Journal of Analytical and Applied Pyrolysis 110 (2014) 470–475

Contents lists available at ScienceDirect

Journal of Analytical and Applied Pyrolysis

journal homepage: www.elsevier.com/locate/jaap

Short communication

Thermally assisted hydrolysis and methylation (THM) analysis: A new perspective for biochemical investigation of fatty acid composition in enchytraeid tissues

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ARTICLE INFO

Article history:
Received 15 May 2014
Accepted 30 August 2014
Available online 8 September 2014

Keywords:
Enchytraeidae
Oligochaeta
Lipids
Phospholipids
Fats
Mass spectrometry

ABSTRACT

Fatty acids are ubiquitous components in all organisms, and their applications in taxonomic and ecological studies of enchytraeids are scarce, and their small size (*ca.* 133 μg fresh weight) may be the main reason. Thermally assisted hydrolysis and methylation (THM) reaction with pyrolysis–gas chromatography (Py–GC) allow determination of fatty acid composition for small size samples. Thus, our objective was to test this methodology with soil enchytraeids cultured in laboratory. We used THM on-line Py–GC using trimethylsulfonium hydroxide (TMSH) reagent to investigate fatty acid composition in tissues of two enchytraeid species: *Enchytraeus crypticus* and *Enchytraeus* n. sp. cultured on soil and agar. A total of 12 fatty acids were consistently identified, ranging from C_{10} to C_{16}. The major fatty acids were $C_{14:0}$ (myristic acid) and an unsaturated $C_{14:1}$. Fatty acid distribution was dependent on species and culturing method, suggesting the need of standardization of the culturing substrate and diet in chemosystematics studies. THM using TMSH provided insight on the fatty acid composition of *Enchytraeus* tissues and may be promising for application in taxonomic and ecological studies of this group of neglected soil animals.

1. Introduction

Fatty acids are ubiquitous components in all organisms, and they occur as constituents of epicuticular tissues and waxes (esters), fats/oils (triacylglicerols) and cell membranes (phospholipids), acting as energy stores, cell wall components, and performing protective functions in some cases [1–4]. Since fatty acid compositions are influenced by environmental condition and vary significantly with life stage, species, and diet, they are used in ecological studies involving trophic interaction, organic matter decomposition, environmental quality and microbial symbioses, as well as chemosystematics [4–8]. One prominent application is the use of iso anteiso C_{15} and C_{17} phospholipid fatty acids to characterize grampositive bacteria populations in soil [9].

Only a few annelids have had their fatty acids studied thus far, including the earthworm *Lumbricus terrestris* [7,10–12], and three enchytraeid species in the *Enchytraeus* genus (*Enchytraeus albidus*, *Enchytraeus fragmentosus* and *Enchytraeus bigeminus*) [13]. The purpose of these studies was to evaluate the fatty acid composition, the presence of odd fatty acid components [10], obtain the distribution of fatty acids in the digestive tract, show microbial symbioses, and verify their usefulness in chemosystematics. However, the study of fatty acid patterns in enchytraeids, a neglected group of soil animals, has not been carried out since the work by Jacob et al. [13]. The small size of enchytraeids may be the main problem in the investigation (*ca.* 133 μg fresh weight), since conventional "wet" fatty acid analysis requires extensive time consuming sample handling, increasing the risk of contamination especially when small sample sizes are used [14].

Thermally assisted hydrolysis and methylation (THM) reactions using organic alkalis such as tetramethylammonium hydroxide (TMAH) and trimethylsulfonium hydroxide (TMSH) are a well-established derivatization process in fatty acid investigations

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http://dx.doi.org/10.1016/j.jaap.2014.08.019
0165-2370/

[15–18]. Nevertheless, TMSH is more effective, since TMAH causes appreciable isomerization/degradation of polyunsaturated fatty acids. Its use has been applied in the investigation of a wide range of synthetic and natural products [15,16,18–21]. Furthermore, TMSH can be used in combination with pyrolysis and gas chromatography (Py–GC) providing the following advantages for analysis of individual organisms: (i) THM occurs in situ, (ii) only small sample size required, (iii) speed of analysis, (iv) no use of solvents, (v) small volume of reagent, and (vi) reduced sample contamination [14,15].

Hence, in the following study, we investigated the use of THM using a heated-filament pyrolyser to obtain the fatty acid composition of enchytraeid tissues of cultured specimens. The usefulness, advantages and disadvantages of this method to enchytraeids are herein discussed.

2. Materials and methods

2.1. Enchytraeids

Enchytraeus crypticus and *Enchytraeus* n. sp. were obtained from single strain cultures maintained in laboratory for more than 2 years in moist defaunated natural soil as substrate (22% sand, 62.5% clay, 2.8% organic carbon and pH 5.2) at 22 ± 1 °C and fed weekly with oatmeal flakes *ad libitum*. *E. crypticus* is one of the most used species in soil ecotoxicological tests, while *Enchytraeus* n. sp. is a new species originally sampled from Piraquara City, Brazil (25°23′2.03″S, 49°7′2.42″W). The taxonomical description of *Enchytraeus* n. sp is in preparation as a separate paper. *E. crypticus* and *Enchytraeus* n. sp. have a short life cycle of about 20 days, and reproduce by cocoon [22,23].

2.2. Preparation of the enchytraeids

Five groups of 10 to 15 adult individuals (5–7 mm length for *E. cypticus* and 3–4 mm length for *Enchytraeus* n. sp.) were separated from the natural soil cultures and transferred to five culture boxes containing 1% agar substrate and supplied weekly with oatmeal flakes (*ca.* 20 mg). After a period of 0, 7, 14 and 21 d, a group of five healthy adult individuals of each species were selected from the different culture boxes and placed in petri dishes (Ø90 mm × 15 mm height) containing deionized water (15 mL) for 24 h to remove intestinal contents (verified by stereomicroscope). Each enchytraeid was inserted individually into a calcined pyrolysis quartz tube (1000 °C, 4 h) filled with quartz wool at both ends, dried in an oven overnight (40 °C) and stored at −20 °C until required for analysis.

2.3. Thermally assisted hydrolysis and methylation (THM) with trimethylsulfonium hydroxide (TMSH)

Approximately 2 μL of TMSH (0.25 mol L^{-1} in methanol, Fluka Sigma-Aldrich Co. Ltd) was added to the quartz tube containing each individual enchytraeid. The methanol was evaporated before insertion into the pyroprobe inlet. The on-line THM reactions were performed by fast pyrolysis to 350 °C and held for 10 s using a pyropobe 5000 (CDS analytical, Oxford, USA), with the coil filament heater set at 10 °C ms^{-1}. The additional pyroprobe conditions were: single step interface programme for transferring volatiles were used from 40 °C to 280 °C (held for 60 s) at 100 °C min^{-1}, oven 290 °C, and transfer line 290 °C. The on-line derivative fatty acid methyl ester (FAME) products were introduced on-line via pyroprobe transfer line into a split/splitless injector at 290 °C using a splitless mode for 1 min. They were separated using a Focus GC gas chromatography and analysed in a Polaris Q ion trap mass spectrometer (Thermo, Waltham, USA). The GC was equipped with a capillary column DB5-ms (60 m × 0.25 mm, 0.25 μm film thickness). The GC oven was programmed from 40° (held for 2 min) to 140 °C at 10 °C min^{-1} then from 140° to 290 °C at 7 °C min^{-1} and held at 290 °C for 1 min. Helium, at a constant flow of 1.0 mL min^{-1}, was the carrier gas. The GC–MS interface and ion source temperatures were 290 °C and 200 °C, respectively. The ion trap mass spectrometer was operated in the positive electron ionization mode at 70 eV scanning the range m/z 50–650 in a 0.58 total scan time and emission current 250 mA.

2.4. Post analysis treatment

Individual fatty acid methyl esters were identified by mass spectra, and their structure confirmed using the molecular ion ($M^{+\cdot}$) and $[M\text{-}32]^+$ for saturated and monoenoic/dienoic compounds, respectively. The superscript letters ($^{a, b, c}$) in the monounsaturated fatty acids through the text indicate different isomers. Integrated chromatograms were normalized and an individual fatty acid was expressed as a relative amount (%) of the sum of all fatty acids detected (100%). Only fatty acids that occurred at >0.5% and detected in all samples were reported and included in the statistical analyses. The results were reported using mean and standard deviation of three samples, and the differences among fatty acid distributions were evaluated by ANOVA at $p < 0.05$.

3. Results and discussion

A total of 12 fatty acids were consistently identified in the pyrograms, and accounted for 91–99% of total fatty acids detected (Table 1). The distribution of major fatty acids, ranging from C_{10} to C_{16}, was characterized by a strong contribution of C_{14} fatty acids in both species (Fig. 1 and Table 1). The major fatty acids were $C_{14:0}$ (myristic acid) and an unsaturated $^{a}C_{14:1}$ (Table 1), here identified by elution order in non-polar column as 9-tetradecanoic acid ($C_{14:1}\omega9$) [24]. Other unsaturated C_{14} fatty acids ($C_{14:2}$, $^{b}C_{14:1}\omega7$, $^{c}C_{14:1}\omega5$) were also identified by retention times and mass spectra [24], albeit in lower abundance (Fig. 1 and Table 1). In general, the presence/absence of fatty acids appeared to be constant after culturing substrate change (soil to agar substrate), with only small differences detected in fatty acids $C_{12:1}$ and $^{b}C_{16:1}$, with the former being absent in the initial stages of the experiment with *E. crypticus* (0 and 7 days), and the later in the *Enchytraeus* n. sp. tissues feeding on soil substrate (Table 1). This fatty acid range for enchytraeid tissues was similar to that observed by Jacob et al. [13] who also found dominance of C_{14} in the fatty acids of *E. albidus*, *E. fragmentosus* and *E. bigeminus*. However, differently from their observations, in our data no C_{18} fatty acids were detected in significant level (≥0.5%), and the relative abundance of C_{16} fatty acid was lower (Table 1). TMSH analysis is effective in the detection of the major classes of acyl lipids present in soil mesofauna [14], and such differences may be related to the *Enchytraeus* species, diet, and methodology, which may be less sensitive for detection of longer chain fatty acids. Interestingly, the remarkably high percentage of C_{14} fatty acid present in enchytraeid tissues was not observed in *L. terrestris* in which the fatty acid $C_{18:1}\omega7$ was the most abundant compound in the body and gut walls [7,11].

Fatty acids are constituents of cell membranes and may be affected by the diet and environmental conditions. So, considering that natural soil is richer in minerals, nutrients and microorganisms and thus a more complex substrate than agar, we expected the two enchytraeid species to show differences in their lipid profiles when reared on soil *versus* agar, even though oatmeal was offered as food source in both culturing methods. In fact, a distinct behaviour was observed in the fatty acid distribution of the two species used in the present study. The mean values (%peak area) for monounsaturated and saturated C_{14} fatty acids of *E. crypticus*