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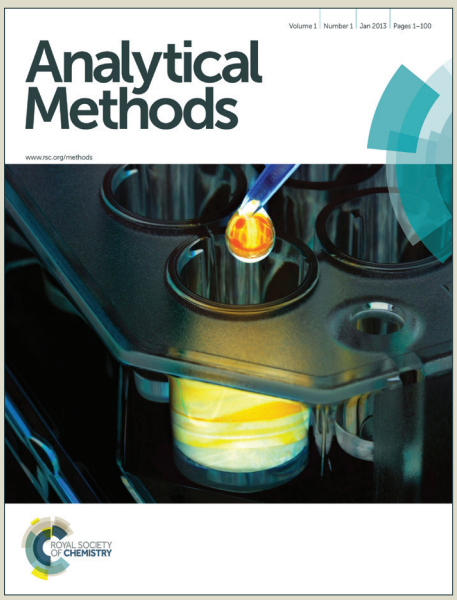

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Microchip Nonaqueous Capillary Electrophoresis of Saturated Fatty Acids Using a New Fluorescent Dye

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Abstract

We demonstrate nonaqueous labeling and separation of the full range of short to long saturated fatty acids (C2 to C30) for the first time on a microfluidic device. A new fluorescent dye, Pacific Blue hydrazide, labels the carboxylic acid in a two-step, one-pot reaction to enable detection via laser-induced fluorescence at 405 nm excitation. Limits of detection for C10 to C30 acids range from 0.9 to 5.7 μM. Fatty acids were successfully quantified in a sediment sample from the 'Snake Pit' hydrothermal system of the Mid-Atlantic Ridge, demonstrating the potential of this method to help characterize microbial communities through targeted biomarker analysis. Such a technique could also be utilized to differentiate between abiotic and biotic compounds in the search for life beyond Earth.

[Main Text]

Fatty acids are found in the cell membranes of all three kingdoms of life on Earth,[1] and normally account for 5-10% of microbial biomass.[2] As fatty acids also have high biological specialty, these amphiphilic molecules are often employed as biomarkers.[3] For example, n-3 polyunsaturated C20 and C22 fatty acids are typically algal-derived,[4] whereas bacterial signatures include n-7 monounsaturated C16 and C18 fatty acids.[5] Total fatty acid concentrations can also be used as biomass indicators.[6]

Long chain fatty acids (C14-C30) have been found in Cretaceous sedimentary rocks and meteorites.[7] Fatty acids have also been used to identify and quantify certain microbial groups in hydrothermal systems

on the sea floor.[8] Importantly, the size fraction distribution and relative abundance of fatty acids provides a highly diagnostic, nearly unambiguous means for differentiating between abiotically produced organics (e.g., meteorites) and those produced by biological processes (e.g., microbes).[9] Sensitive detection and quantitation of fatty acids on a small, portable system would therefore be useful for both microbial biomass monitoring in extreme environments on Earth, as well as in situ discrimination between abiotic and biotic compounds on potentially habitable worlds beyond Earth.

Microchip capillary electrophoresis (μCE) is a liquid-based separation technique that operates with high performance, reagent economy and speed.[10] When coupled to laser-induced fluorescence (LIF) detection, separated species can be identified and quantified with high sensitivity (nM to pM). Microfluidic devices can also be readily automated for remote in situ analysis.[11]

Short chain (C1-C8) carboxylic acids have been detected using μCE-LIF with the Mars Organic Analyzer Microchip Capillary Electrophoresis System in aqueous conditions.[12] However, fatty acids with chain lengths longer than C12 are not soluble in aqueous solution.[13] As a result, a variety of protocols utilizing nonaqueous solvents have been reported.[14] Although most of these nonaqueous capillary electrophoresis (NACE) techniques describe separations with excellent resolution, they are restricted to silica capillaries and also are not capable of separating both short and long chain species.

We recently developed a protocol for the separation and detection of both short and long chain (C1-C18) aliphatic amines using nonaqueous microchip capillary electrophoresis with laser-induced fluorescence detection (μNACE-LIF).[15] We have employed a similar strategy here, and optimized conditions to expand the range of aliphatic chain lengths accessible by this technique.

A new derivative of 3-carboxy-6,8-difluoro-7-hydroxycoumarin (Pacific Blue™) with a hydrazide functionality (PB-NH_2), was synthesized by Invitrogen (Life Technologies, Inc.) specifically for this work (Scheme 1). Fatty acids are first activated by the O-(1H-6-chlorobenzotriazole-1-yl)-1,1,3,3-tetramethyl-uronium hexafluoro-phosphate (HCTU) coupling agent in basic conditions to form an ester (Scheme 2); the active ester then reacts with PB-NH_2 in a one-pot synthesis to form the hydrazide amide (PB-labeled acid).

The μCE-LIF system used for this study was designed for temperature-controlled μNACE analyses and has been described elsewhere.[15] Briefly, a 405-nm laser beam (16.0 mW) passes through excitation and dichroic filters to enter a 20X microscope objective, which focuses the beam ~2 mm above the objective. The microfluidic chip is placed on a custom temperature-controlled stage with the center of the channel at the focal point. Laser-induced fluorescence is directed though an emission filter and focused onto a photomultiplier tube (PMT). The PMT signal is processed using a data acquisition card and a laptop computer running LabVIEW, which also controls a high-voltage sequencer (LabSmith) during μNACE injection and separation.

The microfluidic chip (Micralyne, Inc.) is composed of low-fluorescence Schott Borofloat glass and contains two channels (8.0 mm and 85.0 mm) that intersect perpendicularly to form a 4-port simple cross with two arms of equal length (4.0 mm) and a separation channel 80.0 mm in length. The microchannel cross-section is semicircular with a width of 50 μm and depth of 20 μm. Prior to each analysis, the microchannel was preconditioned with 0.1 M HCl (aq), deionized water (resistivity 18.2 MΩ) and ethanol. The microchannel was filled with separation buffer, and an aliquot of sample pipetted into the sample well. Injection potentials were 2 kV from the sample (negative) to the waste well (positive), with bias applied to the other two wells (-0.4 and -0.8 kV) to pinch the injection plug for 30 s. Separations were performed at 20 °C at a potential of 6 kV along the separation channel. Data fitting was performed with PeakFit (Seasolve Software, Inc., v4.12).

Labeling of fatty acids was performed in dimethylformamide (DMF) using 1 mM PB-NH_2 and 2 mM HCTU with 50 mM diisopropylethylamine (DIEA). It should be noted that labeling with lower concentrations of dye and coupling agent resulted in suboptimal results (see Supporting Information). After 1-3 h, the labeled acids were diluted into separation buffer in ethanol and analyzed using μNACE. As the PB-labeled fatty acids are very similar in structure and charge to the analogous PB-labeled amines,[15] the same background electrolytes (ammonium acetate and acetic acid) were used. The concentrations were optimized for separation of both short and long chain fatty acids (C2-C30). With 100

mM ammonium acetate and 1.0 M acetic acid in ethanol, fatty acids differing by 2 methyl units are baseline resolved (Fig. 1). Peak efficiencies are on average 228,000 plates/meter (see Supporting Information).

Limits of detection were determined for saturated fatty acids labeled over a concentration range of 0.4-120 μM in DMF and diluted 1:4 into the ethanol-based separation buffer. With the exception of palmitic (C16) acid, the detection limits for all fatty acids of length C10 to C30 were 0.9-5.7 μM (0.3-2.2 ppb) in the original, undiluted DMF solution (Table 1). Palmitic acid could not be quantified by this method due to the presence of a contaminant peak at the same migration time in all blanks and controls.

Though fatty acids shorter than C10 were effectively separated, limits of detection were not determined for these species due to the presence of interference peaks, most likely fluorescent by-products of the synthesis of the novel dye. We are currently working to further purify the dye and remove such contaminants. However, we note that other aqueous-based microfluidic protocols already exist for the detection of C1-C9 fatty acids.[12]

Our reported detection limits are comparable to similar CE-based methods using indirect absorbance[14c,16] and capacitively coupled contactless conductivity detection.[14h] Though capillary-based techniques using laser-induced fluorescence have reported LODs in the 10^{-9} to 10^{-10} M range,[14f,17] these methods were not able to separate the full range of C2-C30 fatty acids, and could not be incorporated onto a small, portable microfluidic device. One reported technique[18] was able to separate fatty acids up to C15 on a microfluidic device with LODs in the 10^{-10} M range, though we stress that *separation and quantification of fatty acids up to C30 is vital* if such a technique were to be used for biomarker analysis.

To demonstrate the efficacy of our technique, we analyzed a sample from the 'Snake Pit' hydrothermal field (23°22'N) along the Mid-Atlantic Ridge. The Snake Pit vent field is at a depth of ~3600 m (Fig. 2) and has abundant microbial life and macrofauna.[19] The sample was collected on 12 August 2003 by K. P. Hand from the MIR 2 Russian submersible, which was deployed from the research vessel Academic Keldysh. Samples were stored frozen (-20 °C) prior to use. Fatty acids were extracted via a protocol adapted from Grima et al.,[20] which was modified to eliminate the unnecessary methylation

step (see Supporting Information). The extract was resolubilized in 100 µL of DMF containing 1 mM PB-NH_2 and 2 mM HCTU with 50 mM diisopropylethylamine (DIEA). After 2 hrs, the solution was diluted 1:4 into separation buffer (100 mM ammonium acetate and 1.0 M acetic acid in ethanol) and analyzed using µNACE-LIF.

Several long chain fatty acids were detected and quantified in the Snake Pit hydrothermal sediment sample, including C12, C18, C20 and C22 fatty acids (Fig. 3). This is consistent with another study, which detected C12 to C22 fatty acids in an active hydrothermal chimney in the Mid-Atlantic Ridge.[21] As this sample analysis was a proof-of-concept and not a quantitative study, we report fatty acid amounts assuming 100% extraction efficiency and therefore these values represent lower bounds for the amount of material present.

The composition and distribution of fatty acids can be used to understand phylogenetic and taxonomic classifications in an ecosystem.[22] For example, bacteria contain characteristic lipid fatty acids in the C12-C19 range,[23] and even-numbered straight-chain fatty acids are signatures of gram-negative bacteria.[24] Further, C20 and C22 fatty acids are biomarkers for phototrophic microplankton.[5] We can therefore attribute the C12 and C18 fatty acids to gram-negative bacteria, and the C20 and C22 fatty acids to photosynthetic microplankton, most likely ingested by other organisms in the photic zone and later deposited on the ocean floor.

Conclusions

Fatty acids (C2-C30) were successfully labeled with a newly derivatized fluorescent dye and separated on a microfluidic device in nonaqueous conditions. Labeling was effected in a two-step, one-pot synthesis in DMF. Separations in ethanol allowed for resolution of both short and long chain fatty acids, with those differing by two methyl units baseline resolved. To our knowledge, this is the first separation of fatty acids up to C30 on a microfluidic device. Though contamination from species in the dye hampered detection of certain fatty acids, this work represents an important proof-of-concept in the use of this novel dye to analyze fatty acids in a complex sample.

The non-statistical distribution of the fatty acids measured in the Mid-Atlantic Ridge sediment suggests a biogenic source for this material; such information could help determine a biotic/abiotic origin for fatty acids or other organics detected beyond Earth. Future work will involve protocol development to distinguish between saturated, monounsaturated and polyunsaturated fatty acids. We also aim to adapt the fatty acid extraction protocol to a microfluidic platform, enabling fully automated sample processing and analysis.

Acknowledgment

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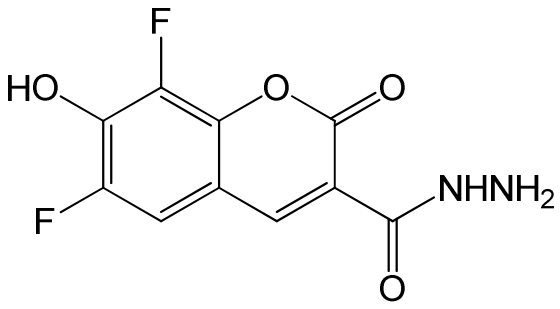
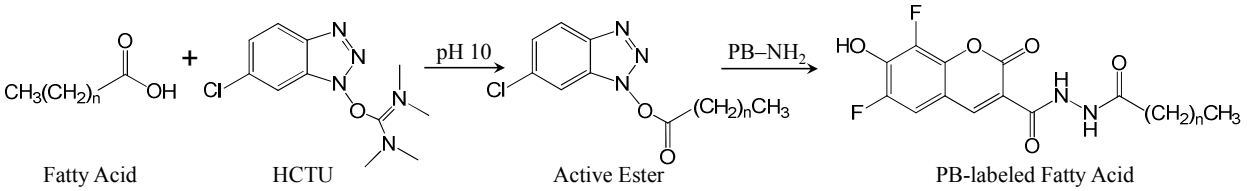
Schemes



Scheme 1. Pacific Blue hydrazide (PB-NH_2), a new dye.



Scheme 2. Two-step, one-pot reaction to form the Pacific Blue labeled fatty acid in DMF.

Figures

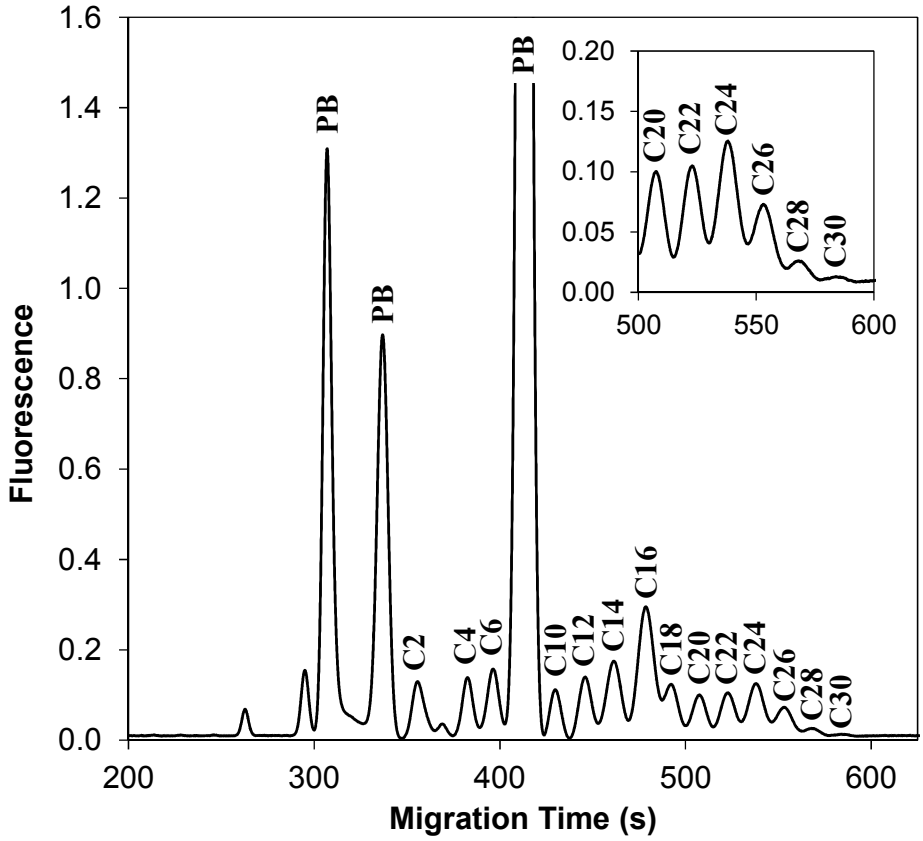


Figure 1. Separation of C2 to C30 fatty acids (2 μM C2-C26, 5 μM C28-C30) in ethanol using μNACE-LIF. Inset: Long-chain fatty acids.

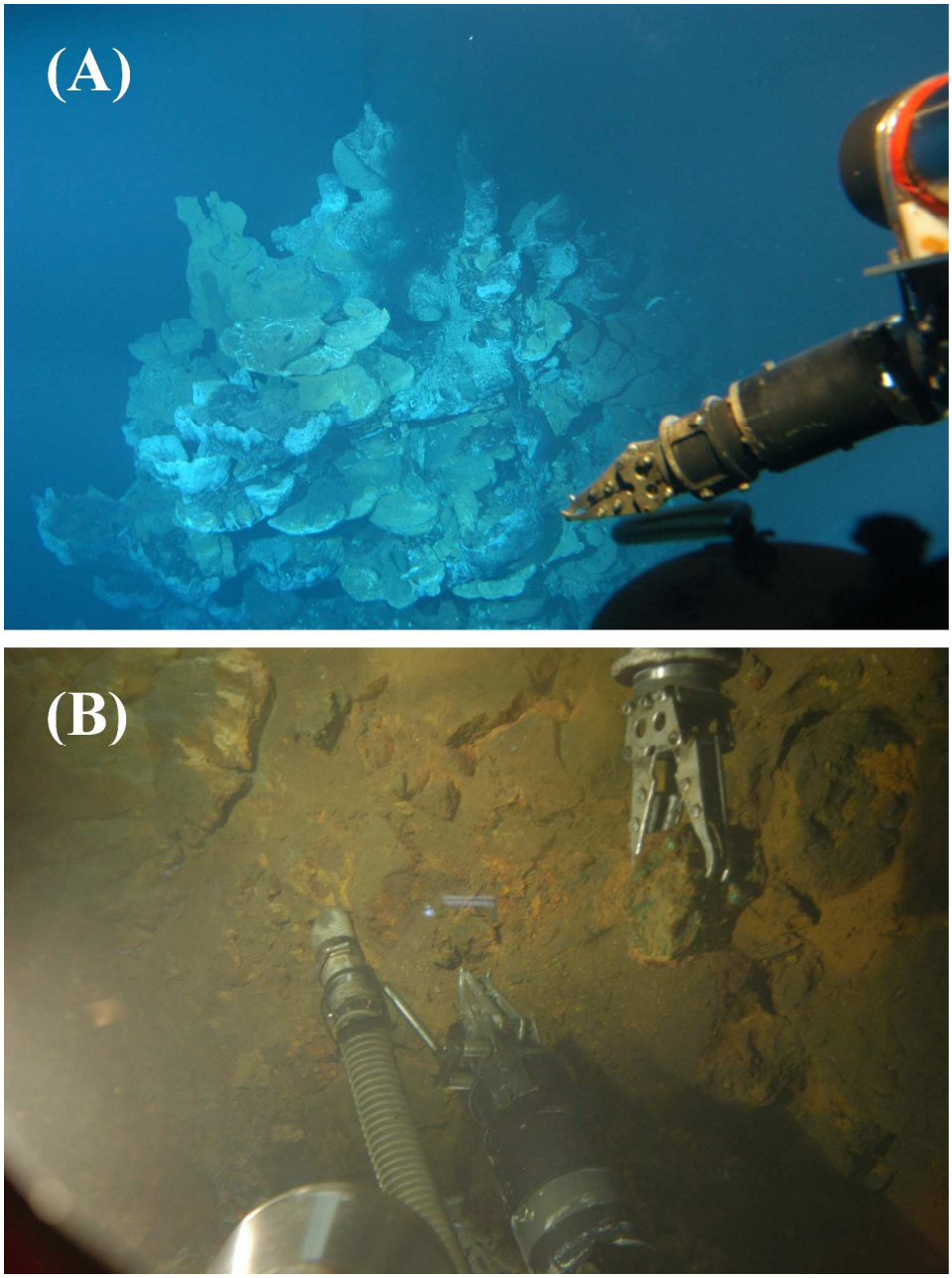


Figure 2. (A) The Snake Pit hydrothermal vent field as seen from the MIR 2 submersible. Active vents can be seen at the top of the structure. The sample site was at the base. (B) Sample collection at the base of the mound. Solid rocks were collected with the manipulator and sediments were collected with a water vacuum connected to a sample chamber. Photos by K. P. Hand.

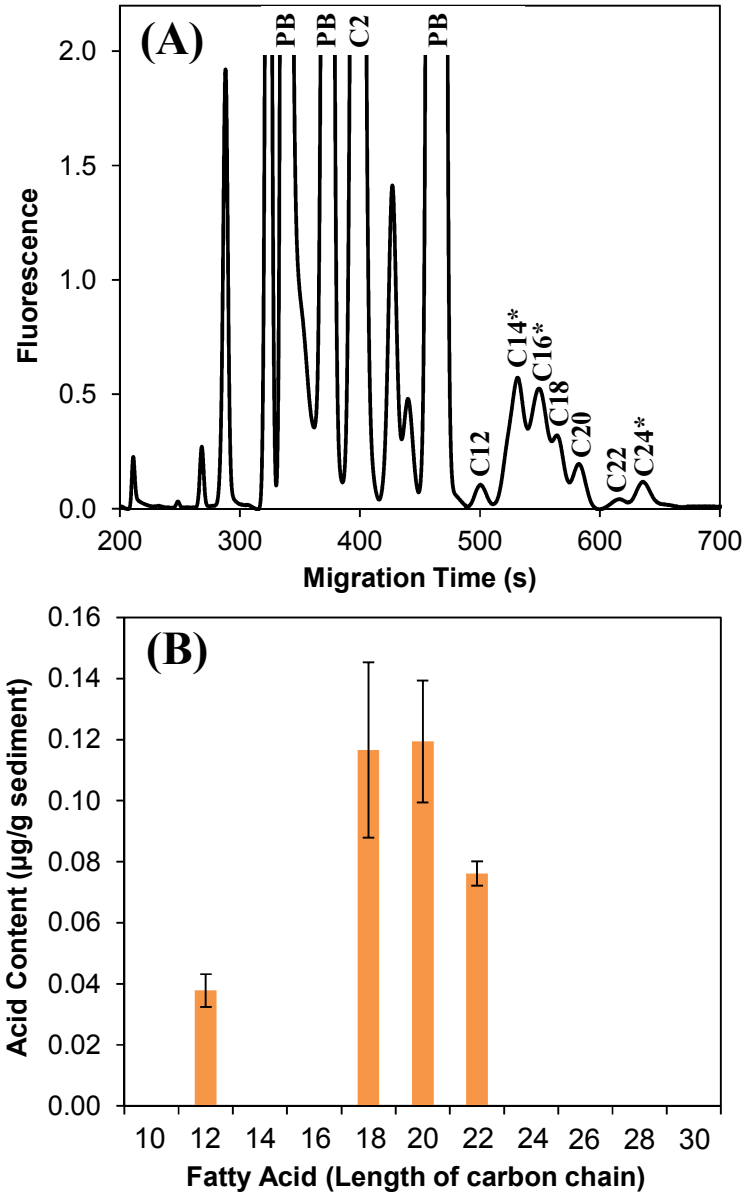


Figure 3. Fatty acids present in a sample from the Snake Pit hydrothermal vent system. (A) Separation of the sediment extract using μNACE yields several long chain fatty acids (starred acids were also present in a procedural blank). (B) Fatty acids are reported in μg per g of sediment prior to extraction.

Tables

Table 1. Limits of detection for fatty acids using μNACE with LIF in 100 mM ammonium acetate and 1.0 M acetic acid in ethanol.

Fatty Acid	LOD (μM)	LOD (ppb)
C10	1.45 ± 0.71	0.32 ± 0.16
C12	1.26 ± 0.28	0.32 ± 0.07
C14	5.67 ± 0.53	1.64 ± 0.15
C16	85.9 ± 18.9*	27.9 ± 6.1*
C18	1.57 ± 0.06	0.57 ± 0.02
C20	2.11 ± 0.32	0.84 ± 0.13
C22	1.57 ± 0.28	0.68 ± 0.12
C24	1.11 ± 0.34	0.52 ± 0.16
C26	0.92 ± 0.06	0.46 ± 0.03
C28	3.77 ± 0.50	2.03 ± 0.27
C30	3.79 ± 1.02	2.18 ± 0.58

* Due to contamination in the dye, we report a limit of quantitation instead of detection for palmitic (C16) acid.

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