOH C16:0) and 9,10,18-trihydroxyoctadecanoic acid (9,10,18-triOH C18:0) (Table 2). These findings highlight the differences in the types of products the two

methods detect.

While methanolysis predominantly yielded aliphatic polyester-derived compounds, pyrolysis with TMAH generated additional signals consistent with products from tannins, lignin, and carbohydrates. As these classes lay outside the study scope, such signals were not structurally assigned or quantified; accordingly, Table 2 is restricted to cutin- and suberin-derived compounds. Both methods released the same series of C16-C22 diacids in root samples (Table 2). However, in needle material, only the C16 diacid was detected using pyrolysis with TMAH (Table 2), whereas C20-C22 diacids were analysed by methanolysis. These patterns are consistent with previously published studies on plant polyesters (Nierop, 2001; Mendez-Millan et al., 2010). Overall, the diversity of aliphatic products was higher with methanolysis, averaging 22

Table 2

Molecular proxies based on individual molecules, using hexadecanoic acid as a reference for base-catalyzed methanolysis (KOH) and TMAH pyrolysis (TMAH) (Abbreviations: SN – spruce needles, PN – pine needles, SR – spruce roots, PR, pine roots).

| Name molecule | KOH | | | | TMAH | | | |
|-----------------------------------------------------|-------|-------|------|------|------|------|------|------|
| | SN | PN | SR | PR | SN | PN | SR | PR |
| n-Carboxylic acids | | | | | | | | |
| Hexadecanoic acid (n-16:0) | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| x methyl Hexadecanoic acid (n-16 + 1:0) | 0.11 | – | 0.74 | 0.12 | 0.08 | 0.03 | 0.27 | 0.05 |
| Heptadecanoic acid (n-17:0) | – | – | 0.09 | 0.10 | 0.11 | 0.05 | 0.18 | 0.05 |
| Heptadecanoic acid (n-17:0) | – | – | 0.13 | 0.21 | – | 0.00 | 0.07 | 0.02 |
| Octadecanoic acid (n-18:0) | 0.11 | 0.26 | 0.32 | 0.17 | 0.41 | 0.28 | 0.55 | 0.33 |
| Octadecanoic acid (n-18:1) | – | – | 0.23 | 0.20 | 0.1 | 0.05 | 0.11 | 0.09 |
| Octadecanoic acid (n-18:2) | – | – | 0.14 | – | – | – | – | – |
| Nonadecanoic acid (n-19:0) | – | – | 0.36 | 0.04 | 0.03 | – | 0.18 | 0.00 |
| x-methyl Nonadecanoic acid (n-19 + 1:0) | – | – | 0.18 | – | – | – | – | – |
| Eicosanoic acid (n-20:0) | 0.07 | 0.22 | 2.45 | 0.61 | – | – | 0.49 | 0.20 |
| Hencosanoic acid (n-21:0) | – | – | 3.25 | – | – | – | 0.10 | – |
| x-methyl Hencosanoic acid (n-21 + 1:0) | – | – | 0.14 | – | – | – | – | – |
| Docosanoic acid (n-22:0) | 0–82 | – | 1.69 | 0.55 | 0.08 | 0.11 | 0.44 | 0.26 |
| x-methyl Docosanoic acid (n-22 + 1:0) | – | – | 0.20 | – | – | – | – | – |
| Tricosanoic acid (n-23:0) | – | – | 0.23 | – | 0.03 | 0.02 | 0.04 | 0.01 |
| Tetracosanoic acid (n-24:0) | – | – | 0.73 | 0.43 | 0.08 | – | 0.2 | 0.06 |
| Hexacosanoic acid (n-26:0) | – | – | – | 0.09 | – | – | – | – |
| n-Alcohols | | | | | | | | |
| Tetradecanol (Alcohol 14:0) | 0.02 | – | – | 0.03 | – | – | – | – |
| Hexadecanol (Alcohol 16:0) | 0.18 | – | – | 0.21 | – | – | – | – |
| Octacosanol (Alcohol 18:0) | 0.03 | – | – | 0.03 | – | – | – | – |
| Eicosanol (Alcohol 20:0) | 0.01 | – | – | 0.01 | – | – | – | – |
| Docosanol (Alcohol22:0) | 0.23 | – | – | – | – | – | – | – |
| Di-Alcohols | | | | | | | | |
| 1,12-Didocosanol | 3.57 | 0.63 | – | – | – | – | – | – |
| 1,14-Ditetrasanol | 1.08 | 0.53 | – | – | – | – | – | – |
| 1,16-Dihexacosanol | 0.4 | – | – | – | – | – | – | – |
| ω-Hydroxy alcanoic acids | | | | | | | | |
| 12-Hydroxy dodecanoic acid | 1.26 | 2.52 | – | – | 0.32 | 0.67 | – | – |
| 14-Hydroxy tetradecanoic acid | 7.06 | 1.45 | – | – | 0.81 | 0.28 | – | – |
| 16-Hydroxy hexadecanoic acid | 6.69 | 6.23 | – | 0.2 | 0.83 | 1.08 | 0.28 | 0.08 |
| 18-Hydroxy octadecanoic acid | – | – | 0.01 | 2.20 | – | 0.14 | 2.61 | 0.8 |
| 20-Hydroxy eicosanoic acid | – | – | 0.13 | 0.74 | – | – | 0.73 | 0.15 |
| 22-Hydroxy docosanoic acid | – | – | 0.34 | 0.69 | – | – | 0.55 | 0.16 |
| α,ω-Alcanedioic acids | | | | | | | | |
| 1,16-Hexadecadioic acid | 0.15 | 0.07 | 0.44 | 0.06 | 0.03 | 0.02 | 0.07 | 0.01 |
| 1,18-Octadecadioic acid | – | – | 0.41 | 0.03 | – | – | 0.32 | 0.09 |
| 1,20-Eicosanedioic acid | – | 0.817 | 2.53 | 0.55 | – | – | 0.29 | 0.11 |
| 1,22-Docosanedioic acid | 0.31 | 0.05 | 1.56 | 0.44 | – | – | 0.19 | 0.09 |
| Midchain hydroxy alcanoic acids | | | | | | | | |
| 9,15-Dihydroxy pentadecanoic acid | 0.37 | 0.25 | – | – | – | – | – | – |
| 9,16 Dihydroxy hexadecanoic acid | 99.09 | 62.75 | – | – | 1.24 | 2.54 | – | – |
| 9,18 Dihydroxy octadecanoic acid | 1.88 | 1.14 | – | – | – | – | – | – |
| 9,10,18 Trihydroxy octadecanoic acid | 0.94 | 10.08 | – | – | 0.26 | 1.37 | – | – |
| 9-Hydroxy pentadecanoic acid | 0.15 | 0.20 | – | – | – | – | – | – |
| 7-Hydroxy pentadecadioic acid | 0.35 | 1.18 | – | – | – | – | – | – |

compounds per sample, compared to pyrolysis with TMAH, which released only 17 aliphatic molecules per sample on average (Table 2).

Several compound classes, such as diols and mono-alcohols, were absent in pyrolysis with TMAH. Extracted-ion chromatograms within expected retention windows showed no evidence of co-elution that

could have masked these compounds, suggesting these molecules were altered during pyrolysis. Pyrolysis processes are known to cause deoxygenation and dehydration of molecules, which likely explains the reduced detection of alcohols (Kawamoto et al., 2007). Furthermore, de Leeuw and Baas (1993) demonstrated that alcohols do not react

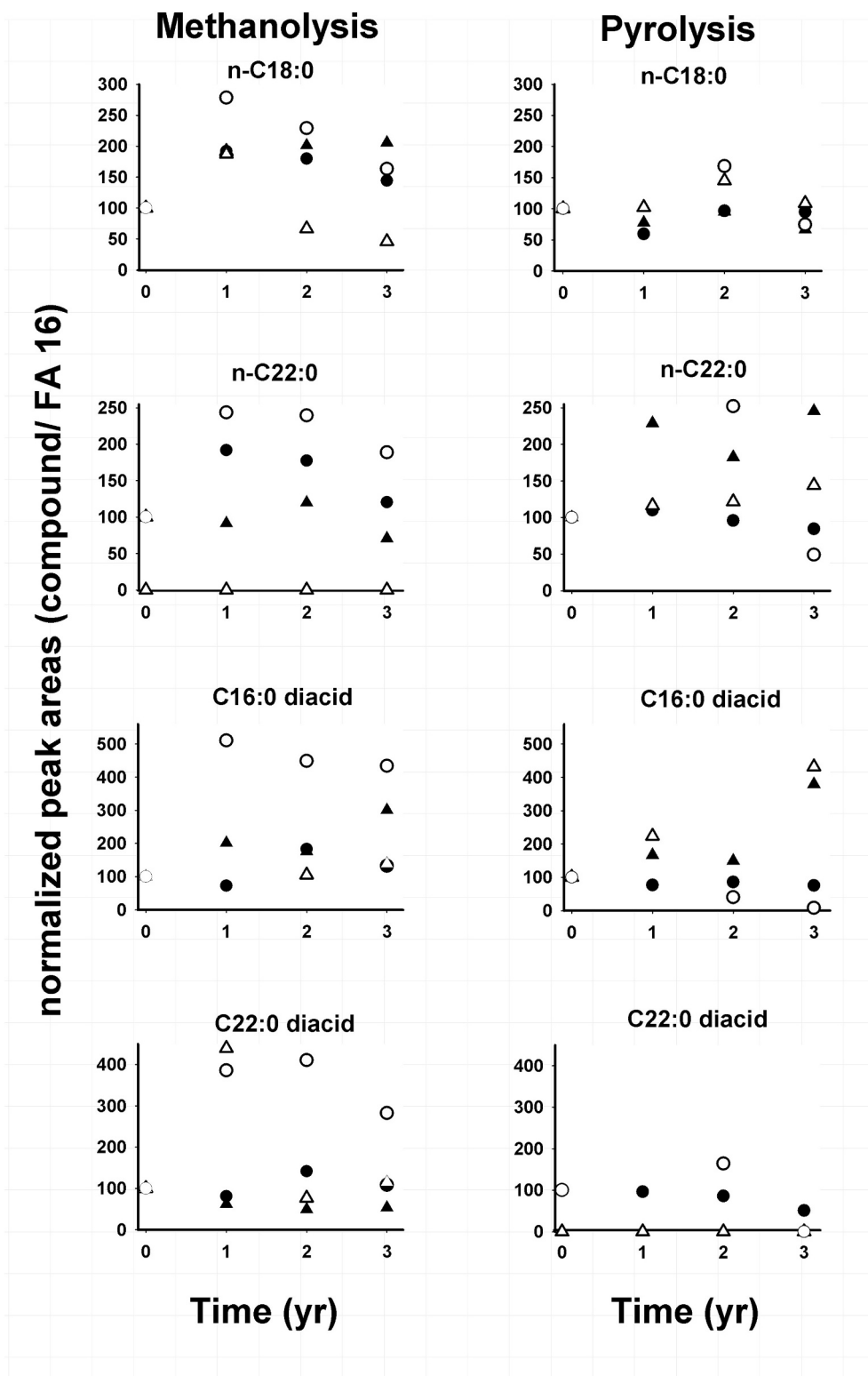


Fig. 2. Stability of polyester derived lipids gained by base catalyzed methanolysis and TMAH pyrolysis. Triangles represent needle derived compounds, circles represent root derived compounds, white symbols represent *Pinus* samples, black symbols represent *Picea* samples. All compounds were measured as methyl esters and TMS ethers for methanolysis and as methyl esters and methyl ethers for pyrolysis ($n = 2$).

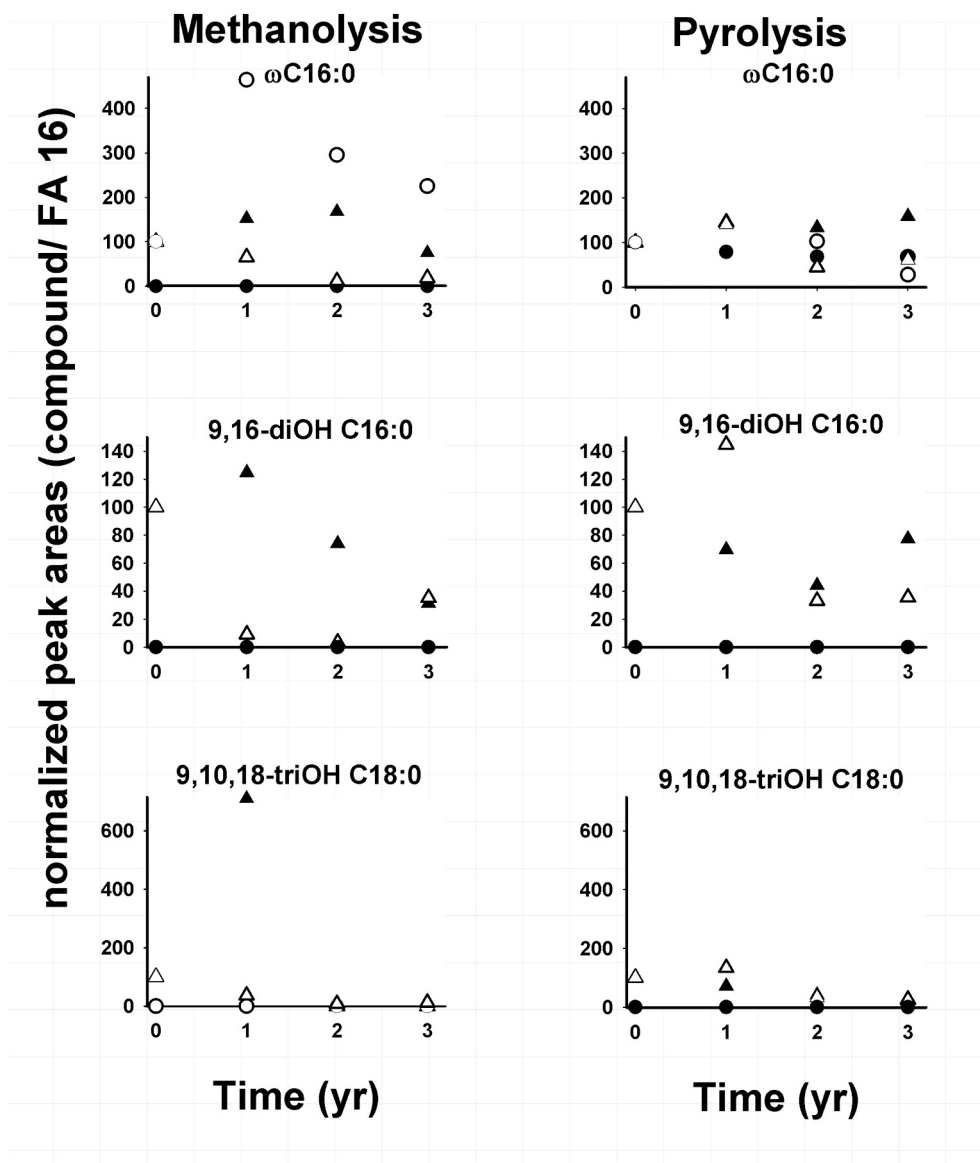


Fig. 2. (continued).

effectively with TMAH at 600 °C. These two mechanisms—low reactivity of alcohols with TMAH and dehydration during pyrolysis—can also explain the absence of methylated or non-methylated alcohols. Additionally, they may account for the low abundance of mid-chain hydroxy fatty acids observed in the chromatograms.

3.2. Biomarker selection of methods

For base-catalyzed methanolysis of the sample suite, 11 compounds were identified as specific to needles and 9 compounds as specific to roots. The following biomarkers were consistently identified as specific to needles (Table 2): 1,12-docosanediol (diol C12:0), 1,14-tetracosanediol (diol C14:0), 1,16-hexacosanediol (diol C16:0), 12-hydroxydodecanoic acid (ω C12:0), 14-hydroxytetradecanoic acid (ω C14:0), 9,15-dihydroxypentanoic acid (9,15-diOH C15:0), 9-hydroxypentadecanoic acid (9-OH C15:0), 7-hydroxypentadecanedioic acid (7-OH C15:0 diacid), 9,16-dihydroxyhexadecanoic acid (9,16-diOH C16:0), 9,18-dihydroxyoctadecanoic acid (9,18-diOH C18:0), and 9,10,18-trihydroxyoctadecanoic acid (9,10,18-triOH C18:0) (Table 2).

The following compounds were exclusively identified in methanolysis products from root samples: heptadecanoic acid (*n*-C17:0), x-

methylheptadecanoic acid (*n*-C17 + 1:0), octadecanoic acid (*n*-C18:1), nonadecanoic acid (*n*-C19:0), tetracosanoic acid (*n*-C24:0), 18-hydroxyoctadecanoic acid (ω C18:0), 20-hydroxyeicosanoic acid (ω C20:0), 22-hydroxydocosanoic acid (ω C22:0), and 1,18-octadecanedioic acid (C18:0 diacid) (Table 2).

From the pyrolyzed samples, four compounds were specific to needles: diol C12:0, diol C14:0, 9,16-dihydroxyhexadecanoic acid (9,16-diOH C16:0), and 9,10,18-trihydroxyoctadecanoic acid (9,10,18-triOH C18:0). All four compounds were detected after methanolysis of the needle samples. Only six compounds from pyrolysis were specific to roots: eicosanoic acid (*n*-C20:0), ω C20:0, ω C22:0, C18:0 diacid, 1,20-eicosanedioic acid (C20:0 diacid), and 1,22-docosanedioic acid (C22:0 diacid). Three of these (ω C20:0, ω C22:0, and C18:0 diacid) had been detected by methanolysis (Table 2).

The trend of a higher number of aliphatic products in methanolysis compared to pyrolysis also extended to biomarker detection. Methanolysis proved more effective in releasing potential biomarkers, such as alcohols, diols, and mid-chain hydroxy fatty acids, in both root and needle samples.

3.3. Molecular changes during litter decomposition

The degradation patterns of all products could be represented by one of seven selected products (Fig. 2). The representative products were rigorously selected based on multiple criteria to ensure comprehensive analytical representation. First, all seven of the representative compounds were consistent to both analytical methods. The products were also specifically chosen to represent key molecular groups relevant to the study: saturated fatty acids, dicarboxylic acids, omega-hydroxy fatty acids, hydroxy fatty acids, and trihydroxy fatty acids. Additionally, all selected molecules feature carbon chain lengths ranging from 16 to 22 carbon atoms, which represents the most informative range for the compounds under investigation. It should be noted that hexadecanoic acid, despite its relevance, was excluded from the selection as it served as the reference compound for semi-quantitative analysis, against which all other compounds were normalized.

Generally, the abundances of the seven representative products (normalized with hexadecanoic acid) remaining detected by TMAH pyrolysis were more stable (staying closer to 100 %) over the three-year timescale of the project than abundances detected by methanolysis, over the three years of the project (Fig. 2). Additionally, differences between different tree species and plant parts were less pronounced for pyrolysis compared to methanolysis. The present results can be interpreted to indicate that TMAH pyrolysis was less sensitive in detecting differences between tree species and plant parts (needles vs. roots). Methanolysed pine roots exhibited the relatively highest product abundances for nearly all of the representative products after one year of decomposition (Fig. 2), followed by a decrease in abundance in the two subsequent years. These findings are consistent with a previous study, in which the same sample set was quantified using a deuterized internal-standard (Altmann, et al, 2021b). Generally, however, no other obvious trends were observed for the different molecules for the same method across the various samples.

The differences between the two methods could be explained by the different reaction mechanisms of base catalyzed methanolysis and pyrolysis with TMAH. In the methanolysis reaction, ester bonds in the polymer are cleaved by nucleophilic attack of methanol—often catalyzed by a base such as KOH—resulting in the formation of methyl esters and the corresponding alcohols (Järvinen et al., 2009; Zhang et al., 2023), whereas in analytical pyrolysis, polymers are thermally decomposed at high temperatures into smaller fragments, which are then chemically derivatized—often by methylation with reagents like TMAH—to yield volatile, analyzable products for chromatographic identification (Del Rio and Hatcher, 1998; Moldoveanu, 1998).

The effectiveness of alkaline methanol in hydrolyzing and subsequently extracting cutin- and suberin-derived monomers is influenced by factors such as the remaining degree of polymerization, surface area, porosity, barrier sheathing, and the lignin and hemicellulose content of the degraded litter material (Alvira et al., 2010). Previous findings suggest that variations in plant material heterogeneity, fiber density, and arrangement affect solvent accessibility and hence the efficacy of methanolysis across different sample types and decomposition stages (Himmel et al., 2007). This effect is particularly pronounced between the fresh and the one-year aged material.

Another influential methodological difference was the energy availability during reaction. Pyrolysis is conducted at a temperature of 600 °C, whereas base-catalyzed methanolysis occurs at 100 °C. These differing reaction conditions imply that pyrolysis can cleave bonds more extensively (and randomly) than methanolysis, especially for fresher material. The relatively stable molecular ratios observed over time in pyrolyzed samples suggest that depolymerization of ester-bound lipids with pyrolysis with TMAH is generally less influenced by plant material structure. Consequently, pyrolysis appears more suitable than methanolysis for analyzing ratios of plant-derived ester-bound lipids independently of sample decomposition state.

Furthermore, biomarkers such as 9,16-dihydroxy hexadecanoic acid

or 1,22-docosanedioic acid exhibit minimal changes during litter decomposition when analyzed via pyrolysis with TMAH. This stability allows semi-quantitative estimation of litter-derived quantities in soils or sediments based on biomarker concentrations, even for samples of unknown age.

4. Conclusions

We demonstrated that the analysis of ester-bound lipids by base-catalyzed methanolysis with KOH and pyrolysis with TMAH released distinct types of molecules. These differences significantly impacted the selection of needle- and root-specific biomarkers. Base-catalyzed methanolysis identified 19 specific molecules, while pyrolysis identified only 10 molecules indicative of roots and needles.

This novel finding from our comparative study highlights that the type and relative abundance of biomarkers detected strongly depends on the analytical method used, making direct comparisons between methods challenging. Furthermore, we showed that the relative amounts of molecules detected by each method did not exhibit consistent trends over three years of litter decomposition. This has important implications for quantifying litter residues in soils and sediments.

We attribute these differences to the distinct reaction mechanisms of base-catalyzed methanolysis with KOH and pyrolysis with TMAH. Pyrolysis does not require solvent access to chemical bonds for cleavage and release of monomers, and the higher available reaction energy compensates for stronger chemical linkages in fresh material. In contrast, methanolysis samples exhibited stronger changes in peak ratios during decomposition and greater variability between different samples.

For qualitative analyses, we recommend using purely chemical degradative reaction techniques, such as methanolysis, as they release more suitable molecules for identifying the origin of litter samples. For semi-quantitative approaches comparing relative abundances of biomarkers across different degradation stages, pyrolysis proved to yield more consistent and reliable results, requiring fewer sample preparation steps and thus being less time-consuming.

CRediT authorship contribution statement

J.G. Altmann: Writing – original draft, Validation, Resources, Investigation, Formal analysis, Data curation, Conceptualization. **B. Jansen:** Writing – review & editing, Supervision, Project administration, Methodology, Conceptualization. **M. Palviainen:** Writing – review & editing, Resources, Investigation. **K. Kalbitz:** Writing – review & editing, Validation, Supervision, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

Data will be made available on request.

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