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Short Paper

Lipid analysis of a ground sloth coprolite

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ABSTRACT

Coprolites can provide detailed information about the nutritional habits and digestive processes of the animals that produced them and may also yield information about the palaeoenvironment in which the animal existed. To test the utility of the lipid biomarker approach to coprolite analysis, lipids were extracted from a coprolite of the Pleistocene ground sloth *Nothrotheriops shastensis*. Gas chromatography/mass spectrometry results revealed a dominant spiroketal sapogenin component identified, using nuclear magnetic resonance spectroscopy, as epismilagenin. The dominance of epismilagenin is probably due to ingestion of *Yucca* spp. and *Agave* spp., which is consistent with previous studies on the diet of this species.

Introduction

Coprolites (fossilised faeces) provide a unique means of investigating the diet of animals in ancient ecosystems and can establish a direct link between the animal and the plants and/or animals it consumed (Chin et al., 1998). Coprolites have been studied previously on the basis of gross morphology (e.g. Mead et al., 1986a,b; Thulborn, 1991) and by identifying preserved fragments of ingested material such as bone (Chin et al., 1998), muscle (Chin et al., 2003), wood (Chin, 2007), plant leaves, stems and seeds (e.g. Eames, 1930; Mead et al., 1986a,b; Ambwani and Dutta, 2005; Kropf et al., 2007), phytoliths (e.g. Prasad et al., 2005) and pollen and spores (e.g. Martin et al., 1961; Mead et al., 1986b; James and Burney, 1997). Various chemical approaches have also been adopted including trace element analysis using emission spectroscopy (Martin et al., 1961), carbon and nitrogen stable isotope analysis (Ghosh et al., 2003), molecular (DNA) analysis (Poinar et al., 1998; Hofreiter et al., 2000) and pyrolysis coupled to gas chromatography mass spectrometry (py–GC/MS) (Hollocher et al., 2001). Radiocarbon dating of coprolites of appropriate age has also been conducted (e.g. Mead and Agenbroad, 1992).

Coprolites therefore possess the potential to provide detailed information about the diet, digestive processes and lifestyle of extinct animals, as well as the wider palaeoenvironment and palaeoclimate. However, when interpreting physical or chemical data from coprolites it is critical to consider the taphonomic processes that the specimen has been subjected to. For example, Pleistocene mammal coprolites from the Southwestern USA are typically preserved by dessication in cave deposits and have not been lithified (e.g. Eames, 1930; Martin et al., 1961; Mead et al., 1986a,b). Consequently, such specimens are likely to exhibit a much higher organic matter content and better preservation of biomolecules than older, lithified coprolites.

Existing techniques of coprolite analysis based on identification of preserved food fragments, while useful, may be biased by the variable resistance of different food materials to the chemical and physical processes of digestion. Existing chemical approaches can overcome these limitations in identifying dietary materials although these methods have their own limitations in that chemical signatures of all kinds may be subject to diagenetic loss or alteration (Peters et al., 2005). The lipid biomarker approach has the specific additional potential to provide information about the digestive processes and microbial communities of extinct animals. To date, biomarker analyses have rarely been applied to coprolites (notable exceptions include Lin et al., 1978; Chin and Brassell, 1993; Chin, 1996), but their successful and wide ranging application in other settings, particularly organic geochemistry, archaeological chemistry, environmental science, biogeochemistry, geomicrobiology and palaeoclimatology, indicates that they could prove a useful tool for the study of coprolites. The work reported herein comprises part of a larger study to assess the utility of the biomarker approach in coprolite analysis. As part of that study a coprolite from the Pleistocene ground sloth *Nothrotheriops shastensis* was analysed to test the twofold hypothesis that faecal biomarkers are present within the coprolite and that these extant compounds are indicative of the ground sloth's dietary preferences.

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Materials and methods

A sample was taken from specimen BRSUG 19569-3, a coprolite from Gypsum Cave, Nevada, USA, attributed to the ground sloth *N. shastensis*. The sample was ground using a pestle and mortar and approximately 1 g of the powdered sample was extracted, using a modified version of the lipid extraction and separation method of Kawamura et al. (2003). Briefly, the powdered sample was sonicated for 15 min and heated at 70°C for 2 h with 10 ml of 0.1 M methanolic potassium hydroxide (KOH) solution with 5% water. The sample was centrifuged for 5 min at 2500 revolutions per minute to separate the liquid extract from the remaining solid particles, transferred to a clean, round-bottomed flask and solvent was removed by rotary evaporation. Neutral lipids were isolated from the total lipid extract by dissolving it in hexane:dichloromethane (DCM) mixture 9:1; the solution was then acidified to pH1–0 and acidic lipids were extracted using DCM. Following removal of the solvent the remaining neutral lipids were separated into "aliphatic," "aromatic," "aldehyde and ketone," and "alcohol" fractions using the silica column chromatography method described by Kawamura et al. (2003). The alcohol fraction was derivatised prior to analysis, by adding 50 μl of *N,O*-bis(trimethylsilyl)trifluoroacetamide with 1% trimethylchlorosilane and 50 μl pyridine and heating at 70°C for 1 h. The samples were dissolved in ethyl acetate prior to analysis by gas chromatography (GC; Carlo Erba HRGC 5300 equipped with a non-polar fused silica capillary column, CPSil-5CB, 50 m × 0.32 mm × 0.12 μm, Varian Chrompack). Gas chromatography-mass spectrometry (GC/MS) was conducted using a Thermoquest TraceMS GC-MS equipped with an identical column. In both cases the following temperature programme was used: initial temperature 70°C, rising to 130°C at 20°C min^{-1}, then rising to 300°C at 4°C min^{-1}, holding at 300°C for 25 min.

NMR spectroscopy

All NMR spectra were recorded on a 600 MHz Varian INOVA spectrometer equipped with a ^{1}H,^{13}C,^{15}N triple resonance inverse probe and shielded Z-pulsed field gradients. The 1D and 2D NMR experiments were recorded on 1.2 mg 600 μL^{-1} in $CDCl_3$ at 25°C. Two-dimensional double quantum filtered DQF-COSY, ^{1}H-^{1}H TOCSY, ^{1}H–^{13}C constant time HSQC and HMBC experiments were used in combination with reported ^{13}C assignments of epismilagenin (Lajis et al., 1993).

Results and discussion

Provenance and age of the specimen

Specimen BRSUG 19569-3 was not directly dated, but previous coprolites from Gypsum Cave, shown by molecular analysis to have derived from *N. shastensis*, have ages of between 11,005 ± 100 ^{14}C yr BP (Ua-12506) and 29205 ^{14}C yr BP (Ua-13224) (Hofreiter et al., 2000). Coprolites attributed to *N. shastensis* from other localities have also been dated within a similar range (e.g. Long et al. (1974) 10,780 ± 200 ^{14}C yr BP (A-1067) to 36,200 ± 6000 ^{14}C yr BP (A-1043); Thompson et al. (1980) 10,500 ± 180 ^{14}C yr BP (A-2174) to 33,910 ± 3720 ^{14}C yr BP (A-1609). Specimen BRSUG 19569-3 is morphologically identical to figured coprolites attributed to *N. shastensis* (e.g. Poinar, 2002, Figs. 5 and 6; Kropf et al., 2007, Fig. 2A) in terms of size, shape, structure and texture and we are therefore confident that the specimen analysed is a coprolite from *N. shastensis*, with a minimum age of approximately 11,000 ^{14}C yr BP.

The original label included with the specimen indicated that the coprolite was "almost entirely composed of yucca leaves," an assessment based on the gross morphology of the fragments comprising the coprolite (J. Attridge, pers. comm., 2008).

GC/MS results

Figure 1 depicts the total ion current (TIC) chromatogram obtained for the alcohol fraction of the sloth coprolite extract and the mass chromatogram for the *m/z* 215 fragment ion. These chromatograms show the unsaturated sterols cholesterol and sitosterol and some of their

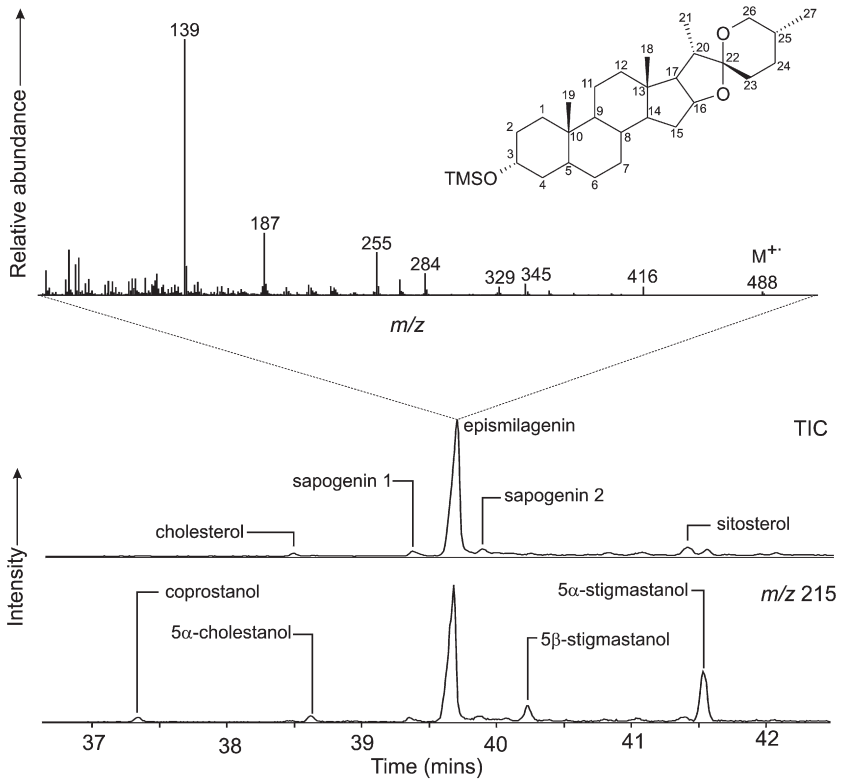

Figure 1. Total ion current (TIC) and an *m/z* 215 (a major fragmentation ion of saturated, trimethylsilylated sterols) mass chromatogram of the alcohol fraction of *Nothrotherium shastensis* coprolite lipid extract and the mass spectrum and structure of the predominant trimethylsilylated (25R)-5β-spirostan-3α-ol (epismilagenin).

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Table 1
^{1}H and ^{13}C chemical shift data for epismilagenin (ppm).

	^{1}H	^{13}C
1	0.97, 1.79	35.3
2	1.32, 1.62	30.4
3	3.63	71.7
4	1.51, 1.74	36.4
5	1.4	41.9
6	1.11, 1.43	26.7
7	1.26, 1.86	27.1
8	1.58	35.4
9	1.42	40.5
10	–	34.8
11	1.28, 1.40	20.7
12	1.15, 1.71	40.1
13	–	41.0
14	1.16	56.2
15	1.25, 1.98	31.8
16	4.4	80.9
17	1.77	62.2
18	0.75	16.5
19	0.95	23.3
20	1.86	41.6
21	0.97	14.4
22	–	109.2
23	1.58, 1.66	31.4
24	1.46, 1.63	28.7
25	1.64	30.3
26	3.38, 3.48	66.9
27	0.8	17.1

saturated stanol analogues, including 5β-cholestan-3β-ol (coprostanol), 5α-cholestan-3β-ol (5α-cholestanol), 24-ethyl-5β-cholestan-3β-ol (5β-stigmastanol) and 24-ethyl-5α-cholestan-3β-ol (5α-stigmastanol). 5β-stanols, such as coprostanol and 5β-stigmastanol, are uniquely formed in the gut by biohydrogenation of unsaturated sterols by digestive tract bacteria (Murtaugh and Bunch, 1967). Their presence, albeit at only trace concentrations, therefore serves to confirm the faecal origin of this material. The presence of cholesterol and sitosterol, derived from body cells and diet respectively, is consistent with this interpretation and the preservation of these unsaturated sterols also confirms the relatively low diagenetic maturity of the sample (Gaskell and Eglinton, 1976). However, these faecal biomarkers are present at relatively low levels (between 0.8% and 6.9%).

The alcohol fraction is dominated by a compound tentatively identified as a spirostanol sapogenin on the basis of its mass spectrum (Fig. 1), exhibiting a characteristic base peak fragment ion of m/z 139 and a molecular ion of m/z 416 (Taylor et al., 1997), and co-injection of authentic standards of (25R)-5β-spirostan-3β-ol (smilagenin; Sigma-Aldrich) and (25S)-5β-spirostan-3β-ol (sarsasapogenin; Sigma-Aldrich). In order to confirm the identity of this predominant compound, ^{1}H and ^{13}C NMR assignments were made using a combination of DQF-COSY, TOCSY and ^{1}H–^{13}C CT-HSQC in combination with ^{1}H-detected long range hetereonuclear multiple bond connectivity (HMBC) experiments. The ^{1}H and ^{13}C chemical shift data are presented in Table 1. The ^{1}H–^{13}C CT-HSQC was well resolved enabling most ^{1}H–^{13}C spin systems to be unambiguously grouped and assigned. Of note was the presence of the *R* configuration at C25 which was assigned by the identical matching of the ^{1}H, ^{13}C chemical shifts of ring F to the published assignments of (25R)-5β-spirostan-3β-ol (smilagenin) (Agrawal et al., 1997). Similarly assignments for the A ring of the molecule confirmed the α-configuration of the hydroxyl group at C3, consistent with identical ^{13}C shifts previously reported for epismilagenin (Lajis et al., 1993), thereby identifying the compound as (25R)-5β-spirostan-3α-ol (epismilagenin).

Discussion

Epismilagenin, the dominant component of the hydroxylated lipids extracted from the coprolite, is a sapogenin, a class of compounds derived from saponins, which are widely distributed secondary plant metabolites (Glasby, 1991). Saponins consist of a glycone (sugar) moiety and an aglycone (non-sugar) moiety, the sapogenin, which may commonly have a spirostane steroid structure (Flåøyen et al., 2002). Epismilagenin may be formed from one of two aglycone moieties, smilagenin and diosgenin (Figs. 2a, b).

Smilagenin and diosgenin are found predominantly in plants in their conjugated forms, i.e. as saponins, with a glycone moiety at the C3 position. When an animal consumes a plant containing such saponins, they are hydrolysed in the digestive tract to give free sapogenins that may then be converted to their epimeric analogues (Flåøyen and Wilkins, 1997). Miles et al. (1992, Fig. 2) proposed a scheme for the conversion of diosgenin and smilagenin to epismilagenin. The first step in the conversion of diosgenin is the stereoselective hydrogenation of the double bond at the C5 position to give smilagenin, with the hydroxyl group at the C3 position in the beta configuration. Smilagenin is then oxidised to smilagenone and reduced to give epismilagenin, with the hydroxyl group in the alpha configuration.

In ruminant animals hydrolysis of saponins and oxidation and reduction of sapogenins occurs predominantly in the rumen, omasum and abomasum (Flåøyen and Wilkins, 1997). In the sheep studied by Flåøyen and Wilkins (1997) episapogenins were re-conjugated in the liver and mainly re-hydrolysed in the lower intestine to give a mixture of free and conjugated episapogenins in the faeces, with the latter dominating. A similar process is invoked to explain the occurrence of epismilagenin in the sloth coprolite, which may have originally been present in both free and conjugated forms. Thus, the high epismilagenin content of the coprolite analysed is interpreted to derive from the digestive processing of a saponin precursor contained in the animal's food. The methodology used in the present study, with a combined hydrolysis and extraction step, ensured that any conjugated sapogenins would be hydrolysed to give free sapogenins for analysis.

The conversion of smilagenin and diosgenin to epismilagenin has only been demonstrated to occur in ruminant animals such as sheep (e.g. Miles et al., 1992, 1993; Flåøyen et al., 2002; Flåøyen and Wilkins, 1997). Although modern three-toed tree sloths, e.g. *Bradypus variegatus*, are not ruminants they have a complex stomach with fore-gut fermentation taking place in the pre-gastric pouch (Pacheco et al., 2007). By analogy and also based on tooth occlusal surface area (Vizcaíno et al., 2006) extinct ground sloths have been interpreted to have had a similar ruminant-like mode of digestion and the presence of epismilagenin in the *N. shastensis* coprolite is consistent with this interpretation.

Plant taxa reported to contain saponins with an epismilagenin precursor aglycone molecule, i.e. smilagenin or diosgenin, are listed in Table 2. In theory, any of these twenty-three taxa could have been the dietary component from which the epismilagenin in the *N. shastensis* coprolite was derived. However, some taxa can be excluded on the

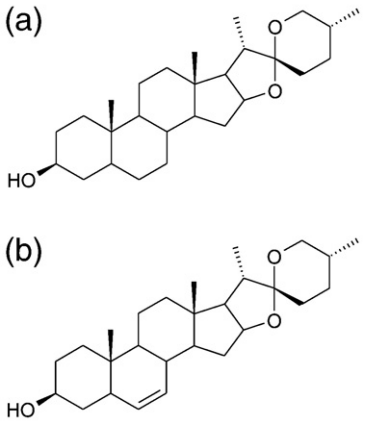

Figure 2. Chemical structures of a) (25R)-5β-spirostan-3β-ol (smilagenin) and b) (25R)-spirost-5-en-3β-ol (diosgenin).

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Table 2
Plant taxa with saponins containing an epismilagenin precursor aglycone moiety i.e. smilagenin or diosgenin.

Taxon	Reference
Agave spp.	Glasby, 1991
Allium spp.	Do et al., 1992
	Fattorusso et al., 1998
	Akhov et al., 1999
Asparagus africanus	Debella et al., 1999
Balanites aegyptiaca	Chapagain and Wiesman, 2007
Brachiaria decumbens	Cruz et al., 2000
Chamaerlerium carolinianum	Marker et al., 1942
Clintonia borealis	Marker et al., 1942
Costus spp.	Lin et al., 1997
	Meagher et al., 2001
Dioscorea spp.	Marker et al., 1942
	Hoyer et al., 1975
	Dacosta and Mukherjee, 1984
	Glasby, 1991
	Haraguchi et al., 1994
	Nino et al., 2007
Dracaena australis	Glasby, 1991
Helonias sp.	Marker et al., 1942
Lycium barbarium	Harsh and Nag, 1981
Narthecium ossifragum	Flåøyen et al., 2002
Panicum miliaceum	Miles et al., 1993
Phoenix humilis	Asami et al., 1991
Radix sarsaparilla	Agrawal et al., 1997
Smilax spp.	Kar and Sen, 1984
	Glasby, 1991
Solanum spp	Sato and Latham, 1953
	Glasby, 1991
	Yahara et al., 1996
Tofeilia garmnifolia	Marker et al., 1942
Tribulus terrestris	Miles et al., 1993
Trigonella foenum-graecum	Taylor et al., 1997
Trillium spp	Marker et al., 1942
Yucca spp.	Glasby, 1991
	Flåøyen et al., 2002

grounds of their geographical distribution, e.g. *Asparagus africanus* (Debella et al., 1999), *Balanites aegyptiaca* (Chapagain and Wiesman, 2007), *Trigonella foenum-graecum* (Taylor et al., 1997). Coprolites of *N. shastensis* have been studied previously in order to deduce its diet, palaeoenvironment and possible causes of extinction (e.g. Eames, 1930; Martin et al, 1961; Hansen, 1978; Thompson et al., 1980; Poinar et al., 1998, Hofreiter et al., 2000). These studies are in broad agreement regarding the diet of *N. shastensis*, which was found to be variable, e.g. Hansen (1978) identified seventy-two genera of plants from over 500 different coprolites, but largely featured the following taxa: *Ephedra nevadensis*, *Sphaeralcea ambigua*, *Atriplex* spp., Pinaceae (probably the genus *Pinus*, pines), Moraceae (probably *Morus microphylla*), Caparales (capers and mustards), Poaceae (grasses), Lilliales (*Yucca* or *Agave* spp.), Lamiales (scrophs and mints), Asteraceae (asters) and *Vitis* (grapes). Of these taxa, only *Yucca* spp. and *Agave* spp. are known to produce saponins with a diosgenin or smilagenin aglycone moiety (See Table 2). *Yucca* spp. and/or *Agave* spp. are therefore interpreted to have formed the bulk of the sloth's diet prior to production of the coprolite. The biomarker evidence is thus in agreement with the original interpretation of the specimen consisting predominantly of yucca fibres.

Conclusions

The hypothesis tested by this study was twofold:

(1). that the N. Shastensis coprolite would contain biomarkers.
(2). that any such biomarker would be indicative of the animal's dietary preferences.

Recovery of diagnostic faecal biomarkers such as 5β-stanols, e.g. coprostanol, from the coprolite upholds Hypothesis 1. The dominant lipid component extracted from the coprolite was epismilagenin, derived from a secondary metabolite found in a limited range of plants. Comparison of dietary plants previously reported from *N. shastensis* coprolites with plants known to contain an epismilagenin precursor (Table 2) reveals that only two genera, *Yucca* spp. and *Agave* spp. appear in both lists. Therefore, the most parsimonious explanation for the presence of epismilagenin in the coprolite is that the sloth had eaten a meal rich in *Yucca* spp. and/or *Agave* spp. prior to defecating. This result supports Hypothesis 2, but is not conclusive, since the epismilagenin could have been derived from another plant taxon not yet reported to contain an epismilagenin precursor.

Our results suggest that biomarker analysis is a useful source of complementary information about the diet of coprolite producers and can be used to corroborate dietary information obtained by other means, such as microhistological analysis (and vice versa). Although epismilagenin cannot be shown conclusively to derive from specific dietary plants, its recovery from the coprolite of *N. shastensis* is encouraging and suggests the possibility that other, more specific, dietary lipid biomarkers may be recovered from other suitably preserved coprolites. The recovery of epismilagenin, which has only been reported to have been formed in extant ruminant animals suggests that lipid biomarkers from coprolites may have the potential to reveal information about the digestive physiology of extinct animals and is therefore a promising area for future study.

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