

PROTOCOL DEVELOPMENT FOR PURIFICATION AND CHARACTERIZATION OF SUB-FOSSIL INSECT CHITIN FOR STABLE ISOTOPIC ANALYSIS AND RADIOCARBON DATING

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ABSTRACT. Reliable radiocarbon dating depends upon well-defined samples. We have been investigating whether or not reliable ¹⁴C dates can be obtained directly from sub-fossil insect cuticle or biochemical fractions derived from it. Initial carbon and nitrogen stable isotope measurements on sub-fossil insect chitin from species with known feeding behaviors found within a single site (St Bees, Cumbria) clustered in a manner reminiscent of trophic level effects seen in terrestrial ecosystems. Although this finding implied some chemical stability, the measurement of CN ratios from the same samples indicated compositional variability. In addition, ¹⁴C dates obtained from these same samples were different from dates obtained from plant macrofossils found at the same depth. We have experimented with protocols designed to biochemically reduce chitin to its principle carbohydrate component glucosamine with the aim of using this compound to generate reliable ¹⁴C dates. Solvent extractions of sub-fossil chitin were carried out to remove both endogenous and exogenous lipid-soluble materials. Base hydrolysis reactions designed to extract polypeptides retained surprisingly high levels of contaminating amino acids. Proteinase K enzyme treatment had little effect on the level of amino acid contamination. Strong acid hydrolysis reactions designed to depolymerize chitin to glucosamine yielded only 5% glucosamine. Clearly alternative methods of chitin depolymerization must be identified before the purification and ¹⁴C dating of glucosamine from sub-fossil chitin becomes practical.

INTRODUCTION

The sub-fossil remains of temperature-sensitive insects, predominantly *Coleoptera*, can be used to generate high resolution palaeoclimate data (Atkinson et al. 1987; Aalbersberg and Litt 1998; Coope et al. 1998). Currently, radiocarbon measurements from associated organic material rather than the insect remains are used to fix the climate data in time. This study investigates the feasibility of directly dating insect remains, so that environmental and time data can be derived from the same material.

The insect cuticle is formed from a complex secretion containing lipids, proteins, and carbohydrates (Richards 1978). The principal component of the cuticle is the carbohydrate polymer chitin. This is hardened by sclerotization, a process resulting in cross-link formation between proteins and carbohydrates within the structure (Peter et al. 1986). Miller et al. (1993) studied the composition of Quaternary *Coleoptera* remains using chemical tests for primary amines and N-acetylglucosamine: protein content appeared to drop more quickly than chitin. Stankiewicz et al. (1997a) using analytic pyrolysis-GC-MS, found well-preserved cuticle within Pleistocene deposits, and demonstrated chitin-derived fragments within the remains of 25-million-year-old fossil insects (Stankiewicz et al. 1997b).

Initial studies using accelerator mass spectrometry (AMS) measurements on insect remains showed significant differences between the dates obtained for insects and those obtained from surrounding macrofossils (Elias and Toolin 1990; Elias et al. 1991). The reason for the offset was unclear, and the problem obscured by the possibility of taphonomic mixing. A recent study (Walker et al. 2001)

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compared AMS dates obtained from insect remains, plant macrofossils, and bulk organic materials, and found insect-derived dates systematically differed from the dates from the stratigraphically associated material.

The chemical pretreatments used for the AMS ^{14}C dating studies by Elias and Toolin (1990), Elias et al. (1991), and Walker et al. (2001) were minimal, no doubt partially because of limited quantities of starting material.

Marine arthropod and insect chitins have been studied as possible source materials for carbon and nitrogen stable isotope studies. Feeding experiments conducted by DeNiro and Epstein (1981) demonstrated a link between diet and both carbon and nitrogen stable isotope ratios in macromolecular insect chitin. Schimmelmann and DeNiro (1986a) developed an ion-exchange chromatography protocol to purify D-glucosamine, the deacetylated monomer from marine arthropod chitin hydrolysates. They found that D-glucosamine $\delta^{13}\text{C}$ values from marine arthropods were on average 9.1‰ heavier than the same material from terrestrial arthropods, and that trophic level shifts occurred in $\delta^{15}\text{N}$ values of D-glucosamine from arthropods within marine ecosystems (Schimmelmann and DeNiro 1986b). Significantly, $\delta^{15}\text{N}$ levels within D-glucosamine purified from insect chitin was found to be about 12‰ lower than $\delta^{15}\text{N}$ levels within the muscle mass.

We have been investigating how chemical pretreatment protocols (Table 1) affect sub-fossil chitin and alter both the carbon and nitrogen stable isotope values and AMS dates derived from it. This study has been conducted using samples from the same site as described in Walker et al. as well as from Redkirk Point, another ^{14}C dated, insect-rich deposit. These experiments are directed toward producing and defining chitin fractions amenable to subsequent chromatographic purification by high pH anion exchange chromatography (also see Hodgins et al. 2001). The ultimate objective is to isolate carbohydrate monomers from chitin in quantities sufficient for ^{14}C dating.

Table 1 Chitin chemical pretreatment protocols

Study	Elias et al. (1991)	Walker et al. (2001)	Schimmelmann and DeNiro (1986a)
Objective	^{14}C dating	^{14}C dating	C and N stable isotopes
Starting material	Sub-fossil insect remains	Sub-fossil insect remains	Modern marine arthropod cuticle
Purified material	Macromolecular chitin	Macromolecular chitin	The chitin-derived monomer glucosamine-HCl
Starting quantity	Milligrams	Milligrams	Grams
Protocol	Acid treatment? Pentane extraction Hot water wash	Water wash 0.5 N HCl @ 80 °C 8 hr water wash	2N HCl @ 22 °C 24 hr water wash 1 N NaOH @ 100 °C 1 hr water wash 7 N HCl @ 100 °C 4 hr ion exchange chromatography

MATERIALS AND METHODS

Insect Remains, *Carex ssp.* Seeds, and Bulk Organic Material

The insect remains and *Carex ssp.* seeds were recovered from a Quaternary peat deposits near St Bees, Cumbria, UK National Grid Reference NX965114, and Redkirk Point, Dumfriesshire, UK National Grid Reference NY301652. The St Bees section, was bounded by ^{14}C dates of $12,560 \pm 100$ BP and $11,500 \pm 120$ BP from bulk organic material. The 40-cm-thick section, (thinned down from 50 cm when sampled by Walker et al. [2001]) was sampled in 5-cm layers. The Redkirk Point

deposit, considered homogeneous, was dated at $11,205 \pm 177$ BP, again from bulk organic material. Both sites are described in Atkinson et al. (1987), and the Redkirk Point site described extensively in Bishop and Coope (1977).

Two-kilogram batches of the deposits were wet sieved through a 1-mm mesh, and the retained portion resuspended in water. Insect fragments and *Carex ssp.* seeds were picked from the suspension using tweezers. Fifteen to 20 mg of *Carex ssp.* seeds recovered from each five centimeter layer were chemically pretreated as cellulosic material using ORAU standard protocol (Hedges et al. 1989). The Redkirk Point peat was devoid of identifiable plant remains. Consequently, small twigs or possibly rootlets (<2 mm diameter) were collected, macerated with a scalpel, and pretreated as cellulose using the ORAU standard protocol.

The diversity and abundance of Coleopteran species varied within different layers of the St Bees section. To obtain sufficient quantities for stable isotopic analysis and dating, species in each 5-cm layer were pooled according to feeding behavior. Only Carabids (terrestrial scavengers) and Dytiscids (aquatic carnivores) were recovered throughout the section in large enough quantities for isotopic analysis, and even then only after pooling adjacent sections. The lower layers of the section were richer in remains and from these, the terrestrial herbivores *Adoxus obscurus*, and *Barynotus squamosus*, and the aquatic herbivore *Donacia versicolore*, were recovered in sufficient quantities to allow species-specific stable isotope measurements.

The initial chemical pretreatment began with deionized water-washed lyophilized remains. These were extracted sequentially with methanol, methanol:chloroform 2:1, chloroform, and then lyophilized. The remains were then suspended in 1.0 N HCl for 3 hr at room temperature, washed repeatedly with deionized water, and lyophilized. Elemental analysis, stable isotope measurements, and ^{14}C dates were obtained for these samples.

Further protocol development was carried out on the mass of unidentified insect remains from the St Bees site, and species recovered from the Redkirk Point peat deposit. These were predominantly the polyphagous weevil *Otiorhynchus nodosus* (see Morris 1997). The initial stage of the pretreatment is described above. This was followed by an incubation in 1 N NaOH at 100 °C for 3–12 hr in order to remove polypeptides within the cuticle structure. The remaining solids were washed repeatedly with deionized water and then lyophilized. In some preparations, Proteinase K enzyme (10U/mg) was added at an enzyme to substrate ratio of 1:40 (either before or after the NaOH treatment) to enhance deproteinization. Samples destined for AMS measurement were treated again with 1 N HCl for 30 minutes, washed repeatedly with deionized water, lyophilized and then combusted. Samples destined for chitin hydrolysis followed by high pH ion exchange chromatography, amino acid analysis, and MALDI-TOF MS were treated with 7N HCl at 100 °C for 4 hr, and then lyophilized.

HPLC

High pH anion exchange chromatography with pulsed amperometric detection was carried out on an HPLC system from Dionex Corp. The system is described in Hodgins, Butters et al. (2001). The column, a Dionex Carbopac PA-1 (9 mm × 250 mm) semi-preparative anion exchange column, was run isocratically, at room temperature, in carbonate-free 200 mM NaOH flowing at 1.7 mL/min. Carbohydrates were detected using a pulsed amperometric detector (PAD) cell (Dionex PAD-1). The elution times and detector response calibration curves of the chitin-derived carbohydrates N-acetylglucosamine and glucosamine were determined using standards obtained from Sigma-Aldrich.

Amino Acid Composition Analysis

Amino acid analysis was carried out on chitin hydrolysates using an Applied Biosystems model 420A derivatizer/analyzer (Foster City, California). Amino acid and amino sugar compositions were determined by precolumn derivatization with phenylisothiocyanate, followed by separation of the phenylthiocarbamyl amino acids and amino sugars by reversed-phase HPLC. The elution of the PTC-derivatized glucosamine standard overlapped with the elution of PTC-asparagine, however, in the chitin hydrolysates, we assumed that the quantity of asparagine would be small compared to that of glucosamine, and so could be disregarded.

Matrix Assisted Laser Desorption Mass Spectrometry

Mass spectrometric analysis was carried out on unfractionated *Otiorhynchus nodosus* chitin hydrolysates using a Lasermat Instrument produced by ThermoBioanalysis Ltd. The hydrolysates were diluted in water to approximately 50 pmol/ μ L and mixed 1:1 with either DHB or α -cyano matrix. One microliter of this mixture was spotted onto aluminium targets and air-dried. The targets were loaded into the instrument, and pulsed with laser light. The resulting vapor was directed into a time of flight mass spectrometer. The principle molecular ion of glucosamine has a m/z ratio of 179. The mass/charge spectra from hydrolysates were compared to spectra from targets containing the DHB and α -cyano matrices alone.

Carbon and Nitrogen Elemental Analysis, Stable Isotope Analysis, and ^{14}C -AMS

All samples were weighed into tin capsules. Combustion and stable isotope measurements were carried out on a Carlo Erba CN Analyzer-Stable isotope Mass Spectrometer and the CO_2 recovered for later ^{14}C -AMS measurement. ^{14}C -AMS was accomplished using the ORAU gas ion source.

RESULTS AND DISCUSSION

Our original objectives targeted terrestrial herbivore species in an effort to avoid the input of detrital carbon and hard water effects. The site yielded terrestrial and aquatic carnivores and scavengers throughout the section, with herbivorous species recovered only in the lower half of the section.

The mass of individual fragments varied from approximately 50–350 μg . This necessitated all measurements being made on pooled samples. Combustion and stable isotope measurement revealed that with one exception, the CN ratios of the material fell between 6 and 8 (Table 2). Plotted as $\delta^{15}\text{N}$ versus $\delta^{13}\text{C}$ the values cluster in a manner consistent with a nitrogen trophic level effect (Figure 1). No trends were visible in the shifts in stable isotope values or the CN ratio as a function of the position within the section.

The AMS dates from plant macrofossils (*Carex spp.*) appeared to agree with the dates obtained on identical material by Walker et al. (2001). The differences in section thickness made precise comparison difficult. ^{14}C dates obtained from the insect remains were found to be younger than dates from stratigraphically associated seeds (with the exception of a single measurement). The fact that the insect-derived dates were younger argues against explanations based upon the hard water effect or the indirect ingestion of detrital carbon through the ingestion of detritus feeders. The offsets between the AMS dates obtained from the terrestrial herbivore insects and plant macrofossils within the lower layers of the section were not as extreme as the offsets observed by Walker et al. (2001).

The data in Figure 1 indicate a possible trophic level effect within insect chitin $\delta^{15}\text{N}$ values. However, alternative explanations are possible. A comparison of Figures 1 and 2 suggests a correlation

Table 2 Insect cuticle CN ratios and stable isotope values for remains recovered from the St Bees section

Group	Feeding behavior	Height above datum (cm)	Atomic CN ratio	$\delta^{13}\text{C}$ (‰)	$\delta^{15}\text{N}$ (‰)
Dytiscidae	Aquatic carnivores	30–40	7.0	–30.3	2.4
		20–30	6.8	–29.8	2.4
		10–20	6.8	–29.4	2.6
		0–10	6.9	–30.1	2.0
Carabidae	Terrestrial	30–40	6.6	–26.8	3.3
	Carnivores/scavengers	20–30	6.4	–26.9	3.0
		10–20	6.5	–26.3	4.0
		5–10	6.7	–26.9	3.5
		0–5	7.1	–27.1	3.9
<i>Barynotus</i> ssp.	Terrestrial herbivores	10–15	7.8	–25.7	0.9
		0–10	8.7	–26.3	0.5
<i>Adoxus</i> obs.	Terrestrial herbivores	10–15	7.7	–24.3	–1.5
		0–10	7.9	–24.5	–1.0
<i>Donacea</i> vers.	Aquatic herbivores	0–10	7.9	–24.5	–1.6

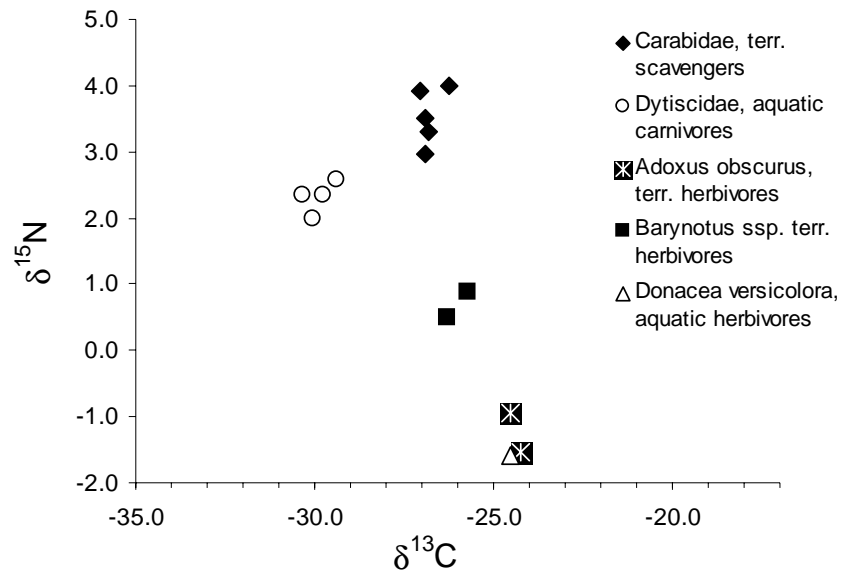


Figure 1 The carbon and nitrogen stable isotope values for St Bees insect remains. The measurement errors are $\delta^{13}\text{C} \pm 0.1$ ‰ for carbon and $\delta^{15}\text{N} \pm 0.3$ ‰ for nitrogen.

between the $\delta^{15}\text{N}$ values within chitin fractions and the magnitude of the AMS date offset between chitin and plant remains. For example, the Carabid species have both the highest $\delta^{15}\text{N}$ and the greatest offset from the *Carex* seed dates. This situation could be explained by the presence of a ^{14}C -enriched, ^{15}N -enriched nitrogen-containing contaminant. A comparison of the CN ratios of the insect fractions (shown in Table 2) and the chitin AMS dates shown in Figure 2 is also consistent with this picture.

Schimmelmann and DeNiro (1986a) pointed out that several factors influence isotopic variability within macromolecular chitin. The acetyl carbon pool is isotopically different than the hexose ring carbon pool, therefore deacetylation during burial and chemical pretreatment will affect $\delta^{13}\text{C}$. They also found that $\delta^{15}\text{N}$ levels within D-glucosamine purified from arthropod chitin were about 12‰ lower than $\delta^{15}\text{N}$ levels within the muscle mass (Schimmelmann and DeNiro 1986b). Webb (1998) observed a similar spacing in locust tissues. This means that the level of deproteinization would influence the $\delta^{15}\text{N}$ of macromolecular chitin. These effects were measured within fresh chitin. When the contamination from the burial environment is added into the system, the unsuitability of macromolecular chitin for ^{14}C dating becomes clear. Given these factors it is surprising that the isotope values within each group of St Bees insect remains clustered so tightly.

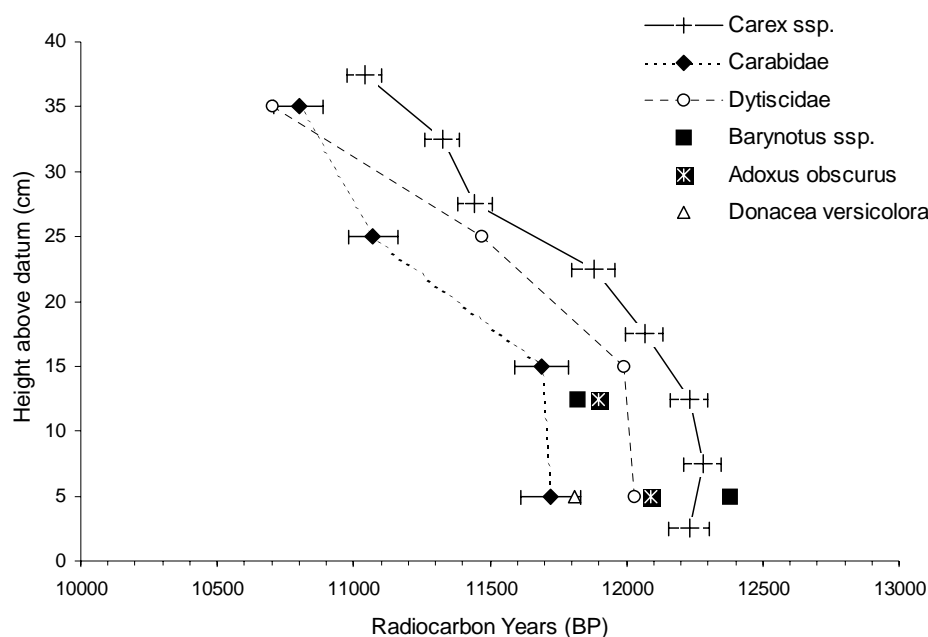


Figure 2 ^{14}C dates from the St Bees insect remains compared to associated plant macrofossils. The errors of all the insect remains are similar but some have been omitted for clarity. The bars show 1σ errors.

Further Purification of the Chitin Fractions

Schimmelmann and DeNiro (1986a) suggested that the most reliable chitin-derived target compound for stable isotope studies of marine arthropod chitin was glucosamine-HCl. Their protocol included a base hydrolysis step, aimed at de-proteinizing the chitin, followed by mineral acid

hydrolysis that hydrolyzed the chitin polymer and deacetylated N-acetylglucosamine to glucosamine-HCl. The glucosamine monomer was separated from the resulting amino acids using Dowex-50 W-X8 ion exchange resin run in 1 N HCl. We investigated their protocol using St Bees insect remains and utilized analytical high pH anion exchange chromatography instead of cation exchange.

Five-milligram batches of insect remains from St Bees and Redkirk Point were carried through solvent, acid extraction, deproteinization, and chitin hydrolysis steps. Various deproteinization protocols were investigated; namely combinations of proteinase K treatment and hot alkaline hydrolysis. The mass of chitin/chitosan after each stage was measured directly until the hydrolysis step. After hydrolysis glucosamine was quantified by analytical high pH anion exchange chromatography, as well as by derivatization with phenylisothiocyanate followed by reverse phase HPLC. The results in Figure 3 shows that 5–10% of the starting mass was soluble in organic solvents, and another 10% of the mass removed by 1N HCl hydrolysis. Thirty to 50% of the mass was removed by alkaline hydrolysis in combination with proteinase K treatment. This result generally agreed with the observations of Miller et al. (1993) that 41–55% of chitin mass was proteinaceous in material of a similar age. Regardless of the method of deproteinization, only 5% of the starting mass was recovered as glucosamine monomer.

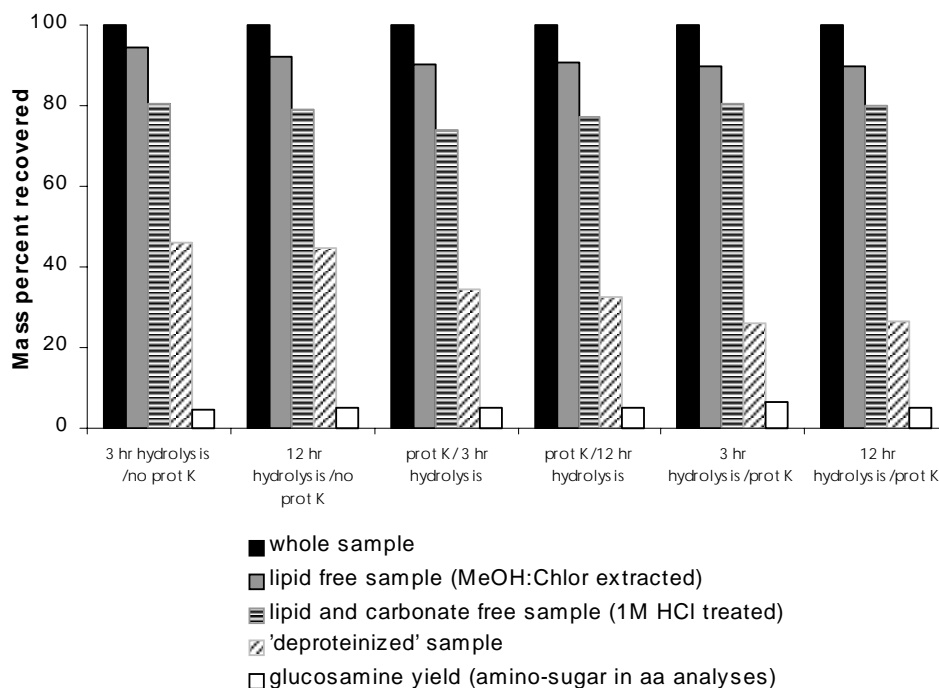


Figure 3 The effect of different extraction protocols on six aliquots of insect chitin recovered from St Bees, Cumbria. Two and one half milligram aliquots were treated sequentially with solvents, 1 N HCl, and the either 1N NaOH alone or 1N NaOH combined with proteinase K digestion as described in the Materials and Methods. Glucosamine yields were determined chromatographically, using pulsed amperometric detection, or UV absorption after phenylisothiocarbamyl derivatization.

Hydrolysis, phenylisothiocarbamyl derivatization and reverse phase HPLC of the “deproteinized” chitin revealed that 3 hr in 1N NaOH at 100 °C (followed by water rinsing), still left amino acids within the structure that accounted for one quarter of the remaining mass. This value was reduced to approximately 1/10 the remaining mass if 12-hr incubations with 1N NaOH were used. Although it is possible that the yield of glucosamine in the chitin hydrolysates has been reduced due to base-catalysed epimerization of glucosamine to mannosamine, the so-called Lobry de Bruyn-van Ekenstein transformation (see Pigman 1957), the fact that the HPLC resolved essentially a single principle component suggests the epimerization reaction is not significant.

Matrix-assisted laser desorption mass spectrometry was also carried out on four of the six chitin hydrolysates. A single peak was common to all and this had a mass/charge ratio of 179, the ratio expected for the principle molecular ion of glucosamine (not shown).

Using the remains of *Otiorhynchus nodosus* recovered from the Redkirk Point site, we investigated how different purification protocols affected elemental ratios, stable isotope values and ^{14}C date, and compared these to the published dates obtained from surrounding organic material (Table 3). The large changes in CN ratios and stable isotope values compared to the relatively small changes in ^{14}C date throughout the purification argue against Redkirk Point being the appropriate site for protocol development. Shown graphically in Figure 4, only methanol/chloroform solvent washes were required to bring the dates in line with all subsequent fractions. Initially these appeared to be systematically offset from the bulk material based upon the published BIRM-41 date. However, repeating the measurement on peat from the site (OxA-9499) indicated that the insect remains and peat were contemporary. The offset seen in the St Bees material was not evident at Redkirk Point.

The purification of N-acetylglucosamine and glucosamine from insect remains for the purpose of compound-specific dating does not currently appear practical because the yield of specific compound after chitin hydrolysis is so low. It is likely that the presence of substantial quantities of amino acids within the cuticle structure even after “deproteinization” promotes the formation of Maillard reaction products during the chitin hydrolysis step. In an associated study (Hodgins et al. 2001), we have been investigating enzymatic methods of carbohydrate polymer hydrolysis and purification using wood cellulose as a model system. This study has shown significant increases in monomer yield over acid hydrolysis. Future experiments on the purification of insect remains will be directed towards investigating the efficiency of chitinase-mediated hydrolysis.

Table 3 Changes in chitin chemistry during pre-treatment for ^{14}C dating

<i>O. nodosus</i> fraction:	Starting mass (mg)	Atomic CN ratio	$\delta^{13}\text{C}$ (‰)	$\delta^{15}\text{N}$ (‰)	Uncal. date (BP)	Error (±)
Untreated	4.1	8.5	−25.7	1.8	11390	70
Solvent	5.2	14.5	−24.7	−0.9	11670	65
Solvent/acid	4.2	9.1	−25.9	2.8	11665	65
Solvent/acid/ alkaline/acid	11.8	7.8	−24.3	−0.5	11645	119
Redkirk Point peat:						
(BIRM-41) ^a					11205	177
(OxA-9499)			−26.9		11690	65

^aFrom Atkinson et al. (1987)

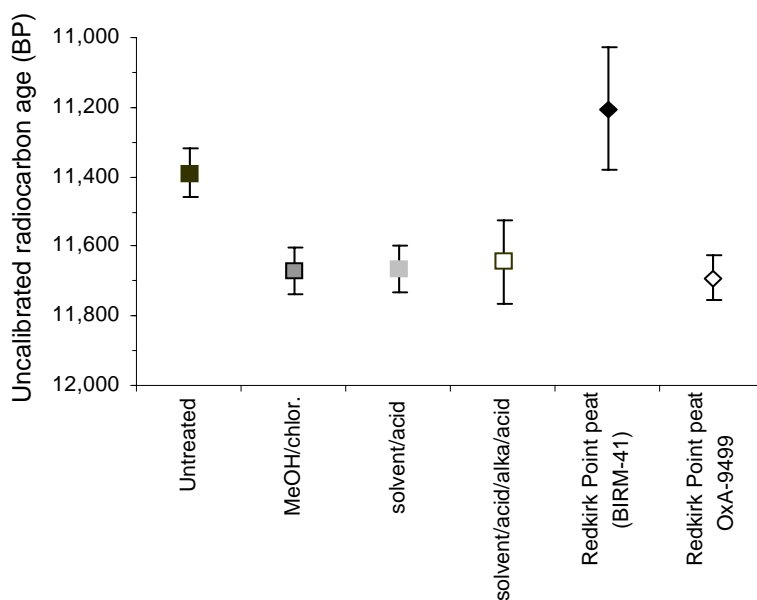


Figure 4 ^{14}C dates from various fractions of *Otiorhynchus nodosus* obtained from Redkirk Point compared to dates from bulk organic material

CONCLUSIONS

The observed dating offset between sub-fossil insect remains and surrounding organic material was shown to be influenced by chemical pretreatment protocols. The offset was not completely eliminated after the isolation of macromolecular chitin, suggesting that either further chitin purification is necessary, or some biological/ecological explanation must be put forward.

Sub-fossil insects with different feeding behaviors displayed nitrogen stable isotopic values reminiscent of trophic levels seen in other terrestrial ecosystems. However, the demonstration of variability in CN ratios and residual amino acids within the chitin preparations indicated that further purification would be necessary before firm conclusions could be reached.

Shimmelfmann and DeNiro (1986a) demonstrated that the CN ratio and carbon and nitrogen stable isotope values are not reliable criteria for macromolecular chitin purity for several reasons. There is inherent variability in the carbohydrate composition. The principle carbohydrate N-acetylglucosamine is susceptible to acid hydrolysis. It is difficult to fully “deproteinize” the sample and this combined with a large spacing between chitin and protein stable nitrogen isotope values means that residual peptides within the chitin structure have a large affect on the measurement.

We have investigated the feasibility of purifying monomer carbohydrates from sub-fossil chitin. We found that only 5% of the cuticle mass can be detected as monomer carbohydrate in acid hydrolysates. Clearly, sample recovery is the greatest hurdle. Chitinase enzymolysis is being investigated as an alternative, and parallel experiments on enzymolysis of cellulose have been encouraging.

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