Investigating the volatilome of ambergris using comprehensive two-dimensional gas chromatography coupled with time-of-flight mass spectrometry

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Abstract

Ambergris is a rare whale coprolith which has captivated human interest for centuries due to its unique olfactory properties and historical use in perfumery, as well in alternative medicine. The complex mixture of volatile organic compounds (VOCs) within ambergris comprises a wide range of chemical classes, including terpenes, aliphatic and aromatic hydrocarbons, and oxygenated derivatives. These compounds contribute to the distinct and alluring scent profile for which ambergris is renowned. The formation of volatile compounds in ambergris is a result of intricate biological processes within the digestive system of sperm whales, involving the interaction of ingested marine materials, enzymatic reactions, and subsequent aging and maturation of the material when deposited on beaches. The distribution and the ratios of these different compounds determine the value of the ambergris. Various methods and protocols have been engaged to assess and determine the composition. The present study focuses on the use of comprehensive two-dimensional gas chromatography coupled with time-of-flight mass spectrometry (GC×GC–TOFMS). This advanced analytical technique provides an increase in separation capacity to resolve the complexity of the ambergris volatile profile and uncover components that have not

previously been reported. This novel technique being rapid, precise and convenient can bring about quick identification of authentic ambergris samples.

Keywords: Coprolith, ambrein, two-dimensional gas chromatography, TOF-MS, volatilome

Introduction

Ambergris is the natural product of the intestinal activity of only some sperm whales (*Physter microcephalus*), formed as a protective barrier in response to, and around, undigestible material such as squid beaks, specifically in the rectum. It can also be found in pygmy (*Kogia breviceps*) and dwarf whales (*K. sima*) and is therefore termed a coprolith (Clarke 2006).

Ambergris has long been recognized for its distinctive aroma and has historical significance in the perfume industry, mainly as a prized ingredient within luxury perfumes. Ambergris has also been included in certain medicines, such as aphrodisiacs and laxatives, as well as in cosmetics (Dugan 2011). It is well reported in the literature that the ambergris, in combination with other medicinal herbs, is still in use by the practitioners of the Ayurveda and Unani systems of medicine to treat general weakness, epilepsy, typhoid, fever, hysteria and other nervous disorders or afflictions (Singh 2018 *et al*.).

The role of bacteria is well established as a key factor in ambergris formation, including gut microbiota of the whale (Clarke 2006; Rowland and Sutton 2017; Ueda 2013 *et al*.).

Ambergris is comprised of both inorganic and organic components. The inorganic component has a high content of mineral salts such as phosphate and ammonium. The use of ethanolic extracts, as well as lipid-soluble extracts, has been reported for the analysis of the organic components, with ambrein found to be the main constituent (Awano *et al*. 2005). Ambrein is not preformed but is the product of the cyclization of squalene (Ueda *et al*. 2013), the enzymes for this conversion being produced by the microbiota present in the whale's intestines (Clarke 2006).

The few published studies on ambergris have revealed that the organic component also comprises faecal steroids, such as pristane and epicoprostanol, which are by-products of cholesterol and bile pigments (Rowland and Sutton 2017). Octadecanoic acid, steroidal diacid, linoleic, archidic and betenic acids are among some of the carboxylic acids also reported (Taha 1989).

The proportions and types of volatile organic compounds (VOCs) in ambergris can vary depending on factors such as the age, origin and processing of the ambergris. For example, fresh ambergris does not exhibit the desirable musky odour associated with aged ambergris, and instead has a characteristic pungent note.

Techniques like gas chromatography-mass spectrometry (GC-MS) (Rowland *et al*. 2017, Wilde *et al*. 2019), Fourier transform infra-red spectroscopy (FTIR) (Rowland and Sutton 2017) and nuclear magnetic resonance (NMR) spectroscopy (Moniz and Hammond 1996) have played crucial roles in deciphering the intricate chemical makeup of ambergris with ambrein often reported as the main constituent.

The quality and resulting market value of ambergris depends on the concentration and ratio of its constituent components. For example, natural photo-oxidation (or autoxidation) of ambrein can result in the formation of odoriferous mono, bi and tricyclic volatile compounds, impacting the perceived aroma of ambergris (Serra 2013). Accurate analysis of the volatile composition, including ambroxan, ambreinolide, ambrox, ambrinol and ionones, is also imperative to classify the nature of the ambergris samples, which has not been possible by all methods employed so far.

As techniques for VOC analysis continue to evolve, further revelations about the complex interactions between these compounds and their contribution to the captivating aroma of ambergris are anticipated.

Here, we evaluate the use of comprehensive two-dimensional gas chromatography coupled with time-of-flight mass spectrometry (GC×GC-TOF MS) as an enhanced separation tool to reveal the hidden complexity of ambergris. The enhanced separation capacity offered by the coupling of two columns of different selectivity, combined with highly sensitive mass spectral identification, provides a comprehensive profiling of the ambergris volatilome. This level of detail is required for thorough quality and authenticity evaluation of ambergris, as well as for the development of synthetic alternatives.

Method and Materials

Sample: Ambergris was obtained from M/S Hamdard Laboratories India and 20mg was dissolved in 4mL of dichloromethane.

GC×GC: A non-polar to polar column setup was utilised to separate components based on both volatility and polarity. Separation was performed on the Agilent 7890B gas chromatograph utilising the following columns: 1D: BPX5-20 m × 0.18 mm i.d. × 0.18 μm film thickness and 2D: BPX90-5m×0.25 mm id. ×0.25 μm film thickness. Oven temperature was 60 °C (1 min), 7 °C/min to 320 °C (0 min), 1 °C/min to 330 °C (15 min). The INSIGHT® modulator (SepSolve Analytical, Peterborough, UK) was used to provide reverse fill/flush flow modulation, with a 25μL sample loop and a bleed line of 5 m × 0.1 mm i.d. The modulation period was 3.5 seconds, with a flush time of 80 ms.

FID: Temperature of 300 ^{0} C with air flow of 300 mL/min. The H_2 fuel flow was 10mL/min and N_2 flow (as the makeup gas) was 5mL/min.

TOF MS: A Bench TOF time-of-flight mass spectrometer (SepSolve Analytical, Peterborough, UK) was used. The ion source temperature was 300 ^{0} C; the transfer line was 300 ^{0} C. The filament voltage was 1.8 V and the ionization energy was 70eV. The mass range was m/z 35-500, with the TOF simultaneously analysing all ions.

Data analysis: ChromSpace® software (SepSolve Analytical, Peterborough, UK) was used for both acquisition and post-analysis data processing. The chromatogram was de-convolved, integrated and mass spectra searched against commercial mass spectral libraries, including the National Institute of Standards and Technology (NIST) 2017 library and the Wiley Registry (12th Edition), for tentative identification.

2. Results & Discussion

Figure 1 shows the GC×GC-TOF MS colour plot (TIC) of the ambergris sample. The view is normalised to the largest peak in the current window in this case, ambrein, which was identified to be the most abundant component in the sample. As compounds were separated in the first dimension by volatility and their affinity to the non-polar stationary phase, and then by affinity to the polar phase in the second dimension, interferences such as non-polar column bleed and siloxane peaks were well-separated from the compounds of interest (Figure 2). This figure also excludes the abundant ambrein peak, enabling the complexity of the more volatile composition of the ambergris to be revealed.

In Figure 3.1, another enhanced region of the colour plot is shown this time focusing on the set of abundant peaks, including ambrein. In Figure 3.2, the acquired TOF MS spectra (red) were compared to commercial libraries (black) for identification of these dominant constituents. The peaks highlighted in Figure 3.1 were identified as (a) ambrein, (b) Chloestan-3-ol, (3α, 5β)-, (c) Chloestan-3-one, (5β)-, and (d) tentatively identified as squalene or another isomer of $C_{30}H_{50}$.

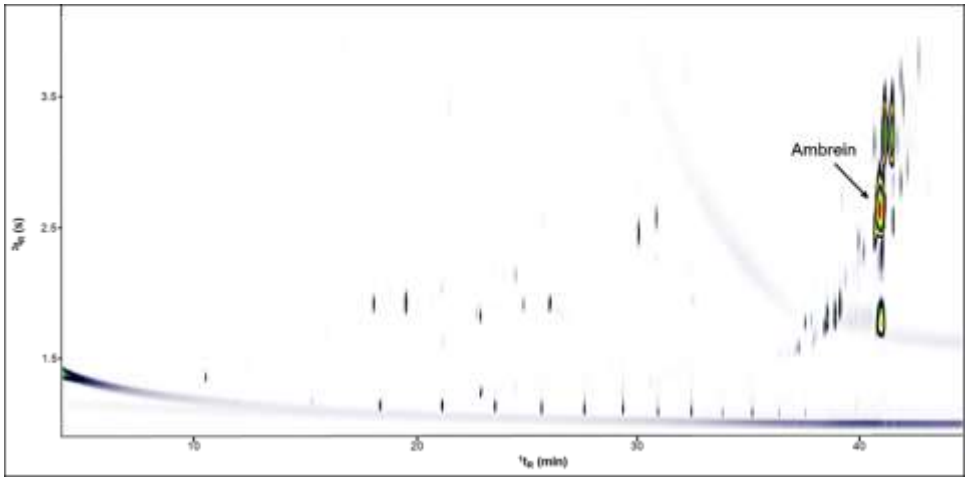

Fig 1: GC×GC-TOF MS colour plot (TIC) of the ambergris sample

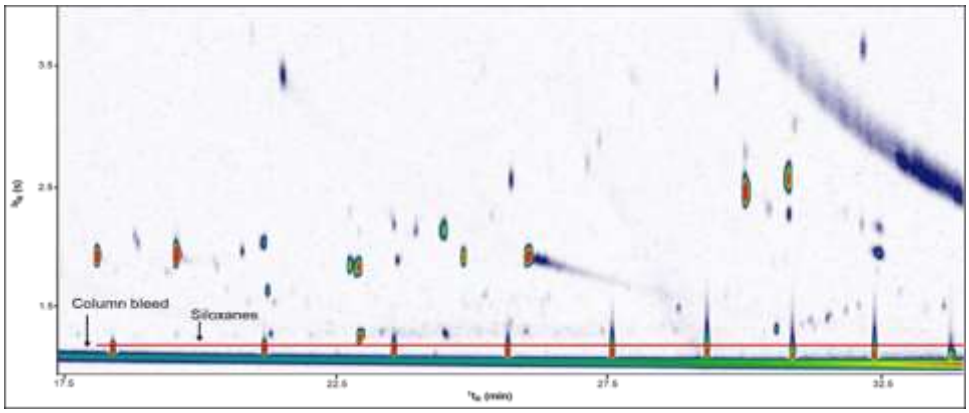

Fig 2: An enhanced region of the GC×GC-TOF MS colour plot (TIC) highlighting the underlying complexity of the ambergris volatile profile that was overshadowed by ambrein in Figure 1

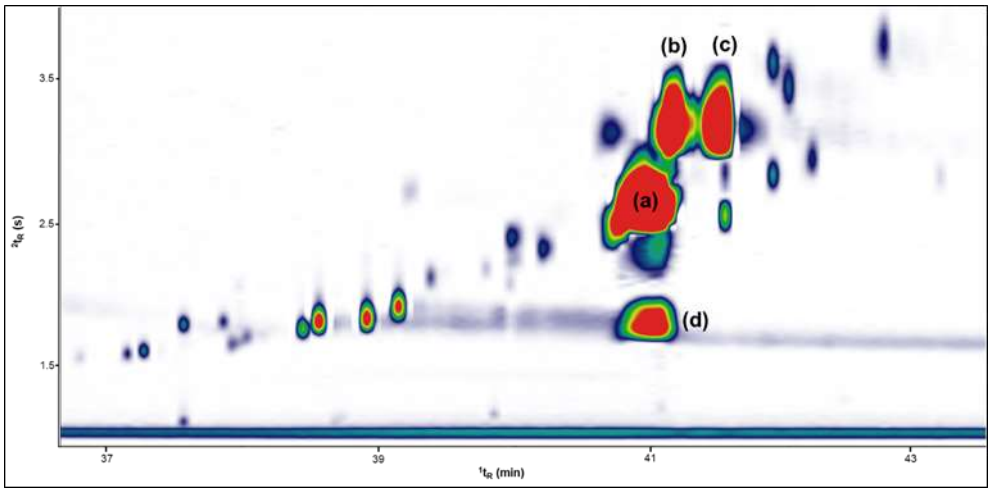

Fig 3.1: An enhanced region of the GC×GC-TOF MS colour plot (TIC)highlighting the four most intense peaks in the ambergris chromatogram-(a) Ambrein, (b) Chloestan-3-ol, (3α, 5β)-, (c) Chloestan-3-one, (5β)- and (d) tentatively identified as squalene. See Figure 3.2 for mass spectral information

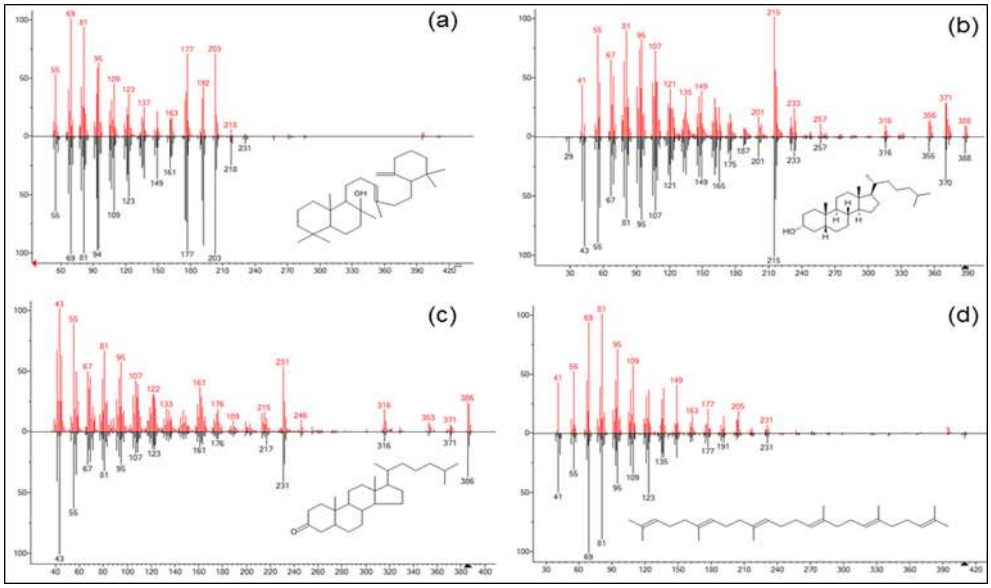

Fig 3.2: The acquired spectra (red) was compared to the NIST library (black). Enhanced separation has allowed for three major components to be separated and identified: (a) Ambrein, (b) Chloestan-3-ol, (3α, 5β)-, (c) Chloestan-3-one, (5β)- and (d) tentatively identified as squalene

Integration of the chromatogram detected a total number of 162 peaks for the ambergris sample. The detected peaks were then identified based on spectral matches to commercial mass spectral libraries. A total of 58 compounds were tentatively identified **(**Table 1**)** with the most abundant peaks representing ambrein (plus isomers of ambrein), and the derivatives of cholesterol metabolism, present as fecal steroids

such as cholestan-3-one, (5β)- and cholestan-3-ol, (3α,5β)-. Various other odour-active compounds were also identified within the sample, such as, γ-ionone, longicamphenylone, β-ionone, boronia, α-ambrinol, sclareolide and muscone, which contribute to the distinctive musky and earthy aroma of ambergris. Squalene isomers and derivatives (from which the ambrein is biosynthesised) were also present in the form of alkanes and fatty acids, such as pentanoic acid.

Table 1: Metabolites identified in Ambergris sample using GC×GC-TOF MS

Ambergris Peak Table							
S. No.	**Compound**	**RMF**	**RT**	**tr1**	**tr2**	**Area**	**Odour description and comments**
1	Cyclohexasiloxane, dodecamethyl-	871	15.3	15.28	1.15	2.97E+05	
2	7,8-dihydro-α-Ionone	823	18.12	18.09	1.91	2.79E+06	Woody, earthy, ambergris, tobacco odour
3	Cycloheptasiloxane, tetradecamethyl-	901	18.4	18.38	1.13	2.58E+06	
4	γ-Ionone	691	18.82	18.78	2.09	8.04E+04	
5	α-Ambrinol	723	18.88	18.85	2.03	9.63E+04	Amber natural musk animal
6	Longicamphenylone	828	19.58	19.55	1.91	7.53E+06	
7	Cyclooctasiloxane, hexadecamethyl-	907	21.19	21.18	1.13	2.94E+06	
8	Boronia Butenal	775	21.19	21.16	2.03	4.35E+05	Sweet tobacco nutty violet boronia dry leaves hay wood acorns
9	Pentanoic acid, 2,2,4-trimethyl-3-carboxyisopropyl, isobutyl ester	838	21.27	21.24	1.63	2.60E+05	
10	Hexadecane	809	21.33	21.31	1.25	1.08E+05	
11	Diethyl Phthalate	883	21.59	21.54	3.42	5.67E+05	
12	β-Ionone Epoxide	775	22.78	22.75	1.83	7.02E+05	Fruity sweet woody powdery
13	Unknown		22.93	22.9	1.82	2.14E+06	Fruity sweet berry woody violet orris powdery
14	Pentadecane, 2,6,10,14-tetramethyl-	926	22.97	22.95	1.23	1.78E+06	Structurally similar to ambriosol
15	Cyclononasiloxane, octadecamethyl-	903	23.59	23.57	1.13	2.79E+06	
16	Ambrial	712	23.66	23.63	1.87	1.24E+05	
17	Muscone	754	24.52	24.48	2.13	1.05E+06	Musk
18	Octadecane	851	24.55	24.53	1.26	2.26E+05	Powerful musky rich

							musk
19	Ambroxide	866	24.88	24.85	1.91	1.16E+06	
20	Cyclodecasiloxane, eicosamethyl-	868	25.69	25.67	1.11	2.33E+06	
21	Isobutyl Phthalate	892	25.78	25.74	2.55	2.92E+05	
22	Ambriosol	802	26.1	26.07	1.91	4.04E+06	Ambergris labdanum amber woody
23	Cyclodecasiloxane, eicosamethyl-	836	27.61	27.59	1.11	2.35E+06	
24	Cyclodecasiloxane, eicosamethyl-	830	29.36	29.34	1.11	2.24E+06	
25	Sclareolide	813	29.57	29.52	3.39	2.05E+05	Dry paper woody ambergris labdanum
26	Unknown		30.09	30.05	2.45	4.36E+06	Structurally similar to labd-diols
27	Unknown		30.89	30.84	2.57	2.56E+06	Structurally similar to labd-diols
28	(±)-Ambreinolide	772	30.89	30.85	2.27	1.75E+05	
29	Cyclodecasiloxane, eicosamethyl-	829	30.94	30.92	1.09	1.49E+06	
30	Cyclodecasiloxane, eicosamethyl-	824	32.45	32.44	1.09	1.91E+06	
31	Eicosamethyl-cyclodecasiloxane	830	33.88	33.86	1.07	1.10E+06	
32	Heptasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11,13,13-tetradecamethyl-	836	35.19	35.17	1.08	1.61E+06	
33	Squalene Isomer		37.18	37.15	1.55	7.40E+05	
34	Squalene Isomer		37.3	37.28	1.57	1.34E+06	
35	Cholest-4-ene	854	37.6	37.57	1.77	1.73E+06	
36	Octasiloxane,1,1,3,3,5,5,7,7,9,9,11,11,13,13,15,15-hexadecamethyl-	820	37.62	37.6	1.07	7.34E+05	
37	Cholest-2-ene	804	37.89	37.86	1.79	7.66E+05	
38	Squalene Isomer		37.97	37.94	1.64	1.17E+06	
39	2,6,10,15,19,23-Pentamethyl-2,6,18,22-tetracosatetraen-10,15-diol	885	38.07	38.05	1.67	7.33E+05	
40	Squalene Isomer		38.47	38.44	1.73	3.28E+06	
41	1,1,6-trimethyl-3-methylene-2-(3,6,9,13-tetramethyl-6-ethenye-10,14-dimethylene-pentadec-4-enyl) cyclohexane	801	38.6	38.57	1.79	1.25E+07	
42	Squalene Isomer		38.95	38.92	1.81	1.20E+07	

43	Ambrein Isomer		39.17	39.14	1.89	1.09E+07	Ambergris labdanum amber woody
44	Squalene Isomer		39.42	39.39	2.11	8.11E+05	
45	Heptasiloxane, hexadecamethyl-	830	39.84	39.82	1.1	6.28E+05	
46	Ambrein Isomer		40.03	39.99	2.39	2.95E+06	
47	Ambrein Isomer		40.25	40.21	2.31	2.47E+06	
48	Ergost-22-en-3-ol, (3α,5β,22E)-	735	40.74	40.69	3.13	5.36E+06	
49	Ambrein	879	40.99	40.95	2.62	4.78E+08	Ambergris labdanum amber woody
50	Squalene or another isomer of C30H50	796	41	40.97	1.75	8.38E+07	
51	Cholestan-3-ol, (3α,5β)-	847	41.21	41.15	3.22	1.65E+08	
52	Cholestan-3-one, (5β)-	832	41.54	41.49	3.19	1.86E+08	
53	Ambrein	794	41.58	41.53	2.55	5.13E+06	
54	Unknown		41.94	41.89	2.83	3.01E+06	
55	Desmosterol	751	41.96	41.95	0.12	3.49E+06	
56	Cholestan-3-one, (5α)-	842	42.06	42	3.47	2.36E+06	
57	Unknown		42.21	42.17	2.95	2.23E+06	
58	Stigmast-24(28)-en-3-one, (5α)-	779	42.75	42.75	0.28	2.74E+06	

Ambergris is highly valued for its odoriferous properties. It gains its odour as it ages due to oxidation of one of its major components, the triterpene ambrein which is odourless. As these volatiles form an integral part of the character of the ambergris it is imperative that they be analysed in a rapid and convenient manner as a high percentage of samples suspected to be ambergris washed up on beaches may only be jetsam. Moreover, the different ratios of the various volatile components impart a unique fragrance to that sample. This information can aid in attributing a more exact value to the samples found around the world.

Here, we have shown that the enhanced separation capacity of GC×GC-TOF MS

provides maximum information on the ambergris volatilome, as compared to any other protocol to date. In a one-dimensional separation it is likely that numerous peaks would co-elute, but the physical separation of the peaks by GCxGC provides cleaner spectra for greater confidence in identification of the metabolites and isomers present in ambergris.

The analytical procedure was straight forward, with no derivatization performed in this case-meaning minimal loss of material. The study also demonstrated for the first time the presence of a large number of odoriferous compounds in a single analytical run and as already mentioned it is these odoriferous compounds which lend the character and uniqueness of each ambergris sample. Cataloguing of ambergris by GCxGC-TOF MS may further our understanding of the biochemical pathways involved in its formation, as well as provide insights into the ageing process and in authenticity evaluation.

3. Conclusion

The present study, using this new technique shows the presence of the maximum number of components both identified as well as new chemical metabolites among studies done so far. The accurate and rapid identification of the ambrein and their isomers using GCxGC-TOF MS may be also proving useful for quality control analysis of the ambergris sample for its medicinal use. The manuscript thus demonstrates that with the use of cutting-edge technology better results can be obtained by developing protocols which are quick and precise.

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Disclosure statement

We confirmed that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

Data availability statement

The data that support the findings of this study are available from the authors upon reasonable request.

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