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SHORT COMMUNICATION

# Optimization of acid digestion conditions on the extraction of fatty acids from stalagmites

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**Abstract** Lipids in stalagmites have been shown the potential for the paleoclimate reconstruction. However, the low lipid content leads to the difficulty in gaining high resolution lipid record in stalagmites because large mass of samples are required. Previous studies have validated that the acid digestion can improve the yield of lipids, especially fatty acids (FAs) and 3-hydroxy fatty acids (3-OH-FAs). In order to obtain more content of FAs and 3-OH-FAs with limited stalagmite sample weight, we investigate here how the acid digestion conditions (HCl concentration, heating temperature and time duration) could affect the yields of FAs and 3-OH-FAs. Under different concentration of HCl, from 2.0 to 6.0 mol/L, the FAs keep the same step in content variation with 3-OH-FAs, and the highest yields of both two appeared under the 3 mol/L HCl. The content of 3-OH-FAs increases positively with the heating temperature from 80°C to 150°C, while FAs showed the highest content at 130°C. Both of FAs and 3-OH-FAs firstly increased to a high content and then decreased as the heating time duration varies from 1.0 to 4.0 h, with the highest yields of both two being at 3.0 h. Consequently, we suggest the optimized acid digestion condition is under 3 mol/L HCl, heating at 130°C for 3 h and 5 g of each stalagmite sample are sufficient for the lipid analysis.

**Keywords** stalagmite, lipids, acid digestion, optimization

## 1 Introduction

Stalagmites have many attractive features for paleoclimate reconstruction. They distribute from low-latitude to high-latitude and can be compared with records of coral and ice core (Henderson, 2006). They can be precisely and accurately dated by uranium-thorium dating method (Lawrence et al., 1987; Fairchild et al., 2006; Zhao et al., 2009). In addition, some stalagmites have annual growth layers which enable us to reconstruct paleoclimate with a resolution of annual scale. With these merits, stalagmites have been widely applied in the reconstructions of paleoclimate changes during the Quaternary. However, most previous studies focused on calcite oxygen and carbon isotope compositions, these proxies are mainly interpreted to reflect the variation of the monsoon intensity (Wang et al., 2010; Lewis et al., 2011; Pausata et al., 2011) and vegetation (Hellstrom et al., 1998). Lipids in stalagmite also have the potential to reconstruct the change of vegetational and microbial ecosystems (Blyth et al., 2008). However, due to their complex origin and low content, paleoclimate records of lipids in stalagmites were rarely reported (Xie et al., 2003; Blyth et al., 2007).

Recently, Blyth et al. (2006) proposed the acid digestion for the lipid extraction from stalagmites, which can obviously improve the yield of lipids especially fatty acids (FAs) and 3-hydroxy fatty acids (3-OH-FAs) (Blyth et al., 2006; Huang et al., 2008). To date, no study has discussed the affection of the acid digestion conditions on the yields of FAs and 3-OH FAs. In this work, we optimized the acid digestion method to obtain higher yields of FAs and 3-OH-FAs extracted from stalagmites.

## 2 Material and methods

### 2.1 Sampling

Samples for lipids analysis were collected from HS4 stalagmite, Heshang Cave (30°27′ N, 110°25′ E, 294 m above sea level, developed in Cambrian limestone) in the middle reach of the Yangtze River, Central China (Hu et al., 2008). This region is dominated by the East Asian Monsoon, with a hot moist summer, but a relatively cold and dry winter. Before digestion processes, the surface of

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the stalagmite samples was removed by hammers and chisels. In the laboratory, samples were ground to powder, followed by passing through a 100 mesh sieve.

### 2.2 Optimization strategy

For the optimization study, the contents of FAs and 3-OH-FAs were used to measure the achievement of optimization. During the acid digestion experiment, three variables, HCl concentration, heating temperature and time duration were optimized. The variables and their levels were shown in Table 1.

### 2.3 Lipids extraction and analysis

To avoid contamination, all the glassware were soaked in detergent overnight, rinsed by deionized water, and finally heated at 500°C for 6 h. HCl was pre-extracted with dichloromethane (DCM, 4×) to exclude organic contaminants. All organic solvents used in this work were purchased from TEDIA with the highest purity. DCM and other reagents were checked by gas chromatography–mass spectrometry (GC-MS) to confirm the absence of contaminants.

Ten grams stalagmite powders were digested by HCl, and refluxed simultaneously in an electric heater for set time. Then left the solution cooled to room temperature. After that, 20 μL pregn-5-en-3*β*-ol (0.484 μg/μL) was added as an internal standard before transfer the solution to a separatory funnel. The solution was extracted by DCM (15 mL ×4), and the organic phase was decanted to a flask. Then the organic solvent was removed by a rotary evaporation under reduced pressure. The condensed lipids were reacted with 1 mL BF3-methanol complex (14% BF3/methanol, Sigma) at 70°C for 2.0 h to get the methylated derivatives. After cooling and destruction of the excessive $BF_3$ complex with ultra purified water (1 mL), the solution was extracted with hexane (2 mL×4). Remove the solvent under nitrogen, and the residue was further derivatized by BSTFA (N, O-bis(trimethylsilyl) trifluoroacetamide, Supelco). The derivative was dried under nitrogen and then resuspended in hexane for analysis by GC–MS.

Lipids were analyzed by a gas chromatograph (GC, HP 6890) combined with a mass spectrometer (MS, HP 5973). Separation was performed on a ZB-5MS fused silica capillary column (60 m×0.25 mm id.; 0.25 μm film thickness). The GC oven temperature was programmed from 70°C to 200°C at 10°C per min, then from 200°C to 300°C at 2°C per min, and finally held at 300°C for 27 min. The carrier gas was He (1 mL/min). The spectrometers were operated in the electron-impact (EI) mode, the ionization energy was set at 70 eV and the scan range was from 50 to 550 aum.

## 3 Results and discussion

### 3.1 Effects of the acid digestion conditions on the lipid yields

The optimization of the acid digestion method was performed in order to enhance the yields of lipids from the limited amount of stalagmite samples (less than 5 g). As it has been mentioned above, HCl concentration, heating temperature and time duration are the main variables that can largely affect the yields of lipids extracted from stalagmites. So the detailed processes of optimization for each variable were shown in Table 1.

To understand the effect of different HCl concentration on the extraction of FAs and 3-OH-FAs from stalagmites, samples were digested by HCl with the following concentration, 2.0, 3.0, 4.0, and 6.0 mol/L respectively, and kept the heating temperature and time constant. The FAs and 3-OH-FAs both were found to obtain the highest yields, with 17.1 and 2.4 μg/g, respectively (Table 2), under the treatment of 3.0 mol/L HCl. The amount of FAs and 3-OH-FAs extracted with 2 mol/L HCl is higher than with 4.0 and 6.0 mol/L, and the lowest yield is with 6.0 mol/L HCl. This suggests that the HCl$\geqslant$4.0 mol/L in concentration will cause a degradation of the FAs and 3-OH-FAs and may result in the low yield. We also found that FAs had dropped significantly in the concentration under 4.0 mol/L HCl compared with 3.0 mol/L, but not for 3-OH-FAs. This may be related to the more stability of 3-OH-FAs in acidic water solution in comparison with FAs. The optimized HCl concentration is 3.0 mol/L.

FAs and 3-OH-FAs present different patterns at four different heating temperature points, i.e., 80°C, 110°C, 130°C and 150°C (Table 2). The concentration of 3-OH-FAs increases with the heating temperature elevates from 80°C to 150°C. As we know, the 3-OH-FAs were ester or amide bonded in the lipopolysaccharides (Wollenweber et al., 1980), and they would be released more easily under higher temperature. The productivity of FAs is 3.6 μg/g at 80°C, and then decreases at 110°C. There is a dramatic

**Table 1** Acid digestion conditions set for optimizing extraction method

| Variables | Levels | | | |
|---|---|---|---|---|
| | 1st | 2nd | 3rd | 4th |
| HCl concentration/($mol \cdot L^{-1}$) | 2.0 | 3.0 | 4.0 | 6.0 |
| Heating temperature/°C | 80 | 110 | 130 | 150 |
| Heating time duration/h | 1.0 | 2.0 | 3.0 | 4.0 |

**Table 2** The yields of FAs and 3-OH-FAs by different acid digestion conditions

| Acid digestion conditions | Yields/(μg·g$^{-1}$) | | |
|---|---|---|---|
| | 3-OH-FAs | FAs | Total |
| HCl concentration/(mol·L$^{-1}$) | | | |
| 2.0 | 2.0 | 8.1 | 10.1 |
| 3.0 | 2.5 | 17.1 | 19.6 |
| 4.0 | 1.9 | 6.1 | 8.0 |
| 6.0 | 1.3 | 4.7 | 6.0 |
| Heating temperature/°C | | | |
| 80 | 0.5 | 3.6 | 4.1 |
| 110 | 0.7 | 2.9 | 3.5 |
| 130 | 0.9 | 5.1 | 6.0 |
| 150 | 1.1 | 4.1 | 5.2 |
| Heating time duration/h | | | |
| 1.0 | 0.4 | 2.0 | 2.4 |
| 2.0 | 0.8 | 5.5 | 6.3 |
| 3.0 | 1.7 | 5.8 | 7.5 |
| 4.0 | 1.3 | 4.7 | 6.0 |

increase when the temperature changes to 130°C, followed by a decrease when the temperature increases to 150°C. When the temperature $>$ 110°C, the release and degradation of FAs coexist, and the final amount gained is the net effect. Taking both 3-OH-FAs and FAs into consideration, the optimized heating temperature is set at 130°C.

Finally, under the HCl concentration (3.0 mol/L) and the heating temperature (130°C) as optimized above, the effect of heating time is tested. The yields of 3-OH-FAs and FAs show the same trends when the heating time varied from 1.0 to 4.0 h, and both of them gained the maximum yields with the processing time period of 3.0 h (Table 2). As the heating time duration increases, the release and degradation of FAs and 3-OH-FAs occur at the same time. So the contents of FAs and 3-OH-FAs increase when the heating time duration varies from 1.0 h to 3.0 h. However, when the heating time returns to 4.0 h, the release of FAs and 3-OH-FAs is terminated. Consequently, the optimized heating time duration is set at 3.0 h.

From the different treatments processed above, we can summarize that the optimized acid digestion condition for stalagmite samples is under 3 mol/L HCl, heating at 130°C for 3 h. According to the detection limit of GC-MS, we can conclude that 5 g of each stalagmite sample are sufficient for lipids analysis when using acid digestion method.

### 3.2 FAs distribution and origins

In the present study, abundant FAs have been identified from stalagmite samples (Fig. 1), including normal fatty acids (*n*-FAs) and branched fatty acids. The *n*-FAs are dominated by saturated fatty acids, ranging from $C_{14}$ to $C_{26}$ in carbon number with maximizing at $C_{16}$. A less amount of unsaturated fatty acids is gained, including $C_{16:1}$ and $C_{18:1}$. The branched fatty acids are dominated by isomer (*i*-) and ante-isomer (*a*-) homologues of $C_{15}$, $C_{16}$, $C_{17}$. The distributions of the FAs in stalagmite samples are similar to those in drip water of Heshang Cave (Li et al., 2011). The *n*-FAs in stalagmite samples may come from the overlying soil and cave organisms (Blyth et al., 2008). The branched fatty acids may originate from sulfate reducing bacterial (Edlund et al., 1985) and *Bacillus* (Kaneda, 1967).

### 3.3 3-OH-FAs distribution and origins

The 3-OH-FAs were identified from their characteristic mass spectra (Fig. 2), which show prominent ions at *m*/*z* 175 ($[CH_3]_3SiO = CHCH_2CO_2CH_3$) due to the cleavage between $C_3$ and $C_4$, and $M^+$-15 (base peak) results from a loss of a $CH_3$ group. Other minor ions include *m*/*z* 103, 89, 133, 159, and $M^+$-31 (Mielniczuk et al., 1993; Volkman et al., 1999; Szponar and Larsson, 2001).

The 3-OH-FAs include normal 3-hydroxy fatty acids (*n*-3-OH-FAs) and branched fatty acids (Fig. 3). The *n*-3-OH-FAs range in carbon number from $C_{10}$ to $C_{18}$, maximizing at $C_{12}$. The branched hydroxy fatty acids are dominated by *i*-$C_{11}$, $C_{13}$, $C_{14}$, $C_{15}$, $C_{16}$, $C_{17}$ and *a*-$C_{13}$, $C_{15}$, $C_{17}$. The 3-OH-FAs are predominantly found as constituents of lipopolysaccharides in gram-negative bacteria and require acid hydrolysis for release from amide bonds (Edlund et al., 1985; Mendoza et al., 1987; Wakeham, 1999). However, some other species, such as yeasts and other fungi, and gram-positive actinomycetes (Dyk et al., 1994; Keinänen et al., 2003) can also contribute 3-OH-FAs.

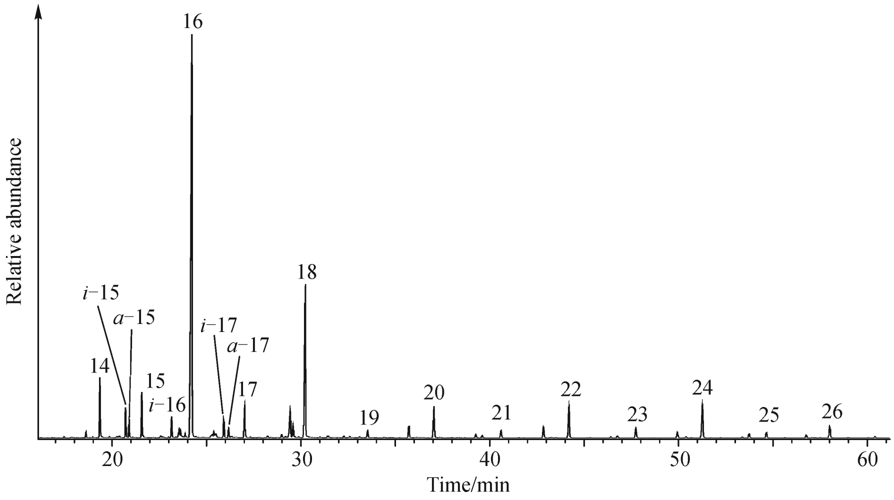

**Fig. 1** *m*/*z* 74 mass chromatogram of FAs in a stalagmite sample

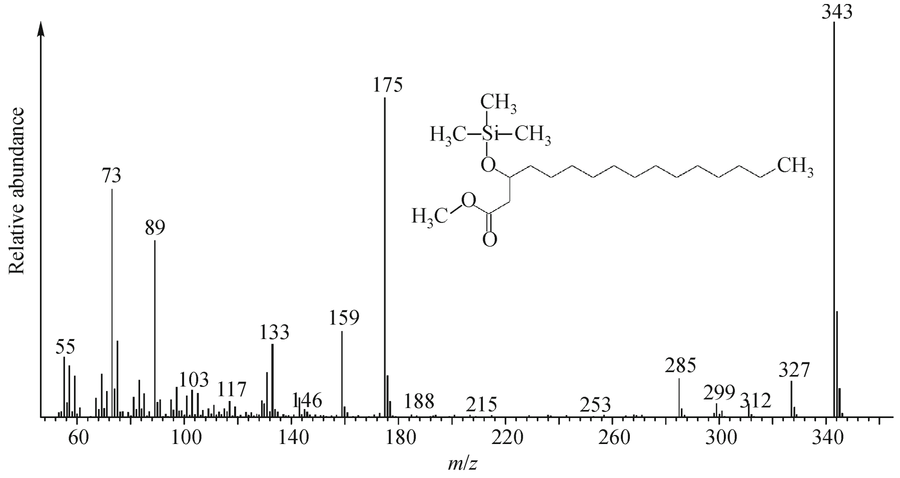

**Fig. 2** Mass spectrum of 3-OH $C_{16}$ fatty acid as Me/TMS derivative

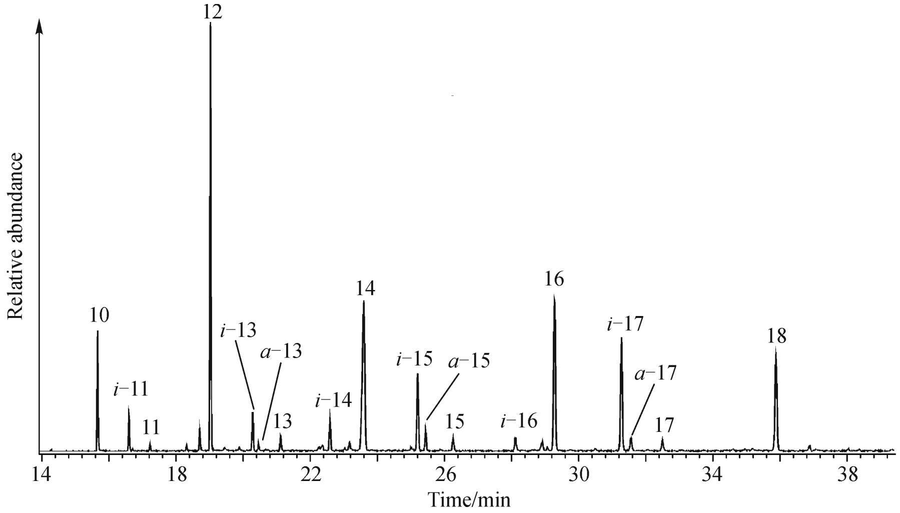

**Fig. 3** *m*/*z* 175 mass chromatogram of 3-OH-FAs in a stalagmite sample

## 4 Conclusions

Acid digestion conditions have been shown to exert great effects on the lipids extracted from stalagmite samples. Obtaining more content of lipids from less samples weight could produce high resolution records, especially when sample weights are limited in some stalagmites. In this work, we optimize the acid digestion methods to obtain higher yields of FAs and 3-OH-FAs extracted from a stalagmite in Central China. The optimized HCl concentration is set at 3.0 mol/L. High concentration of HCl will degrade both of the FAs and 3-OH-FAs. High temperature will promote the release of 3-OH-FAs, but decrease the content of FAs, and 130°C is proposed to be the optimized heating temperature when we take the FAs and 3-OH-FAs into consideration. The content of FAs and 3-OH-FAs will initially increase as the heating time duration increases, but when heating over 3.0 h, both the FAs and 3-OH-FAs will begin to degrade, and so the optimized heating time duration is set at 3.0 h. The optimized acid digestion condition is under 3 mol/L HCl, heating at 130°C for 3 h, and 5 g of each stalagmite sample are sufficient for lipid analysis.

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