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TECHNIQUES IN MICROBIAL ECOLOGY

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New York Oxford
Oxford University Press
1998

Oxford University Press

Oxford New York
Athens Auckland Bangkok Bogota Bombay Buenos Aires
Calcutta Cape Town Dar es Salaam Delhi Florence Hong Kong
Istanbul Karachi Kuala Lumpur Madras Madrid Melbourne
Mexico City Nairobi Paris Singapore Taipei Tokyo Toronto Warsaw

and associated companies in
Berlin Ibadan

Published by Oxford University Press, Inc.
198 Madison Avenue, New York, New York 10016

Oxford is a registered trademark of Oxford University Press

Library of Congress Cataloging-in-Publication Data
Techniques in microbial ecology / edited by Robert S. Burlage . . . [et al.].
p. cm. Includes bibliographical references and index.
ISBN 0-19-509223-6
1. Microbial ecology—Laboratory manuals.
2. Molecular microbiology—Laboratory manuals.
I. Burlage, Robert S.
QR100.T44 1997 579'.17—dc21
DNLM/DLC for Library of Congress 96-49666

9 8 7 6 5 4 3 2 1

Printed in the United States of America
on acid-free paper

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Signature Lipid Biomarker Analysis

The classical microbiologic approach that was so successful in public health for the isolation and culture of pathogenic species is clearly not satisfactory for many environmental samples. Microbes may still be infectious and antigenic, even though they are not culturable. It has been repeatedly documented in the literature that viable or direct counts of bacteria from various environmental samples may represent only 0.1% to 10% of the extant community [12–15, 17]. Finally, classical microbial tests are time-consuming and provide little indication of the nutritional status or evidence of toxicity that can affect pathogenicity, and these tests do not give accurate estimates of microbial fragments or other components that can act as antigens or immune potentiators.

The signature lipid biomarker (SLB) assay described in this chapter does not depend on growth or morphology. Instead, the microbial biomass is determined in terms of universally distributed biomarkers that are characteristic of all cells. The extraction process provides both a purification and concentration of these lipid biomarkers.

The SLB method is based on the extraction of "signature" lipid biomarkers from the cell membranes and walls of microorganisms [4]. Lipids, which are recoverable by extraction in organic solvents, are an essential component of the membrane of all cells and play a role as storage materials. They can be extracted directly from a wide range of materials, such as ceiling tiles, soils, and filters. Even polycarbonate filters can be extracted using a modified one-phase hexane/isopropanol procedure [5].

LIPID EXTRACTION

SLB analysis can be useful to the microbiologic researcher by quantitatively providing (1) an estimate of viable microbial biomass by measuring the amount of cellular membrane, (2) an outline of community structure by identifying signature biomarkers indicative of prokaryotic and eukaryotic taxa, and (3) an indication of

microbial physiologic status by analyzing for known stress indicators. This procedure uses the single-phase chloroform:methanol:water extraction system of Bligh and Dyer [1] as modified by White et al. [16] to quantitatively extract the lipid soluble components from viable cells.

Samples to be analyzed must be stored at −20°C or fixed in buffered formalin until extraction. Ideally, samples are lyophilized and weighed before extraction. If this is not possible, wet weights are recorded. If the samples have been grown in culture, the medium should be centrifuged and the resulting cell pellet rinsed twice with 0.05 M phosphate buffer (pH 7.5) before lyophilization.

The apparatus needed for the extraction consists of glass separatory funnels with Teflon stopcocks and ground-glass apertures (see Note 1). The standard size is 250 ml, but smaller volumes require smaller separatory funnels. A glass test tube is taped under the stopcock drain to catch drips. Round-bottom flasks (of the size appropriate to the volume being reduced, usually 250 ml) with glass stoppers and cork ring seats are used to recover the lower organic phase.

The lipids are filtered and dried by passing the organic phase through Whatman #2[v] folded paper filters (12.5 or 18.5 cm) placed in glass funnels to fit round-bottom flasks. The lipid is recovered in a rotating glass solvent evaporator with a temperature-controlled water bath, which removes the solvent. It is transferred into Pyrex test tubes with Teflon-lined screw caps. Solvent is removed in a stream of nitrogen in a gas blow-down apparatus in test tubes with a temperature-controlled water bath. The lipids are stored under nitrogen in the dark at −20°C until processed further.

The reagents used in the extraction are organic solvents, which include chloroform and methanol. All solvents should be of the purest grade possible (Burdick and Jackson GC[2] or equivalent). An aliquot of each new lot of chromatography solvents should be concentrated by a suitable factor (e.g., 1000) and analyzed by capillary gas chromatography to be sure it meets the manufacturer's specifications for organic residue. The water is nanopure-filtered organic-free deionized distilled water. All distilled water should be chloroform-extracted using approximately 200 ml chloroform per 4 liters of distilled water. Any aqueous solution used in these analyses should be stored over chloroform in this same ratio. In the extraction 50 mM phosphate buffer, pH 7.4: phosphate buffer is made to a working concentration by dissolving 8.7 g K_2HPO_4 (dibasic) in 1 liter nanopure distilled water and adjusting to pH 7.4 with approximately 3.5 ml 6 N HCl. Phosphate buffer should be chloroform-extracted using 50 ml chloroform per 1 liter buffer.

The Modified Bligh/Dyer (mB/D) First-Phase Solvent

Reagent

A combination of chloroform, methanol, and 50 mM phosphate buffer mixed to a volume ratio of methanol:chloroform:phosphate buffer, 2:1:0.8.

Procedure: Cells

1. Weigh lyophilized cells and add directly to a separatory funnel where they are extracted.
2. Add extraction solvents in this order: buffer, chloroform, and methanol. Observe the ratio of 1 mg lyophilized cells to 1 ml chloroform in the first-phase mB/D extraction (i.e., 37 mg cells in 75 ml methanol, 37.5 ml chloroform, and 30 ml phosphate buffer).
3. Allow first-phase extractions to proceed for a minimum of 2 h (up to 18 h) at room temperature.

Procedure: Sediments and Soils

1. Before extraction, using a mortar and pestle, lyophilize and thoroughly homogenize sediment samples.
2. Weigh and transfer samples to glass centrifuge bottles where they are extracted. Observe the ratio of 1 g lyophilized sediment to 1 ml chloroform in the first-phase mB/D extraction (i.e., 37 g sediment in 75 ml methanol, 37.5 ml chloroform, and 30 ml phosphate buffer). If lyophilization is not possible, subtract the amount of water in the sample from the amount of buffer added.
3. Sonicate sediments for no more than 2 min.
4. Once the extraction is complete, centrifuge the bottles (30 min at 2000 rpm) to separate sediment from solvent, and decant the mB/D first phase into a separatory funnel.

The Modified Bligh/Dyer (mB/D)

Procedure

1. Once the first-phase extraction is complete (2–18 h), add chloroform and water to split the phase and to provide a final solvent volume ratio of 1:1:0.9 for chloroform:methanol:water/buffer. Portions of water and chloroform are added to equal the amount of chloroform added in the first phase [4, 6].
2. Shake the separatory funnel vigorously, vent it, and allow to separate overnight (approximately 18 h) or until the aqueous (upper) phase is no longer cloudy.
3. Remove the organic phase (lower) via the stopcock through a Whatman #2^{v} filter supported by a glass funnel into a glass round-bottom flask.
4. Drain the organic phase until the interface between water and solvent just meets the stopcock, making certain none of the aqueous phase drains through. If analysis of lipopolysaccharide hydroxy fatty acids (LPS OH-FA) is to be performed, retain the aqueous phase.
5. Remove the solvents from the organic fraction in the round-bottom flask under vacuum (<37°C) with a rotary evaporator. Take care not to exceed 37°C, since heat breaks down the unsaturated fatty acids. In addition, never expose lipids to air, as oxygen will react with the double bonds, further breaking down the unsaturation. Christie [2] and Kates [7] recommend using nitrogen gas to evacuate the rotary evaporator. Avoid exposure to light, especially from fluorescent lights, as much as possible.
6. Transfer the dried total lipid in the round-bottom flask to test tubes with 3 × 2 ml chloroform washes.
7. Sample cloudiness upon the addition of chloroform indicates the presence of water. Add methanol (approximately 0.5 ml) until the cloudiness disappears, and redry the sample on the rotovap. This procedure may need to be repeated.
8. Once transferred, remove the solvent from the test tube under constant nitrogen flow using the dry-down with a water bath temperature of less than 37°C.
9. Store the total lipid under nitrogen at −20°C until lipid class separation is achieved.

Notes

1. All glassware used for lipid analysis must be ion- and organic-free. Scrupulous cleaning of all glassware is necessary for contaminant-free analysis.

If glassware is made dirty, immediately immerse it fully into a washtub full of hot water and phosphate detergent (S/P Micro or equivalent). Scrub the glassware with a brush and rinse four times each with cold tap water and deionized water. Allow glassware to dry completely before wrapping in aluminum foil and heating in a clean muffle furnace for a minimum of 4 h at 450°C. Disposable glassware, such as pipettes and silicic acid columns, need not be washed, but should be fired similarly. Take care not to recontaminate fired glassware. Rinse items that will not tolerate heating to 450°C with methanol and then chloroform, and allow to dry.

2. Organic-free technique is different from sterile technique. Meticulous technique must be practiced to ensure contaminate-free analyses. This is the cardinal rule; No materials other than fired glass and solvent-rinsed Teflon may come into contact with lipid solvents. Finger lipids, hair, stopcock grease, oils, and hydrocarbons are all potential contaminants.

SILICIC ACID COLUMN CHROMATOGRAPHY

Silicic acid column chromatography is used to separate total lipid extracts into general lipid classes (neutral lipid, glycolipid, and polar lipid). This procedure uses three solvents of increasing polarity (chloroform < acetone < methanol) to selectively elute the lipid classes from the silicic acid stationary phase. Reagents for this procedure include chloroform, acetone, methanol, and silicic acid (powder). Safe handling of these materials is described in the material safety data sheet (MSDS) literature. Samples—the total lipid extract obtained from the lipid extraction—should be stored in a test tube at −20°C until use. The test tubes must warm slowly to room temperature before the caps are opened.

Equipment

Glassware includes test tubes with Teflon-lined screw caps, 10-ml beakers, Pasteur pipettes. All glassware is fired in a muffle furnace (450°C for at least 4 h).

Chromatography columns are constructed from large-volume dispo-pipets (Fisher #13-678-8) packed with glass wool plugs inserted into the bottom and fired as above. Prepacked silicic acid columns (Burdick and Jackson inert solid-phase extraction system #7054G) are a commercial alternative.

Suitable racks to hold the assembled columns.

Nitrogen gas blow-down for solvent removal from test tubes with a temperature-controlled water bath.

Reagents

1. Organic solvents include chloroform, methanol, and acetone. All solvents should be of the purest grade possible (Burdick and Jackson GC[2] or equivalent)
2. Silicic acid, 100–200 mesh powder—Unisil (Clarkson Chemical Co., Williamsport, PA) or equivalent—activated at 100°C for a minimum of 1 h in a fired test tube or flask (see Note 1)

Procedure

1. If commercial columns are not used, construct a suitable column from dispo-pipets by wetting the glass wool in the bottom of the dispo-pipet with chlo-

roform and transferring a silicic acid slurry (0.5 g silicic acid suspended in 5 ml chloroform in a 10-ml beaker) by Pasteur pipette. There should be no sign of air pockets within the bed. If there are, add additional chloroform, and agitate the bed with a Pasteur pipette until the air bubbles rise to the surface. Whichever column is used, do not allow the packing to dry or disturb the surface of the bed once the procedure has begun.

2. Resuspend the total lipid in a minimal volume of chloroform (100–200 ml), and load onto the top of the silicic acid bed with a Pasteur pipette. Repeat three times for a quantitative transfer. Take care not to disturb the surface of the bed once total lipid has been applied (see Note 2).
3. Once the column is loaded, use a series of three solvents of increasing polarity to separate the lipid classes: neutral lipids, 5 ml chloroform; glycolipids, 5 ml acetone; and phospholipids, 5 ml methanol [4]. Collect lipid classes in separate test tubes set up below the column.
4. Once each fraction is collected, remove the solvent with the nitrogen gas blow-down, and store the lipid under nitrogen at −20°C for further analysis.
5. If necessary, perform sterol analysis on the neutral lipid fraction and PHA analysis on the glycolipid fraction. Phospholipid fatty acid methyl esters are prepared from the phospholipid fraction.

Notes

1. Silicic acid is slightly acidic precipitated silica. Silanols (active sites on the silicic acid granules) contain -OH groups directly bound to the silicon atom. The silanols interact with the polar groups of the lipid classes, whereas the non-polar end of the lipid molecule contributes little to separation. As the polarity of the solvents increases, the lipid classes are selectively eluted from the silanols, thereby effecting separation. Silicic acid is easily hydrated and must be dehydrated at 100°C for at least 1 h before use.
2. Do not overload the silicic acid columns. By saturating the active sites with lipid, quantitative recovery of lipid fractions is diminished, and a lower biomass estimate will result. It may be necessary to increase the amount of silicic acid if the samples are of exceptionally high biomass or are highly pigmented. If the mass of silicic acid is increased, volumes of eluting solvents must also increase accordingly to retain a 1:10 ratio (g silicic acid: ml eluting solvent).

PREPARATION OF FATTY ACID METHYL ESTERS FROM ESTERIFIED LIPIDS

Mild alkaline methanolysis is used to cleave the fatty acids from the phospholipid glycerol backbone and replace the glycerol bonds with methyl groups, creating fatty acid methyl esters (FAMEs). The purpose of this procedure is to prepare FAMEs from the esterified lipids found in either total lipid extracts or the individual lipid classes. Reagents for this procedure include chloroform, hexane, methanol, toluene, acids, and bases. Safe handling of these materials should be followed as described in the MSDS literature. Samples are either the total lipid extract or the individual lipid classes. Most commonly, the polar lipid fraction is used.

Equipment

a 37°C heating block for incubation
table-top centrifuge

vortx mixer
nitrogen gas blow-down
litmus paper

Reagents

1. Methanolic potassium hydroxide: 0.2M KOH in methanol, made fresh before each use; 0.28 g KOH per 25 ml methanol (or direct proportion thereof); guard against aqueous contamination (see Note 1)
2. 1 N glacial acetic acid: 5.72 ml concentrated (17.5 N) glacial acetic acid per 100 ml nanopure distilled water
3. Organic solvents: toluene:methanol (1:1, vol/vol; i.e., 125 ml toluene to 125 ml methanol) and hexane:chloroform (4:1, vol/vol; i.e., 200 ml hexane to 50 ml chloroform)
4. Nanopure distilled water

Procedure

1. Prepare fatty acid methyl esters from esterified lipids in the lipid fraction by mild alkaline methanolic transesterification as reported by Guckert et al. [4].
2. Redissolve the dried lipid in 1 ml toluene:methanol (1:1, vol/vol) and 1 ml methanolic KOH.
3. Vortex the mixture briefly, and incubate the samples for at least 15 min at no greater than 37°C.
4. After the samples have cooled to room temperature, add 2 ml hexane:chloroform (4:1, vol/vol), and mix the sample.
5. Then neutralize the sample (pH 6-7) with approximately 200 ml 1 N acetic acid, and analyze the pH with litmus paper (see Note 2).
6. Add 2 ml nanopure distilled water to break, phase, and mix the sample for at least 30 s on a vortex mixer. The phases (upper: organic containing the fatty acid methyl ester (FAME), lower: aqueous) are separated by centrifugation (5 min at approximately 2000 rpm).
7. Transfer the upper phase to a clean test tube (see Note 3).
8. Re-extract the lower phase with 2 ml hexane:chloroform (4:1, vol/vol), centrifuge, and transfer as above, twice more.
9. Remove the solvent with the nitrogen gas blow-down, and store the FAME under nitrogen at −20°C until separation and quantification.

Notes

1. Any water in the reaction will act as a reagent by attacking double bonds in the long-chain fatty acids. Water will also compete with the methanol for the fatty acids, yielding free fatty acids rather than methyl esters. Because potassium hydroxide is hygroscopic and will absorb water out of the air, it must be stored in a sealed container, and a quick transfer from the balance to the methanol is required.
2. The sample is neutralized because (1) methanolysis is incomplete at a higher pH and (2) FAMEs have a higher affinity for water at a higher pH. Usually, 200 ml 1 N acetic acid is sufficient to neutralize the sample. One sample from the set is pH-analyzed by drawing a small amount of the lower phase into a Pasteur pipette and spotting the sample onto litmus paper. The litmus paper should indicate that pH = 7.

3. For this operation, it is best to hold both test tubes in one hand while pipetting with the other hand. Take care to avoid transferring any water with the organic phase. It is not necessary to retrieve all of the organic phase each time, since the aqueous phase is rinsed three times.

SEPARATION, QUANTIFICATION, AND IDENTIFICATION OF ORGANIC COMPOUNDS

The purpose of this procedure is to separate, quantify, and identify the organic compounds isolated through the various procedures by gas chromatography (GC) and to confirm identification of these compounds by gas chromatography/mass spectrometry (GC/MS). Reagents for this procedure are iso-octane, cholestane, and nonadecanoic acid. Safe handling of these materials should be followed as described in the MSDS literature. Samples are either (1) fatty acid methyl esters (FAMEs), (2) poly-β-hydroxyalkanoates (PHAs) from (3) trimethyl-silyl (TMSi) derivatives of 3β-ol sterols, (4) TMSi derivatives of lipopolysaccharide hydroxy fatty acids (LPS OH-FA), or (5) dimethyl disulfide (DMDS) derivatives of mono-unsaturated FAMEs.

Equipment

Separation: nitrogen gas blow-down and capillary gas chromatograph (GC) with optional autosampler and controller; the mobile phase for the GC is hydrogen gas, obtained from the purest possible source (99.999% pure or above).

Quantification: signal output from the GC retained and initially processed by a data system (P.E. Nelson or equivalent).

Identification: preliminary identification of compounds based on comparison to retention times of standards; mass spectrometry used for verification of compound structure.

Reagents

1. Iso-octane: This solvent should be of the purest grade possible (Burdick and Jackson GC[2] or equivalent). An aliquot of each new lot should be concentrated by a suitable factor (e.g., 1000) and analyzed by capillary GC for any organic contaminants
2. 50 pmol/ml C19:0 internal standard: 15.6 mg C19:0 (nonadecanoic acid methyl ester, M.W. 312) in 1.000 liter iso-octane
3. 50 pmol/ml cholestane internal standard: 18.7 mg cholestane (M.W. 287.86) in 1.000 liter iso-octane

Procedure Separation

1. Separate the compounds to be analyzed for quantification using capillary GC with flame ionization detection. Use a 60-meter non-polar cross-linked methyl silicone column (i.e., Restek RTX-1) with a suitable temperature program (see Note 1).
2. Before dilution with internal standard solution, shoot a rangefinder using iso-octane as the solvent to determine the correct dilution of the sample (see Note 2).

3. Before injection, remove any remaining solvent with the nitrogen gas blowdown, and dilute the sample in the appropriate internal standard solution. Generally, 1 ml is injected.

Procedure: Quantification

1. Base quantification on a comparison to an internal injection standard (FAME, C19:0, LPS OH-FA, C19:0; sterols, cholestane; PHAs, malic acid). Equimolar responses are generally assumed within the range of microbial FAMEs (12:0–24:0) and sterols (22C–30C); however, tables of molecular weight correction factors are available [2].
2. Results obtained from the GC will be quantified areas under each sample peak, including the internal injection standard. For each peak, do the following calculation to obtain molar or weight amounts per sample. To normalize these amounts to a per gram dry weight basis, use appropriate dilution factors and mass measurements. The calculations done for each compound are

$$C_X = (A_X/A_{ISTD}) * C_{ISTD} * D$$

where C_X is the calculated concentration of compound X (moles or weight per unit volume), A_X is the GC area of compound X (unitless), A_{ISTD} is the GC area of the internal injection standard as determined by the GC data system (unitless), C_{ISTD} is the concentration of the internal injection standard as given above, and D is the appropriate dilution factor.
3. Assuming an average phospholipid content of 10^{-4} moles of PLFA per 5.9×10^{12} bacterial cells (based on *E. coli*) and 10^{-4} moles PLFA per 1.2×10^{10} algal cells (based on *Chlorella*), obtain an estimate of bacterial and algal cells by multiplying calculated picomolar concentrations of PLFAME by the appropriate factor (1 to 5.9×10^4 cells/pmol for bacteria, 1.2×10^2 cells/pmol for algae), yielding cells per gram.

Procedure: Identification

1. FAMEs. The use of a linear temperature program for the separation of FAMEs permits the use of equivalent chain length (ECL) analysis for FAME identification. This technique, detailed by Christie [2], is based on the linear relationship between the retention times of a homologous series of straight-chain saturated FAMEs against the number of carbons in the FAME chain. Because ECLs are a constant property of a specific FAME as long as the temperature program is linear, published ECLs in a library of FAMEs can be used to help identify specific FAME.

   This identification is preliminary, however, and selected samples should be further analyzed by (1) GC/MS as detailed in Guckert et al. [4] and [2] DMDS derivatization of monounsaturated double bonds [10].

   Fatty acid nomenclature is of the form A:BwC where A designates the total number of carbon atoms, B the number of double bonds, and C the distance of the closest unsaturation from the aliphatic end of the molecule. The suffixes **c** for *cis* and **t** for *trans* refer to geometric isomers. The prefixes **i** and **a** refer to iso and anteiso methyl-branching, respectively [7].
2. PHAs. Comparison of unknown peaks to a prepared standard (usually poly-β-hydroxybutyrate) allows preliminary identification of PHAs. However, structural identification requires GC/MS analysis as detailed by Findlay and White [3].

3. Sterols. Due to variations in chromatographic variables, identification of sterols requires the calculation of relative retention times (RRT) based on cholesterol and sitosterol [8]. The RTT for each peak is calculated by the following formula:

$$RRT_X = 1 + [0.63 * (RT_X - RT_c)]/(RT_S - RT_C)$$

where RRT_X is the relative retention time of the unknown peak, RT_X is the retention time of the unknown peak, RT_C is the retention time of cholesterol, and RT_S is the retention time of sitosterol.

By comparing the calculated RRT_X of an unknown sterol to a library of RRT for known sterols under the given chromatographic conditions, preliminary identification of individual compounds is possible. This identification is preliminary, however, and selected samples should be analyzed further by GC/MS as detailed in Nichols et al. [9].

4. LPS OH-FA. A bacterial fatty acids standard mixture containing a- and β-hydroxy fatty acids may be obtained from Matreya (cat# 1114) and preliminary identification of hydroxy fatty acids achieved by comparison to the standard mixture. Identification should be considered tentative, however, and GC/MS analyses should be performed according to Parker et al. [11].

Notes

1. FAME temperature program: 100°C for 0 min, 10°/min to 150°C for 1 min, 3°/min to 282°C for 5 min. Injector temperature = 270°C, detector temperature = 290°C. Total run time = 55 min.
2. Sterol temperature program: 200°C for 0 min, 10°/min to 280°C for 0 min, 2°/min to 310°C for 5 min. Injector temperature = 290°C, detector temperature = 290°C. Total run time = 28 min.
3. PHA temperature program: 60°C for 10 min, 10°/min to 280°C for 0 min. Injector temperature = 220°C, detector temperature = 290°C. Total run time = 32 min.
4. LPS OH-FA temperature program: Same as FAME temperature program.
5. Rangefinders are shot to ensure that the internal standard is within a factor of the sample peaks. Generally, for 37 mg lyophilized bacterial isolate or 37 g dry sediment, a rangefinder is shot at 1:1000 μl iso-octane (no internal standard). Adjustments are made and the sample diluted in internal standard only when the proper dilution is determined.

PREPARATION OF POLY-β-HYDROXYALKANOATES FROM GLYCOLIPIDS

Poly-β-hydroxyalkanoates (PHAs) are bacterially synthesized endogenous storage polymers. During their preparation, an acid ethanolysis is used to cleave the polymer and form ethyl esters of the constituent monomers found in the glycolipid fraction and to prepare these compounds for quantification and identification. Reagents for this procedure include absolute ethanol, diethyl ether, malic acid, chloroform, and strong acids. Safe handling of these materials should be followed as described in the MSDS literature. Samples are dried glycolipid collected during silicic acid chromatography.

Equipment

a 100° heating block
table-top centrifuge
vortex mixer
nitrogen gas blow-down

Reagents

1. 2 mM malic acid internal standard: 0.1341 g malic acid (MW 134.1) in 500.00 ml nanopure distilled water
2. Organic solvents: diethyl ether, absolute ethanol, chloroform
3. Concentrated hydrochloric acid (12M); no dilution from the stock bottle required
4. Nanopure distilled water

Procedure

1. Prepare ethyl esters of PHAs from the glycolipid fraction by a strong acid ethanolysis as reported by Findlay and White [3].
2. To the dried glycolipid, add 100 ml diethyl ether and 100 ml (accurate) 2 mM malic acid internal standard, and completely dry the sample under a stream of nitrogen.
3. Once dry, dissolve the sample in 500 ml chloroform, cap tightly, and heat at 100°C for 10 min to dissolve the PHAs (see Note 2).
4. After removal from the heating block, gradually release pressure from the test tube by slowly unscrewing the cap.
5. To the hot chloroform mixture add 1.7 ml absolute ethanol and 200 ml concentrated HCl, and replace the cap tightly.
6. Vortex the test tube briefly, and return to the heating block for at least 4 h at 100°C.
7. After the samples have cooled to room temperature, add 2 ml chloroform and 2 ml nanopure distilled water to split phase.
8. Vortex the sample for at least 30 s, and separate the phases (upper: aqueous, lower: organic, containing the PHAs) by centrifugation (5 min at approximately 2000 rpm).
9. Remove and discard the upper phase (see Note 3).
10. Add an additional 1 ml nanopure distilled water, vortex, and centrifuge the sample as above.
11. Remove the lower organic phase to a clean test tube (see Note 4).
12. Re-extract the remaining upper phase with 1 ml chloroform, centrifuge, and transfer as above, twice more.
13. Remove the solvent from the combined organic phases under nitrogen until approximately 100 ml sample is left (see Note 5).
14. Store the PHA under nitrogen at −20°C until separation and quantification.

Notes

1. If the sample is of exceptionally high biomass or is highly pigmented, the dried glycolipid must be rinsed with 2 × 2 ml absolute ethanol washes followed by 2 × 2 ml diethyl ether washes. Solvent is gently applied to the top of the test tube and allowed to wash down the side of the tube. It is removed from the test tube with a Pasteur pipette, taking precaution not to remove any of the PHA. If any white flocculent material is present in the

pipette, the sample is redried on the blow-down and the rinsing procedure resumed.

2. PHAs dissolve completely in hot chloroform. Using Teflon-lined screw caps with complete circular indentations from the test tube in the Teflon lining should prevent any sample from escaping during the heating process. If a test tube does leak, replacing the cap with another one usually curbs this loss; however, transferring the sample to a different test tube may be necessary.
3. Since no lipid soluble organic compounds are present in the upper aqueous phase, this phase may be discarded with no fear of losing valuable sample. This step washes the organic phase, removing most water-soluble contaminants.
4. This is accomplished by bubbling air through a Pasteur pipette as it passes through the upper phase, drawing off the lower phase, and dripping solvent through the upper phase as the pipette is removed from the liquid.
5. Loss of volatile ethyl esters will occur if the sample is allowed to dry completely.

PREPARATION OF STEROLS FROM ESTERIFIED LIPIDS

Sterols are a stable class of compounds used as signature biomarkers for microeukaryotes, such as fungus and algae. During this procedure, an alkaline saponification is used to derivatize sterols found in either the neutral lipid or total lipid fraction and to prepare these compounds for quantification and identification. Reagents for this procedure include methanol, chloroform, hexane, bases, and BSTFA. Safe handling of these materials should be followed as described in the MSDS literature. Samples are either neutral lipids or total lipid extract.

Equipment

a temperature-controlled 60°C heating block for derivatization
table-top centrifuge
vortex mixer
nitrogen gas blow-down

Reagents

1. Organic solvents: hexane:chloroform (4:1, vol/vol; i.e., 200 ml hexane to 50 ml chloroform)
2. Methanolic potassium hydroxide: 5% (w/v) KOH in methanol:water (80:20); 10 g KOH in 160 ml methanol and 40 ml nanopure distilled water
3. Nanopure distilled water
4. N,O-*bis*(Trimethylsilyl)trifluoroacetamide (BSTFA); Pierce Chemical Company, Rockford, IL)

Procedure

1. Form trimethyl-silyl (TMSi) derivatives of 3β-ol sterols from either the neutral lipid or total lipid fraction by alkaline saponification as described by Nichols et al. [9].
2. To the dried lipid, add 3 ml 5% KOH in methanol:water (80:20, vol/vol), and heat the samples for 2 h at 60°C.

3. After the samples have cooled to room temperature, add 1 ml nanopure distilled water and 2 ml hexane:chloroform (4:1, vol/vol), and vortex the sample for a minimum of 30 s.
4. Separate the phases (upper: organic containing the sterols, lower: aqueous) by centrifugation (5 min at approximately 2000 rpm).
5. Transfer the upper phase to a clean test tube.
6. Re-extract the lower phase with 1 ml hexane:chloroform (4:1, vol/vol), centrifuge, and transfer as above, twice more.
7. Remove the solvent with the nitrogen gas blow-down, and store the sterols under nitrogen at −20°C.
8. Within 24 hours before GC analysis, add 100 ml BSTFA (see Note 1), and heat the sterols for 30 min at 60°C.
9. Remove the sample from the heating block, dry under nitrogen, and store at −20°C until separation and quantification.

Notes

1. BSTFA replaces -OH groups with trimethyl-silyl groups on the sterol molecule, improving separation and identification during gas chromatography. This bond is unstable, however, and samples must be analyzed before the reverse reaction occurs.

PREPARATION OF LIPOPOLYSACCHARIDE HYDROXY FATTY ACIDS

Lipopolysaccharide hydroxy fatty acids (LPS OH-FA) are signature biomarkers found in gram-negative bacteria that are useful in community structure investigations. During this procedure, the lipopolysaccharide present in the aqueous phase of the separated modified Bligh/Dyer (mB/D) extraction is hydrolyzed by mild acid hydrolysis, and the conjugal fatty acids are methylated by acid methanolysis before purification by thin-layer chromatography (TLC). The hydroxy fatty acids are silylated just before separation and quantification. Reagents for this procedure include chloroform, methanol, hexane, diethyl ether, fatty acid standards, BSTFA, rhodamine 6G, hydroxy fatty acid standards, and acids. Safe handling of these materials should be followed as described in the MSDS literature. Samples are the aqueous phase collected from the separated Bligh/Dyer lipid extract.

Equipment

Extraction: nitrogen gas blow-down, round-bottom flasks and separatory funnels, roto-vap solvent evaporator.

Hydrolysis: reflux apparatus, round-bottom flasks and separatory funnels, nitrogen gas blow-down, rotary solvent evaporator, and Teflon-lined screw-cap test tubes.

Acid methanolysis: 100°C heating block for incubation, Teflon-lined screw-cap test tubes, vortex mixer, and table-top centrifuge.

Thin-layer chromatography: TLC plates (Whatman LK[6] silica gel, 250 mm thick with preabsorbent zone), chromatography paper (Whatman 4 mm), solvent-rinsed TLC developing tanks, drying oven, Manostat, 100-ml capillary pipettes, rhodamine spraying apparatus, large-volume dispo-pipets (Scientific Products #P5240-1 or equivalent) packed with glass wool plugs

inserted into the bottom of the pipette and fired, vacuum pump, ultraviolet lamp, 60°C heating block.

Reagents

1. Magic methanol: MeOH:$CHCl_3$:conc. HCl (10:1:1, vol/vol/vol); 10 ml each chloroform and concentrated HCl in 100 ml methanol
2. 1 N hydrochloric acid: 8.3 ml concentrated (12 N) HCl per 91.6 ml nanopure distilled water
3. Organic solvents: iso-octane, hexane:diethyl ether (1:1, vol/vol; i.e., 25 ml hexane to 25 ml diethyl ether), hexane:chloroform (4:1, vol/vol; i.e., 200 ml hexane to 50 ml $CHCl_3$), and chloroform:methanol (1:1, vol/vol; i.e., 125 ml $CHCl_3$ to 125 ml MeOH)
4. N,O-*bis* (trimethyl-silyl) trifluoroacetamide (BSTFA; Pierce Chemical Company, Rockford, IL)
5. 14C a- and β-hydroxy fatty acid plating standard: 10 mg each of methyl 3-hydroxytetradecanoate (3-OH14:0) and methyl 2-hydroxytetradecanoate (2-OH14:0) in 10 ml chloroform for a final concentration of 1 mg/ml
6. Rhodamine 6G spray reagent: 0.01% (wt/vol) rhodamine 6G in water; 25 mg rhodamine 6G (chloride salt, M.W. 479) in 250 ml nanopure distilled water

Procedure

1. Prepare fatty acid methyl esters from lipopolysaccharide lipid A present in the residue of the aqueous portion of the separated Bligh/Dyer lipid extraction by acid hydrolysis and acid methanolysis as reported by Parker et al. [11].
2. Extract samples using the Bligh/Dyer lipid extraction, and separate according to procedures outlined earlier.
3. Remove the lower chloroform phase and collect for analysis.
4. Drain the upper aqueous phase containing the lipid A into a 250 ml round-bottom flask and evaporate to dryness on a rotary evaporator (see Note 1).
5. The hydrolysis is done as follows: To the dried residue, add 30 ml 1 N hydrochloric acid, and heat the sample at reflux at 100°C for a minimum of 2 h. After the samples have cooled to room temperature, transfer the acid mixture to a 250-ml separatory funnel. Rinse the reflux apparatus with two 10-ml portions methanol then add two 25-ml portions chloroform, adding each rinse to the separatory funnel. Allow the phases to separate overnight, and collect the organic phase (lower) into a round-bottom flask. Remove the solvents under vacuum with a rotary evaporator. Resuspend the hydrolyzed LPS in 3 × 2 ml chloroform washes and transfer to Teflon-lined screw-cap test tubes. Remove the solvent under nitrogen, and store the hydrolyzed LPS at −20°C until acid methanolysis.
6. The acid methanolysis is done using the following procedure: To the dried sample, add 2 ml magic methanol (MeOH:$CHCl_3$:conc. HCl 10:1:1, vol/vol/vol), and heat at 100°C for 1 h. Once the samples cool to room temperature, add 2 ml hexane:chloroform (4:1, vol/vol), and vortex the sample for a minimum of 30 s. Separate the phases (upper: organic containing the LPS FA, lower: aqueous) by centrifugation (5 min at approximately 2000 rpm), and transfer the upper phase to a clean test tube. Re-extract the lower phase with 2 ml hexane:chloroform (4:1, vol/vol), twice more. Remove the solvent with the nitrogen gas blow-down, and store the methylated LPS

FAME under nitrogen at −20°C until purification by thin-layer chromatography.

7. Thin-layer chromatography (TLC): Rinse lipopolysaccharide FAMEs from the test tube and apply to a prepared and cleaned (see Note 2) TLC plate with three 75-ml washes of chloroform. Apply the sample in a straight line to the preabsorbent zone using a manostat fitted with a 100-ml capillary pipette. The OH-FA plating standard (100 ml) is similarly applied to narrow lanes on both sides of the TLC plate. TLC plates are developed in 50 ml hexane:diethyl ether (1:1, vol/vol). When the solvent front is within 2 cm of the top of the plate (approximately 20 minutes), note the farthest extent of the front, and remove the plate from the tank and allow to dry.
8. Spray the narrow lanes containing plating standard with rhodamine G6, and scrape and recover the hydroxy fatty acid bands (0.25 cm above the 2-OH FA and 0.5 cm below the 3-OH FA standards) in an inverted Pasteur pipette plugged with glass wool by suction.
9. Elute hydroxy fatty acids from the silica gel scrapings with 5 × 1 ml portions of chloroform:methanol (1:1, vol/vol).
10. Remove the solvent with the nitrogen gas blow-down, and store the LPS OH-FAME under nitrogen at −20°C.
11. Within 24 hours of gas chromatographic analysis, add 100 ml BSTFA, and heat the sample for 30 min at 60°C.
12. Remove the sample from the heating block, dry under nitrogen, and store at −20°C until separation and quantification.

Notes

1. There are two acceptable procedures for accomplishing this task: (1) Methanol present in the sample is removed by rotary evaporation with the water bath set at 45°C. The water bath temperature is then increased to 55°C and the remaining water removed to dryness. Do not exceed 55°C in the water bath. (2) Methanol is removed by rotary evaporation at 40°C, and the remaining liquid is frozen and lyophilized.
2. TLC developing tanks are solvent-rinsed with hexane:diethyl ether (1:1, vol/vol) before use. Chromatography paper is cut to fit the tank (20 × 55 cm) and placed inside with 50 ml hexane: diethyl ether (1:1, vol/vol) to saturate the paper with solvent. Before precleaning, a line is scraped free of silica gel down the middle of the plate to allow for two samples to be plated at the same time. TLC plates are precleaned by developing the plate in solvent before spotting with sample. Once dry, plates are activated in a drying oven at 100°C for 15 min.

PREPARATION OF DIMETHYL DISULFIDE ADDUCTS OF MONOUNSATURATED FAME

Verification of monounsaturated fatty acid double bond position is made possible by dimethyl disulfide (DMDS) derivatization and subsequent GC/MS analysis. During this procedure, DMDS derivatives are prepared for GC/MS analysis by an iodine-catalyzed addition of DMDS to monounsaturated FAMEs. Reagents for this procedure include hexane, iodine, sodium thiosulfate, chloroform, DMDS, and iso-octane. Safe handling of these materials should be followed as described in the MSDS literature. Samples are fatty acid methyl esters.

Equipment

a table-top centrifuge
vortex mixer
nitrogen gas blow-down
2-ml autosampler vials with Teflon-lined screw-cap lids

Reagents

1. Organic solvents: hexane, hexane:chloroform (4:1, vol/vol; i.e., 200 ml hexane to 50 ml chloroform)
2. Sodium thiosulfate solution: 5% (wt/vol) sodium thiosulfate in water; 0.5 g $Na_2S_2O_4$ in 10 ml nanopure distilled water
3. Iodine solution: 6% (wt/vol) iodine in diethyl ether; 0.6 g elemental iodine in 10 ml diethyl ether
4. Dimethyl disulfide (DMDS; Gold label, Aldrich Chemical, Milwaukee)

Procedure

1. Form DMDS adducts of monounsaturated FAMEs according to procedures outlined by Nichols et al. [9].
2. Quantitatively transfer samples with 3 × 0.5 ml washes of hexane:chloroform 4:1 (vol/vol) to standard 2-ml autosampler vials fitted with Teflon-lined screw-cap lids and dried under nitrogen.
3. Dilute dried FAME in 50 ml hexane, and add 100 ml DMDS and one to two drops of 6% iodine solution (wt/vol).
4. Heat the sample in a GC oven at 50°C for a minimum of 48 h.
5. Once cool, dilute the sample in 500 ml hexane.
6. Remove the iodine by adding 500 ml sodium thiosulfate solution (5%, wt/vol) and vortexing until none of the original iodine color is present.
7. Separate the phases (upper: organic containing the FAME, lower: aqueous) by centrifugation (5 min at approximately 2000 rpm), and remove the upper organic layer to a clean 2-ml autosampler vial.
8. Re-extract the lower phase with 500 ml hexane:chloroform (4:1, vol/vol), centrifuge, and transfer as above, twice more.
9. Remove the solvent under nitrogen, and dilute the DMDS derivatives in the original volume of iso-octane before GC/MS analysis.

PREPARATION OF ARCHAEBACTERIAL OR *THERMODESULFOBACTERIUM COMMUNE* ETHER LIPIDS FOR SUPERCRITICAL FLUID CHROMATOGRAPHY

Ether membrane lipids are not cleaved from their phosphate head groups by the mild alkaline methanolysis used to prepare fatty acid methyl esters (FAMEs) for GC. In this method, a modification of a mild alkaline methanolysis is used to prepare FAMEs for GC analysis, the FAMEs are separated from phospho-ethers by a second silicic acid column, and a strong acid methanolysis is used to cleave the ether lipids from their phosphate groups for supercritical fluid chromatography (SFC) analysis. Reagents for this procedure include chloroform, hexane, methanol, toluene, silicic acid, acids, and bases. Safe handling of these materials should be followed as described in the MSDS literature. Most commonly, the polar lipid fraction from silicic acid column chromatography are used as samples.

Equipment

a table-top centrifuge
vortex mixer
nitrogen gas blow-down
2-ml autosampler vials with Teflon-lined screw-cap lids
100°C heating block

Reagents

1. Methanolic potassium hydroxide: 0.2M KOH in methanol, made fresh before each use; 0.28 g KOH per 25 ml methanol (or direct proportion thereof); guard against aqueous contamination (see Note 1)
2. 1 N glacial acetic acid: 5.72 ml concentrated (17.5 N) glacial acetic acid made to 100 ml with nanopure distilled water
3. Organic solvents: toluene:methanol (1:1, vol/vol; i.e., 125 ml toluene to 125 ml methanol) and hexane:chloroform (4:1, vol/vol; i.e., 200 ml hexane to 50 ml chloroform)
4. Nanopure distilled water
5. Magic methanol: methanol:chloroform:concentrated hydrochloric acid (10:1:1, vol/vol/vol); 10 ml each chloroform and concentrated hydrochloric acid in 100 ml methanol, made fresh daily

Procedure

1. Utilize FAMEs prepared from esterified lipids in the lipid fraction by mild alkaline methanolic transesterification as reported by Guckert et al. [4] and described above. Only the differences are explained here.
2. After the addition of methanolic KOH and incubating for 15 min at 40°C, remove the samples from the heating block, and allow to cool to room temperature.
3. To the sample, add 2 ml *chloroform* (not hexane:chloroform), and vortex the sample.
4. Then neutralize the sample.
5. Separate the phases (lower: organic chloroform containing the FAMEs: and the unaffected phospho-ethers, upper: aqueous) by centrifugation (5 min at approximately 2000 rpm).
6. Transfer the lower phase to a clean test tube.
7. Re-extract the aqueous phase with 2 ml chloroform, centrifuge, and transfer as above, twice more.
8. Remove the solvent with the nitrogen gas blow-down and the FAMEs, and store phospho-ethers under nitrogen at −20°C until fractionation by silicic acid column chromatography.
9. Separate the FAMEs from the unchanged phospho-ethers by silicic acid column chromatography, except that the acetone elution is omitted.
10. Recover FAMEs in the neutral lipid (chloroform) fraction and phospho-ethers in the polar lipid (methanol) fraction. Separate and quantify FAMEs as described above.
11. Use strong acid methanolysis to cleave the ether lipids from the methanol eluate from their phosphate head groups.
12. Add 1 ml magic methanol to each sample in a screw-top test tube with a Teflon-lined cap (see Note 2).
13. Screw the cap on tightly, and heat the tube on the 100°C heating block for 1 h.

14. After the tubes cool, add 2 ml each water and hexane:chloroform (4:1).
15. Transfer the upper organic phase to a clean test tube, and extract the lower aqueous phase twice more with hexane:chloroform (4:1). Blow-down the combined organic extracts, and store at −20°C until analysis by SFC.

Notes

1. Any water in the reaction will act as a reagent by attacking double bonds in the long-chain fatty acids. Water will also compete with the methanol for the fatty acid, yielding free fatty acids rather than methyl esters. Because potassium hydroxide is hygroscopic and will absorb water out of the air, it must be stored in a sealed container, and a quick transfer from the balance to the methanol is required.
2. The condition of the test tube and cap is very important in the strong acid methanolysis step. A very slight leak will allow the solvent and the ether lipids to escape. Each test tube should be examined for chips, cracks, or deformities in the lip or threads. The Teflon cap liner must be free of cuts or gouges. The test tube and cap must screw together freely and snug down tight. If a high-pitched crack is heard while tightening the cap, it should be removed to look for cracks in the tube's lip.

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