ResearchGate

See discussions, stats, and author profiles for this publication at: https://www.researchgate.net/publication/260842323

Quantification of low molecular weight fatty acids in cave drip water and speleothems using HPLC–ESI–IT/MS–development and validation of a selective method

ARTICLE *in* ANALYTICAL AND BIOANALYTICAL CHEMISTRY · MARCH 2014

Impact Factor: 3.44 · DOI: 10.1007/s00216-014-7743-6 · Source: PubMed

CITATIONS
5

READS
28

5 AUTHORS, INCLUDING:

Simon André Mischel
Johannes Gutenberg-Universität Mainz
3 PUBLICATIONS **9** CITATIONS
SEE PROFILE

Thorsten Hoffmann
Johannes Gutenberg-Universität Mainz
175 PUBLICATIONS **5,911** CITATIONS
SEE PROFILE

Available from: Simon André Mischel
Retrieved on: 02 February 2016

Anal Bioanal Chem (2014) 406:3167–3177
DOI 10.1007/s00216-014-7743-6

RESEARCH PAPER

Quantification of low molecular weight fatty acids in cave drip water and speleothems using HPLC-ESI-IT/MS — development and validation of a selective method

Janine M. Bosle · Simon A. Mischel · Anna-Lena Schulze · Denis Scholz · Thorsten Hoffmann

Received: 20 December 2013 / Revised: 25 February 2014 / Accepted: 3 March 2014 / Published online: 16 March 2014
© Springer-Verlag Berlin Heidelberg 2014

Abstract This study presents a novel method for the analysis of low molecular weight (LMW) fatty acids in cave drip water and speleothems. The method development included optimization of sample preparation procedures, e.g., blank reduction, solid phase extraction, concentration of extracts as well as liquid chromatography coupled to electrospray ion-trap mass spectrometry (HPLC-ESI-IT/MS) measurement parameters. Retention times for five analytes (lauric acid, myristic acid, palmitic acid, stearic acid and arachidic acid) were between 5 and 13.5 min. Spiking experiments were performed to accomplish external calibrations which ranged from 12.5 to 75 ng per spiked water sample. The correlation coefficient ranged from 0.9558 to 0.9989. Inter-batch precision, expressed as the relative standard deviation of three replicates, was <7 %. Limits of detection ranged from 0.77 to 55.97 ng for the diverse analytes; obtained recoveries varied from 30 to 103 %. For a first application, cave drip water and stalagmite samples from Herbstlabyrinth-Adventhöhle cave system were analyzed. Concentrations ranged from 38.37 to 9,982.54 ng L^{-1} for water samples and 2.52 to 1,344.96 ng g^{-1} for the stalagmite. Thereby, the different fatty acids showed a distinctive variation. Whereas shorter-chained fatty acids exhibited similarities, arachidic acid showed opposite trends. Diverse correlations were found, which could improve the understanding of different organic sources of the lipids transported by drip water and preserved in speleothems. This new method provides a more selective extraction process, particularly adjusted to LMW fatty acids and therefore reduces the required sample size. Furthermore, it is applicable to stalagmite as well as cave drip water samples.

Keywords Fatty acid · SPE · HPLC-MS · Speleothem · Cave drip water

J. M. Bosle · A.-L. Schulze · T. Hoffmann (✉)
Institute of Inorganic and Analytical Chemistry, Johannes Gutenberg-University of Mainz, Duesbergweg 10-14, 55128 Mainz, Germany
e-mail: t.hoffmann@uni-mainz.de

S. A. Mischel · D. Scholz
Institute for Geosciences, Johannes Gutenberg-University of Mainz, J.-J.-Becher-Weg 21, 55128 Mainz, Germany

Introduction

Speleothems such as stalagmites and flowstones are calcareous secondary mineral deposits in caves, which occur in karstified carbonate rocks, typically limestone and dolomite [1]. Their growth can be shortly summarized as follows: Percolating rainwater is enriched in CO_2 in the soil zone producing carbonic acid in the water and dissolving the carbonate bedrock. Due to the usually lower partial pressure of CO_2 inside the cave, CO_2 degasses from the drip water and precipitation of $CaCO_3$ occurs [2]. Speleothems have been widely used for the investigation of both climatic changes (e.g., [3–5]) and reconstruction of fluctuations in vegetation regimes [6]. They provide important paleoclimate information because they grow continuously over long time scales, preserve a record of past climate, and hydrological changes in the surrounding environment [7, 8] and can be accurately dated using the ^{230}Th/U-method [9]. While most previous studies have focused on the measurement of stable carbon and oxygen isotope ratios ($\delta^{13}C$, $\delta^{18}O$) in the calcite [7, 10, 2], or in the bulk organic matter [11] and other inorganic trace elements [12, 13], interest in the preservation of lipid biomarkers in speleothems and cave drip water has recently increased [14–19].

Lipids are biologically derived compounds, which are indicatory of different organisms. Therefore, they can identify and trace different organic sources to an environmental record [6, 20, 21]. In earlier studies of soil and sediment samples,

lipid biomarkers have been proven to be suitable as proxies for environmental and ecosystem change [22–25]. However, compared to those studies, the analysis of lipid biomarkers in stalagmites has received little previous investigation. Fatty acids were selected to be the targeted analytes of this study because they are the most elementary components of the lipid biomarkers and they have been investigated in various sample media in previous studies (e.g., [25, 26], including stalagmite samples [18, 27]. The selected compounds are saturated fatty acids with even hydrocarbon chain lengths C_{12}–C_{20}. They are low molecular weight (LMW) compounds which are mainly known to be derived from microorganisms in the overlying soil or the cave system such as bacteria, fungi and algae [15, 28], whereas high molecular weight (HMW) compounds (C_{21} and above) are indicative of higher plants [20]. However, as the vegetation composition and the microbial activities are directly correlated [29], LMW fatty acids have the potential to be used as paleoenvironmental proxies. Furthermore, another group of lipid biomarkers, glycerol dialkyl glycerol tetraethers (GDGTs), have been highlighted by recent studies [30, 31], which are also derived from microbial sources, as the LMW fatty acids. These studies indicate that the GDGTs partly originate from bacteria in the cave system or in the drip water film on the surface of the stalagmite rather than the overlying soil. Therefore, it has to be shown, if the microbial activity in the cave system is also a source [15] for the LMW fatty acids in response to changes in temperature. This demonstrates that further research has to focus on these analytes and their complex potential sources, rather than the HMW compounds, which are known to originate from higher plants. Other previous studies have also focused on fatty acids with an odd hydrocarbon chain length or branched LMW isomers, which are more specific bacterial biomarkers [15, 18, 27], preserved in stalagmite samples. These showed lower concentrations than the analytes with an even carbon number. Therefore, the method development of this study does not focus on them, although it is also applicable to this kind of analytes. Generally, fatty acids are more sensitive to alteration in sediments than other lipids (e.g., *n*-alkanes), although saturated fatty acids are less prone to degradation than unsaturated acids with the same chain lengths [21]. As more complex structures, such as triglycerides and phospholipids, are based on fatty acids, an improved understanding of fatty acids can enhance our understanding of the formation, and consequently the interpretation of these compounds as environmental proxies.

Previous studies of lipids in speleothems were based on soxhlet extraction and measurement by gas chromatography coupled to mass spectrometry (GC-MS). This method proved to be suitable for the analysis of a wide range of analytes, as demonstrated for a number of lipid groups in a Chinese stalagmite [14, 15]. However, due to the low analyte concentration and contamination problems, large samples (70–100 g) of the stalagmite were necessary. An improvement was achieved by Blyth et al. [32] and Wang et al. [33] who prepared samples using acid digestion coupled with liquid-liquid extraction resulting in substantially smaller sample sizes of up to 10 g of stalagmite. However, prior to the GC-MS analysis, a derivatization of the analytes has to occur. This is a time-consuming procedure which can lead to losses of target compounds. Furthermore, this additional sample preparation step and the necessary use of additional reagents provide a further source of contamination.

In addition to using stalagmites as paleoenvironmental archives, further research to explain how lipid biomarkers are transported by cave drip water and are preserved in the speleothems is essential. There are previous studies which describe the bulk organic matter (i.e., [34, 35]), however, studies of lipids occurring in cave drip water are limited. Yang et al. [30] analyzed GDGTs in stalagmites and drip water by LC-MS. Nevertheless, the drip water sample size required for their method was unstated. Another study by Li et al. [19] focused on fatty acids using a GC-MS method similar to Blyth et al. [32]. Their sampling of eleven drip water samples occurred over a time period of two years and required sample volumes up to 4 L. Alternative analytical methods have to be developed to reduce the required sample size for this sample material as well. Therefore, the purpose of this study was to develop and validate a selective and accurate method for both stalagmite and cave drip water samples using HPLC-MS, which does not require a derivatization of the analytes prior to measurement. While the existing GC-MS methods are able to identify a wide range of compounds, the aim of this study was to reduce the required sample size by using a more selective extraction process, particularly adjusted to LMW fatty acids.

Experimental section

Chemicals and materials

Analytical standards (≥99.5 %) of different fatty acids (lauric acid (C_{12}), myristic acid (C_{14}), palmitic acid (C_{16}), stearic acid (C_{18}) and arachidic acid (C_{20})), formic acid (additive for LC-eluents; 98 %) and hydrochloric acid (≥37 %) were obtained from Fluka. Dichloromethane (DCM), methanol, and acetonitrile (ACN) (HPLC grade solvents) were purchased from Sigma-Aldrich. An 18-MΩ water was obtained using a Milli-Q water system from Millipore (Bedford, USA). Solid phase extraction (SPE) columns (Discovery DSC-18, 3 mL tubes, 500 mg packing material) were obtained from Supelco.

Preparation of standards and spiked water samples

Stock solutions of fatty acid standards (Table 1) with concentrations of 1 mg mL^{-1} were produced by dissolving 50 mg of the solid analytical standards in 50 mL DCM followed by 5 min of sonication.

The stock solutions were further diluted (1:1,000, *v*/*v*) and stored at room temperature. Distinct volumes of these standard solutions were provided in 1.5 mL brown glass vials. They were evaporated to complete dryness at 30 °C under a gentle stream of N_2 using a gassing manifold (Reacti-Vap Evaporator, Thermo Fisher Scientific, Germany). A volume of 0.5 mL of a mixture of ACN and DCM (3:1, *v*/*v*) was added and sonicated at 35 °C for 10 min. These standards were used to optimize the LC parameters.

In order to simulate the extraction process different spiking experiments were performed. Distinct volumes of standard solutions were prepared as described above, expect for redilution which was carried out in 0.5 mL ACN. This mixture was added to 9.5 mL ultrapure water to generate a spiked water sample. These spiked water samples were used to optimize the extraction procedure during method development. The external calibration was a multi-point calibration using spiked water samples prepared with 12.5, 25, 50, and 75 ng of each analyte. Additionally, a blank of ultrapure water was used to exclude contaminations in solvents or vessels as well as to determine the analytical limits.

Reduction of contamination

To minimize potential contamination several precautions were taken. All glass vessels used for sampling, sample preparation or storage of fatty acid solutions were rinsed with ACN, deionized water and ultrapure water and then heated overnight for at least 12 h at 450 °C. As DCM was one of the main sources of contamination, it was redistilled three times prior to use in order to further decrease the blank values. All HCl solutions were freshly prepared with ultrapure water and precleaned using fivefold solvent extraction with DCM. Formic acid was stored in 1 mL aliquots to prevent cross contamination.

Table 1 Fatty acid standards and their structure

Common name	Structure	$[M-H]^-$ *m*/*z* ratio	Abbreviation
Lauric acid	$CH_3(CH_2)_{10}COOH$	199	C_{12}
Myristic acid	$CH_3(CH_2)_{12}COOH$	227	C_{14}
Palmitic acid	$CH_3(CH_2)_{14}COOH$	255	C_{16}
Stearic acid	$CH_3(CH_2)_{16}COOH$	283	C_{18}
Arachidic acid	$CH_3(CH_2)_{18}COOH$	311	C_{20}

Sampling and sample preparation

Sampling of drip water samples

Drip water samples were collected in the framework of a monthly cave monitoring program performed in the Herbstlabyrinth-Adventhöhle cave system, central Germany, since September 2010. The monitoring program is set up in a small cave chamber. Samples were taken from two instantaneous or fast dripping sites (T1 and T5, discharge approximately 21.6 L/month) and one slowly dripping site (T2, discharge around 60–150 mL/month). This study focuses only on samples from the fast dripping site T5. Thereby, 80–100 mL were sampled in pre-cleaned glass vessels over a period of some minutes to hours, depending on the drip rates.

Sampling and ^{230}Th/U-dating of stalagmite samples

Stalagmite NG01 was sampled in the Nordgang-passage of the Herbstlabyrinth-Adventhöhle cave system. The sample was taken prior to the destruction of the passage during nearby limestone quarry operations. The Nordgang-passage had a length of about 1 km and was well decorated with different types of speleothems, mostly composed of clear, transparent calcite.

The stalagmite was cut in half using a diamond-blade saw, and from one half a 1 cm thick slab was cut. This slab was again cut along the growth-axis of the speleothem. The samples for ^{230}Th/U-dating were taken from one half using a diamond-wired saw and had an average mass of 250 mg. ^{230}Th/U-dating was performed by multi-collector inductively coupled plasma mass spectrometry (MC-ICPMS). MC-ICPMS samples were prepared similarly as described by Hoffmann et al. [36] and analyzed at the Max-Planck-Institute for Chemistry (MPIC), Mainz, Germany. Details of the calibration of the mixed ^{233}U–^{236}U–^{229}Th spike are described in [37]. Analytical MC-ICPMS techniques involved a standard-sample bracketing procedure to derive correction factors for mass fractionation and Faraday cup to ion counter gain, as described in [36, 38]. All activity ratios were calculated using the decay constants from Cheng et al. [39] and corrected for detrital Th assuming a bulk Earth ^{232}Th/^{238}U weight ratio of 3.8 for the detritus and ^{230}Th, ^{234}U and ^{238}U in secular equilibrium.

Drip water and stalagmite sample preparation for fatty acid analysis

ACN (5 % (*v*/*v*)) was added to the entire drip water sample (80–100 mL) in order to prevent microbial activity during storage (4 °C), which occurred over a period of some days to months. Prior to the extraction procedure each sample was sonicated at 35 °C for 5 min.

Small pieces of stalagmite samples were drilled along a central longitudinal slice of NG01. Due to the given inaccuracy of the drilling process, the sample weight ranged between 0.5 and 3.5 g. The pieces were cleaned to remove surface contamination by sonication in a mixture of DCM and methanol (9:1, *v*/*v*) at 35 °C (2×5 min), similar to Blyth et al. [32]. Pre-cleaned 4 M HCl was then added to the samples for digestion. The mixture was allowed to stand for several hours at room temperature until the stalagmite pieces were dissolved completely. Due to the small sample sizes, it was not necessary to boil the pieces under reflux [32] in order to achieve a complete digestion, what simplified the sample preparation procedure.

Comparative method: liquid-liquid extraction (LLE)

During method development, the extraction of spiked water samples was tested using LLE, based on previous work by Blyth et al. [32]. The spiked samples were extracted with 4× 1.75 mL DCM using 5 min of sonication at 35 °C. The obtained extracts were collected in a 20 mL pre-cleaned glass vessel. After solvent removal at 30 °C under a gentle stream of N_2 using a gassing manifold, 1 mL ACN/DCM (3:1, *v*/*v*) was added and sonicated at 35 °C for 5 min. The redissolved samples were transferred to 1.5 mL brown glass vials for analysis using HPLC-MS.

Solid phase extraction

The spiked water samples, cave drip water and digested stalagmite samples were extracted by SPE using a Visiprep solid phase extraction vacuum manifold (Supelco, Germany). Prior to the extraction process, 30 % (*v*/*v*) ACN was added to the samples as an organic modifier. Furthermore, 0.4 M HCl was added to reduce the pH value below 4.

The C_{18}-SPE column was cleaned and conditioned with 3 mL DCM, ACN and ultrapure water. Using a Visiprep large-volume sampler (Supelco, Germany), the sample was passed through the SPE cartridge. Afterwards, 1 mL of a mixture of methanol and ultrapure water (2:1, *v*/*v*) was rinsed through as well to wash the packing. Prior to elution, the SPE column was dried under N_2 for 20 min. The analytes were eluted with 6× 1 mL DCM, which was stepwise collected in a 1.5 mL brown glass vial followed by removing the solvent. Afterwards, 525 μL ACN/DCM (3:1, *v*/*v*) was added and sonicated at 35 °C for 5 min.

Furthermore, a standard addition method was applied to each natural sample. Five 100 μL aliquots were spiked with 0, 12.5, 25, 50, and 75 μL of the standard solution of each fatty acid. The solvent was evaporated again, and the analytes were redissolved in 150 μL ACN/DCM (3:1, *v*/*v*). The samples were transferred to vial inlets for analysis using HPLC-MS.

HPLC-ESI-IT/MS conditions

Each HPLC-ESI-IT/MS experiment was performed using a HCT-Plus ion-trap (IT) mass spectrometer (Bruker-Daltonics GmbH, Bremen, Germany) equipped with an HPLC system (Agilent 1100 series: auto sampler, gradient pump, degasser; Agilent Technologies GmbH, Germany). The separation was achieved using a reversed phase Pursuit XRs 3 C8 150× 2.0 mm analytical column with 3 μm particle size (Varian, Germany) constantly heated at 50 °C using a CO20 HPLC column oven (Torrey Pines Scientific, USA).

Eluents were ultrapure water with 400 μL L^{-1} formic acid and 2 % ACN (eluent A) and ACN with 2 % of ultrapure water (eluent B). The applied gradient program started with 80 % of eluent B, which was increased within 5 min to 99 %. This composition was held for further 20 min and during the post run time (10 min) eluent B decreased to the initial conditions of 80 % again. The injection volume was 20 μL and all measurements were carried out at a flow rate of 200 μL min^{-1}.

Mass spectrometric detection was performed operating the ESI source in negative ion mode. The conditions used were: nebulizer pressure (N_2) 35 psi, dry gas flow (N_2) 9 L min^{-1}, dry gas temperature 300 °C, and capillary voltage −4.5 kV. The ESI source generated deprotonated molecular ions ($[M-H]^-$) of different *m*/*z* ratios, which are listed in Table 1. Selectivity of the detection was enhanced using multiple reaction monitoring (MRM).

Results and discussion

Method development

Separation and MS detection

The HPLC method was optimized to accomplish an appropriate chromatographic separation of the analytes and short retention times (Fig. 1). As the solubility of fatty acids in polar solvents is low due to their nonpolar hydrocarbon chain a starting concentration of 80 % eluent B was required to avoid peak broadening and shorten the total run time of the measurement. By using the gradient program, as described in the "Experimental section", baseline separation within 13.5 min was achieved.

The extracted ion chromatogram (EIC) of C_{20} (*m*/*z* 311) shows another signal additional to the actual analyte signal (No. 5 in Fig. 1). System blanks demonstrated that this signal originates from the ACN in eluent B. During experiments in which ACN was replaced by methanol, this noise signal was eliminated. However, as methanol reduces the ionization efficiency in the ESI source, ACN produced improved overall results. As shown in Fig. 1, a sufficient separation of C_{20} from the noise signal was accomplished by heating the column during separation to 50 °C.

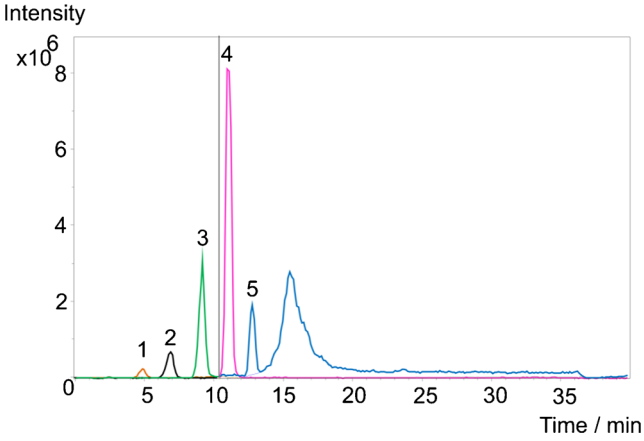

Fig. 1 Chromatographic separation of the fatty acids (*1* C_{12}, *2* C_{14}, *3* C_{16}, *4* C_{18}, *5* C_{20}); shown as their extracted ion chromatograms (EIC) in ESI(−)-mode (MRM *m*/*z* see Table 1)

As described above, a detection of the targeted compounds as deprotonated molecular ions ($[M-H]^-$) in ESI(−)-mode was possible. Consequently, no time-consuming derivatization of the fatty acids was necessary. This procedure is typically needed for measurements using GC-MS as described in earlier studies [14, 19, 32]. An additional sample preparation step as derivatization includes the danger of analyte losses and poorer reproducibility. Furthermore, derivatization can introduce contamination from the used reagents. Thus, for the analysis of the targeted fatty acids, the use of HPLC-MS in ESI(−)-mode has an advantage over GC-MS measurements.

Blank optimization

High blank values of C_{16} and C_{18} indicate a contamination of the glass vessels and organic solvents. A comparison of the EICs of C_{18} acquired by sample preparation with merely rinsed and heated glassware proofed, that heating resulted in a drastic reduction of the background values of the C_{18} acid. These blank values were quantified by external calibration and the heated glassware provided a reduction from originally about 350 ng for rinsed glassware to approximately 10 ng. However, the background signal of all analytes could be substantially reduced. Hence, in order to achieve reproducible and low background values, the pre-cleaning and heating of all glass vessels, as described in the "Experimental section", is essential. Additionally, DCM was redistilled three times prior to use, which resulted in a further decrease of 70–75 % of the blank values of C_{16} and C_{18}.

SPE

SPE conditions were optimized for conditioning, washing and drying the SPE columns as well as elution. After the sample passed through the SPE column, a mixture of methanol and ultrapure water (2:1, *v*/*v*) was used to remove the remaining matrix components, which are of higher polarity than the investigated compounds. Afterwards, the cartridge was dried under a gentle N_2 stream for 20 min to clear the packing material from residual water, which would not be soluble in DCM during elution. This residual water would lead to incomplete elution of the analytes and considerably longer time to evaporate the solvent in the subsequent sample preparation step. Drying for longer than 30 min resulted in loss of target compounds. Elution was accomplished with 6× 1 mL DCM. A 1 mL volume is optimal, providing enough solvent to cover the packing material without using too large a volume that blank values are increased. As demonstrated in measurements of pure DCM samples redissolved in ACN, the solvent is one of the main sources of contamination and a higher amount of DCM showed an increase of the peak area of the blank signal.

After SPE, the solvent of the eluate was removed. Therefore, conditions of the concentration procedure were optimized for evaporation temperature, composition of the solvent for redilution as well as run time and temperature of the ultrasonic bath. Solvents were tested for their efficiency to dissolve fatty acid residues after removal of DCM. The most efficient solvent composition was observed to be ACN/DCM (3:1, *v*/*v*). Whereas pure ACN showed less solubility for the analytes with longer hydrocarbon chains (C_{18}, C_{20}), higher percentages of DCM resulted in severe peak fronting during the separation using HPLC. Subsequently, sonication carried out for longer than 5 min offered no further increase in the amount of extracted analyte. The temperature of the ultrasonic bath was set to 35 °C since the solubility of C_{20} was raised by slightly higher temperatures.

The extraction procedure as described above is more specific for the investigated analytes than LLE. Investigations using LLE showed that this extraction method does not offer the required selectivity to remove matrix interferences. Due to this, matrix effects in the ESI source occurred, leading to a decrease in the measured intensity of target analytes. For this reason, the LLE method was not used for further experiments with natural samples. Our results show that the SPE method provides a more efficient removal of matrix interferences.

Certainly, the lipid concentration varies between stalagmites from different sampling sites and there exists a previous study by Blyth et al. [6] using approximately 1 g sample sizes of a stalagmite with a growth rate of about 0.2 to 0.3 mm per year. However, by focusing on the LMW fatty acids, the extraction process and concentration procedure as described above enables to work with smaller sample sizes (min. 0.5 g) than in most other previous studies [14, 18, 32] improving the temporal resolution of analyzing the targeted fatty acids in speleothems as climate archives. The efficiency of this method has to be confirmed by analyzing different, independent samples from various locations. Additionally, the procedure also reduces the required sample volume for drip water in comparison to an earlier study [19].

Table 2 Tested modifier compositions and their corresponding RSD values

Addition modifier/% in 10 mL	Composition modifier/mL	RSD of three replicates/%
20	1.5 ACN+0.5 methanol	15–40
25	1.5 ACN+1 methanol	8–15
30	1.5 ACN+1.5 methanol	0.3–11
30	1 ACN+2 methanol	16–31
30	2 ACN+1 methanol	5–20
30	3 ACN	0.7–7

Validation

Reproducibility

To ensure adequate inter-batch precision of the procedure, three spiked water replicates with the same amount of target compounds were generated and treated identically. The compositions of an ACN/methanol mixture, which was added as an organic modifier, were systematically changed as shown in Table 2. These modifiers prevent loss of analytes during the SPE procedure by keeping the analytes in solution instead of adsorption to the glass surface. The results are listed in Table 2.

By adding 30 % (*v*/*v*) ACN the relative standard deviation (RSD) of the replicates ranged from 0.7 % to 7 %. Furthermore, for optimum analyte retention during SPE, the pH value of the spiked and natural samples had to be adjusted below 4 to guarantee that the fatty acids are not dissociated.

Calibration

Calibrations with spiked water samples extracted by SPE have been performed. The linear regression analysis was carried out by plotting the respective peak areas against the spiked amount of the analytes in nanograms. The results are summarized in Table 3.

The correlation coefficient R expresses linearity whereas the goodness of fit is demonstrated by the coefficient of determination R^2. The limits of detection (LOD) for each fatty acid were calculated by three times the standard deviation of the blank samples, divided by the slope of the calibration function (3σ/m). In the same manner, limits of quantification (LOQ) were determined (10σ/m). The RSD was calculated for all spiked concentrations, except for the blank, as a measure of precision.

Table 3 Results of linear regression analysis

	C_{12}	C_{14}	C_{16}	C_{18}	C_{20}
Slope	4.37E+04	1.40E+05	5.65E+05	1.36E+05	1.10E+05
Intercept	6.36E+04	1.36E+06	1.47E+08	2.81E+07	6.63E+05
R	0.9989	0.9936	0.9973	0.9558	0.9974
R^2	0.9979	0.9873	0.9946	0.9136	0.9948
RSD/%	0.9–1.4	0.1–6	1.1–2	3–8.6	0.1–3.4
LOD/ng	1.23	0.77	3.81	55.97	2.54
LOQ/ng	4.08	2.57	12.69	186.56	8.48

Recovery

Determination of the recovery (*R*) was accomplished using spiked water samples with different amounts of fatty acids (25, 75 and 150 μL of the standard solution) and a blank sample. The measured masses were compared with the actual spiked masses. Therefore, the values obtained by the blank sample were subtracted from the spiked samples. The mean of the results from different concentrated spiked water samples was calculated to determine the recovery of each target compound. Table 4 lists the obtained results.

C_{12}, C_{14}, C_{16}, and C_{18} show adequate recoveries while the recovery for C_{20} is lower. Because of its long hydrocarbon chain and therefore most nonpolar character, the C_{20} acid possesses the lowest solubility in our chosen solvents. Therefore, the redilution steps prior to the spiking procedure and measurement by HPLC-MS in ACN/DCM (3:1, *v*/*v*) are critical steps for C_{20}. Particularly, at low concentrations of the C_{20} compound, the low recovery can be problematic. Therefore, sonication is essential during these steps in order to improve the solubility of C_{20} as described during the discussion of the SPE procedure. However, the conditions were optimized to achieve the best possible results and the determined concentrations (c_0) of natural drip water and stalagmite samples were corrected for the obtained recovery. An improvement of the recovery also for longer-chained HMW fatty acids (C_{20} and higher) could be realized via additions of higher amounts of DCM, although this comprises the risk of increasing blank values. Another possibility could be the application of longer sonication times and the use of nonpolar solvents.

Application

Drip water samples

The method introduced in this paper was applied to cave drip water samples sampled monthly from drip site T5 in the Herbstlabyrinth-Adventhöhle cave system as described

Table 4 Calculated recovery

	C_{12}	C_{14}	C_{16}	C_{18}	C_{20}
R/%	97±4	103±8	101±8	68±8	30±7

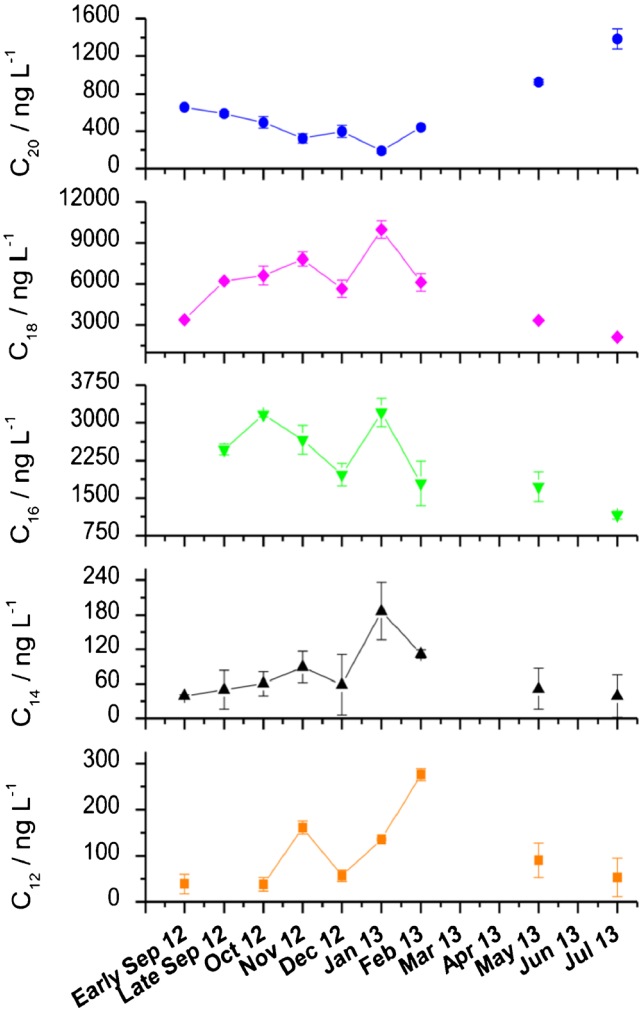

Fig. 2 Measured fatty acid concentrations (C_{12}–C_{20}) in cave drip water sampled at fast drip site T5, Herbstlabyrinth-Adventhöhle cave system

in the "Experimental section". Fatty acid concentrations were determined using a standard addition method to compensate remaining matrix effects. Figure 2 and Table 5 illustrate the obtained results and the corresponding standard deviations (SD).

All targeted compounds were observed in the samples, at a varying range of concentrations. Whereas C_{12}, C_{14}, and C_{20} were present at concentrations between 38.37 and 1,384.47 ng L^{-1} with a RSD range between 1.5 and 95 %, C_{16} and C_{18} show much higher concentrations between 1,157.26 and 9,982.54 ng L^{-1} with RSDs between 1.3 and 25 %. There is a variation in the obtained standard deviations for the different fatty acids. These variations in the standard deviations could be caused by different processes. On the one hand, matrix components can variably influence the ionization in the ESI source and lead to these deviations. On the other hand, the stability of the spray in the ionization source can be altered and thereby affects the ionization process. Therefore, standard addition is the best option for quantification in order to minimize these effects.

Nonetheless, for each analyte, a seasonal variation over the sampling period was observed. Considering the error bars, certain fatty acids generally show similar trends. Hence, C_{14} and C_{18} were most abundant in January 2013, with concentrations declining by summer (early September 2012, also seen in July 2013) and reaching a local minimum in December 2012. The similar pattern for these two analytes is supported by the strong positive correlation coefficient of 0.83 (p=0.007). In addition, a positive correlation of 0.87 (p=0.005) between C_{16} and C_{18} was observed, while C_{12} and C_{14} show a similar trend, but no significant positive correlation.

The opposite behavior was observed for C_{20}. The highest concentrations occurred in September 2012 and May/July 2013 and a distinctive negative correlation to C_{18} and C_{16}, with correlation coefficients of -0.88 (p=0.002) and -0.76 (p=0.028) respectively was found. The corresponding correlation plots are illustrated in Fig. 3.

Table 5 Results of drip water analysis and corresponding SD

	Concentration (c)[a]/ng L^{-1} (SD/ng L^{-1})				
	C_{12}	C_{14}	C_{16}	C_{18}	C_{20}
Early Sep 12	39.13 (21.76)	39.04 (1.90)	–	3387.76 (68.86)	658.12 (13.77)
Late Sep 12	–	49.61 (33.77)	2469.48 (108.64)	6222.49 (133.72)	590.15 (27.08)
Oct 12	38.37 (14.78)	60.28 (21.17)	3161.35 (46.73)	6631.59 (689.90)	493.96 (65.12)
Nov 12	161.62 (14.09)	89.35 (27.22)	2661.47 (286.13)	7829.40 (515.31)	322.92 (48.88)
Dec 12	56.82 (12.31)	58.32 (52.81)	1965.70 (226.96)	5661.29 (635.28)	395.77 (66.62)
Jan 13	135.80 (2.04)	186.46 (50.02)	3200.90 (279.96)	9982.54 (654.88)	190.56 (25.78)
Feb 13	276.11 (12.98)	111.71 (7.12)	1794.42 (443.58)	6126.37 (651.52)	439.81 (14.92)
May 13	90.75 (37.58)	51.25 (35.48)	1728.61 (293.82)	3339.38 (43.29)	923.78 (29.48)
Jul 13	53.46 (41.90)	38.73 (36.96)	1157.26 (80.21)	2119.67 (40.53)	1384.47 (108.65)

[a] Correction for recovery: $c = c_0 \times \left(\frac{100}{R}\right)$

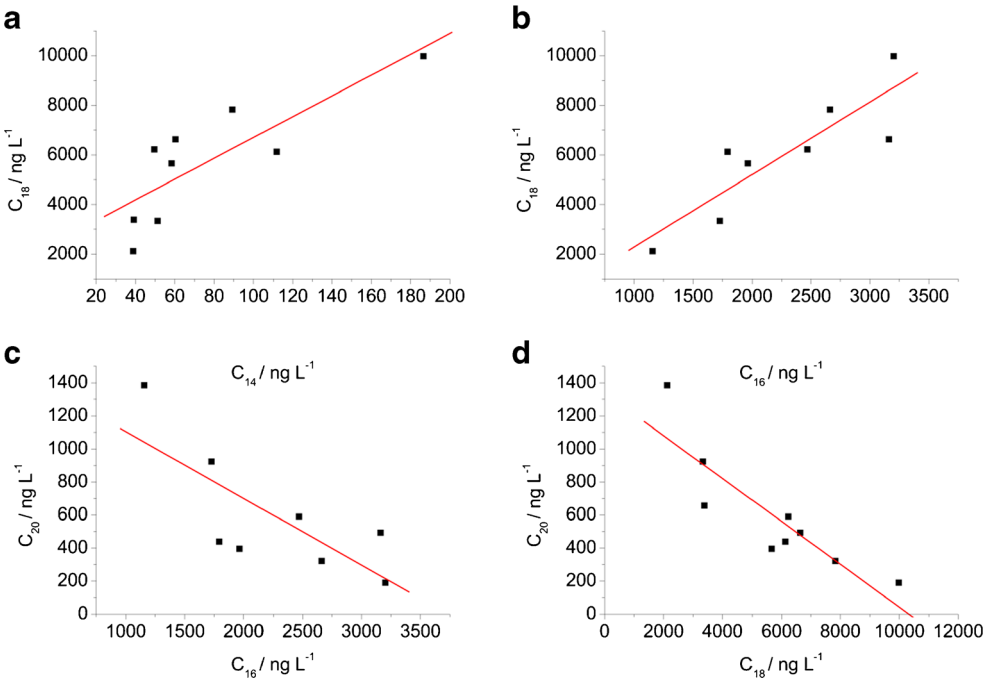

Fig. 3 Correlation plots of **a** C_{14} and C_{18}, **b** C_{16} and C_{18}, **c** C_{16} and C_{20}, **d** C_{18} and C_{20} in drip water from drip site T5

Additionally, the correlation between C_{20} and both C_{12} and C_{14} is also negative, but no significant correlation coefficients were calculated. In general, the concentration of C_{20} decreases during winter whereas the concentrations of the other four fatty acids increase. Although C_{20} is categorized as a LMW compound, just as C_{12}–C_{18} [15, 28], our results indicate that C_{20} found in the drip water samples has a source not shared with the other fatty acids. Potentially, this fatty acid does not originate from the same microorganisms, as previously stated, but is derived from higher vegetation or the cave system. Another possibility is that C_{20} decomposes differently in the soil than C_{12}-C_{18}. Further work to understand sources and removal of C_{20} in other regions would enhance our understanding in this area.

Stalagmite samples

Another application was the stalagmite NG01 sampled from the same cave system. The stalagmite was dated as described in the "Experimental section" and the samples for fatty acid analysis were quantified using the same standard addition method as for the drip water. The results are illustrated in Fig. 4 and Table 6. NG01 shows three major growth phases and consequently two growth stops between approximately 8,450 and 7,750 as well as between 4,900 and 2,560 years before present.

Generally, C_{16} and C_{18} also exhibit much higher concentrations than the other four target compounds. Obtained concentrations of C_{12}, C_{14}, and C_{20} ranged from 2.52 to 86.92 ng g^{-1} with RSD values between 0.1 and 57.5 %. In contrast, C_{16} and C_{18} show concentrations

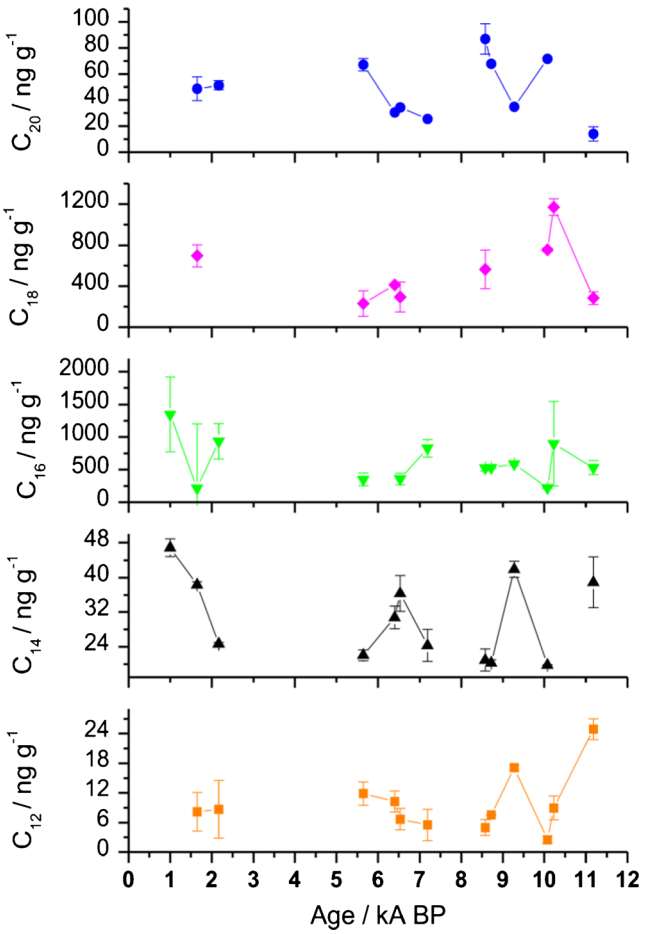

Fig. 4 Fatty acid records (C_{12}-C_{20}) of stalagmite NG01, Herbstlabyrinth-Adventhöhle cave system

Table 6 Results of stalagmite NG01 analysis and corresponding SD

Depth/cm	Age/kA BP (+/−error/kA)	Age/kA BP	c^a/ng g^{-1} (SD/ng g^{-1})				
			C_{12}	C_{14}	C_{16}	C_{18}	C_{20}
1.9	1.00 (0.54/−0.16)	1.00	–	46.85 (1.97)	1344.96 (571.71)	–	–
3.4	1.65 (0.19/−0.12)	1.65	8.17 (3.91)	38.35 (0.72)	214.61 (986.64)	697.02 (107.91)	48.70 (9.09)
4.8	2.17 (0.15/−0.19)	2.17	8.68 (5.86)	24.68 (0.31)	936.44 (272.41)	–	51.40 (3.50)
9.9	5.65 (0.18/−0.07)	5.65	11.86 (2.38)	22.06 (1.25)	349.25 (98.27)	231.16 (125.67)	67.05 (4.74)
14.8	6.40 (0.05/−0.05)	6.40	10.27 (2.15)	30.75 (2.61)	–	413.57 (9.76)	30.58 (0.03)
15.8	6.53 (0.23/−0.06)	6.53	6.69 (2.17)	36.33 (4.15)	357.19 (88.89)	295.22 (146.25)	34.36 (0.59)
21	7.19 (0.14/−0.11)	7.19	5.51 (3.17)	24.31 (3.68)	828.41 (135.55)	–	25.65 (0.75)
28.5	8.58 (0.13/−0.15)	8.58	4.99 (1.62)	20.97 (2.55)	526.36 (48.00)	564.49 (188.15)	86.92 (11.68)
29.6	8.72 (0.12/−0.13)	8.72	7.54 (0.05)	20.29 (0.69)	528.34 (15.20)	–	67.74 (0.95)
34	9.28 (0.08/−0.08)	9.28	17.12 (0.52)	41.91 (1.84)	584.84 (19.81)	–	34.90 (0.66)
40	10.08 (0.13/−0.07)	10.08	2.52 (0.67)	19.81 (0.02)	218.47 (6.94)	755.09 (18.34)	71.69 (0.95)
41.1	10.23 (0.13/−0.07)	10.23	8.96 (2.44)	–	897.17 (649.20)	1169.90 (80.64)	–
48.9	11.18 (0.10/−0.09)	11.18	24.88 (2.08)	38.88 (5.88)	530.92 (106.98)	284.22 (61.13)	14.12 (5.51)

[a] Correction for recovery: $c = c_0 \times \left(\frac{100}{R}\right)$

from 214.61 to 1,344.96 ng g^{-1} with a RSD range between 2.4 and 459 %. The error range of C_{16} in the stalagmite samples is larger than for the drip water samples, because of the high SD value at 1,650 years before present. For unknown reasons, this sample showed an exceptionally high RSD of 459 %. The SD values of the other analytes coincide with the range of the drip water samples.

The fatty acid distribution in the stalagmite samples matches the results of the drip water samples and also reflects the natural occurrence of these fatty acids. This distribution is a consistent finding throughout the literature (e.g., [15, 17]. However, the different fatty acids still show similar trends in different periods. C_{14} and C_{20} show a negative correlation with a correlation coefficient of −0.71 (p=0.014) over all three periods (Fig. 5a). This is similar to their correlation in the drip water samples as discussed above.

C_{12} on the other hand is positively correlated to C_{20} during the two youngest periods (between 7,750 and 200 years before present). In contrast, during the oldest period (between 11,200 and 8,450 years before present), they show a distinctive negative correlation with a correlation coefficient of −0.96 (p= 0.009) as can be seen in Fig. 5b. The results obtained from C_{16} exhibit an entirely different record as there is no significant correlation to C_{12}, C_{14}, and C_{20}. C_{16} and C_{18} seem to be positively correlated during the oldest period, although there is not enough data of C_{18} to calculate a reliable correlation coefficient. Nevertheless, there is a significant variation in fatty acid composition in different depths of the stalagmite. These results show that the method described in this paper permits quantification of fatty acids in both drip water and speleothem $CaCO_3$. As already demonstrated in previous studies, the internal variation of the fatty acids shows that they can be used to obtain information about microbial and environmental influences on speleothems and drip water processes. This study provides an alternative analytical method to analyze these proxies. In addition, the extraordinary attitude of C_{20} in this sample

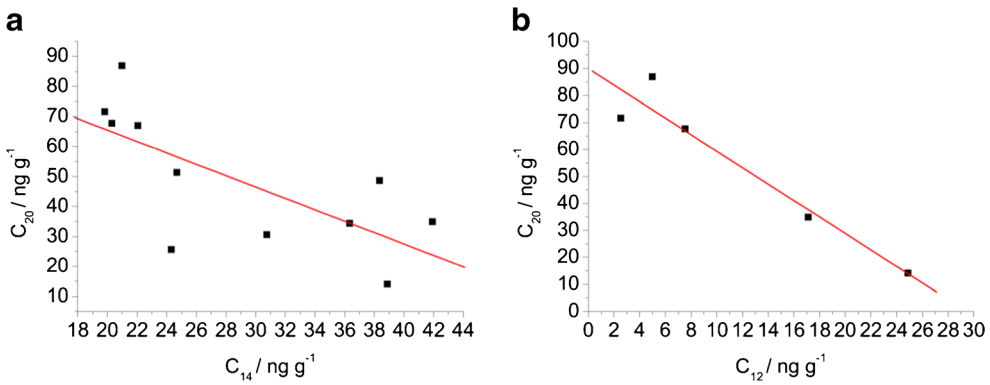

Fig. 5 Correlation plots of **a** C_{14} and C_{20}, **b** C_{12} and C_{20} in stalagmite NG01

material matching the drip water results needs further research with stalagmite samples from different sampling sites.

Conclusions

A selective method for quantification of LMW fatty acids in cave drip water and stalagmite samples using HPLC-ESI-IT/MS was developed. The validation of the method ensured precise and accurate results. The method provides an optimized approach for the extraction and analysis of the targeted fatty acids present in stalagmites and cave drip water, which can be used in paleoenvironmental studies. First applications proved the procedure to be effective. In comparison to earlier studies, it was not necessary to derivatize the targeted compounds, which is time consuming and can result in a loss of analyte and further contamination. Furthermore, sample sizes of the stalagmite samples have been reduced in comparison to most previous studies, which directly translates into an improvement in temporal resolution for these targeted LMW compounds. The extraction method is not limited to stalagmites and cave drip water; it may also be operated on other water samples with a similar matrix, such as water inclusions in sediments for example.

First applications indicate an unexpected behavior of C_{20}, which partly shows opposite results than the shorter chained fatty acids. Further experiments with a larger sample number are in progress to investigate these findings. Future work should focus on correlations of the concentrations of the target analytes with selected climate proxies, such as carbon and oxygen isotopes, which will provide an even better insight in the interpretation of fatty acids as environmental proxies.

Acknowledgments J. M. Bosle and D. Scholz acknowledge support by the Geocycles-Earth System Research Center, University of Mainz, Germany. S. A. Mischel and D. Scholz are thankful for funding of the German Research Foundation (DFG grant SCHO 1274/3-1). The authors also thank the anonymous reviewers, who provided useful comments on the manuscript.

References

1. Fairchild I, Frisia SBA, Tooth A (2007) Speleothems. In: Nash DJ, McLaren SJ (eds) Geochemical sediments and landscapes. Blackwell, Oxford
2. Fairchild IJ, Smith CL, Baker A, Fuller L, Spötl C, Mattey D, McDermott F, E.I.M.F. (2006) Modification and preservation of environmental signals in speleothems. Earth-Sci Rev 75(1–4):105–153
3. Lauritzen S, Lundberg J (1999) Speleothems and climate: a special issue of The Holocene. The Holocene 9(6):643–647
4. McGarrry SF, Baker A (2000) Organic acid fluorescence: applications to speleothem palaeoenvironmental reconstruction. Quatern Sci Rev 19:1087–1101
5. Scholz D, Frisia S, Borsato A, Spötl C, Fohlmeister J, Mudelsee M, Miorandi R, Mangini A (2012) Holocene climate variability in north-eastern Italy: potential influence of the NAO and solar activity recorded by speleothem data. Clim Past 8:1367–1383
6. Blyth AJ, Asrat A, Baker A, Gulliver P, Leng MJ, Genty D (2007) A new approach to detecting vegetation and land-use change using high-resolution lipid biomarker records in stalagmites. Quat Res 68(3):314–324
7. McDermott F (2004) Palaeo-climate reconstruction from stable isotope variations in speleothems: a review. Quat Sci Rev 23(7–8):901–918
8. Rushdi AI, Clark PU, Mix AC, Ersek V, Simoneit BRT, Cheng H, Edwards RL (2011) Composition and sources of lipid compounds in speleothem calcite from southwestern Oregon and their paleoenvironmental implications. Environ Earth Sci 62(6):1245–1261
9. Richards DA, Dorale JA (2003) Uranium-series chronology and environmental applications of speleothems. Rev Mineral Geochem 52:407–460
10. Dykoski C, Edwards R, Cheng H, Yuan D, Cai Y, Zhang M, Lin Y, Qing J, An Z, Revenaugh J (2005) A high-resolution, absolute-dated Holocene and deglacial Asian monsoon record from Dongge Cave, China. Earth Planet Sci Lett 233(1–2):71–86
11. Blyth AJ, Shutova Y, Smith C (2013) δ13C analysis of bulk organic matter in speleothems using liquid chromatography–isotope ratio mass spectrometry. Org Geochem 55:22–25
12. Fairchild IJ, Borsato A, Tooth AF, Frisia S, Hawkesworth CJ, Huang YM, McDermott F, Spiro B (2000) Controls on trace element Sr–Mg/compositions of carbonate cave waters: implications for speleothem climatic records. Chem Geol 166:255–269
13. Wassenburg JA, Immenhauser A, Richter DK, Niedermayr A, Riechelmann S, Fietzke J, Scholz D, Jochum KP, Fohlmeister J, Schröder-Ritzrau A, Sabaoui A, Riechelmann DFC, Schneide L, Esper J (2013) Moroccan speleothem and tree ring records suggest a variable positive state of the North Atlantic Oscillation during the Medieval Warm Period. Earth Planet Sci Lett 375:291–302
14. Xie S, Yi Y, Huang J, Hu C, Cai Y, Collins M, Baker A (2003) Lipid distribution in a subtropical southern China stalagmite as a record of soil ecosystem response to paleoclimate change. Quat Res 60:340–347
15. Xie S, Huang J, Wang H, Yi Y, Hu C (2005) Distributions of fatty acids in a stalagmite related to paleo-climate change at Qingjiang in Hubei, southern China. Sci China Ser D 48(9):1463
16. Blyth AJ, Baker A, Collins MJ, Penkman KE, Gilmour MA, Moss JS, Genty D, Drysdale RN (2008) Molecular organic matter in speleothems and its potential as an environmental proxy. Quat Sci Rev 27(9–10):905–921
17. Blyth AJ, Watson JS, Woodhead J, Hellstrom J (2010) Organic compounds preserved in a 2.9million year old stalagmite from the Nullarbor Plain, Australia. Chem Geol 279(3–4):101–105
18. Blyth AJ, Baker A, Thomas LE, van Calsteren P (2011) A 2000-year lipid biomarker record preserved in a stalagmite from north-west Scotland. J Quat Sci 26(3):326–334
19. Li X, Wang C, Huang J, Hu C, Xie S (2011) Seasonal variation of fatty acids from drip water in Heshang Cave, central China. Appl Geochem 26(3):341–347
20. Brassel SC, Eglinton G, Marlowe IT, Pflaumann U, Sarnthein M (1986) Molecular stratigraphy: a new tool for climatic assessment. Nature 320:129–133
21. Meyers PA (1997) Organic geochemical proxies of paleoceanographic, paleolimnologic, and paleoclimatic processes. Org Geochem 27:213–250

22. Kögel-Knabner I (2002) The macromolecular organic composition of plant and microbial residues as inputs to soil organic matter. Soil Biol Biochem 34:139–162
23. Nott CJ, Xie S, Avsejs LA, Maddy D, Chambers FM, Evershed RP (2000) n-Alkane distributions in ombrotrophic mires as indicators of vegetation change related to climatic variation. Org Geochem 31: 231–235
24. Bull ID, van Bergen PF, Nott CJ, Poulton PR, Evershed RP (2000) Organic geochemical studies of soils from the Rothamsted classical experiments-V. The fate of lipids in different long-term experiments. Org Geochem 31:389–408
25. Zocatelli R, Lavrieux M, Disnar J, Milbeau C, Jacob J, Bréheret JG (2012) Free fatty acids in Lake Aydat catchment soils (French Massif Central): sources, distributions and potential use as sediment biomarkers. J Soils Sediments 12(5):734–748
26. van Dongen BE, Rijpstra WIC, Philippart CJM, de Leeuw JW, Sinninghe Damste JS (2000) Biomarkers in upper Holocene Eastern North Sea and Wadden Sea sediments. Org Geochem 31:1533–1543
27. Huang X, Cui J, Pu Y, Huang J, Blyth AJ (2008) Identifying "free" and "bound" lipid fractions in stalagmite samples: an example from Heshang Cave, Southern China. Appl Geochem 23(9):2589–2595
28. Carrie RH, Mitchell L, Black KD (1998) Fatty acids in surface sediment at the Hebridean shelf edge, west of Scotland. Org Geochem 29(5–7):1583–1593
29. Ohtonen R, Väre H (1998) Vegetation composition determines microbial activities in a boreal forest soil. Microb Ecol 36:328–335
30. Yang H, Ding W, Zhang CL, Wu X, Ma X, He G, Huang J, Xie S (2011) Occurrence of tetraether lipids in stalagmites: Implications for sources and GDGT-based proxies. Org Geochem 42(1):108–115
31. Blyth AJ, Schouten S (2013) Calibrating the glycerol dialkyl glycerol tetraether temperature signal in speleothems. Geochim Cosmochim Acta 109:312–328
32. Blyth AJ, Farrimond P, Jones M (2006) An optimised method for the extraction and analysis of lipid biomarkers from stalagmites. Org Geochem 37(8):882–890
33. Wang C, Zhang H, Huang X, Huang J, Xie S (2012) Optimization of acid digestion conditions on the extraction of fatty acids from stalagmites. Front EarthSci 6(1):109–114
34. Baker A, Barnes WL, Smart PL (1997) Variations in the discharge and organic matter content of stalagmite drip waters in Lower Cave, Bristol. Hydrol Process 11:1541–1555
35. Ban F, Pan G, Zhu J, Cai B, Tan M (2008) Temporal and spatial variations in the discharge and dissolved organic carbon of drip waters in Beijing Shihua Cave. China Hydrol Proc 22(18):3749–3758
36. Hoffmann DL, Prytulak J, Richards DA, Elliott T, Coath CD, Smart PL, Scholz D (2007) Procedures for accurate U and Th isotope measurements by high precision MC-ICPMS. Int J Mass Spectrom 264(2–3):97–109
37. Žák K, Richter DK, Filippi M, Živor R, Deininger M, Mangini A, Scholz D (2012) Coarsely crystalline cryogenic cave carbonate—a new archive to estimate the Last Glacial minimum permafrost depth in Central Europe. Clim Past 8(6): 1821–1837
38. Jochum KP, Wilson SA, Abouchami W, Amini M, Chmeleff J, Eisenhauer A, Hegner E, Iaccheri LM, Kieffer B, Krause J, McDonough WF, Mertz-Kraus R, Raczek I, Rudnick RL, Scholz D, Steinhoefel G, Stoll B, Stracke A, Tonarini S, Weis D, Weis U, Woodhead JD (2011) GSD-1G and MPI-DING reference glasses for in situ and bulk isotopic determination. Geostand Geoanal Res 35(2): 193–226
39. Cheng H, Edwards RL, Hoff J, Gallup CD, Richards DA, Asmerom Y (2000) The half-lives of uranium-234 and thorium-230. Chem Geol 169:17–33