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Technical Note

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Improved Polycyclic Aromatic Hydrocarbon and *n*-Alkane Determination in Speleothems through Cleanroom Sample Processing

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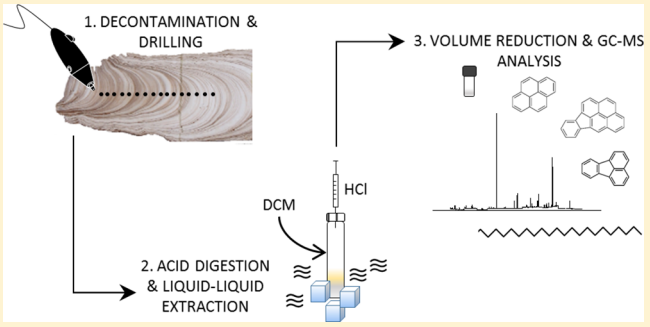
Supporting Information

ABSTRACT: Interest in paleoenvironmental reconstructions from biomarkers in speleothems is increasing, thanks in part to the capacity of speleothems to grow continuously and to resist postdepositional 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 hand-held drill, complete dissolution of the matrix in hydrochloric acid, then liquid−liquid extraction, and GC-MS analysis. Sample preparation was carried out in a 10 000 class clean room built entirely 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 in only one sample, which dates to the year ∼2004 CE, 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 because of their capacity for continuous growth, high temporal resolution, precise and accurate age dating by U/Th methods, and strong postdepositional 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, because of 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 is 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,[5−7] and their persistence in the environment allows detection in several paleoenvironmental archives, including speleothems.[4,8] Numerous studies, especially on lake sediments and peat, have demonstrated the relation of *n*-alkanes with vegetation changes and associated climatic conditions.[9−12]

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Relating fire activity and vegetation composition to temperature and precipitation reconstruction achieved through the oxygen and carbon isotopic ratios of speleothems allows 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.[13−15] Although previously published methods for the analysis of lipid biomarkers in speleothems allowed accurate and high-resolution determination and have become quite well-established,[14] only one method has involved the quantification of PAHs in stalagmites.[8] 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 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 prewashed 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.[16,17] Three samples (Figure 1) of ∼1 g were drilled from the fragment with a hand-held Dremel tool at the Department of Geology, University of Padua. 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.

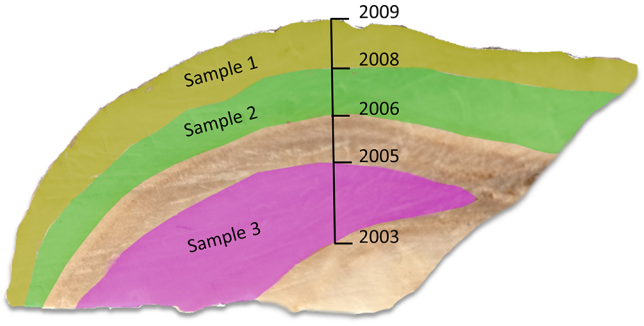

Figure 1. Subsampling and approximate age relations (in years, CE) based on U/Th dating and the zero age of the active-growth surface of the stalagmite fragment.

PAH and *n*-Alkane 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 addition of 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 dissolution was complete to avoid heating and thus volatilization of lighter analytes.[8] Dissolved samples were then vortex-extracted three times with 10 mL of 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 Figure 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.

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

Quality Control. Procedural blanks were analyzed together with samples in order to assess possible contamination. All C_{10}−C_{35} *n*-alkanes were detected in the range 1−54 abs ng, whereas for PAHs, only naphthalene, phenanthrene, fluoranthene, pyrene, retene, benzo(*e*)pyrene, benzo(*a*)pyrene, and benzo(*ghi*)perylene were detected in the range 0.2−4 abs ng.

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Blank concentrations resulted in values generally lower than those reported in the literature,[8,18] which underlines the importance of preventing laboratory contamination through "clean" procedures.

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

Recovery, precision (in terms of relative standard deviation of replicates), and accuracy (percentage relative error with respect to the spiked amounts) were estimated by spiking Carrara calcite with known amounts of native compounds and internal standards; the PAH percent recovery was between 90 and 163%, the precision range was 0.2−11%, and the accuracy was 0.2−8%. For *n*-alkanes, recovery was between 43 and 140%, the precision was 0.3−34%, and the accuracy was 0.1−12%. Overall, the analytical performances for PAHs are significantly better than the ones of the method by Perrette et al.,[8] 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, because to the best of our knowledge, none report such information.

RESULTS AND DISCUSSION

Fortified Samples. The background concentrations of analytes in the Carrara calcite were evaluated through repeated analyses, and the results for the fortified samples were corrected by the mean background values. No PAHs were detected in nonfortified Carrara calcite, while *n*-alkanes were present in low amounts (3−818 ng 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 1896 CE, including three dates between 1999 and 2009 CE.[16,19] An age model based on these dates suggests the sampled intervals correspond approximately to 2008, 2006−2007, and 2003−2004 CE. 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.[8] was complicated by the great depth of the cave and the thick and PAH-rich soil that overlie it. In contrast, soils on the hillslope over KNI-51 are thin, organic-poor, and sparse; 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 remobilization in the soil-karst system that plagued Perrette et al.[8] 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 (~2004 CE), 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 also detected in sample 1 (~2008 CE, Table 1 and Figure 2). Thus, it appears that PAHs formed during the 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),[19] quickly eroded residual PAHs from the land surface, such that dripwater contained high PAH abundances only for a short time (perhaps only ~1 year in some cases) after each fire.

Table 1. PAH Concentrations (ng g^{-1}) in 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

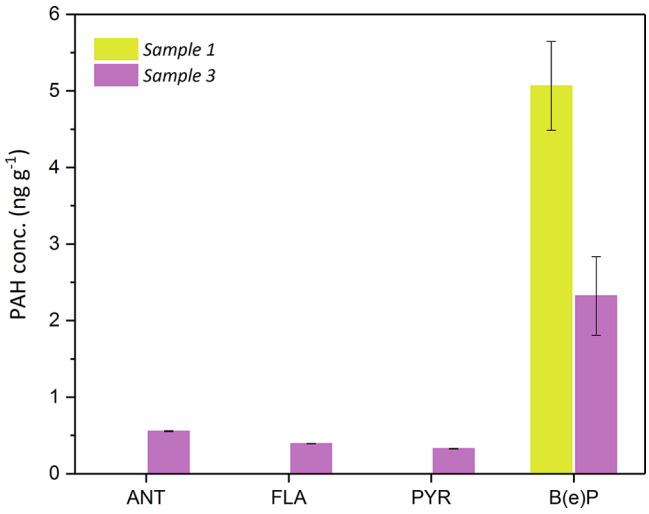

Figure 2. PAHs concentrations (ng g^{-1}) in samples from the stalagmite fragment.

The presence of indeno(1,2,3-*cd*)pyrene, produced by high-temperature combustion,[20,21] 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 the 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 those of the other PAHs considered here, which probably enhanced its precipitation together with calcite, thus resulting in an enrichment of this compound in the stalagmite.

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In sample 2 (2006−2007), all compounds were below the limit of detection, whereas 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 residues were present after digestion in HCl. In addition, PAH compounds detected in the stalagmite samples do not fully coincide with the ones present in sediments (Tables 1 and S1). For instance, no retene or benzo(*a*)pyrene were found in the calcite. This suggests a differentiation in the deposition dynamics and only a partial influence of sediments on the PAH compositions of stalagmites.

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 (Table 2 and Figure 3).[9] 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 hydrocarbon stalagmite data and likely indicate the presence of another source beside plant residues in soil.[1,22] Caves are known to host bacterial and fungal communities, which are able to produce and rework lipid compounds[23] and are involved in the precipitation of calcium carbonate.[24,25] As pointed out by Xie et al.,[22] 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.

Table 2. *n*-Alkane Concentrations (ng g^{-1}) in the Stalagmite Fragment

	sample 1 (2008)	sample 2 (2006−2007)	sample 3 (2004)	external layer
C_{22}	<LOD	2	39	20
C_{23}	77	130	97	115
C_{24}	131	269	221	213
C_{25}	320	629	346	429
C_{26}	600	1274	765	752
C_{27}	917	1802	879	1049
C_{28}	1147	2217	1191	1316
C_{29}	1126	2103	950	1276
C_{30}	1059	1969	946	1236
C_{31}	885	1616	675	1061
C_{32}	513	1008	397	644
C_{33}	226	513	124	299
C_{34}	95	282	64	139
C_{35}	52	196	26	89

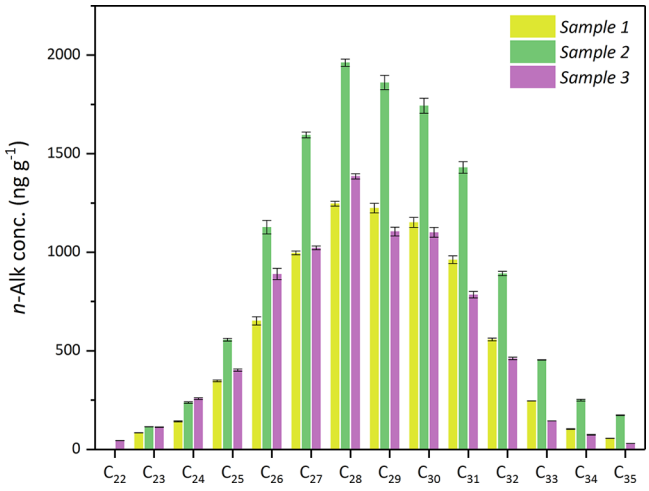

Figure 3. *n*-Alkane concentrations (ng g^{-1}) in samples from the stalagmite fragment.

CONCLUSIONS

We have refined a method for the analysis of organic compounds in aragonite speleothems on the basis of 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.[8] A quality check showed that compared with the latter method, our protocol has considerably better analytical performance. The method is also quite easily and quickly applicable on a routine basis in any chemical laboratory, because 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 on the basis of the preliminary results appear to trace documented fire events. In the future, this technique will be applied to multidecadal- and centennial-scale intervals to obtain high-resolution fire reconstruction to be compared with vegetation history and hydroclimate information provided by isotopic ratios in the same stalagmites.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.9b00767.

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Extraction procedure and results table of PAH concentrations in cave sediments (PDF)

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Notes

The authors declare no competing financial interest.

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