

## The biochemical transformation of oak (*Quercus robur*) leaf litter consumed by the pill millipede (*Glomeris marginata*)

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### Abstract

Soil macrofauna play an essential role in the initial comminution and degradation of organic matter entering the soil environment and yet the chemical effects of digestion on leaf litter are poorly understood at the molecular level. This study was undertaken to assess the selective chemical transformations that saprophagous soil invertebrates mediate in consumed leaf litter. A number of pill millipedes (*Glomeris marginata*) were fed oak leaves (*Quercus robur*) after which the biomolecular compositions (lipids and macromolecular components) of the leaves and millipede faeces were compared using a series of wet chemical techniques and subsequent analysis by gas chromatography (GC) and gas chromatography-mass spectrometry (GC/MS). It was found that the concentrations of short chain (<C<sub>20</sub>) *n*-alkanoic acids, sterols and triacylglycerols reduced dramatically in the millipede faeces relative to the leaf litter. Hydrolysable carbohydrates and proteins both decreased in concentration in the faeces, whereas similar yields of phenolic components were observed for the cupric oxidation products of lignin, although the oxygenated functionalities were affected by passage through the millipede gut, yielding a more highly condensed state for lignin. This shows that the chemical composition of fresh organic matter entering the soil is directly controlled by invertebrates feeding upon the leaf litter and as such that they are key contributors to the early stages of diagenesis in terrestrial soils.

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### 1. Introduction

Soil organic matter (SOM) is currently estimated to comprise a carbon reservoir of ca. 1760 Pg (O'Neill, 1993). Whilst this constitutes less than 0.01% of the global carbon budget (over 99% of carbon is stored in the ocean and marine sediments), it represents 24% of the terrestrial carbon pool. The relatively high turnover of carbon in soils means that any changes in assimilation and mineralisation fluxes for this dynamic system will have important ramifications for the global carbon cycle as a whole. Current concerns over climate change have conferred an increased impetus to research concerned with factors affecting the biological immobilisation of organic matter and the feedback effect that rising CO<sub>2</sub> concentrations are having on the soil fauna that mediate this process (e.g. Verhoef and Brussard, 1990; O'Neill, 1994; Yeates et al., 1997). Whilst there has been much work

concerning the role that microorganisms play in this process (e.g. Cheng and Johnson, 1998; Cardon et al., 2001; Cheng, 1999) surprisingly little attention has focussed on the role of meso- and macrofauna operating within the soil environment. Indeed, for the majority of soils the importance of these larger organisms in the pre-processing and comminution of deadfall cannot be overstated. For example, in the top F layer of a northern temperate forest soil up to 90% of the residual organic matter will exist in the form of arthropod faecal material (Bocock, 1963; Nicholson et al., 1966). The exact changes in the biochemical composition of litter resulting from digestion by arthropods of fresh organic matter entering the soil are mostly unknown and as such represent a significant gap in our understanding of the initial stages of diagenesis in terrestrial soils.

Of the larger animals present in soil ecosystems, earthworms (Annelida, Oligochaeta) have been most widely investigated and contribute to the biodegradation of natural residues primarily through the physical alteration of the structure of plant tissue and the soil matrix enhancing microbial activity (Coleman and Crossley, 1996). This paper focuses on the role of the pill millipede (Diplopoda, *Glomeris*

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*marginata*) in the decomposition of leaf litter. *Glomeris* are important detritivores that inhabit the litter layer of forests, particularly those overlying calcareous soil, and are found in densities of 2.5–7.5 individuals per m<sup>2</sup> although they have been recorded in densities of up to 24 per m<sup>2</sup> (Thiele, 1956). *Glomeris* ingest leaf litter, assimilating 0.3–7% w/w of the ingested material in the process (Byzov et al., 1996) and the resultant faecal material is an important food source for other saprophagous animals such as earthworms (Scheu and Wolters, 1991). *Glomeris* are important in the comminution of litter since they increase the surface area available for microbial growth and their associated decomposition processes during gut passage and in faecal pellets (Striganova, 1971). The presence of *Glomeris* in soil broadens microbial diversity whilst indirectly stimulating nitrogen mineralisation and mobilisation of cations such as potassium and sodium (Anderson et al., 1983; Visser, 1985). Furthermore, faecal pellet formation results in an increase in availability of nutrients such as nitrogen and phosphorous to microorganisms. However, the uptake of these nutrients is dependent on the availability of labile organic matter in the fragmented litter (Maraun and Scheu, 1996).

Food preference experiments have been conducted for *Glomeris* feeding on *Quercus ilex* litter. David and Gillon (2002) reported a preference for decomposed leaf material over freshly fallen litter. It has been reported that for the leaf litter to be palatable to *Glomeris*, colonisation of the litter by microorganisms must occur to soften the leaves (Hassall and Rushton, 1984) and accelerate the loss and degradation of phenolic feeding inhibitors (Edwards, 1974). Mean weight consumption rates of 14 g dry weight of leaf litter per gram of millipede per year have been reported (David and Gillon, 2002), however, laboratory experiments with the millipede *Harpaghe haydeniana* reported daily consumption of conifer litter of between 10 and 20% of millipede fresh biomass that could equate to 36% of total annual litter fall (Carcamo et al., 2000). Soil saprophagous invertebrates (millipedes, woodlice and Diptera larvae) consume between 20 and 100% of plant litter input in a year with consumption being highest during spring (David and Gillon, 2002). However, despite the obvious importance of this pathway for the flux of terrestrial carbon, very little is understood of its effect on the biochemical composition and the associated biogeochemical ramifications.

A variety of non-destructive chemical techniques have been used to analyse the chemical changes that occur when leaf litter is consumed by saprophagous invertebrates. Gillon and David (2001) have used near infrared spectroscopy (NIRS) in conjunction with calibration equations to study *Glomeris* consuming *Q. ilex* leaf litter while <sup>13</sup>C nuclear magnetic resonance (NMR) has been used to study other saprophagous soil invertebrates such as termites and earthworms and the transformations they effect on organic matter (Guggenberger et al., 1996; Hopkins et al., 1998). The advantages of using non-destructive techniques lies with the ease of analysis and speed at which samples can be processed, although obtaining quantitative data is non-trivial with <sup>13</sup>C NMR analysis being semi-quantitative at best (Preston, 1996). However, these

techniques do yield an overview of the changes in chemical composition when used in conjunction with other techniques. NIRS studies concluded that the faeces contained significantly higher concentrations of lignin, but contained less non-structural compounds and nitrogen (Gillon and David, 2001). A significant disadvantage of such approaches is that important changes in composition at the molecular level may be undetectable.

Previous studies have all reported that decomposability is not necessarily greater in millipede faeces than in leaf litter (e.g. Scheu and Wolters, 1991; Maraun and Scheu, 1996; Gillon and David, 2001). *Glomeris* digest a proportion of the cellulose and hemicellulose ingested and readily assimilate compounds such as amino acids (e.g. Bignell, 1989; Scheu and Wolters, 1991). Although *Glomeris* have been extensively studied, the chemical transformations occurring due to the digestive process are poorly understood. Changes in microbial biomass, respiration and nutrient status of beech (*Fagus sylvatica*) leaf litter processed by *Glomeris* have been studied. Using C/N ratios and ergosterol as a fungal indicator, the investigation concluded that *Glomeris* reduced fungal biomass in beech leaf litter more than the bacterial biomass (Maraun and Scheu, 1996). There has been no molecular characterisation of the biochemical transformation of leaf litter consumed by *Glomeris* hence this study was initiated to test the hypothesis that the pre-processing of leaf litter by this arthropod has specific defined effects on organic matter at the molecular level that are likely to affect the subsequent stages of biodegradation of organic matter in soil.

## 2. Experimental

Pill millipedes and oak leaf litter were collected from a woodland that was formerly associated with the Centre of Ecology and Hydrology Merlewood, Cumbria, UK (Grid reference 341,000, 479,600) and cultured in a single food source microcosm. Briefly, oak leaf litter was placed in a pre-furnaced glass jar and a number of pill millipedes added. The jar was placed in the dark, in a temperature-controlled room held at 20 °C. The millipede faeces were removed from the jar and analysed according to the methods described below.

### 2.1. Sample preparation and solvent extraction

All samples were crushed with a pestle and mortar facilitated by the addition of liquid nitrogen and subsequently passed through a 2 mm and a 75 µm sieve. Hexadecan-2-ol, nonadecane, heptadecanoic acid, 5β-pregnan-3α-ol and hexadecyloctadecanoate were added as internal standards. Samples were extracted ultrasonically at room temperature (5×) using dichloromethane (DCM):acetone (9:1 v/v); total volume 50 ml. The extracts were combined to form a total lipid extract (TLE) and solvent was removed by evaporation under reduced pressure.

## 2.2. Initial fractionation of the total lipid extract

The TLEs were separated into two fractions, 'acid' and 'neutral', using an extraction cartridge containing a bonded aminopropyl solid phase (500 mg sorbent, 2.8 ml eluent capacity, Varian). Extracts dissolved in DCM:isopropanol (2:1 v/v) were slowly flushed through a cartridge pre-eluted with hexane. Further addition of DCM/isopropanol (2:1 v/v, 8 ml) eluted a 'neutral' fraction and finally the cartridge was washed with 2%v/v acetic acid in diethylether (8 ml) to elute an 'acid' fraction. Solvent was removed from both fractions under a gentle stream of nitrogen.

## 2.3. Column chromatography of neutral lipids

Columns (120 mm length  $\times$  8 mm i.d.) were packed with dried activated (160 °C, >24 h) silica gel 60 (Fluka) and pre-eluted with hexane. Samples were applied to the column as a mixture of dissolved and finely suspended particulates in hexane. Gradient elution was performed under positive pressure supplied by a stream of nitrogen providing an elution rate of approximately 15 ml min<sup>-1</sup>. The eluents used comprised five separate solvent systems: hexane, hexane:DCM (9:1 v/v), DCM, DCM:methanol (1:1 v/v), and methanol, applied in elutropic order to give five fractions: 'hydrocarbon', 'aromatic', 'ketone/wax-ester', 'alcohol', and 'polar', respectively. The relative volumes of solvents applied were determined by the ratio 2:1:3:2:2, following the above elutropic series, and the size of the column being used for a particular separation. Column fractions were collected and dried in an identical manner to fractions from the initial fractionation.

## 2.4. Carbohydrate analyses

Alditol acetate derivatives were prepared using a modified version of the Blakeney et al. (1983) procedure. Carbohydrate hydrolysis was performed by the addition of 100  $\mu$ l of 72% (v/v) sulphuric acid to the lipid-extracted samples (10 mg) at room temperature (1 h) under vacuum to prevent monosaccharide degradation while hydrolysing cellulose. Double distilled water (900  $\mu$ L) was added and the tube heated under vacuum at 100 °C for 2.5 h. The hydrolysate containing the monosaccharides was filtered and neutralised using ammonia solution (0.2 ml, 18 M). An aliquot was taken and reduced to the corresponding alditols by the addition of 2 ml of sodium borohydride solution (2 g NaBH<sub>4</sub> in 100 ml dimethyl sulphoxide) with heating at 40 °C (90 min). The alditols were acetylated using 1-methyl imidazole (200  $\mu$ l) and acetic anhydride (1 ml). Excess acetic anhydride was removed by the addition of double distilled water (5 ml) and the derivatives extracted with three portions of diethylether (2 ml). The solution was dried over magnesium sulphate, blown to dryness under a gentle stream of nitrogen and re-dissolved in DCM for GC analysis.

## 2.5. Amino acid analyses

Protein hydrolysis was performed using a modified version of the Hirs et al. (1954) procedure. Hydrochloric acid (2.5 ml, 6 M) was added to the lipid extracted samples (5 mg) followed by heating (100 °C, 24 h) to yield the amino acid monomers. The hydrolysate was blown to dryness under nitrogen. An aliquot of the hydrolysate was taken and derivatised to form *N*-trifluoroacetate-isopropyl ester derivatives of the hydrolysed amino acid. The carboxylic acid functionality was esterified by the addition of 0.5 ml acidified isopropanol (made by mixing 1 ml isopropanol and 250  $\mu$ L of acetyl chloride) and the solution heated at 100 °C (1 h). The reaction was quenched in a deep freeze (-20 °C) and excess acidified isopropanol removed under nitrogen. The amino group was acetylated by the addition of 0.5 ml trifluoroacetic anhydride and 0.5 ml DCM heating at 100 °C (10 min). Solvent was removed under a gentle stream of nitrogen and samples re-dissolved in DCM prior to analysis by GC and GC/MS.

## 2.6. Lignin analyses

The CuO oxidation methodology was adapted from Hedges and Ertel (1982). Samples (20 mg) were placed in a mini bomb with CuO powder, ammonium iron (II) sulphate hexahydrate (Fe(NH<sub>4</sub>)<sub>2</sub>(SO<sub>4</sub>)<sub>2</sub>·6H<sub>2</sub>O and 2 M aqueous NaOH solution (1/0.1/0.7, v/v/w) under a N<sub>2</sub> atmosphere and heated at 170 °C (3 h). After cooling, ethylvanillin was added as an internal standard, and the supernatant transferred for extraction. The residue was washed with 1 M aqueous NaOH solution, added to the supernatant and acidified to pH 1. This was then extracted three times with diethyl ether, the combined extract dried over an anhydrous magnesium sulphate column and the oxidation products derivatised to their respective trimethylsilyl (TMS) ethers and/or esters.

## 2.7. Trimethylsilylation

Lipid fractions containing functional and polyfunctional compounds were derivatised to form their respective TMS ethers and/or esters by adding 30  $\mu$ L of *N,O*-bis(trimethylsilyl) trifluoroacetamide containing 1% trimethylchlorosilane (BSTFA + 1% v/v TMCS, Sigma-Aldrich) to sample aliquots and heating for 30 min at 70 °C. Excess derivatising agent was removed under a gentle stream of nitrogen and samples redissolved in 50  $\mu$ l hexane.

## 2.8. Gas chromatography and high temperature-gas chromatography (HT-GC)

Derivatized fractions were analyzed using a Hewlett-Packard 5890 series II gas chromatograph equipped with a fused-silica capillary column (Chrompack CPSil-5CB, 50 m length  $\times$  0.32 mm i.d., film thickness 0.12  $\mu$ m). Samples dissolved in hexane were injected (1.0  $\mu$ l) on-column. The temperature was programmed from 40 °C (1 min isothermal) to 200 °C at a rate of 10 °C min<sup>-1</sup> and finally to 300 °C at

3 °C min<sup>-1</sup> (20 min isothermal). The flame ionisation detector (FID) temperature was held at 350 °C. Hydrogen was used as carrier gas (10 psi head pressure).

Analyses of the TLEs by HT-GC were carried out using a column capable of performing at elevated temperature (J&W, DB1, 15 m length × 0.32 mm i.d., film thickness 0.1 µm). The temperature program used was 50 °C (2 min isothermal) to 350 °C at 10 °C min<sup>-1</sup> (10 min isothermal). The FID temperature was held at 350 °C. Hydrogen was used as carrier gas (10 psi head pressure)

## 2.9. Gas chromatography/mass spectrometry

Gas chromatography/mass spectrometry analyses were carried out using a ThermoFinnigan Trace MS equipped with a fused silica capillary column (Phenomenex, ZB1; 60 m length × 0.32 mm i.d., film thickness 0.1 µm). Samples were introduced using a programmable temperature vapourising injector (PTV) with a transfer line maintained at a temperature of 300 °C. The source temperature was held at 200 °C and ionisation energy was set at 70 eV with the quadrupole mass analyser scanning the range *m/z* 50–650 with a cycle time of 0.6 s<sup>-1</sup>. The temperature program used was: 40 °C (2 min isothermal) to 300 °C at 10 °C min<sup>-1</sup> (20 min isothermal). Helium was employed as carrier gas with a constant flow of 2 ml min<sup>-1</sup>.

## 2.10. Pyrolysis/gas chromatography/mass spectrometry (Py/GC/MS)

Analyses by Py/GC/MS were performed using a CDS 2500 pyrolysis autosampler interfaced to a Perkin Elmer Turbomass Gold equipped with a fused silica capillary column (J&W; DB1; 30 m length × 0.25 mm i.d., film thickness 0.25 µm). Samples were pyrolysed at 610 °C then introduced to the GC column via a split/splitless injector with a 20:1 split. The transfer line was held at 280 °C and the source temperature maintained at 180 °C and ionisation energy was set at 70 eV. The GC oven temperature was programmed: 40 °C (4 min isothermal) to 320 °C at 5 °C min<sup>-1</sup> (15 min isothermal). Pyrolysis products were identified by interpretation, library searches and referring to published spectra (Ralph and Hatfield, 1991; Stankiewicz et al., 1996; Stankiewicz et al., 1997).

## 2.11. <sup>13</sup>C cross polarisation-magic angle spinning (CPMAS) NMR

<sup>13</sup>C spectra were collected on a Bruker Avance 300 NMR Spectrometer operating at 75.53 MHz with a standard Bruker 7 mm CPMAS probe. The spectra were collected using CP-MAS at 4.5 kHz and dipolar decoupling (ca. 90 kHz). Typically, between 4000 and 8000 scans were collected with a CP time of 2 ms and a recycle time of 4 s. The <sup>13</sup>C chemical shifts were referenced with respect to tetramethylsilane (=0 ppm) using solid adamantane as a secondary standard. Intensity distribution in CPMAS spectra can be distorted by spinning sidebands with the effect more severe for carbons in

anisotropic environments (aromatic, carboxyl and carbonyl carbons) and with increasing field. To overcome this, spectra were obtained at two field strengths (4.5 and 5.4 kHz) to identify differences in the spectra that could be attributed to spinning sidebands although no spinning sidebands were observed.

## 3. Results

### 3.1. Lipids

#### 3.1.1. Total lipid extract

Fig. 1a depicts a partial gas chromatogram of the total lipid extract of oak leaf litter. The extract is dominated by *n*-tetracosanol (C<sub>24</sub>) with peripheral homologues observed at lower abundance from C<sub>22</sub> to C<sub>30</sub>. Other dominant homologous series observed are *n*-alkanoic acids which exhibit a much wider distribution than the *n*-alkanols (C<sub>14</sub>–C<sub>30</sub>, C<sub>16</sub> maximum) and *n*-aldehydes (C<sub>24</sub>–C<sub>32</sub>, C<sub>28</sub> maximum). A series of *n*-alkanes with homologues ranging from C<sub>25</sub> to C<sub>29</sub> are observed but are not particularly prominent relative to other lipid components. Sitosterol (24-ethylcholest-5-en-3β-ol) is the only sterol observed at appreciable abundance in the litter extract. A series of wax esters is also present at low abundance exhibiting a maximum at C<sub>44</sub> with homologues ranging from C<sub>38</sub> to C<sub>46</sub>. Two triacylglycerol peaks (TAGs; C<sub>52</sub> and C<sub>54</sub>) are also observed (dominant fatty acid moieties: C<sub>16:0</sub> and/or C<sub>18:1</sub>, C<sub>18:2</sub> and C<sub>18:3</sub>) as well as two unidentified triterpenyl esters and a range of uncharacterised triterpenoids present at relatively low abundance.

Fig. 1b shows the partial gas chromatogram of the total lipid extract of the millipede faeces. The extract is again dominated by *n*-tetracosanol (C<sub>24</sub>) with peripheral *n*-alkanol homologues observed ranging from C<sub>22</sub> to C<sub>30</sub>. Other dominant homologous series are *n*-alkanes (C<sub>25</sub>–C<sub>31</sub>, C<sub>29</sub> maximum), *n*-aldehydes (C<sub>26</sub>–C<sub>30</sub>, C<sub>30</sub> maximum) and *n*-alkanoic acids (C<sub>14</sub>–C<sub>32</sub>, C<sub>28</sub> maximum). At late retention time, a homologous series of wax esters is observed with components ranging from C<sub>38</sub> to C<sub>60</sub> exhibiting a maximum at C<sub>44</sub>, with the C<sub>38</sub> and C<sub>46</sub> components being other dominant homologues. A series of uncharacterised triterpenoids are prominent in the chromatogram of the millipede faeces relative to other lipid components, while TAGs and triterpenyl esters are noticeable by their absence from the millipede faeces.

#### 3.1.2. *n*-alkanes

Fig. 2a depicts the distribution and abundance of *n*-alkane homologues detected in the extract obtained from leaf litter. The distribution is monomodal and ranges from C<sub>18</sub> to C<sub>33</sub> about a maximum at C<sub>29</sub>; the C<sub>25</sub>, C<sub>27</sub> and C<sub>29</sub> homologues are particularly prominent relative to the whole distribution. Overall, the distribution of *n*-alkanes describes a strong odd-over-even predominance considered as indicative of the higher terrestrial plant origin (Eglinton and Hamilton, 1967). Fig. 2b depicts the corresponding extract obtained from the millipede faeces. The distribution observed for the leaf litter is largely maintained ranging from C<sub>18</sub> to C<sub>33</sub> again maximising at C<sub>29</sub>

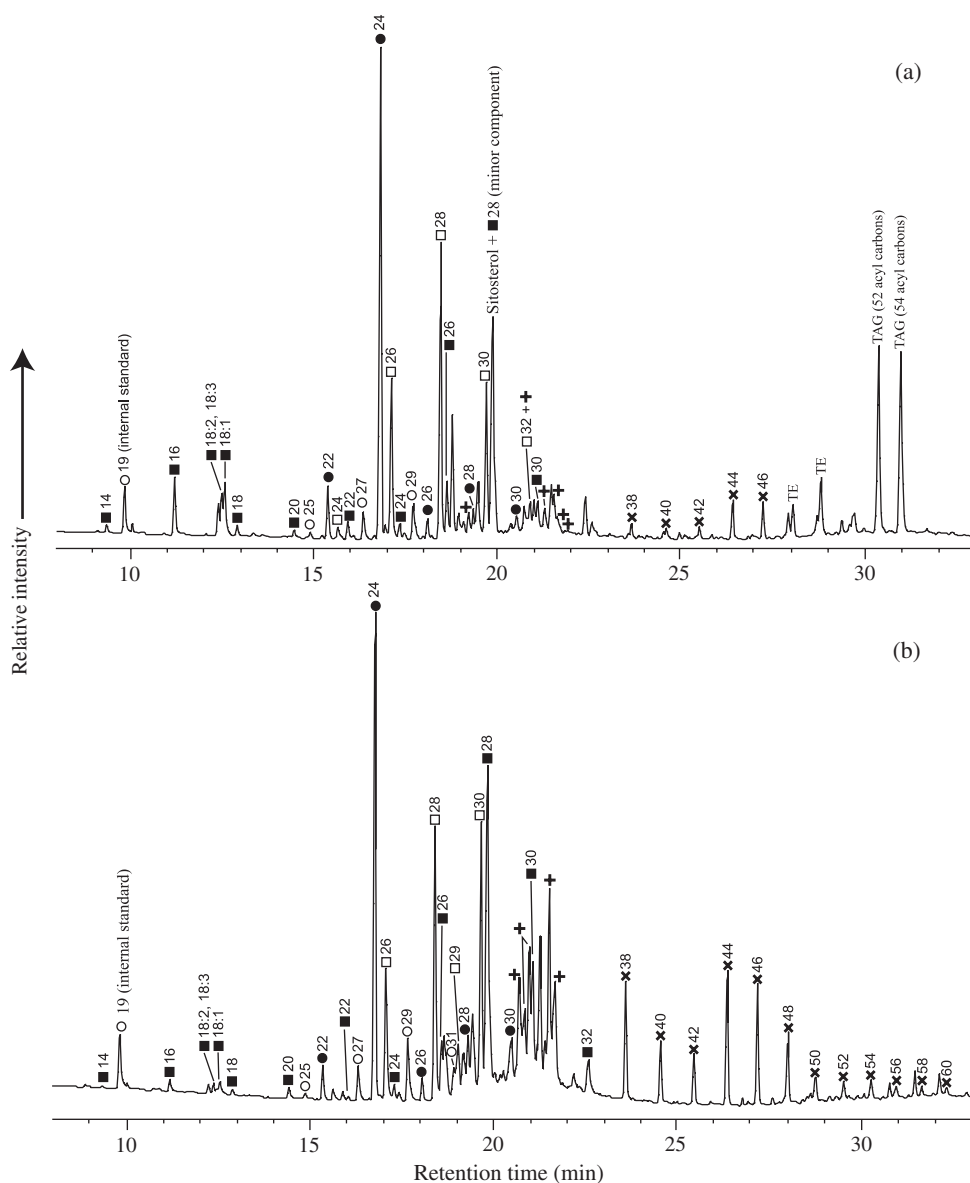


Fig. 1. Partial gas chromatograms of the total lipid extracts of: (a) oak leaf litter, and (b) pill millipede faeces. (Key: ○, *n*-alkane; ●, *n*-alkanol; □, *n*-aldehyde; ■, *n*-alkanoic acid; +, uncharacterised triterpenoid; ×, *n*-alkyl wax ester; TE, triterpenyl ester; TAG, triacylglycerol; values above symbols indicate the number of carbon atoms in a compound, values after colons indicate the number of double bonds).

with particularly prominent preceding  $C_{25}$  and  $C_{27}$  homologues. The strong odd-over-even predominance is also maintained.  $17\beta(H),21\beta(H)$ -hop-22(29)-ene (diploptene) was observed in the hydrocarbon fraction extracted from the millipede faeces.

### 3.1.3. *n*-alkanols

Fig. 2c shows the distribution and abundance of *n*-alkanol homologues detected in the extract obtained from leaf litter. The distribution is monomodal and ranges from  $C_{18}$  to  $C_{30}$  about a maximum at  $C_{24}$ . The  $C_{22}$  and  $C_{24}$  homologues are prominent relative to the whole distribution with the  $C_{24}$  homologue markedly more abundant than the other *n*-alkanol homologues. There is a strong even-over-odd predominance frequent in extracts originating from higher terrestrial plants

(Eglinton and Hamilton, 1967). Fig. 2d depicts the corresponding extract obtained from the millipede faeces. The distribution from the leaf litter is largely maintained with homologues ranging from  $C_{18}$  to  $C_{30}$  again about a maximum at  $C_{24}$  with a monomodal distribution. The even-over-odd predominance is also maintained.

### 3.1.4. *n*-alkanoic acids

Fig. 2e shows the distribution and abundance of *n*-alkanoic acid homologues detected in the extract obtained from the leaf litter. The distribution essentially is monomodal and ranges from  $C_9$  to  $C_{32}$  about a maximum at  $C_{18:1}$ ; the  $C_{16}$  and  $C_{18:1}$  homologues are particularly prominent relative to the whole distribution. There is a strong even-over-odd predominance, typical of those detected in higher terrestrial plants. Fig. 2f

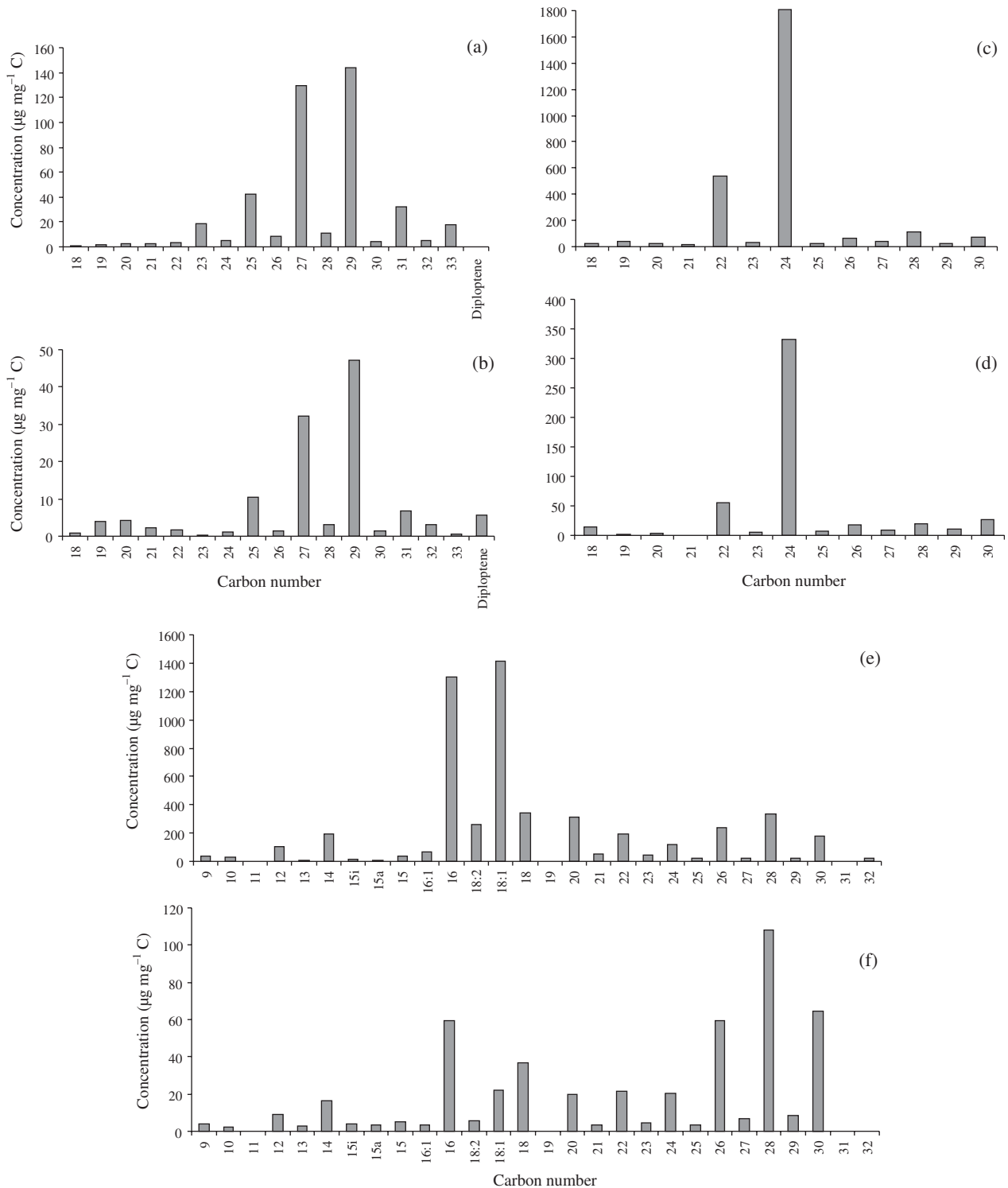


Fig. 2. Carbon number distribution and concentrations of *n*-alkanes of (a) oak leaf litter and (b) pill millipede faeces. Carbon number distribution and concentrations of *n*-alkanols of (c) oak leaf litter and (d) pill millipede faeces. Carbon number distribution and concentrations of *n*-alkanoic acids of (e) oak leaf litter and (f) pill millipede faeces.

depicts the corresponding extract from the millipede faeces with a maximum at C<sub>16</sub>. The latter part of the homologous series is dominated by the C<sub>28</sub> component thereby producing a bimodal distribution; the C<sub>16</sub>, C<sub>26</sub>, C<sub>28</sub> and C<sub>30</sub> homologues are prominent to the whole distribution and the strong even-over-odd predominance is maintained.

### 3.1.5. Sterols and triterpenols

Fig. 3a depicts the distribution and abundance of sterols detected in the extract obtained from the leaf litter. Four  $\Delta^5$  sterols are detected in appreciable quantities, with 24-ethylcholest-5-en-3 $\beta$ -ol (sitosterol) being the most abundant, while the other sterols detected are: cholest-5-en-3 $\beta$ -ol

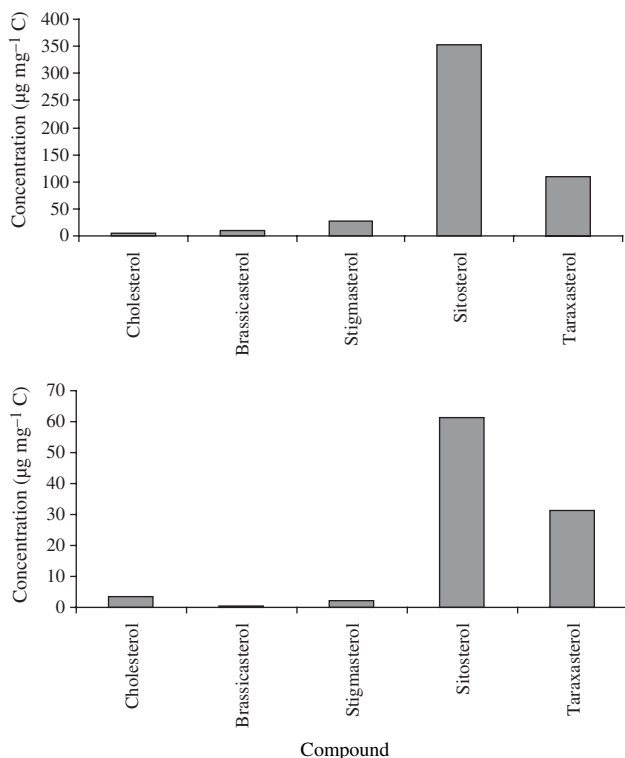


Fig. 3. Distribution and concentrations of sterols detected in: (a) oak leaf litter and (b) pill millipede faeces.

(cholesterol), (22E)-ergosta-5,22-dien-3 $\beta$ -ol (brassicasterol), 24-ethylcholest-5,22(E)-dien-3 $\beta$ -ol (stigmasterol). The triterpene alcohol 5 $\alpha$ -taraxast-20(30)-en-3 $\beta$ -ol (taraxasterol) is present at appreciable concentration (110  $\mu\text{g mg}^{-1}$  C). Fig. 3b depicts the distribution and abundance of sterols in the millipede faeces. Again, the same four  $\Delta^5$  sterols and taraxasterol are detectable and sitosterol is the most abundant sterol while cholesterol, brassicasterol and stigmasterol are present in low abundance, as is taraxasterol.

### 3.2. Carbohydrates

Fig. 4a depicts the abundance and distribution of monosaccharides derived from the hydrolysis of the extracted leaf litter. The distribution is dominated by the presence of glucose (136  $\mu\text{g mg}^{-1}$  C) and other C<sub>6</sub>-monosaccharides (galactose and mannose) with C<sub>5</sub>-monosaccharides also detected (arabinose and xylose). Fig. 4b depicts the abundance and distribution of monosaccharides derived through the hydrolysis of the extracted millipede faeces. Again the distribution maximises at glucose (28  $\mu\text{g mg}^{-1}$  C) with the abundance of C<sub>6</sub>-monosaccharides dominating over the C<sub>5</sub>-monosaccharides, with the distribution of monosaccharides maintained from the leaf litter into the millipede faeces.

### 3.3. Proteins

Fig. 5 depicts the distribution and abundance of hydrolysable amino acids detected in the leaf litter and millipede faeces. The distribution shows ten amino acids in the leaf litter

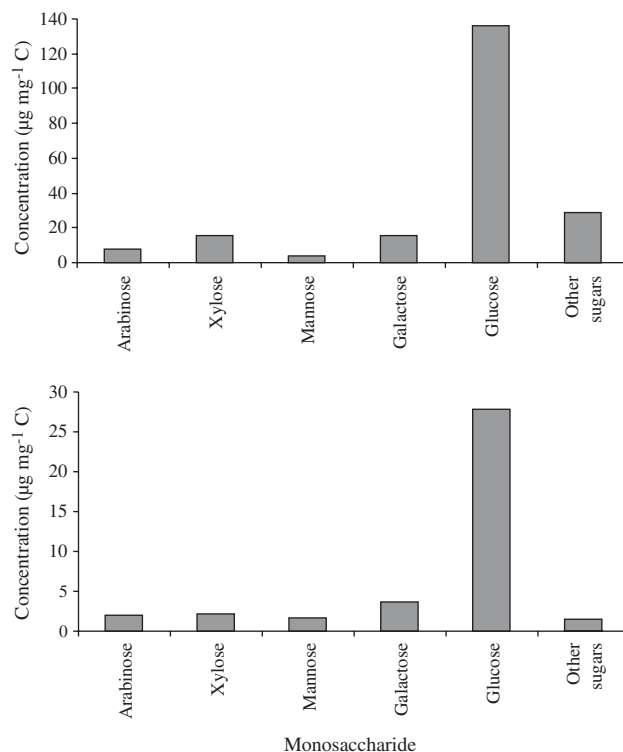


Fig. 4. Distribution and concentrations of hydrolysable monosaccharides of: (a) oak leaf litter and (b) pill millipede faeces.

maximising with glutamic acid. Of the 10 amino acids detected, five are deemed essential amino acids in animals (isoleucine, leucine, phenylalanine, threonine and valine) and five non-essential amino acids (alanine, aspartic acid, glutamic acid, proline and serine).

The distribution of hydrolysable amino acids in the millipede faeces is dominated by phenylalanine. The five non-essential amino acids detected in the leaf litter all decrease in concentration in the millipede faeces and are found in concentrations ranging from 0.7 to 2.6  $\mu\text{g mg}^{-1}$  C, all in lower concentration than that detected in the leaf litter. Four essential amino acids (valine, leucine, isoleucine and threonine) are observed to occur at near trace concentrations (0.1–1.2  $\mu\text{g mg}^{-1}$  C) in the faeces, as would be expected, however, phenylalanine an essential amino acid is the most abundant of all amino acids in the millipede faeces.

### 3.4. Lignin

Characteristic oxidation products of lignin were detected in both litter and faecal pellets (Fig. 6) with similar yields (67.5 and 59.8 mg VSC  $\text{g}^{-1}$  C). The distribution of the products is different showing an increase of vanillin and the occurrence of *p*-hydroxyacetophenone in the millipede faeces. However, only the vanillyl, syringyl and cinnamyl units can be used as true indicators of lignin since phenolic moieties can have different origins, especially in the millipedes' faeces. The acid:aldehyde (Ac/Al) ratios of both vanillyl and syringyl units decreases in the millipede faeces compared to the leaf litter.

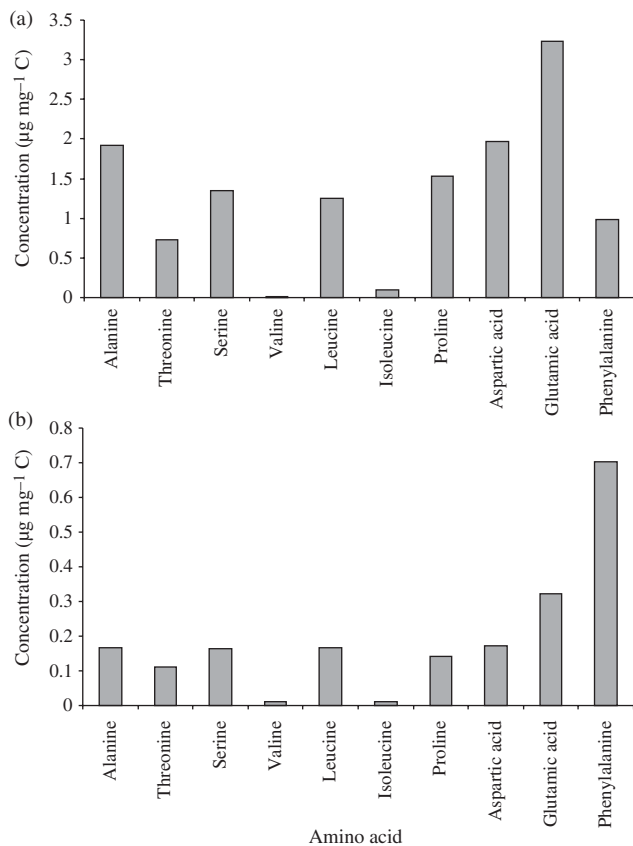


Fig. 5. Distribution and concentrations of hydrolysable amino acids of: (a) oak leaf litter and (b) pill millipede faeces.

### 3.5. Py/GC/MS

Fig. 7a depicts the partial pyrogram of the *Quercus* leaf litter. Thirty-six pyrolysis products are identifiable in significant abundance (Table 1), originating from carbohydrates, lignin, protein and lipids. The pyrogram is dominated by lignin markers vinylphenol (16) and 4-vinylguaiacol (18) with broad carbohydrate peaks from 1,6-anhydro- $\beta$ -D-glucopyranose (27) and 1,6-anhydro- $\beta$ -D-glucofuranose (28).

Fig. 7b depicts the partial pyrogram of the millipede faeces with 34 pyrolysis products identifiable originating from lignin, lipids, proteins or carbohydrates (Table 1). The pyrogram is dominated by 3 lignin markers; vinylphenol (16), 4-vinylguaiacol (18) and *cis*-isoeugenol (25) and more lignin products are identifiable. Carbohydrate pyrolysis products are less abundant; particularly 1,6-anhydro- $\beta$ -D-glucofuranose (28) although other carbohydrate products such as 1,6-anhydro- $\beta$ -D-glucopyranose are still detected. A variety of protein pyrolysis products were detected in the millipede faeces, with changes in abundance of these compounds occurring relative to other pyrolysis products.

### 3.6. <sup>13</sup>C solid state NMR

Fig. 8a depicts the <sup>13</sup>C CPMAS NMR spectra of oak leaf litter which is dominated by peaks at 75 and 105 ppm. The peak at 75 ppm corresponds to carbon atoms in pyran moieties

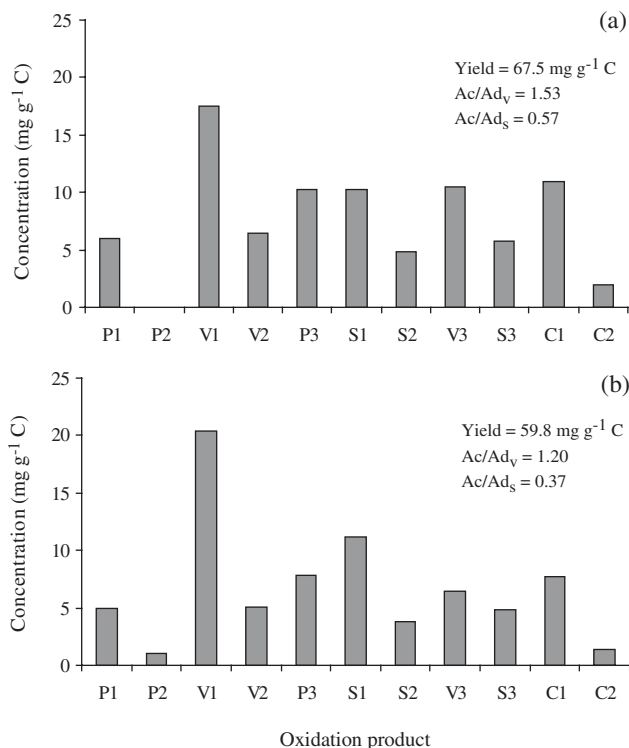


Fig. 6. Distribution of lignin index phenols from the cupric oxidation of: (a) oak leaf litter and (b) pill millipede faeces. (Key: P1, *p*-hydroxybenzaldehyde; P2, *p*-hydroxyacetophenone; P3, *p*-hydroxybenzoic acid; V1, vanillin; V2, acetovanillone; V3, vanillic acid; S1, syringaldehyde; S3, syringic acid; C1, *p*-coumaric acid; C2, ferulic acid; Ac/Ad<sub>v</sub>, acid to aldehyde ratio of vanillyl units; Ac/Ad<sub>s</sub>, acid to aldehyde ratio of syringyl units).

from C<sub>2</sub>, C<sub>3</sub> and C<sub>5</sub> in sugars while the peak at 105 ppm is from anomeric carbons in glycosidic linkages and tannins (Table 2). Other important peaks are at 35–45 ppm (aliphatic C), 65 ppm (*O*-alkyl C (carbohydrate derived)) and the sharp peak at 170 ppm (carbonyl-C).

Fig. 8b depicts the <sup>13</sup>C CPMAS NMR spectrum of the millipede faeces. The spectrum is very similar to that of the leaf litter, again being dominated by pyran moieties at 75 ppm, anomeric carbons at 105 ppm with an aliphatic-C signal between 35 and 45 ppm and a more defined region between 50 and 70 ppm showing clearly defined peaks at 56 ppm (methoxyl-C from lignin and C<sub>α</sub> in amino acids) and a double peak at 63 ppm corresponding to *O*-alkyl C (C<sub>6</sub> in sugars) signals. The carbonyl-C region at 170 ppm is of higher intensity than the corresponding resonance observed for leaf litter.

## 4. Discussion

### 4.1. Lipids

#### 4.1.1. TLE

When comparing the partial gas chromatograms of the total lipid extracts of the leaf litter and millipede faeces (Fig. 1a and b), there is a substantial loss of sitosterol. Although sterols are reportedly more resistant to degradation than other lipid components, such as *n*-alkanols and *n*-alkanoic acids (Cranwell, 1981), it is most probable that this loss is due to

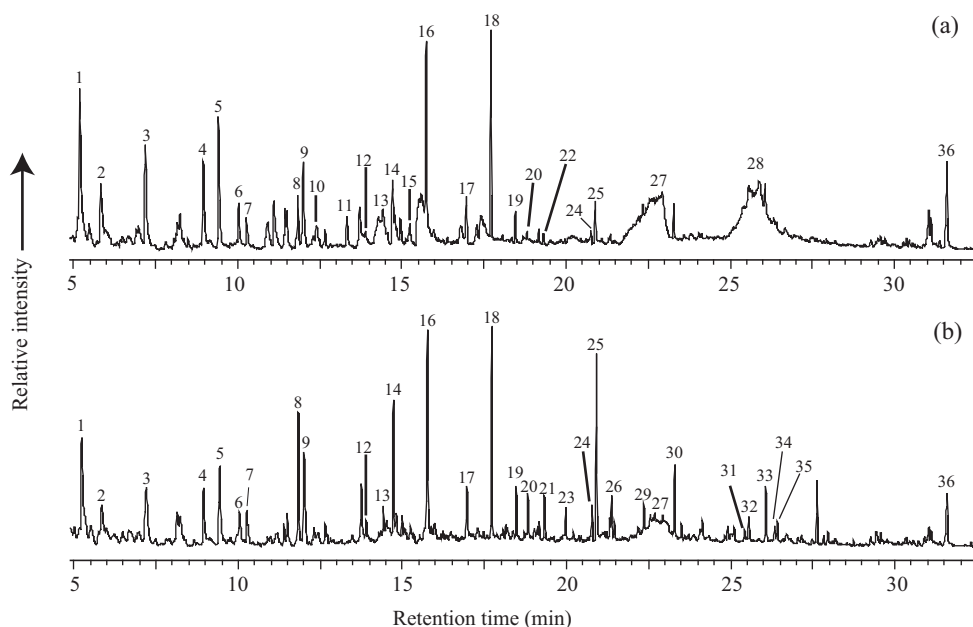


Fig. 7. Pyrolysis products of: (a) oak leaf litter and (b) pill millipede faeces.

direct assimilation by the millipede. Invertebrates are reported to be unable to biosynthesise sterols *de novo* (Svoboda and Thompson, 1985) so many arthropods produce cholesterol from higher sterol homologues, such as sitosterol, by dealkylation at the C-24 position. Several TAGs are detected in high abundance relative to other lipids in the leaf litter yet virtually absent in the millipede faeces. The high-energy value of TAGs would lead to the rapid utilisation of these compounds by hydrolysis and subsequent assimilation and/or catabolic breakdown mediated by lipases inherent in soil microfauna (Hita et al., 1996). Wax esters increase in relative abundance in the millipede faeces compared to the leaf litter. Composed of a long chain *n*-alkanoic acid linked to a long chain *n*-alkanol, wax esters constitute an important component of epicuticular waxes present on the leaf surface that form a protective physiochemical barrier against environmental degradation (Kolattukudy, 1975); the recalcitrance of these compounds to assimilation during passage through the *Glomeris* gut is of little surprise. A range of uncharacterised triterpenoids are observed to increase in abundance relative to other lipids in the faeces. The cyclic nature of these compounds appears to make them more resistant to degradation in the gut of the millipede than other lipids.

#### 4.1.2. *n*-alkanes

There is an overall 71% decrease in abundance of *n*-alkanes in the millipede faeces relative to the leaf litter. The relative abundance distribution of homologues from the leaf litter is maintained in the faeces suggesting that *n*-alkanes are degraded via non-discriminant  $\beta$ -oxidation, of first order rate, mediated by microflora in the millipede gut; possible losses due to degradation prior to ingestion or post excretion by the millipede must also be considered. Diploptene, a well established bacterial product (Ourisson

et al., 1987) detected in the faeces infers an intimate association with gut bacteria during the early stages of organic matter degradation and is supported by the notion that bacteria are significantly more abundant in the faeces of the millipede than in the diet (Anderson and Bignell, 1980; Ineson and Anderson, 1985).

#### 4.1.3. *n*-alkanols

Overall, there is an 82% decrease in the abundance of homologous *n*-alkanols in the millipede faeces relative to the leaf litter. However, the relative abundance distribution of the homologous series is maintained between the leaf litter and the millipede faeces suggesting that *n*-alkanols are also degraded via  $\beta$ -oxidation at a rate determined by first order kinetics. The *n*-alkanols are important constituents of epicuticular leaf waxes, comprising in the region of 36% of the composition of some epicuticular waxes (Prasad et al., 1990) and appear to have little or low nutritional value for the pill millipede.

#### 4.1.4. *n*-alkanoic acids

The distribution of *n*-alkanoic acids exhibits a shift in relative abundance to the higher molecular weight homologues in the millipede faeces suggesting preferential degradation and assimilation of the shorter chain homologues (C<sub>9</sub>–C<sub>20</sub>) and particularly the C<sub>16</sub> and C<sub>18</sub> components; the C<sub>16</sub> and C<sub>18</sub> homologues could be readily utilised as an energy source or for cell wall phospholipid biosynthesis. The C<sub>24</sub>–C<sub>32</sub> homologues originating from leaf epicuticular waxes (Kolattukudy, 1975) are still degraded by  $\beta$ -oxidation probably by the gut microflora of the millipede, albeit at a lower rate than the shorter chain homologues where the reduction on concentration is likely to be a combined result of assimilation and degradation by  $\beta$ -oxidation.

The relative reduction in concentration of the *n*-alkanoic acids comprising C<sub>16:1</sub>, C<sub>18:1</sub> and C<sub>18:2</sub> reflects the metabolic

Table 1

Pyrolysis products of oak leaf litter and pill millipede faeces (numbers refer to peak annotations given in Fig. 7; Ralph and Hatfield, 1991; Stankiewicz et al., 1996, 1997)

Peak #	Source	Compound
1	Carbohydrate	Furfural
2	Carbohydrate	2-hydroxymethylfuran
3	Carbohydrate	2,3-dihydro-5-methylfuran-2-one
4	Carbohydrate	4-hydroxy-5,6-dihydro-(2H)-pyran-2-one
5	Protein	Phenol
6	Lignin	2-hydroxy-3-methyl-2-cyclopenten-1-one
7	Carbohydrate	2-(propan-2-one)tetrahydrofuran
8	Lignin	Guaiacol
9	Lignin	4-methylphenol
10	Protein	Ethylcyanobenzene
11	Carbohydrate	3,5-dihydroxy-2-methyl-5,6-dihydro-4H-pyran-4-one
12	Lignin	2-methoxytoluene
13	Lignin	4-methoxytoluene
14	Lignin	2-acetyl-5-ethylfuran
15	Carbohydrate	3-hydroxy-2-methyl-(4H)-pyran-4-one
16	Lignin	Vinylphenol
17	Protein	Indole
18	Lignin	4-vinylguaiacol
19	Lignin	Vinylguaiacol
20	Lignin	2,6-dimethoxyphenol
21	Lignin	Eugenol
22	Protein	C <sub>1</sub> -indole
23	Lignin	Vanillin
24	Protein	2,5-diketopiperazine
25	Lignin	Cis isoeugenol
26	Lignin	Acetovanillone
27	Carbohydrate	1,6-anhydro-β-D-glucopyranose
28	Carbohydrate	1,6-anhydro-β-D-glucofuranose
29	Lignin	2,6-dimethoxy-4-vinylphenol
30	Lignin	Propiovanillone
31	Lignin	Syringaldehyde
32	Lignin	1-(3,5-dimethoxy-4-hydroxyphenyl)propyne
33	Lignin	4-allyl-2,6-dimethoxyphenol
34	Lignin	Trans coniferaldehyde
35	Lignin	Acetosyringone
36	Lipid	C <sub>16:0</sub> fatty acid

importance of these compounds to the pill millipede resulting in the direct incorporation of the *n*-alkenoic acids into phospholipid biosynthesis or catabolisation as an energy source. The *n*-alkanoic acids are degraded at a lower rate than the *n*-alkenoic acids as reflected by the presence of long chain homologues up to C<sub>32</sub>. The degradation of the long chain homologues is slower than the short chain homologues as degradation of the long chain homologues is likely to proceed via β-oxidation while short chain homologues could be degraded and assimilated directly by the pill millipede thus the reduction in concentration is significantly greater for the short chain homologues.

#### 4.1.5. Sterols and triterpenols

As discussed above (4.1.1) invertebrates are reportedly unable to synthesise cholesterol *de novo* (Svoboda and Thompson, 1985) and must utilise dietary cholesterol or convert higher dietary sterols to satisfy their physiological requirement for cholesterol. Sitosterol, stigmasterol and brassicasterol could all be potentially converted to cholesterol via de-alkylation at the C-24 position and with additional

reduction of the double bond at C-22 in the case of stigmasterol and brassicasterol. Rather surprisingly, cholesterol is detected in the faeces of the millipede with more than 50% of the concentration of cholesterol detected in the leaf litter still present in the faeces. The cholesterol in the millipede faeces might originate from the leaf litter with cholesterol not assimilated during passage through the gut of the millipede but more likely arises from the excretion of dead cells and gut lining by the millipede. Taraxasterol, a triterpene alcohol, is more recalcitrant than the sterols presumably due to its low physiological value to the millipede and the inherent stability of the pentacyclic ring system.

#### 4.2. Carbohydrates

Monosaccharides are detected at lower concentration in the millipede faeces than in the leaf litter but the relative abundance distribution and concentrations of monosaccharides in the leaf litter is maintained in the millipede faeces. Hydrolysable monosaccharides likely to originate from cellulose and hemicellulose are clearly being degraded by the millipede and presumably utilised as an energy source. Maintaining the distribution of monosaccharides from the leaf litter into the millipede faeces infers that glucose is being utilised indiscriminately compared to other monosaccharides. This suggests that monosaccharides are degraded at rates adhering to first order rate kinetics irrespective of their source. The degradation of glucose, predominantly sourced from cellulose suggests the presence of appreciable cellulolytic activity in the gut of the millipede, associated with either endogenous cellulases or symbiotic gut bacteria capable of cellulose degradation. Bacteria are reported to be more abundant in the gut of the millipede and in the faecal matter than in leaf litter that they feed upon (Anderson and Bignell, 1980) while the presence of cellulolytic enzymes in millipedes capable of cellulose degradation has been suggested (Striganova, 1971). By which of these pathways cellulose degradation proceeds is unclear. This is additionally supported by examining the xylose-to-mannose ratio, since mannose is of microbial origin and xylose of phytostructural origin. The X/M ratio decreases from 3.9 in the litter to 1.4 in the faeces reflecting the assimilation of xylose, thereby emphasising the importance of the millipede gut in leaf litter polysaccharide degradation and/or transformation.

#### 4.3. Proteins

The relative abundance distribution and concentrations of amino acids in the millipede faeces is significantly different to that observed in the leaf litter suggesting that amino acids are degraded and assimilated, or excreted differently in the millipede gut. Consumption of leaf litter will provide the pill millipede with the bulk of its amino acid requirements. However, consuming leaf litter would also mean the consumption of bacteria and fungi growing on the leaf litter, which are stimulated by passage through the gut of the pill millipede (Anderson and Bignell, 1980). Other organisms assimilate essential amino acids from symbiotic gut bacteria (Torrallardona et al., 2003; Metges, 2000; Pokarzhevskii

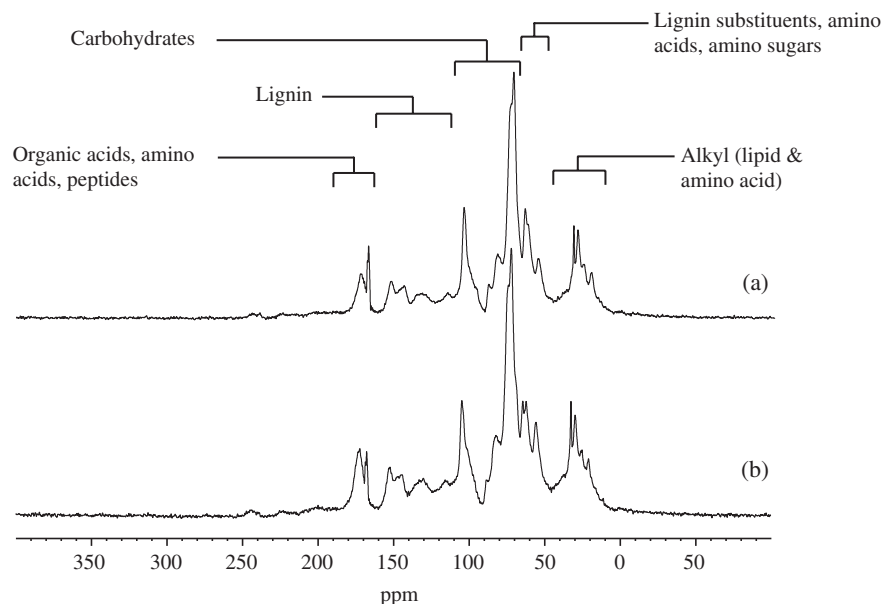


Fig. 8.  $^{13}\text{C}$  CPMAS NMR spectra of: (a) oak leaf litter and (b) pill millipede faeces.

et al., 1997). The presence of symbiotic gut bacteria in the millipede is also a possible source of the amino acids detected in the faecal pellets due to the excretion of the gut lining, and associated symbiotic microorganisms.

The decrease in abundance of amino acids in the millipede faeces relative to the leaf litter is indicative of them being essential building blocks for the body and a nitrogen source for millipede growth and maintenance. Despite the utilisation of amino acids, the overall concentration of nitrogen is greater in the faeces than in the leaf litter ( $2.5 \text{ g } 100 \text{ g}^{-1}$  compared to  $1.2 \text{ g } 100 \text{ g}^{-1}$ , respectively). This is possibly due to the assimilation and/or degradation of compounds by the pill millipede (C has fallen from  $47.2 \text{ g } 100 \text{ g}^{-1}$  to  $45.4 \text{ g } 100 \text{ g}^{-1}$ ) or as a result of excretion of excess nitrogen by the pill millipede in a different form to that in which it is digested, probably in the form of ammonia (Bocock, 1963). The pill millipedes used for this study were mature and hence little protein would be required for body growth.

#### 4.4. Lignin

The Ac:Al ratios of vanillyl and syringyl units are used to describe the microbial alteration of lignin, which is thought to start by the oxidation to carboxylic acids of the phenylpropanoid units without cleavage of the ring structure (Haider, 1986). Thus, an increase of these ratios is usually observed in soils and sediments compared to the values of fresh plants (Ertel and Hedges, 1984; Ziegler et al., 1986). In this study, a decrease was observed between the oak leaves and the millipede faeces for both the vanillyl and syringyl units' ratios (Fig. 6), which suggests a lower acidity of the remaining lignin. This suggests the lignin functionalities underwent a change in the millipede gut, which might be either the use of the accessible oxygen of the acidic functionalities of lignin or a

mechanism leading to a more highly condensed state of the lignin macromolecule reducing the yield of the cupric oxidation. The S:V ratio is representative of the vegetation type when bulk samples are studied and can also be used as an index for degradation due to V units having a more condensed structure than S units. However, several studies of particle-size fractions showed different values for this ratio between the fractions within a soil (Amelung et al., 1999 and references therein) indicating the preferential biodegradation of the vanillyl or the syringyl units. The S:V ratios of the oak leaves and the millipede faeces were similar, 0.59 and 0.58, respectively. In summary, the compositions indicate that only the oxygenated functions are affected by the millipede gut.

#### 4.5. Py/GC/MS

Carbohydrate pyrolysis products are dramatically reduced in concentration in the faeces. 1,6-anhydro- $\beta$ -D-glucofuranose (28) and 3,5-dihydroxy-2-methyl-5,6-4H-pyran-4-one (11) are not detected in the faeces but are abundant in the leaf litter

Table 2  
Recovery of lipid classes in the oak leaf litter and pill millipede faeces

Compound class	Concentration ( $\mu\text{g mg}^{-1} \text{C}$ )	
	Leaf litter	Millipede faeces
<i>n</i> -alkanes	0.430	0.126
<i>n</i> -alkanols	2.806	0.505
<i>n</i> -alkanoic acids	5.394	0.495
Sterols	0.506	0.099
Carbohydrates	207	39
Proteins	13.1	1.97
C	$47.2 \text{ g } (100 \text{ g})^{-1}$	$45.4 \text{ g } (100 \text{ g})^{-1}$
N	1.2	2.5

while 1,6-anhydro- $\beta$ -D-glucopyranose is reduced in abundance in the millipede faeces. This suggests that the carbohydrates are being degraded by passage through the millipede gut and probably catabolised as an energy source. This correlates well with the wet chemical analyses of carbohydrates (Sections 3.2 and 4.2) and the reduction in concentration of carbohydrates in the faeces compared to the litter. Protein pyrolysis products are seen to decrease in abundance relative to other pyrolysis products. Phenol (5) is seen to decrease in abundance relative to 4-hydroxy-5,6-dihydro-(2H)-pyran-2-one (4) and 2-hydroxy-3-methyl-2-cyclopenten-1-one (6). Ethylcyanobenzene (10) and C<sub>1</sub>-indole (22) are not detected in the millipede faeces while indole (17) can still be detected in the faeces. This most likely represents selective degradation of particular amino acids as observed in the data from the acid hydrolysis of proteins in the litter and faecal pellets, where individual amino acids appear to be selectively degraded by passage through the millipede gut. There is a change in relative abundance and distribution of lignin markers in the faeces compared to the leaf litter, with 2,6-dimethoxyphenol (20), eugenol (21), vanillin (23), *cis*-isoeugenol (25) and propiovanillone (30) all becoming more prominent in the faeces suggesting that lignin is a more significant constituent of faeces than leaf litter. However, lignin products in general do not exhibit a dramatic increase in abundance that would be expected if labile compounds such as proteins, carbohydrates and some lipids were significantly degraded by the millipede. This correlates well with the results from the cupric oxidation of lignin where a change in the distribution of lignin markers is observed, along with a decrease in the acid-to-aldehyde ratio for both syringyl and vanillyl units in the faeces relative to the leaf litter.

#### 4.6. <sup>13</sup>C CPMAS NMR

A number of subtle changes can be seen in the intensity of the signals in <sup>13</sup>C CPMAS NMR spectra. The spectrum is dominated by the peak at 75 ppm, which is attributable to carbon atoms in pyran moieties (C<sub>2</sub>, C<sub>3</sub> and C<sub>5</sub> in sugars) with strong signals at 35 ppm (alkyl C) and 105 ppm (anomeric carbons in glycosidic linkages (Preston, 1996)). This suggests the presence of significant concentrations of intact or weakly altered polysaccharides in both the leaf litter and millipede faeces, however, tannins also produce a strong signal at around 105 ppm. The peak at 175 ppm is a carbonyl signal from esters or amides and the region of low intensity between 110 and 140 ppm is an olefinic or aromatic carbon-carbon double bonds signal or both (Knicker et al., 1996). Differences in signal intensity in these regions are noticeable particularly at 175 ppm representing an increase in the abundance of organic acids and/or esters or protein in the millipede faeces. Changes in the intensity of the peak at 56 ppm are attributed to a change in the distribution of constituents of lignin in the faeces compared to the leaf litter (Poirier et al., 2003). Subtle changes in the intensity of peaks are observed in the spectra although not as dramatic as changes observed for other coprophagous litter dwellers such as dipteran larva (Hopkins et al., 1998)

emphasising the need for the more detailed approach at the molecular level presented above to elucidate the changes that occur when leaf litter is consumed by invertebrates.

## 5. Conclusions

Quantitative and qualitative analyses of the lipids, carbohydrates, proteins and lignin constituents of the leaf litter and millipede faeces reveals that a number of important changes occur when leaf litter is consumed and processed by a pill millipede.

The physiological requirements for growth, reproduction and maintenance of the pill millipede explain the dramatic decrease in concentration in the faecal pellets of:

- (i) Sterols that could be converted to cholesterol to fulfil biochemical demands.
- (ii) Short chain fatty acids that could be directly converted into phospholipid membranes or catabolised as an energy source.
- (iii) Triacylglycerols that could be catabolised for energy or stored.
- (iv) Carbohydrates are reduced in concentration by 85%, most likely utilised for energy.
- (v) Amino acids decrease in concentration and change in distribution suggesting selective assimilation.

A distinction can be seen between the utilisation and assimilation of metabolically useful compounds and those that are relatively more recalcitrant originating from the epicuticular waxes. *n*-alkanes and *n*-alkanols are degraded, probably via  $\beta$ -oxidation, while more refractory lipids such as triterpenoids and wax esters increase in relative abundance in the faeces compared to other lipid components. Originating from epicuticular waxes, these compounds are inherently resistant to degradation and would be expected to better survive passage through the gut.

Since the millipede faeces contain only low concentrations of physiologically useful compounds, such as sterols and carbohydrates, but a high abundance of recalcitrant components, such as lignin, wax esters and triterpenoids further decomposition is likely to be mediated by microorganisms, such as white rot fungi and ascomycetes. This investigation emphasises the biochemical effects associated with the comminution of leaf litter by macroinvertebrates, and shows the key role they play in the early stages of organic matter cycling and/or sequestration in soils. Furthermore, our study serves to further illustrate the importance of developing a molecular-based understanding of the transformation processes underlying organic matter degradation in soil (Poirier et al., 2005).

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