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Ambrein: a minor, but common constituent of mammalian faeces?

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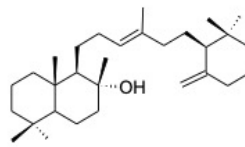


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AMBREIN

338x190mm (54 x 54 DPI)

Running Head: Faecal ambrein. To be submitted to Natural Product Research

Ambrein: a minor, but common constituent of mammalian faeces?

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Abstract

For nearly 200 years, the only natural source of the alcohol, ambrein, has been coproliths produced in about 1% of sperm whales and in related jetsam. However, the finding of ambrein in adipocere/faeces of human corpses, led us to hypothesise that ambrein might occur in the faeces of other mammals.

Herein, we used a recently developed gas chromatography-mass spectrometry method, with suitable derivatisation of the hindered hydroxy group of ambrein, to screen a number of extracts of mammalian faeces.

Minor proportions of ambrein were detected in digested human sewage sludge and in the dung of elephant, domestic cattle, giraffe and buffalo, as well as the faeces of a sperm whale. Whether ambrein formation in the terrestrial species is associated with coprolith formation, is unknown, but solid deposits known as enteroliths and fecaliths occur in humans and some domestic animals.

Keywords: Faeces, mammals, ambrein, humans, sewage, sperm whale.

1. Introduction

The natural product ambrein (I; Figure 1) was, for nearly two hundred years, known only in ambergris (Pelletier and Caventou 1820 through Rowland et al. 2019), which is a coprolith which occurs in the rectum of about 1% of sperm whales (Clarke, 2006).

In such coproliths, which can be very large (e.g. 420 kg; Clarke, 1954) and in related jetsam samples, ambrein is usually one of the major components of solvent extracts ($81 \pm 22\%$; $n=43$ for jetsam ambergris; Rowland et al. 2018a).

Determination of the isotopic carbon ratios of ambrein and of co-occurring faecal 5β -stanols in natural ambergris samples, including in those taken directly from whales, indicated that ambrein was biosynthesised from a different squalene source to that of the sterols (Rowland et al. 2019). Examination of extracts for possible intermediates in biosynthesis suggested a route involving conversion of acyclic squalene to tricyclic ambrein *via* bicyclics with the polypodane skeleton, presumably by bacteria (Rowland et al. 2019). A wide range of bacteria are certainly indicated by recent studies of the microbiome DNA of whale and jetsam ambergris samples (Macleod et al. 2020).

However, the serendipitous finding of minor proportions of ambrein in human adipocere, which is a waxy substance comprising mainly fatty acid salts formed post-mortem during incomplete anaerobic decomposition of soft tissues, indicates that the natural occurrence of traces of ambrein may be more widespread (von der Lühe et al. 2019). The association with adipocere recovered from the abdominal region of human corpses suggested that perhaps ambrein was produced during the microbial decomposition of human faecal residues. Assuming that the adipocere matrix of saturated fatty acids aided the preservation of ambrein over extended periods of time, von der Lühe et al. (2019) suggested that the association of ambrein with ageing faecal material, preserved under moist oxygen-depleted environments, required more attention in studies of other mammalian samples. Thus, a growing body of evidence suggests that ambrein may be a more common product of mammalian anaerobic bacterial processes than realised previously.

In the present study, we therefore examined the extracts of the faeces of a variety of mammals, including humans, for the presence of ambrein, using recently developed analytical methods (Rowland and Sutton 2017).

2. Results and discussion

Field samples of dung were collected from a variety of terrestrial mammals (elephant, hippopotamus, buffalo, giraffe and domestic cattle) in Mara Conservancy, Narok County, Kenya on 14-17th January 2016 (cf Vane et al. 2010).

A sewage sample was obtained from Göttingen, Germany in November 2018 and freeze-dried in the laboratory.

A sample of faeces was also collected from a sperm whale in south Abaco, Bahamas on September 12th 2018 and freeze-dried in the laboratory.

Trimethylsilylated (TMSi) fractions of the dichloromethane extracts of all samples were examined herein by gas chromatography-mass spectrometry (GC-MS) in full data collection (m/z 50-550) or selected ion monitoring (SIM; m/z 143, 203 and 500) modes, or both, using a published method (Rowland and Sutton 2017). Samples of previously authenticated ambrein (Rowland and Sutton 2017) and solvent and procedural blanks, were co-examined.

GC-MS total ion current (TIC) chromatograms of the trimethylsilylated total organic extracts of African mammal faeces revealed several peaks when operating in SIM mode (Figure S1) and intriguingly, one eluted at the same time as the ambrein TMSi ether peak in a derivatised authentic ambergris sample extract (retention time 34.30 minutes). This occurred in all but the sample of hippopotamus faeces extract (MMF4A, Figure S1). However, good quality mass spectra could not be obtained from the data, due to the low amounts present. Therefore, a fractionation procedure was designed in order to provide more concentrated samples from which better mass spectra could be obtained, as follows:

Separation of the buffalo faeces total organic extract MMF7A using adsorption chromatography on silica (method adapted from Rowland et al. 2018b) and elution with *n*-hexane (fraction coded Si F1) followed by *n*-hexane:diethylether (1:1 v/v; fraction Si F2), resulted in two fractions. A GC-MS TIC chromatogram (Figure S2) revealed that fraction Si F1 was dominated by odd carbon number *n*-alkanes with 25-35 carbon atoms, whilst fraction Si F2 contained more polar compounds, one of which eluted at the same retention time as the ambrein TMSi ether identified previously in an authentic ambergris sample (34.30 minutes; Figure S2). The mass spectrum of this peak also compared well with the mass spectrum of ambrein TMSi ether from a previously authenticated ambergris extract (Figure S3). SIM GC-MS data were also consistent with the presence of a component assigned to ambrein TMSi ether in the fraction from this

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3 faeces extract (Figures S4 and S5). The retention index for ambrein TMSi ether on this
4 stationary phase (Agilent DB5 ms; cf Rowland and Sutton 2017 for a different phase), was
5 calculated by comparison with the total ion current chromatogram of the *n*-alkane series.
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9 Similar treatment of all the African mammal extracts produced GC-MS data (Figures S6-S7)
10 indicating that ambrein TMSi ether was present in all the corresponding Si F2 fractions, except
11 the fraction of hippopotamus faeces. SIM GC-MS (m/z 143 + 203 + 500) of the fractions of
12 derivatised mammalian extracts and an authentic ambergris extract (Figure 1 and Figure S8)
13 supported this conclusion. (Possibly detection in the hippopotamus extract would have required
14 a lower limit of detection in the method).
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19 Following detection of traces of ambrein (as the TMSi ether) in most of the African mammal
20 faeces extracts, a similar investigation was made of extracts of three subsamples of digested
21 human sewage sludge. Total extracts of a freeze-dried sample derivatised with 'Sylon BTZ',
22 were examined by GC-MS in full data collection mode (m/z 50-550). Ambrein TMSi ether was
23 detected in trace amounts relative to the expected faecal steroids, in each of the three
24 subsamples and assigned from the retention positions of authenticated ambrein TMSi ether
25 examined at the same time and from weak mass spectra (notably ions m/z 143,
26 175/176/177,192, and 203 were present (Figure S9)). No ambrein was detected in the
27 procedural blank.
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34 Finally, for comparison, a freeze-dried sample of sperm whale faeces excreted from a whale in
35 the Bahamas in September 2018, was extracted and derivatised in the same manner as those
36 of the African mammal samples and human sewage sludge. As expected, ambrein (TMSi ether)
37 was detected and assigned from the retention position of authenticated ambrein TMSi ether
38 examined at the same time, and from the mass spectrum (Figure S10; cf Rowland and Sutton
39 2107). Again, no ambrein was detected in the procedural blank.
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47 Ambrein has long been recognised to occur in the rare solid coproliths found in a minority of
48 sperm whales (Pelletier and Caventou, 1820; Suzuki 1925; Clarke 1954). In the whales, the
49 nucleating pieces of partially digested squid beaks, the large sizes and the long lives of the
50 whales, probably facilitate accumulation of the large solid boulders (Clarke 1954; 2006). It is
51 therefore perhaps unsurprising that ambrein was also detected herein in the whale faeces. Its
52 presence is presumably a reflection of the action of bacteria in the colon/rectum of the whale
53 (Ueda et al. 2013; Rowland et al. 2019) and its non-detection previously may have been due to
54 use of unsuitable analytical methods. Sperm whale faeces are an important part of the
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3 'biological pump' of the oceans and have been well studied in respect of the inorganic
4 constituents (Lavery et al. 2010), but less so in terms of the natural organic products (Smith and
5 Whitehead 2000). Also present in the extract (Figure S10), were the two unknown compounds
6 (A1, A2) reported, but not assigned, previously in numerous jetsam ambergris extracts
7 (Rowland and Sutton 2017). Indeed, these are common or ubiquitous in extracts of ambergris
8 derivatised with 'Sylon' (e.g. Rowland et al. 2018b). The mass spectra of the unknowns
9 (Rowland and Sutton 2017) were similar to those reported by Benfenati et al. (1994) for the
10 TMSi-ether derivatives of the enol forms of coprostanone (5 β (H)-cholestan-3-one). The spectra
11 of the enols herein and previously only differed in having higher abundances of the M⁺, M-15
12 and M-29 ions, which may be due to differences in mass spectrometers used in the different
13 studies. Benfenati et al. reported data for enolisation and derivatisation of both 5 α (H) and 5 β (H)
14 isomers, (viz: cholestanone and coprostanone). These were produced by partial enolization
15 from samples of the pure ketones using bistrimethylsilyltrifluoroacetamide (BSTFA) as a
16 derivatising reagent at 100 °C for 1h, whilst further enolization occurred with at room
17 temperature for 24h. Complete enolisation was obtained using N-trimethylsilyl-N-
18 methyltrifluoroacetamide (MSTFA). This is an example of the capture of the enol form of the
19 ketones as TMSi-derivatives, leading, effectively, to artefactual formation of 3-OH TMSi sterols
20 with Δ^2 and/or Δ^3 double bonds. The authors could not differentiate between the Δ^2 and Δ^3 ,
21 5 α (H) and 5 β (H) isomers, which had very similar spectra (in contrast to spectra of the
22 corresponding acetates (Autier et al. 1963)). Benfenati et al. conducted ring A deuterium
23 labelling experiments which supported the proposed identifications and particularly showed that
24 the *m/z* 143 ion in the mass spectra resulted from fragmentation of the derivatised, enolised A
25 ring. The elemental composition of this ion, also postulated by Rowland and Sutton (2017) for
26 the spectrum of ambrein-TMSi ether, was confirmed by accurate mass studies (Borges et al.
27 2005). Benfenati et al. (1994) reported that derivatisation of the Δ^2 and/or Δ^3 5 β (H) isomers
28 produced a compound which eluted before the corresponding ketone, whereas derivatisation of
29 the Δ^2 and/or Δ^3 5 α (H) isomer produced a compound which eluted after the corresponding
30 ketone.

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48 Thus, it is now clear from a comparison of the retention indices of the unknowns in the
49 ambergris extracts (Rowland and Sutton 2017, Rowland et al. 2018 a, b) and in the whale
50 faeces studied herein, with the data of Benfenati et al. (1994), that the unknowns in the
51 derivatised ambergris and faeces extracts are enols of coprostanone. Given the known relative
52 retention times of the TMSi ethers of Δ^4 , Δ^5 , Δ^6 , Δ^8 and Δ^7 cholestenols (Gerst et al. 1997), it is
53 likely that the first-eluting unknown is the Δ^2 and the second the Δ^3 5 β (H) isomer. Indeed, after
54 complete derivatization of coprostanone using MSTFA, Chou and Liu (2004) obtained a
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3 chromatogram with two peaks (1:10 ratio in abundance) with similar spectra to each other (i.e.
4 spectra with m/z 458 and m/z 143 ions) which they also attributed to cholest-2-en-5 β -ol TMSi
5 ether and the Δ^3 isomer. Coprostanone (for which the presence of the unknowns now provides
6 evidence) is a well-known intermediate in the formation of coprostanol from cholesterol. The
7 presence of enol forms of coprostanone in derivatised extracts of ambergris, are also consistent
8 with the evidence of carbonyl compounds detected by infrared spectroscopy before
9 derivatisation (Rowland and Sutton 2017; Rowland et al. 2018b).

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15 The finding of ambrein as a major component of extracts of the faeces of the sperm whale is
16 somewhat expected (although unreported previously, to our knowledge). The discovery of
17 traces of ambrein in the faeces of other mammals is more surprising, but there has long been a
18 suggestion that, at least the *smell* of ambergris, can develop in human faeces. Thus, Beale
19 (1839), cited Brande (1819) as saying "Mr. Homberg found that a vessel in which he had made
20 a long digestion of human faeces, acquired a very strong and perfect smell of ambergris,
21 inasmuch that any one would have thought that a great quantity of essence of ambergris had
22 been made in it, the perfume was so strong and offensive that the vessel had to be removed
23 from the laboratory!". This last phrase was repeated in 1855 by Piesse, who cited Homberg's
24 publications in Mem. Acad. Paris, 1711 and certainly Homberg published at that time on a '
25 suite des observations sur la matiere fecale'. The finding herein, of traces of ambrein in human
26 sewage sludge might be an explanation for these very early physical observations. This is also
27 consistent with the recent report of traces of ambrein in the adipocere of human corpses,
28 perhaps contaminated with faecal matter (von der Lühe et al. 2019). It is unlikely that the
29 ambrein in the sewage is a result of the very minor amounts of ambrein used in perfumes, but
30 this cannot be entirely excluded.

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41 What *is* without any precedent, as far as we are aware, is the report herein of traces of ambrein
42 in a range of other mammal faeces, including that of giraffe, elephant, domestic cattle and
43 buffalo. These data suggest that ambrein, which is likely a product of bacterial metabolism from
44 squalene (Mori and Tamura 1990; Ueda et al 2013; Rowland et al. 2019), is a more common
45 constituent of mammalian faeces than recognised previously. Indeed, ambrein may now be a
46 useful additional chemical marker of recently deposited faecal material (cf McCalley et al. 1981;
47 Leeming et al. 1996), if it has sufficient chemical longevity in the environment. It is known to
48 survive in the sea, at least in solid coproliths, for at least 1000 years (Rowland et al. 2018a).

54 3. Conclusions

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57 Use of a recently developed gas chromatography-mass spectrometry method, with suitable
58 derivatisation of the hindered hydroxy group of ambrein, to screen a number of extracts of
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mammalian faeces, revealed minor proportions of ambrein in the faeces of humans (digested sewage sludge), elephant, domestic cattle, giraffe and buffalo. These data suggest that ambrein, is a more common, though perhaps minor, constituent of mammalian faeces, than hitherto recognised. Whether ambrein formation in the terrestrial species is associated with coprolith formation, is unknown, but solid deposits known as enteroliths and fecaliths occur in humans and some domestic animals.

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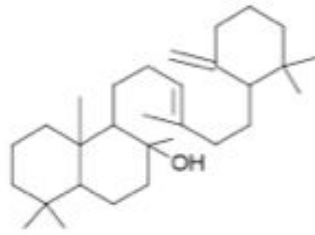
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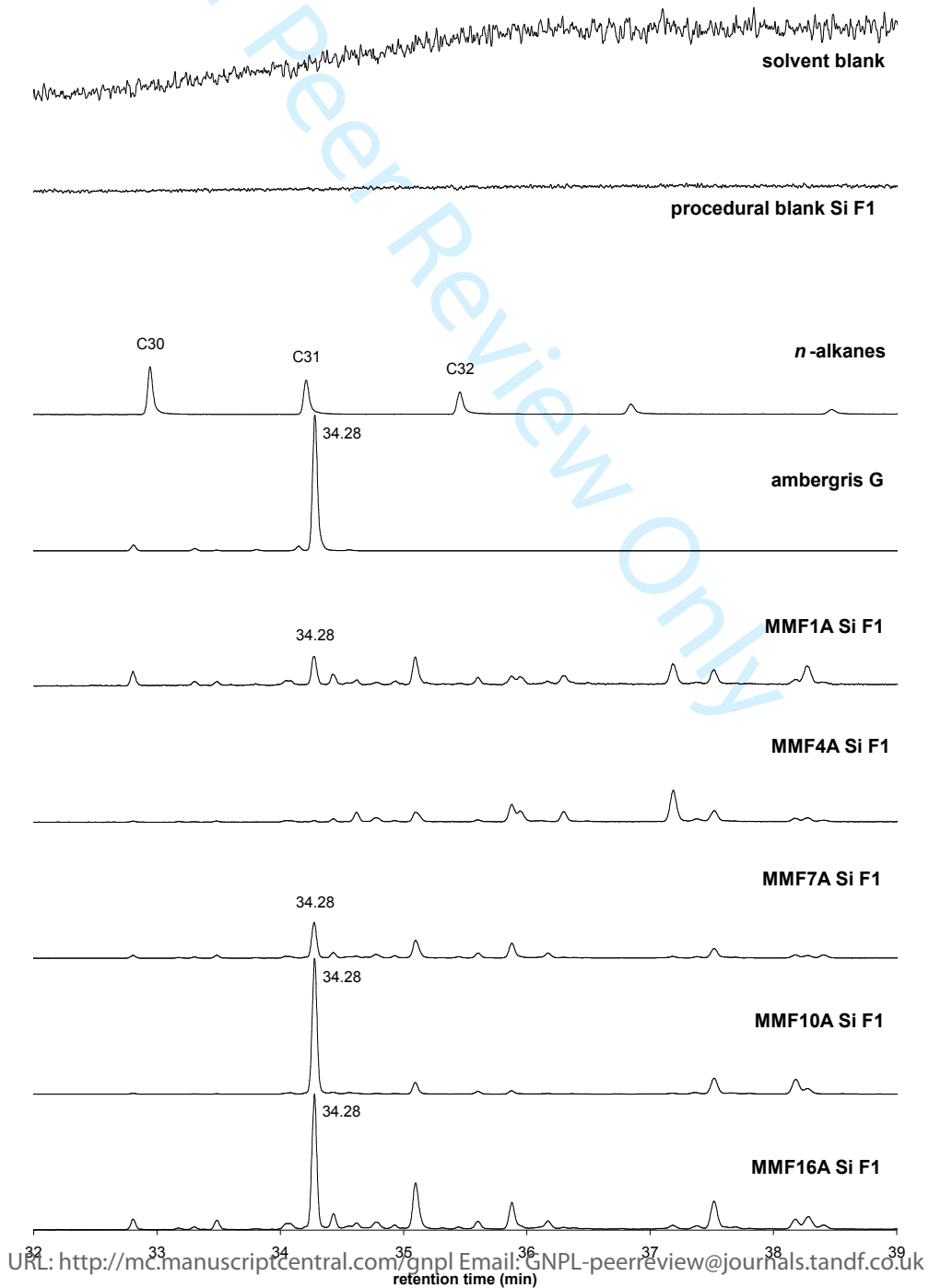
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3 **Figure 1. I:** Structure of ambrein. Partial total ion current (TIC) GC-MS
4 chromatograms of solvent and procedural blanks, an *n*-alkane series and extracted
5 ion chromatograms (*m/z* 143 + 203 + 500) of a trimethylsilylated, previously
6 authenticated, ambergris extract (ambergris G = sample 5 of Rowland and Sutton
7 2017) and of trimethylsilylated fractions from separation of total organic extracts of
8 mammalian faeces samples using silica: MMF-1A Elephant; MMF -4A Hippo; MMF-
9 7A Buffalo; MMF-10A Giraffe; MMF-16A Domestic cattle. Component with retention
10 time of 34.28 minutes assigned as ambrein TMSi ether.
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I: ambrein



Ambrein: a minor, but common constituent of mammalian faeces?

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Abstract

For nearly 200 years, the only natural source of the alcohol, ambrein, has been coproliths produced in about 1% of sperm whales and in related jetsam. However, the finding of ambrein in adipocere/faeces of human corpses, led us to hypothesise that ambrein might occur in the faeces of other mammals.

Herein, we used a recently developed gas chromatography-mass spectrometry method, with suitable derivatisation of the hindered hydroxy group of ambrein, to screen a number of extracts of mammalian faeces.

Minor proportions of ambrein were detected in digested human sewage sludge and in the dung of elephant, domestic cattle, giraffe and buffalo, as well as the faeces of a sperm whale. Whether ambrein formation in the terrestrial species is associated with coprolith formation, is unknown, but solid deposits known as enteroliths and fecaliths occur in humans and some domestic animals.

Keywords: Faeces, mammals, ambrein, humans, sewage, sperm whale.

Sample Collection

Fresh dung samples (coded MMF-1A Elephant; MMF -4A Hippo; MMF-7A Buffalo; MMF-10A Giraffe; MMF-16A Domestic cattle) were collected 14-17th January 2016 in the Mara Conservancy, Narok County, Kenya (latitude S 1.452015, longitude E 34.91223). Upon return to the laboratory samples were freeze dried for 72 h and approximately 30 g (dry wt) ground to a coarse powder using a blender (De Longhi KG40). All samples were then ground in agate ball mill (Retsch PM400) to pass a 63 μm brass sieve.

A fresh sample of sperm whale faeces (coded Pm 18-01; 180912-Pmol), was collected off Abaco, the Bahamas, on 12th September 2018. No preservative was added.

A sample of human sewage was collected from Gottingen, Germany in November 2018.

Extraction of mammal faeces

Methods: Five samples of dried mammal faeces were weighed out (Table 1) and extracted with dichloromethane (DCM; 2 x 2 mL) with sonication (5 min) and centrifugation (2500 rpm/5 min). The liquid phase was filtered through a cotton wool plug in a Pasteur pipette and solvent removed under a gentle stream of nitrogen ($\text{N}_2/50^\circ\text{C}$) before weighing the extract (Table S1). Extracts were reconstituted in DCM (10 mg mL^{-1}). An aliquot (nom. 10 μL) of each extract was diluted (x5), transferred (nom. 10 μL) to a GC vial, dried (heater block; 60°C) and derivatised (nom. 50 μL SYLON BTZ; 70°C ; 1 h), before reconstitution in DCM (1 mL) for analysis using GC-MS.

A test separation using silica was undertaken using the extract from sample MMF7A. The dried extract was dissolved in a small volume of *n*-hexane (vortex and sonication) and transferred with washings on to silica gel (510 mg; Davisil, grade 645, pore size 150 \AA , 60-100 mesh; Sigma-Aldrich; packed in a 3 mL glass tube with bottom frit and top frit added over silica) pre-conditioned with diethyl ether (6 mL) and *n*-hexane (6 mL) and eluted with *n*-hexane (4 mL; Si F1), *n*-hexane/diethyl ether 1:1 (v/v; 4 mL; Si F2) and methanol (4 mL; Si F3; not analysed). Solvent was removed from Si F1 and Si F2 fractions ($\text{N}_2/50^\circ\text{C}$) and fractions weighed (Table S1) and reconstituted to 1 mg mL^{-1} (DCM). An aliquot (nom. 100 μL) was transferred to a GC vial, dried (heater block; 60°C) and derivatised (nom. 50 μL SYLON BTZ; 70°C ; 1 h), before reconstitution in DCM (1 mL) for analysis using GC-MS (Figures 5 and 6).

Extraction of sewage sludge

Three sub-samples (each 25-35 mg) of a sample of freeze-dried human sewage sludge from Gottingen, Germany, and a procedural blank, were extracted with dichloromethane (DCM; 2 x 2.5 mL) with sonication (5 min). The liquid phase was filtered through a cotton wool plug in a Pasteur pipette and solvent removed under a gentle stream of nitrogen ($\text{N}_2/50^\circ\text{C}$) before weighing the extract (Table S2). Extracts were reconstituted in DCM (10 mg mL^{-1}). An aliquot (nom. 10 μL) of each extract was diluted (x5), transferred (nom. 10 μL) to a GC vial, dried (heater block; 60°C) and derivatised (nom. 50 μL SYLON BTZ; 70°C ; 1 h), before reconstitution in DCM (1 mL) for analysis using GC-MS.

Extraction of sperm whale faeces

A sample of freeze-dried faeces (23.2 mg) from the whale, along with a procedural blank and sample of previously authenticated ambergris, were extracted with dichloromethane (DCM; 2 x 2.5 mL) with sonication (5 min). The liquid phase was filtered through a cotton wool plug in a Pasteur pipette and solvent removed under a gentle stream of nitrogen ($\text{N}_2/50^\circ\text{C}$) before

weighing the extract (0.5mg). The extracts were reconstituted in DCM (10 mg mL⁻¹). An aliquot (nom. 10 µL) of each extract was diluted (x5), transferred (nom. 10 µL) to a GC vial, dried (heater block; 60°C) and derivatised (nom. 50 µL SYLON BTZ; 70°C; 1 h), before reconstitution in DCM (1 mL) for analysis using GC-MS.

GC-MS: A 1 µL aliquot of sample was injected split/splitless (250°C; Agilent 7683C Series autosampler) onto an Agilent DB-5ms Ultra Inert (30 m x 0.25 mm x 0.25 µm) column with helium carrier gas (constant flow mode; 1 mL min⁻¹). The column was interfaced (280°C) with an Agilent 5975C Inert XL EI/CI MSD (ion source 230°C; MS quad 150°C) and the Agilent 7890A GC oven was programmed from 40 – 300°C at 10°C min⁻¹ with a 10 minute isothermal hold period. Mass spectra were recorded from *m/z* 50-550 or in selected ion monitoring (SIM) mode (tune optimised on *m/z* 219) using *m/z* 143, 203 and 500.

Table S1. Gravimetric data (mg) and percentage recovery in brackets from extraction of mammal faeces (note increased recoveries from ground-up samples).

	MMF1A	MMF4A	MMF7A	MMF10A	MMF16A
Sample weight	64.7	56.1	78.8	48.1	53.8
Extract	1.4 (2%)	3.0 (5%)	7.6 (10%)	5.2 (11%)	4.2 (8%)
Si F1	0.5 (0.8%)	0.6 (1.1%)	0.2 (0.3%)	0.2 (0.4%)	0.1 (0.2%)
Si F2	0.3 (0.5%)	0.6 (1.1%)	1.2 (1.5%)	1.0 (2.1%)	0.7 (1.3%)

Table S2. Gravimetric data (mg) and percentage recovery in brackets from extraction of human sewage sludge.

	SS1	SS2	SS3
Sample weight	25.3	34.7	33.7
Extract	0.9 (4%)	<0.1 (<1%)	0.4 (1%)

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5 **Figure S1.** GC-MS TIC chromatograms of dichloromethane solvent (DCM) and procedural
6 (Sylon BTZ) blanks, a C₂₅₋₃₄ n-alkane mixture and selected ion monitoring (SIM)
7 chromatograms (*m/z* 143+203+500) of trimethylsilylated ambergris (ACL454A) and mammal
8 faeces total extracts. A component is present in all mammalian extracts at the retention time
9 of ambrein TMSi ether, which also dominates the chromatogram of the ambergris extract
10 (ACL 454A) at ~ 34.3 minutes retention time, except for the extract from hippopotamus
11 (MMF4A). However, good quality mass spectra could not be obtained from data for these
12 total extracts, which were then further fractionated (as described in the text).
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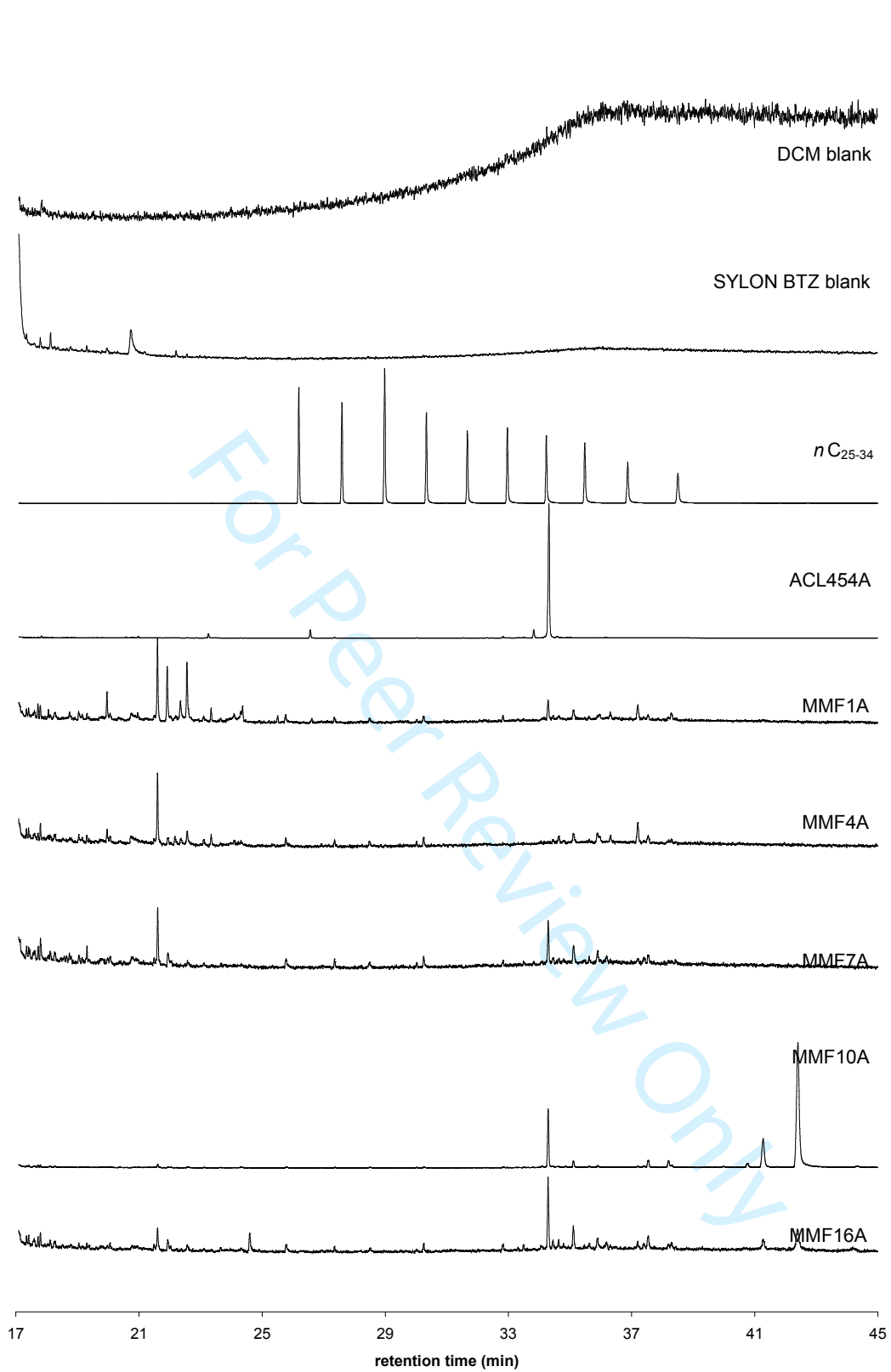


Figure S2. GC-MS TIC chromatograms of blanks and an *n*-alkane mixture and of trimethylsilylated ambergris extract (Ambergris G) and of trimethylsilylated silica chromatography fractions of a buffalo faeces extract (MMF 7A). Components in fraction F1 of the buffalo faeces extract are *n*-alkanes with carbon numbers as shown. A component is present in fraction Si F2 at the retention time of ambrein TMSi ether. A mass spectrum of reasonable quality mass spectra could be obtained from these data (Figure S3).

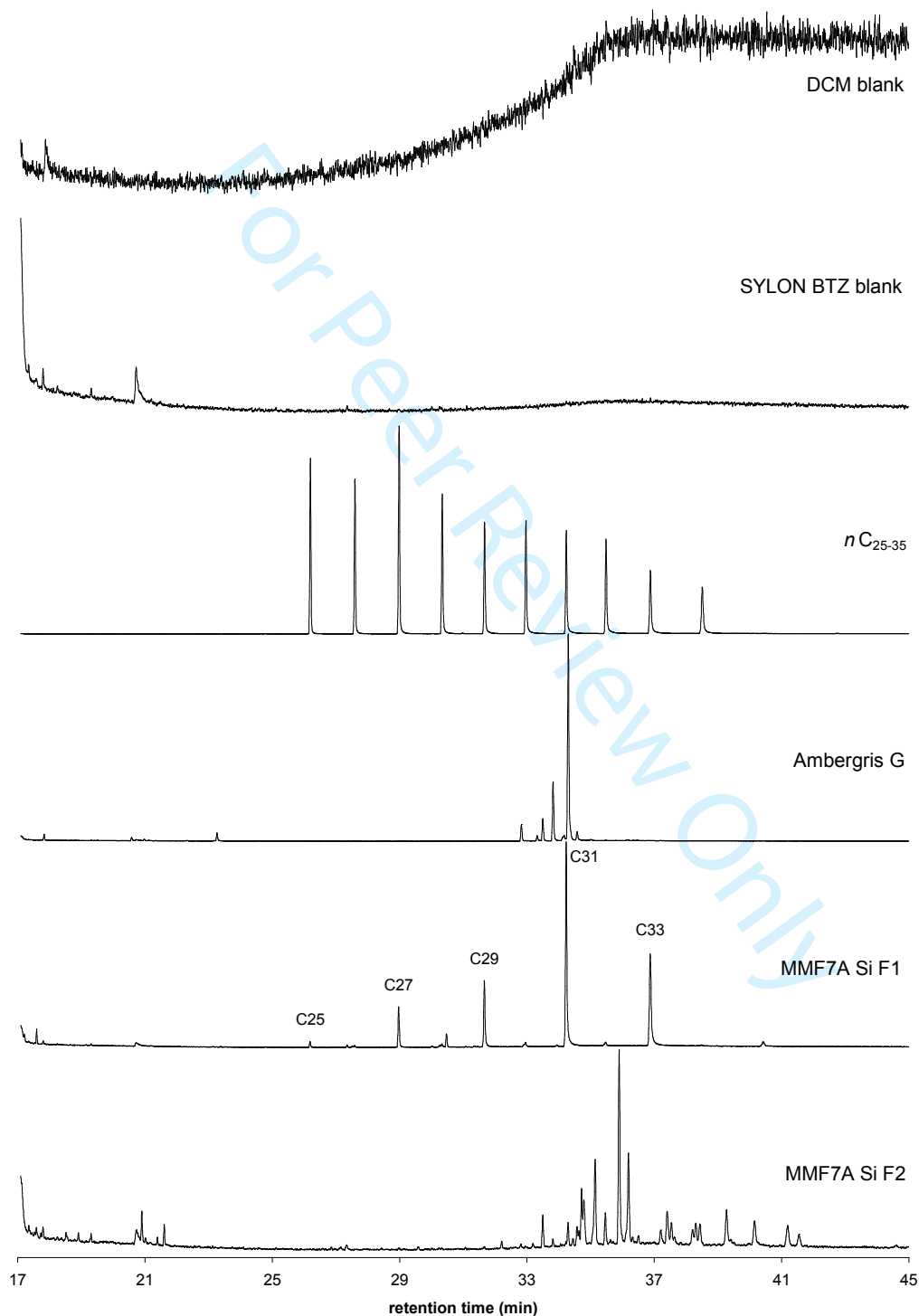


Figure S3. Mass spectra of peaks at 34.30 minutes in GC-MS TIC chromatogram of trimethylsilylated fraction Si F2 of extract of buffalo faeces MMF7A (upper) and authenticated ambrein TMSi ether at same scale (lower). Ions due to stationary phase background (e.g. m/z 207, 281) are present in the upper spectrum, but key ions in spectrum of ambrein TMSi ether (e.g. m/z 143, 175/176/177, 192 and 203) are also present.

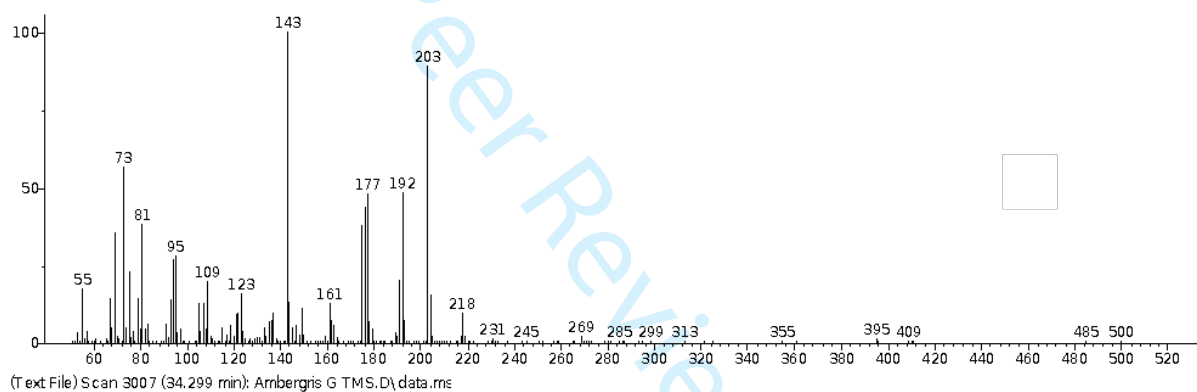
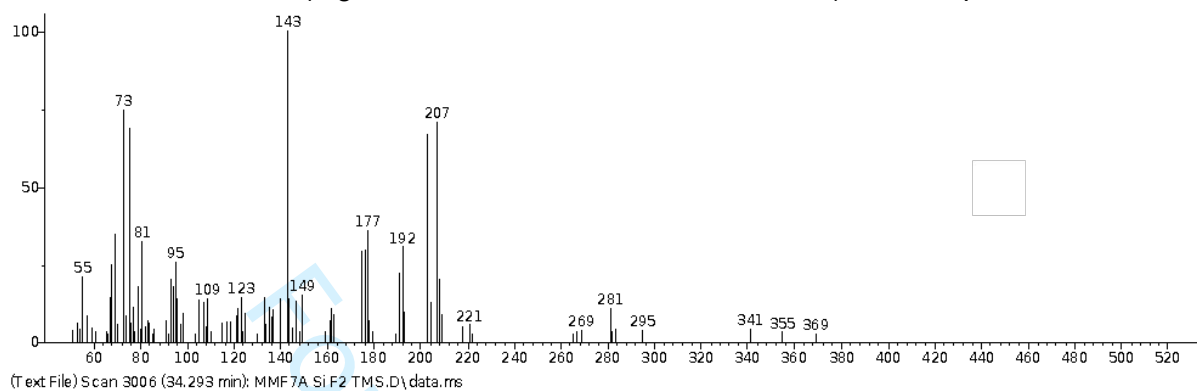


Figure S4. GC-MS TIC chromatogram of blanks and an n-alkane mixture and SIM chromatograms (m/z 143+203+500) of trimethylsilylated authentic ambrein in ambergris (Ambergris G) and silica chromatography fractions (Si F1 and F2) of buffalo faeces extract. A component is present in fraction Si F2 at the retention time of ambrein TMSi ether.

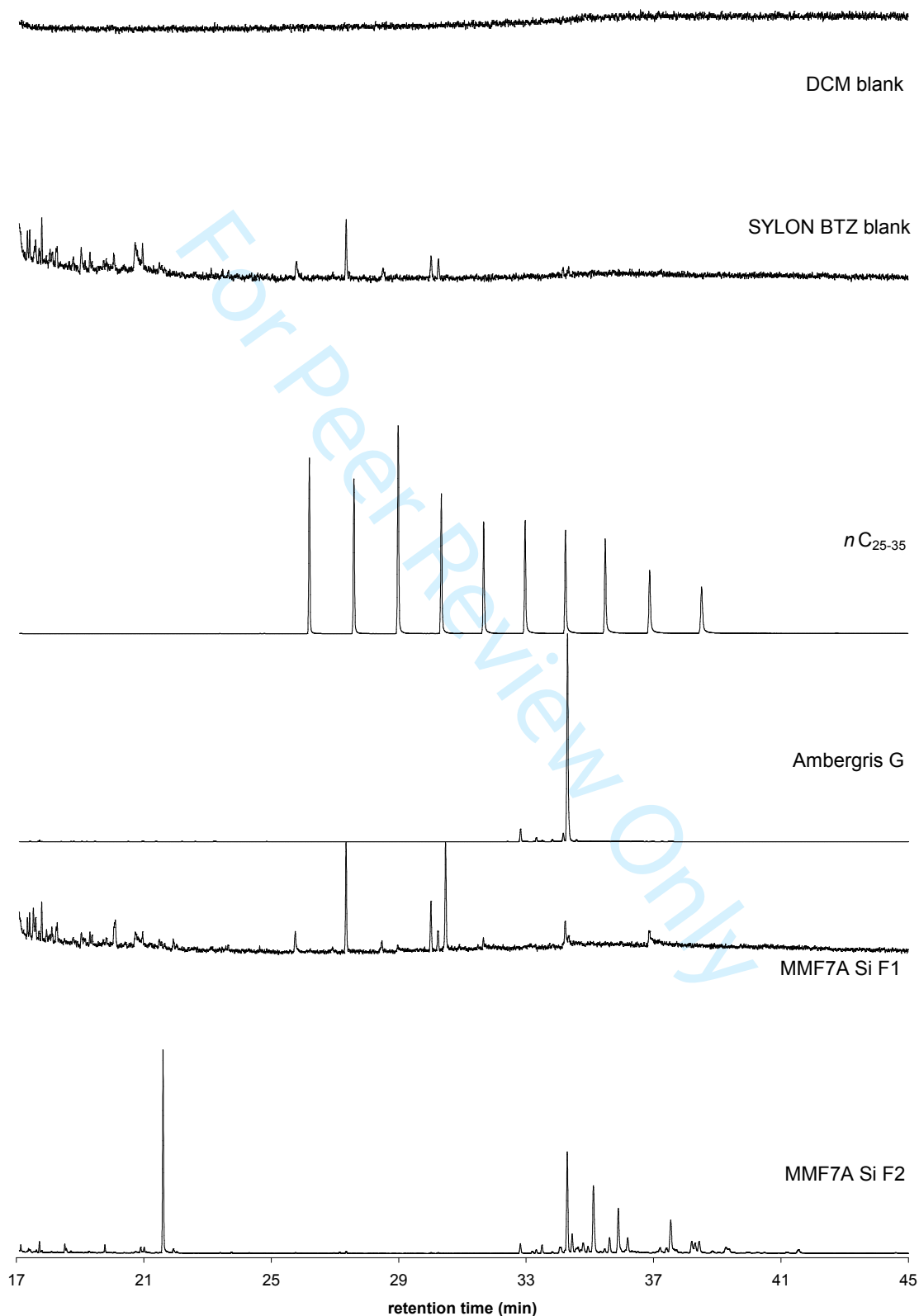


Figure S5. Zoomed (33-37 minutes) GC-MS TIC of blanks and an *n*-alkane mixture and SIM chromatograms (m/z 143+203+500) of trimethylsilylated authentic ambrein in ambergris (Ambergris G) and silica chromatography fractions (Si F1 and F2) of buffalo faeces extract. A component is present in fraction Si F2 at the retention time (34.3 minutes) of ambrein TMSi ether.

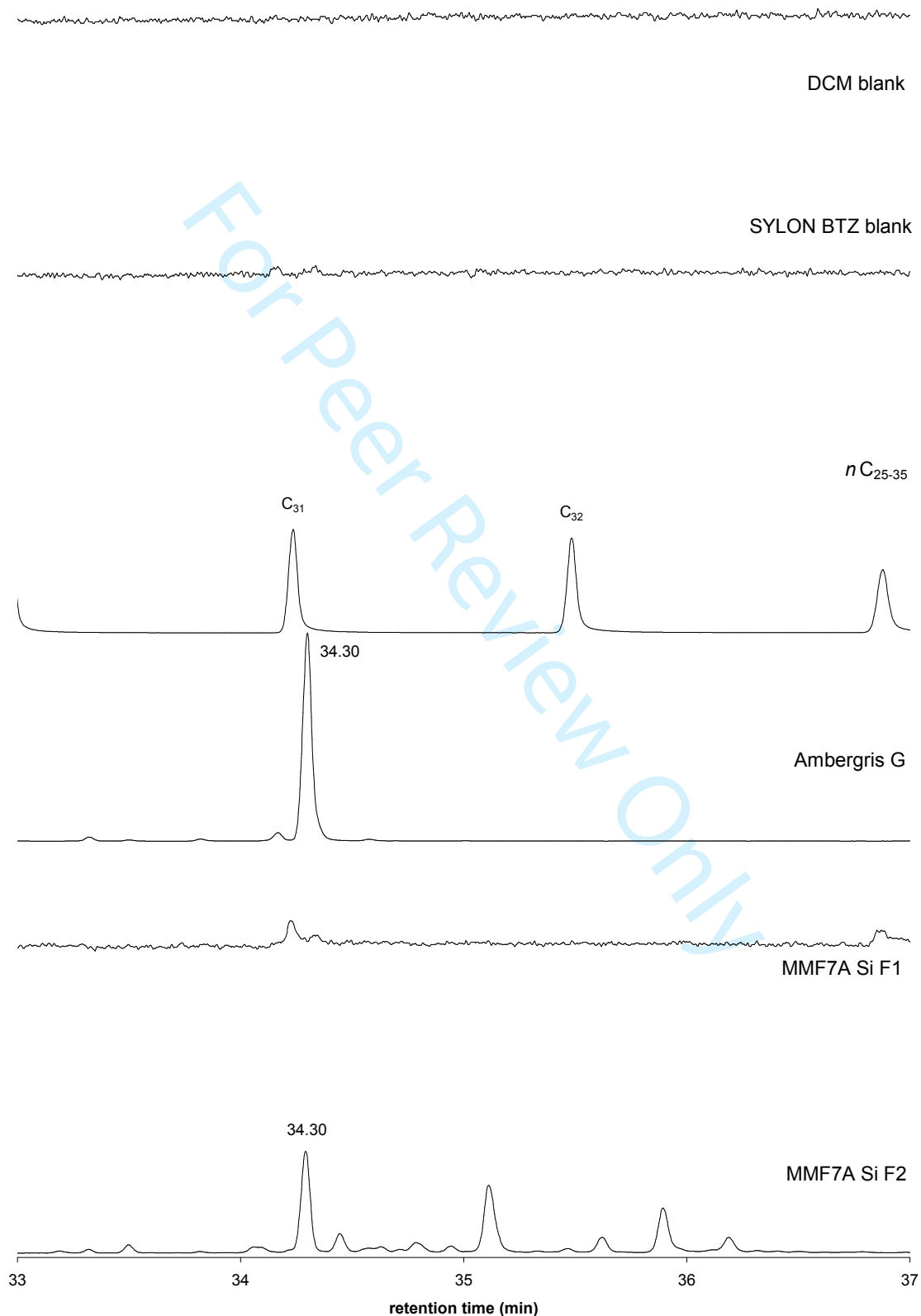


Figure S6. GC-MS total ion chromatograms (m/z 50-550) of blanks, standards and fractions (Si F1) from separation of total organic extract of MMF7A using silica.

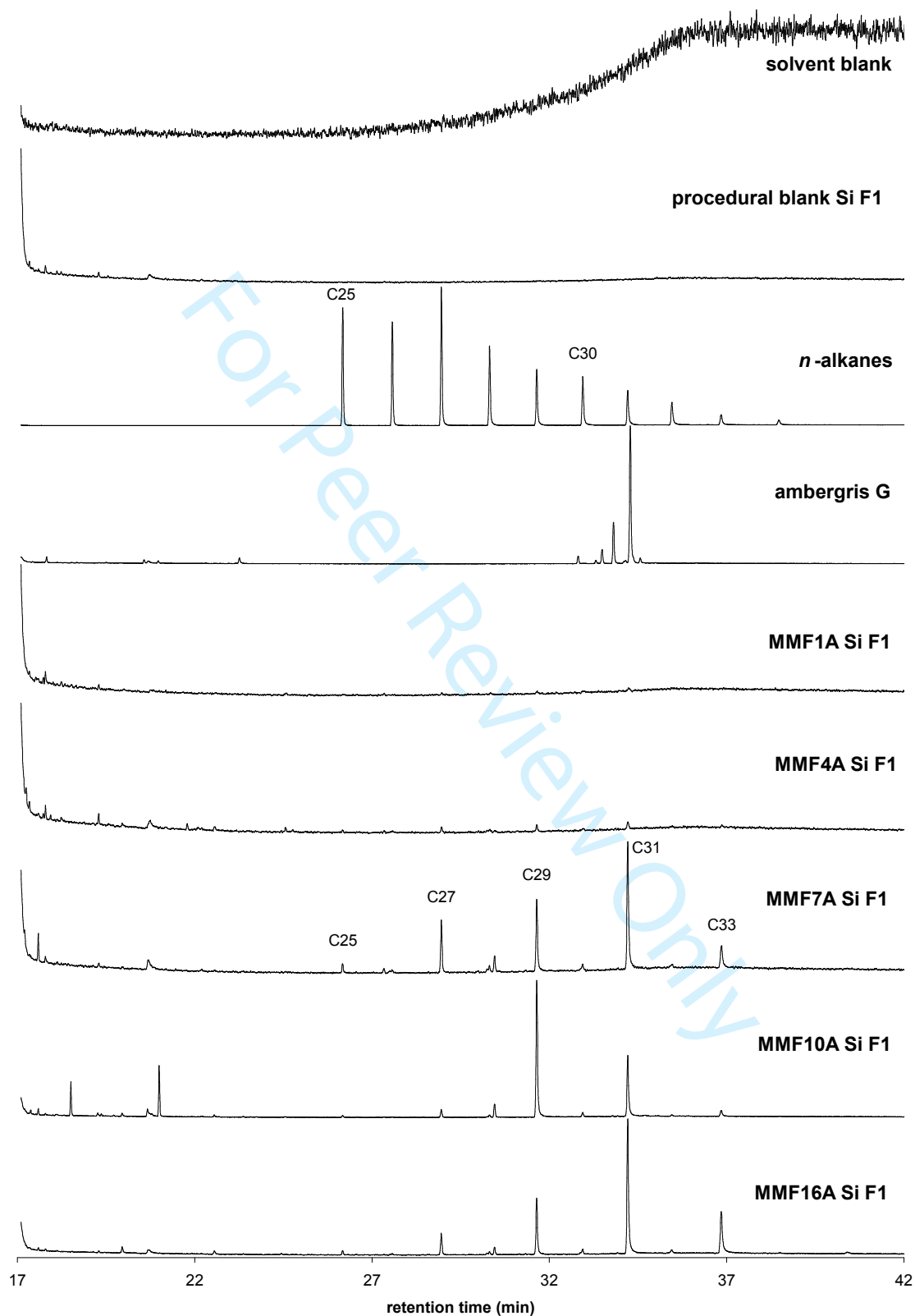


Figure S7. GC-MS TIC (m/z 50-550) of GC-MS total ion chromatograms of blanks, authentic *n*-alkanes, an ambergris extract (Ambergris G) and for trimethylsilylated silica chromatography fractions (Si F2) from separation of total organic extracts of African mammalian faeces.

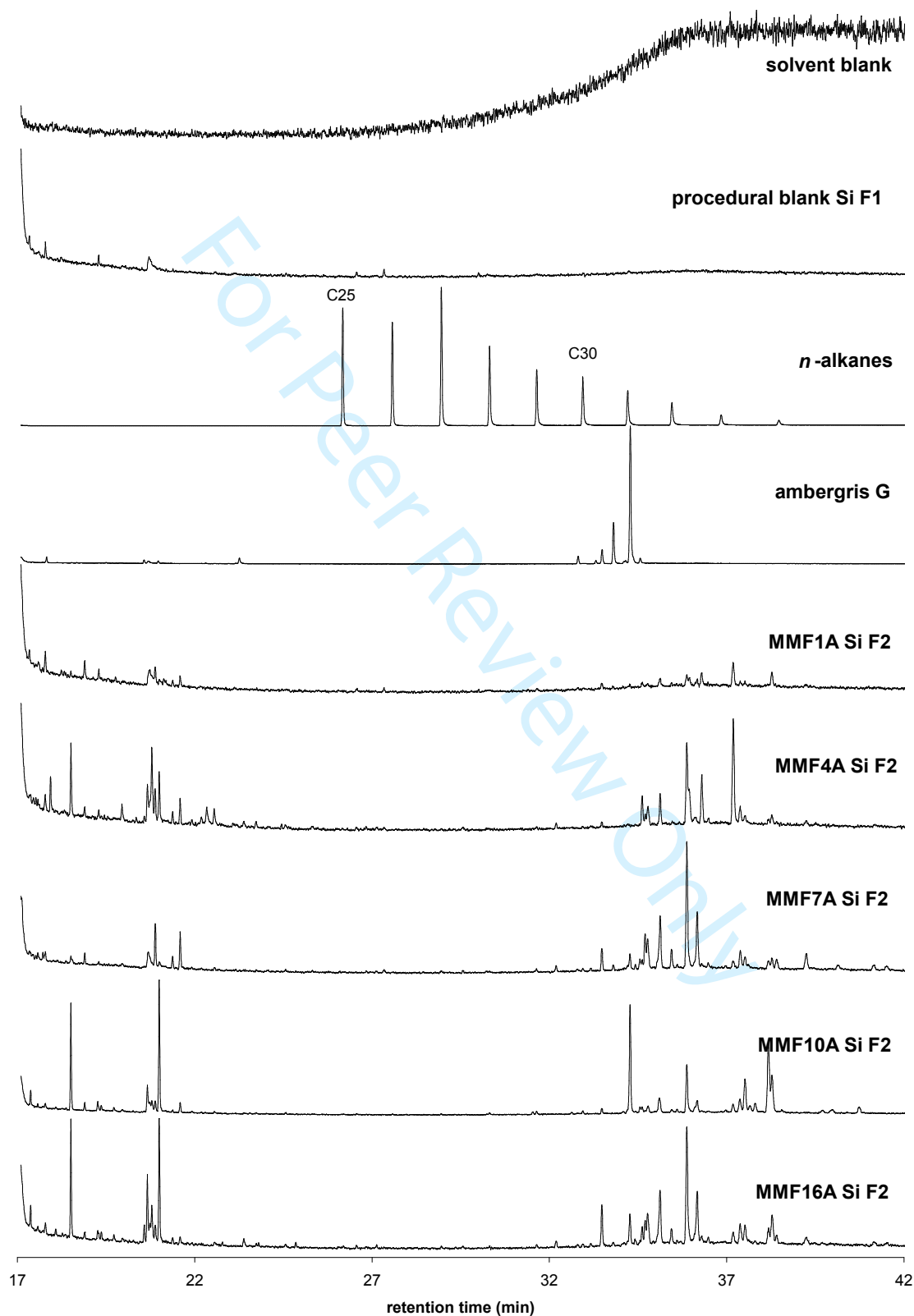


Figure S8. GC-MS total ion chromatograms of blanks, standards and GC-MS SIM (m/z 143, 203, 500) data for trimethylsilylated silica chromatography fractions (Si F2) from separation of total organic extracts of African mammalian faeces (zoomed chromatograms are shown in main text Figure 1).

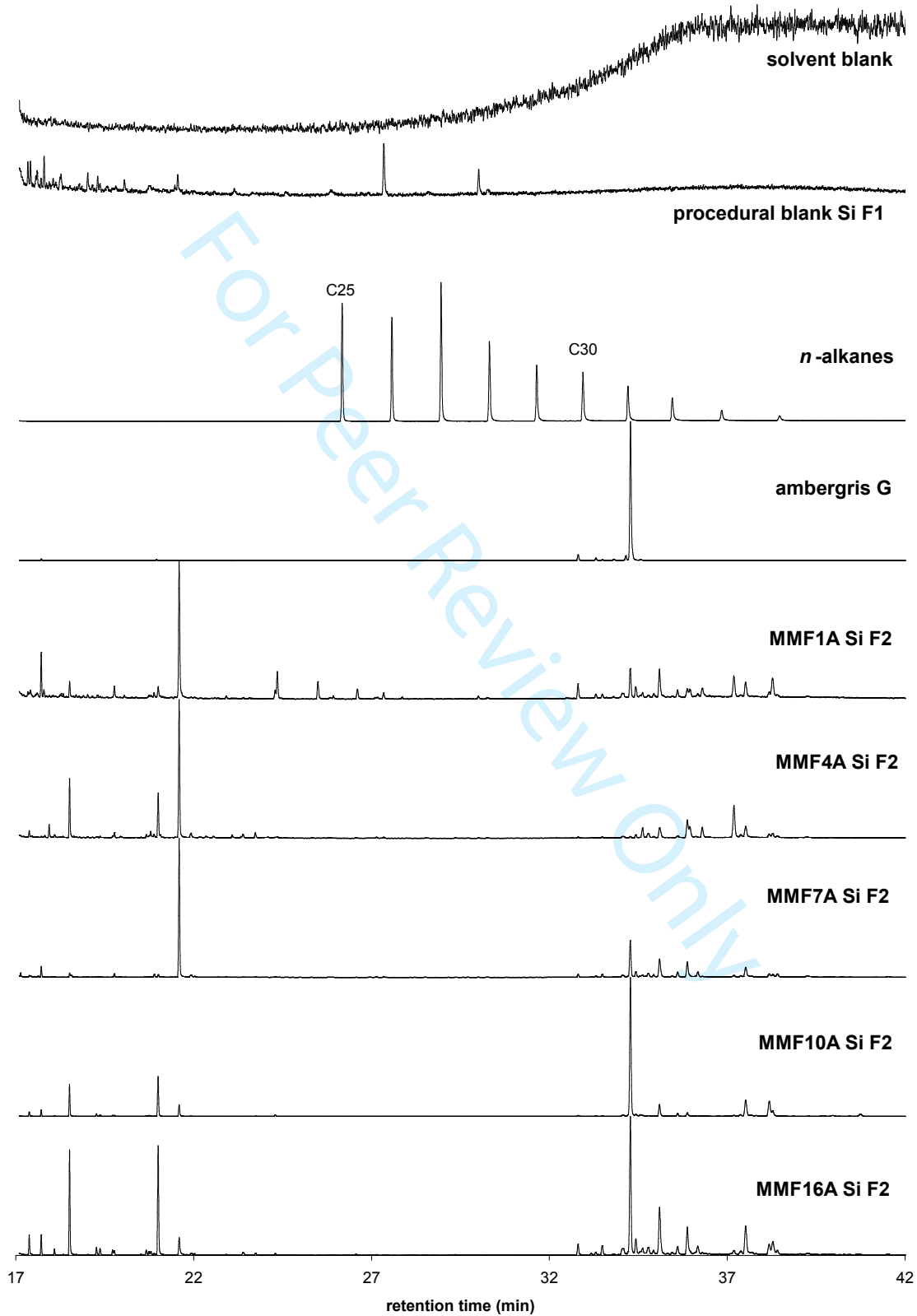
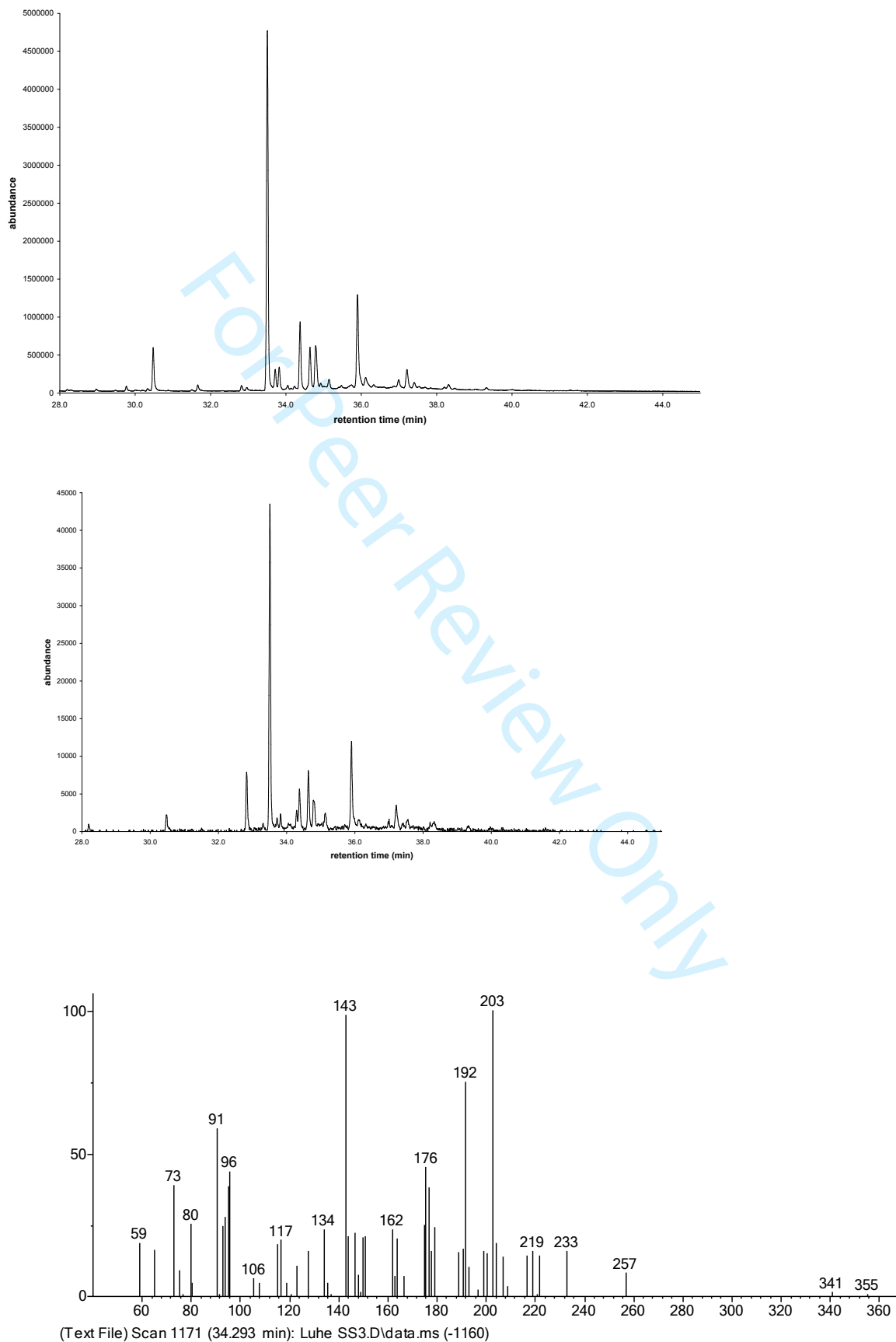


Figure S9. GC-MS total ion current (upper) and (middle) extracted ion chromatograms (m/z 143+203+500) of trimethylsilylated total organic extracts of human sewage sludge and (lower) mass spectrum of component assigned as ambrein TMS ether.



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5 **Figure S10.** GC-MS total ion current (TIC) chromatogram of trimethylsilylated total organic
6 extract of sperm whale faeces. A=ambrein TMSi ether; A1, A2 are unknowns also reported
7 by Rowland and Sutton (2107) in ambergris extracts and now identified herein as TMSi enol
8 ether artifacts of coprostanone derivatisation. Inset shows mass spectrum of component A
9 (ambrein TMSi ether). Other unlabelled (major) peaks in the chromatogram are due to
10 coprostanol and epicoprostanol (TMSi ethers) faecal sterols.
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