Title

An improved method for the GC-MS determination of polycyclic aromatic hydrocarbons and *n*-alkanes in speleothems

Authors

Elena Argiriadis[1]*, Rhawn D. Denniston[2], Carlo Barbante[1,3]

Affiliation

[1]Ca' Foscari University of Venice, Department of Environmental Sciences, Informatics and Statistics, Via Torino 155, 30172 Venice, Italy

[2]Cornell College, Department of Geology, 600 First Street SW Mount Vernon, IA 52314-1098, USA

[3]Institute for the Dynamics of Environmental Processes CNR-IDPA, Via Torino 155, 30172 Venice, Italy

*Corresponding author, email: elena.argi@unive.it, telephone: +39 041 234 8658, ORCID iD: 0000-0001-7227-405X

Abstract

The interest in paleoenvironmental reconstructions from biomarkers in speleothems is increasing, thanks in part to the capacity of speleothems to grow continuously and to resist post-depositional alteration. In particular, the possibility exists to link high-resolution and accurately dated fire and vegetation records with isotopic data of climatic and paleoenvironmental interactions at the local and regional scale. However, the scarcity of existing methods for the quantification of organic molecules in stalagmites, together with the issues of sample availability, contamination, and low concentrations, complicate this approach. In this work, we developed a novel method for the simultaneous determination of 18 polycyclic aromatic hydrocarbons (PAHs) and 26 *n*-alkanes (C_{10}-C_{35}) and then tested it on "clean" calcite and aragonite stalagmite samples from cave KNI-51 in the Australian tropics. The method involves subsampling by using a handheld drill, then the complete dissolution of the matrix in hydrochloric acid, followed by liquid-liquid extraction and GC-MS analysis. Sample preparation was carried out in a 10,000 class clean room, entirely built in stainless steel, to avoid contamination. Detection limits were 0.3-9 ng for PAHs and 6-44 ng for *n*-alkanes. Measurable concentrations of fire-derived PAH compounds, namely phenanthrene, pyrene, benzo(*e*)pyrene and indeno(123-*cd*)pyrene, were detected only in one sample, which dates to the year ~AD 2004, when a fire burned vegetation over the cave; *n*-alkanes were detected in all samples in the range C_{23}-C_{35}, with no odd-even preference.

The knowledge and use of biomarkers as proxies for paleoenvironmental change is rapidly expanding in concert with a growing demand for such records. Speleothems are important paleoenvironmental proxies due to their capacity for continuous growth, high temporal resolution, precise and accurate age dating by U/Th methods, and strong post-depositional stability[1]. In addition, speleothems record a variety of paleoenvironmental events through numerous chemical, mineralogical, and sedimentological signals[2,3]. Thus, improving our understanding of biomarkers in speleothems represents an important goal. However, due to the dynamics of the deposition process and the inorganic nature of the matrix, concentrations of organic compounds in speleothems are usually very low, close to method and instrumental detection limits[4]. As a result, high amounts of extractable material are required in order to perform a reliable analysis, which in the case of speleothems can be a substantial challenge. In this context, the sources of contamination from sampling, cutting and laboratory operations must be minimized. The need for high sensitivity, low contamination techniques in speleothems are therefore undeniable. In addition, little work has tested the capacity of karst hydrologic systems to transport organic compounds through the bedrock and into cave dripwater at sufficient temporal scales so as to allow for identification of discrete events, such as fires.

We focused our research on developing a method for the simultaneous analysis of two classes of hydrocarbons in speleothems, namely 18 polycyclic aromatic hydrocarbons (PAHs) and 26 *n*-alkanes, with the aim of minimizing external contamination and improving the sensitivity of instrumental determination. The use of PAHs as tracers of past combustion processes is well-established[5], and their persistence in the environment allows detection in several paleoenvironmental archives, including speleothems[4,6]. Numerous studies, especially on lake sediments and peat, have demonstrated the relation of *n*-alkanes with vegetation changes and associated climatic conditions[7–10]. Relating fire activity and vegetation composition to temperature and precipitation reconstructions achieved through the oxygen and carbon isotopic ratios of speleothems will allow a more in-depth understanding of the interplay between climate and fire at local and regional scales.

To date, protocols for the extraction of organic molecules from speleothems include several steps to prevent contamination and maximize recovery, such as solvent washing and acid digestion[11–13]. Although previously published methods for the analysis of lipid biomarkers in spelothems allowed accurate and high-resolution determination and have become quite well-established[12], only one

method has involved the quantification of PAHs in stalagmites[6] . Briefly, the latter involved crushing the stalagmite fragments into a powder and extracting them with ultrasounds, before Florisil purification and HPLC-fluorescence analysis. Here, we describe the development of a new analytical method based on the complete dissolution of the matrix, followed by a liquid-liquid extraction and GC-MS determination. All steps aim at minimizing contamination while maximizing the analyte yield and thereby allow for a more routine measurement of target compounds in speleothems.

Experimental Section

Materials

Pesticide-grade dichloromethane, *n*-hexane, and acetone and 34-37% SpA hydrochloric acid from Romil Ltd. were employed. Isotope labeled standard solutions ($^{13}C_6$-acenaphthylene, $^{13}C_6$-phenanthrene, $^{13}C_4$-benzo(a)pyrene and $^{13}C_6$-chrysene) were obtained from Cambridge Isotope Laboratories. Native PAHs were acquired from Dr. Ehrenstorfer (PAH Mix-9) and *n*-alkanes (C_{10}-C_{35} solution and hexatriacontane) were acquired from Sigma-Aldrich. All tools and glassware were pre-washed with a 5% Contrad aqueous solution and rinsed three times with *n*-hexane and dichloromethane (DCM), respectively. Where possible, glassware was also muffled at 400 °C.

Sampling Methodology

Preliminary tests were run on two types of sample: calcite from Carrara, Italy (granulometry 80-150 µm), and portions of an aragonite stalagmite from cave KNI-51, located in the Kimberley region of tropical Western Australia[14,15]. Three samples (fig. 1) of ~1 g were drilled from the fragment with a handheld Dremel tool at the University of Padua, Department of Geology. Before drilling, the fragment was sonicated three times with *n*-hexane and three times with dichloromethane in order to decontaminate the outer surface. The external layer was removed with the drill and analyzed in order to assess its analyte content. Each tool was cleaned with *n*-hexane and DCM before use and drilling was performed under a fume hood.

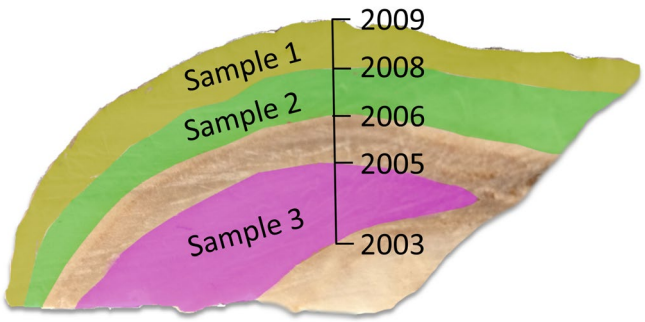

Figure 1. Subsampling and approximate age relations (in years CE) based on U/Th dating and the zero age of the active growth surface of the stalagmite fragment.

PAHs and n-alkanes extraction

Each sample was transferred to a sealed, clean 60 mL vial with a pierceable cap and spiked with a 50 ng of $^{13}C_6$-acenaphthylene, $^{13}C_6$-phenanthrene, $^{13}C_4$-benzo(*a*)pyrene and hexatriacontane as internal standards before adding 10 mL of DCM. The calcium carbonate matrix was dissolved with 37% Super Purity HCl pre-extracted with dichloromethane to guarantee the absence of organic contaminants. HCl was added to the samples using a disposable syringe through the vial septum to avoid evaporation. Samples were kept in an ice bath and mixed by shaking every few minutes until the reaction was complete, to avoid heating and thus volatilization of lighter analytes[6]. Dissolved samples were then vortex-extracted three times with 10 mL DCM each. The first aliquot of DCM was added before the HCl to guarantee the immediate dissolution of the samples in the solvent and prevent evaporation with the gas developed by the reaction. The extracts were finally collected in clean glass tubes and evaporated to ~200 µL under a gentle stream of nitrogen.

In order to test the recovery of the method, several ~0.5 g aliquots of Carrara calcite were fortified with known amounts of all native compounds and extracted as described. The method was then employed for the analysis of stalagmite samples drilled from the fragment shown in fig. 1, together with several procedural blanks. All operations were run in the class 10,000 cleanroom of Ca' Foscari University, built entirely in stainless steel and equipped with fume hoods and air filters to guarantee the lowest external contamination.

120 *GC-MS analysis*

121 PAHs and *n*-alkanes were analyzed with a 7890A GC system coupled to a 5975C single quadrupole
122 mass spectrometer (Agilent Technologies). Compounds were separated through a HP-5ms column
123 (60 m, id 0.25 mm, 0.25 µm film thickness, Agilent Technologies). Operating conditions of the GC
124 for PAHs were as follows: injector temperature 300 °C; transfer line temperature 300 °C; oven
125 temperature program 70 °C for 1.5 min, then 10 ° C min^{-1} to 150 °C for 10 min, then 3 °C min^{-1} to
126 300 °C for 15 min, 305 °C for 30 min (postrun); carrier gas (helium) 1 ml min^{-1}; injection mode,
127 splitless with split valve open after 1.5 min at 50 mL min^{-1}. For *n*-alkanes, the GC program was the
128 following: injector temperature 300 °C; transfer line temperature 300 °C; oven temperature
129 program 50 °C for 5 min, then 18 ° C min^{-1} to 315 °C for 16 min, 315 °C for 15 min (postrun); carrier
130 gas (helium) 1.2 ml min^{-1}; injection mode, splitless with split valve open after 1 min at 50 mL min^{-1}.
131 The mass spectrometer was operated in single ion monitoring mode, using an electron impact
132 source set at 70 eV and 230 °C. The quadrupole temperature was 150 °C. Chromatograms were
133 processed by Agilent MSD Chemstation software. Analytes were quantified by the isotope dilution
134 technique and results for each compound were corrected by the instrumental response factors,
135 obtained by repeated injections of solutions containing all native compounds and internal standards
136 in equal concentrations.

137

138 *Quality control*

139 Procedural blanks were analyzed together with samples in order to assess possible contamination.
140 All C_{10}-C_{35} *n*-Alkanes were detected in the range 1-54 abs ng, while for PAHs only naphthalene,
141 phenanthrene, fluoranthene, pyrene, retene, benzo(*e*)pyrene, benzo(*a*)pyrene and
142 benzo(*ghi*)perylene were detected in the range 0.2-4 abs ng. Blank concentrations resulted
143 generally lower those reported in the literature[6,16], which underlines the importance of preventing
144 laboratory contamination through “clean” procedures.

145 The detection limits for PAHs, expressed as three times the standard deviation of the blank, range
146 from 0.3 to 9 ng. For *n*-alkanes, the detection limit range is 6-44 ng. Absolute quantities of analytes
147 in samples were corrected by the mean blank values plus three times the standard deviation.

148 Recovery, precision (in terms of relative standard deviation of replicates) and accuracy (percentage
149 relative error with respect to the spiked amounts) were estimated by spiking Carrara calcite with

known amounts of native compounds and internal standards: PAH percent recovery was between 90 and 163%, precision ranges 0.2-11% and accuracy was 0.2-8%. For *n*-alkanes, recovery was between 43 and 140%, precision was 0.3-34% and accuracy was 0.1-12%. Overall, analytical performances for PAHs are significantly better than the ones of the method by Perrette et al. (2008), confirming that tests carried out on real matrix and complete dissolution of the sample bring significant improvements to the quantification of analytes. Conversely, a direct comparison with literature methods is not possible for *n*-alkanes, since, to the best of our knowledge, none reports such information.

Results and discussion

Fortified samples

The background concentration of analytes in the Carrara calcite was evaluated through repeated analyses and results for the fortified samples were corrected by the mean background values. No PAHs were detected in non-fortified Carrara calcite, while *n*-alkanes were present in low amounts (0.03-0.8 $\mu g\ g^{-1}$).

Stalagmite samples

Samples were drilled from the uppermost section of stalagmite KNI-51-11. This sample was collected while actively growing in June 2009 and has been dated by 16 high-precision U/Th methods (two standard deviation errors averaging ±1.3 year) from CE 1896, including three dates between CE 1999-2009[14,17]. An age model based on these dates suggests the sampled intervals correspond approximately to CE 2008, 2006-7, and 2003-4. We aimed to test whether burning of the eucalypt savanna over the cave produced PAHs that were transferred via cave drip water into KNI-51 and then incorporated into the stalagmite carbonate. The study of PAHs in stalagmites by Perrette et al. (2008) was complicated by the great depth of the cave and the thick and PAH-rich soil that overlay it. In contrast, soils on the hillslope over KNI-51 are thin, organic-poor, and sparse; rillenkarren limestone is exposed over much of the landsurface above the cave. In addition, the limestone is fractured, leading to rapid infiltration with little storage of water in the epikarst. As a consequence, issues of PAH storage and re-mobilization in the soil-karst system that plagued Perrette et al. (2008) are not apparent at this site. Fire activity over the cave was assessed for each year between 2001 and 2008 using monthly satellite maps of burn scars created and archived

through the North Australia and Rangelands Fire Information website (http://www.firenorth.org.au/nafi3/). These maps reveal that 2004 was the only one of these years to experience a fire directly over the cave. PAHs were present in measurable abundances only in sample 3 (~CE 2004), with fluoranthene, pyrene, benzo(*e)*pyrene and indeno(1,2,3-*cd*)pyrene in the 0.3-2 ng g^{-1} range. The latter was detected also in sample 1 (~CE 2008) (table 1). Thus, it appears that PAHs formed during burning of the overlying savanna were transported by infiltrating rainwater into the underlying cave where they were incorporated into stalagmite carbonate, forming a chemical marker of the fire. In addition, we hypothesize that the thin soils and sloped hillside over the cave, coupled with the intense monsoonal rainfall regime of the eastern Kimberley (~850 mm yr^{-1}, with ~80% falling in the austral summer, DJF)[17], quickly eroded residual PAHs from the land surface such that dripwater contains high PAH abundances only for a short time (perhaps only ~1 yr in some cases) after each fire.

The presence of indeno(1,2,3-*cd*)pyrene, produced by high-temperature combustion[18], in two out of the three samples requires further investigation to identify the sources and processes involved. No indeno(1,2,3-*cd*)pyrene was detected in blanks, nor are the sawing and drilling operations likely to produce such a heavy compound, as they do not reach the high temperatures required. Indeed, a possible interpretation could be based on its high molecular weight relative to the other PAHs considered here, which probably enhances its precipitation together with calcite, thus resulting in an enrichment of this compound in the stalagmite.

Table 1. PAHs concentrations (ng g^{-1}) in the stalagmite fragment.

	Sample 1 2008	**Sample 3 2004**	**External Layer**
Anthracene	nd	nd	4
Fluoranthene	<lod	0.6	nd
Pyrene	<lod	0.4	nd
Benzo(*a*)anthracene	nd	nd	1
Chrysene	nd	nd	1
Retene	<lod	<lod	nd
Benzo(*b*)fluoranthene	nd	nd	nd
Benzo(*k*)fluoranthene	nd	nd	1
Benzo(*e*)pyrene	<lod	0.3	nd
Benzo(*a*)pyrene	nd	nd	nd
Benzo(*ghi*)perylene	nd	nd	<lod
Indeno(1,2,3-*cd*)pyrene	5	2	nd
Dibenzo(*ah*)anthracene	nd	nd	nd

In Sample 2 (2006-7) all compounds were below the limit of detection, while the external layer contained detectable amounts of anthracene, benzo(a)anthracene, chrysene and benzo(k)fluoranthene between 1 and 4 ng g^{-1}. Such compounds differ completely from the ones detected in samples and blanks, indicating that, if contamination is the source of PAHs in the external layer, it did not affect the inner part and is likely not ascribable to laboratory operations.

Cave KNI-51 fills with floodwaters multiple times per decade, and sediments mobilized during these flood events become deposited on stalagmite caps when flood waters recede and then trapped within stalagmites when cave dripwaters resume. As these flood layers can be difficult to avoid when milling stalagmite carbonate, we conducted a test of the impact of these sediments on the PAH abundances measured in KNI-51-11 in case they, rather than the carbonate itself, were the source of the PAHs identified in our analysis. Ten sediment samples from the cave passage were collected from the stalagmite room of KNI-51 and these samples were processed according to the same methods. As expected, given the higher organic content of sediments, PAH abundances in these sediments were higher than those measured in the stalagmite carbonate (Table S1) and thus incorporation of flood detritus within the stalagmite could increase the overall measured PAH concentration. However, no visible flood detritus was present in Samples 1-3 and no insoluble residue was present after digestion in HCl. In addition, PAH compounds detected in the stalagmite samples do not fully coincide with the one present in sediments (Table 1 and S1). For instance, no retene or benzo(*a*)pyrene were found in calcite. This suggests a differentiation in the deposition dynamics and only a partial influence of sediments on the PAH composition of stalagmites.

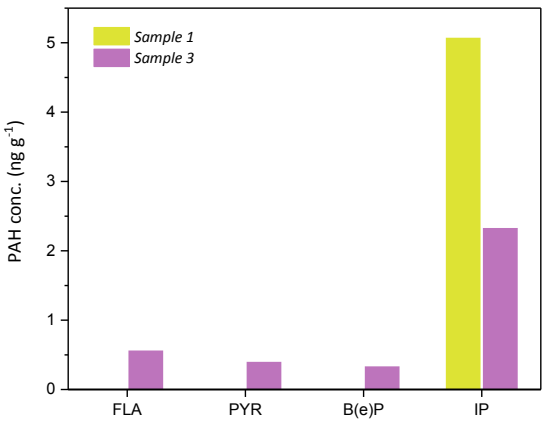

Figure 2. PAHs concentrations (ng g^{-1}) in samples from the stalagmite fragment.

Only high molecular weight (HMW) *n*-Alkanes in the range C_{23}-C_{35} were present in all samples and in the external layer and no marked odd-even preference was present[7]. The *n*-alkane distribution is the same in all samples and centered on the C_{27}-C_{31} interval; sample 2 displayed the highest concentrations (0.1-2 µg g^{-1}). These findings are coherent with literature aliphatic hydrocarbons stalagmite data and likely indicate the presence of another source beside plant residues in soil[1,19]. Caves are known to host bacterial and fungal communities, which are able to produce and rework lipid compounds[20] and are involved in the precipitation of calcium carbonate[21,22]. As pointed out by Xie et al. (2003), the presence of microbes contributing to the distribution of *n*-alkanes complicates the interpretation of these data. Future assessment of longer time series from KNI-51 stalagmites and the use of specific indexes therein will be required to disentangle the two signals and help in source attribution.

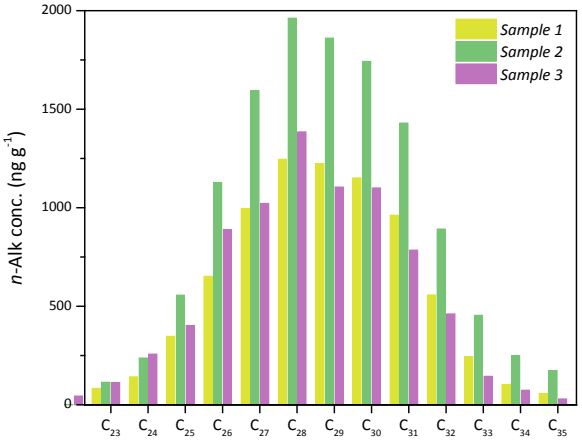

Figure 3. *n*-Alkane concentrations (ng g^{-1}) in samples from the stalagmite fragment.

Table 2. *n*-Alkanes concentrations (ng g^{-1}) in the stalagmite fragment.

	Sample 1 2008	Sample 2 2006-7	Sample 3 2004	External Layer
C22	<lod	2	39	20
C23	77	130	97	115
C24	131	269	221	213
C25	320	629	346	429
C26	600	1274	765	752
C27	917	1802	879	1049
C28	1147	2217	1191	1316
C29	1126	2103	950	1276
C30	1059	1969	946	1236
C31	885	1616	675	1061
C32	513	1008	397	644
C33	226	513	124	299
C34	95	282	64	139
C35	52	196	26	89

Conclusions

We have refined a method for the analysis of organic compounds in aragonite speleothems, based on complete digestion of the matrix followed by liquid-liquid extraction. This approach minimizes external contamination and sample amount while increasing analyte recoveries. To our knowledge, this is the only method for PAH determination in stalagmites besides the one proposed by Perrette et al. (2008). Quality check showed that, compared to the latter, our protocol has considerably better analytical performance. The method is also quite easily and quickly applicable on a routine basis in any chemical laboratory, since it does not require complex instrumentation. In addition, the wide number of analytes included increases the amount of information obtained from the record without further sample demand, a significant added value in terms of resolution and considering the scarce sample availability and the sampling effort required for speleothems.

The purpose of this work was to provide a valid tool to the developing field of organic proxy determination in speleothems. Our method was thus tested on samples from an aragonite stalagmite from cave KNI-51, which based on the preliminary results, appear to trace documented fire events. In the future, this technique will be applied to multi-decadal/centennial-scale intervals to obtain a high-resolution fire reconstruction to be compared with vegetation history and hydroclimate information provided by isotopic ratios in the same stalagmites.

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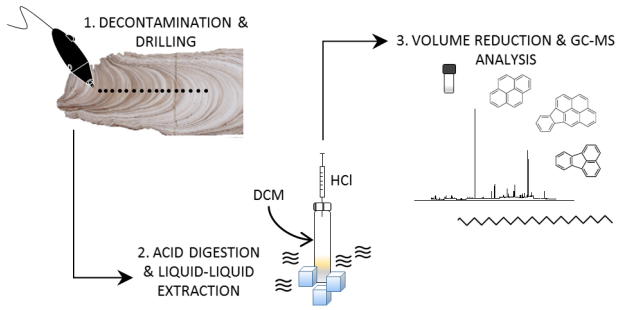

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