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Rapid extraction of lipid biomarkers from pure culture and environmental samples using pressurized accelerated hot solvent extraction

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Abstract

Lipid biomarker analysis is a quantitative and sensitive method for the in situ analysis of microbial communities in environmental samples (e.g. soil, water, air). The one-phase modified Bligh and Dyer solvent extraction is a commonly used method for obtaining phospholipid fatty acid biomarkers used in such community analysis. This method, however, is relatively labor intensive and slow, often taking up to 24 h for the initial extraction. Using a pressurized hot solvent extractor, we have been able to extract lipid biomarkers from selected vegetative and/or sporulated biomass (*Escherichia coli*, *Staphylococcus aureus*, *Mycobacterium fortuitum*, *Bacillus subtilis*, *Saccharomyces cerevisiae* and *Aspergillus niger*) as well as from environmental samples collected from water, soil and air. Depending on sample type, the automated extraction procedure took ~35–45 min per sample. Compared to the modified Bligh and Dyer extraction, phospholipid fatty acid lipid yields obtained using the pressurized hot solvent extraction were not significantly different for the vegetative biomass or water and soil samples ($P>0.05$), but were significantly higher for the spores and the airborne biomass ($P<0.05$ for both sample types). The advantage of using accelerated hot solvent extraction is that by increasing the speed and decreasing the labor involved, pressurized hot solvent extraction should enable the rapid and improved extraction of lipids from large numbers of environmental samples providing data essential for total microbial community analysis.

Keywords: Lipid biomarkers; Accelerated solvent extraction

1. Introduction

Classical methods for microbial community analysis have severe limitations when used in environmental samples. As viable counts are dependent on microbial growth, they tend to consistently underestimate the microbial community [1–4]. Direct counts of microorganisms in environmental samples such as soil are highly subjective and difficult to quantify as cell assemblages and soil aggregation can mask microorganisms [5–7]. Additionally, such traditional methods of microbial community analysis

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provide little or no indication of the in situ nutritional/metabolic status of the microbial community [8].

Using the modified Bligh and Dyer solvent extraction, direct quantification of lipids has been used to estimate microbial biomass in situ for over 15 years [9–12], however, this method is labor intensive and slow, often taking up to 24 h for the initial extraction. Pressurized accelerated hot solvent extraction offers the possibility of significantly improving the speed and extraction efficiency of lipid analysis. The higher temperature increases the extraction kinetics while high pressure keeps solvents below their boiling point, thereby enabling safe rapid extraction [13,14].

The accelerated hot solvent extractor (ASE 200; Dionex) was developed for the extraction of compounds such as PCBs or PAHs from solid waste samples [13,14]. Whereas supercritical fluid extraction (SFE) of polar analytes is a complex, time consuming, and matrix-dependent procedure [15,16], the accelerated hot solvent system offers the possibility of increasing the automation, speed and efficiency of polar lipid extraction. This paper presents a comparison of the efficiency of the conventional modified Bligh and Dyer and accelerated hot solvent extraction for the extraction of PLFA from both pure biomass and environmental samples.

2. Materials and methods

2.1. Pure biomass samples

Escherichia coli (ATCC 8739), and *Staphylococcus aureus* (obtained from Mr. Jon Geiger, Olin Research Center, Cheshire, CT), were grown overnight at 30°C in Luria broth (Becton Dickinson; Cockeysville, MD 21030). *Mycobacterium fortuitum* (ATCC 6841) was grown in glucose yeast extract broth (yeast extract, 10; glucose, 5; g/l distilled water) over 72 h and harvested in stationary phase. Growth medium from all three bacteria was removed by centrifugation at 8000×*g* for 20 min and the cells were washed three times in cold sodium phosphate buffer (0.1 M, pH 7.4). Cell suspensions were diluted to an optical density of 0.5 at 610 nm (Diode Array Spectrophotometer 8450A: Hewlett Packard). *Aspergillus niger* (obtained from Ms. Judith Noble, Georgia State University) was grown on Malt extract agar for 14 days at 30°C to enhance sporulation. The plates were washed with 15 ml sterile distilled water containing Triton X-100 (0.05% v/v). The spore-containing liquid was transferred into a flask containing sterile glass beads (3 mm diameter) and shaken vigorously to suspend the spores. *Bacillus subtilis* (obtained from Mr. Andrew Arrage at the Center for Environmental Biotechnology, Knoxville, TN) was grown in sporulation medium (yeast extract, 1.5 g; beef extract, 1.5; peptone, 5; and K_2HPO_4, 1.74 g/l distilled water (pH 7.2) to which was added 1% of sporulation salts: 14.7 g of $CaCl_2.2H_2O$, 10 g of $MgCl_2.6H_2O$, 2.8 g of $MnSO_4.7H_2O$, 0.5 g of $ZnSO_4.7H_2O$, 0.1 g of $FeSO_4.7H_2O$, and 0.1 g of $CuSO_4.5H_2O$ in 1 l of 2 mM HCl [17]. Spores were cultured by heating a starter culture (10 ml) at 60°C for 10 min, following which the starter culture was added to the sporulation media and incubated at 30°C for 72 h. Sporulation was confirmed microscopically. Growth medium was removed by centrifugation at 8000×*g* for 20 min and the bacteria were washed three times in cold sodium phosphate buffer. The bacterial suspension was diluted to an optical density of 0.5 at 610 nm. *Saccharomyces cerevisiae* was obtained from Sigma (St. Louis, MO).

Escherichia coli, *S. aureus*, *M. fortuitum*, *B. subtilus* and *A. niger* (10 ml) were filtered through glass fiber filters (GF/F, Whatman Labsales Hillsboro, Oregon), prior to chemical extraction while 25 μg of the dry *Saccharomyces cerevisiae* (Sigma) was extracted per analysis.

2.2. Environmental samples

Water samples (1 l) were collected from Fort Loudon lake, Knoxville, TN, and filtered through glass fiber filters (GF/D, 2.7 μ; GF/F, 0.7 μ) and Anodiscs (anopore™ filters peripherally bonded to an annular polypropylene ring, 0.2 μ; Whatman International Ltd., Maidstone, UK). Filters were then cut into ~0.5 inch squares prior to lipid extraction. The soil, a fine sandy loam, (Sequatchie series, pH 6.4), was obtained from the University of Tennessee Knoxville Agricultural Experiment Station, sieved through a 2-mm sieve and stored at 4°C prior to use.

Prior to lipid extraction in the pressurized solvent extractor, the soil (5 g) was mixed with a drying agent (diatomaceous earth ~1 g). Air samples were collected concurrently outdoors using triplicate portable high volume samplers able to pull air at over 1 m^3/min over a glass fiber filter (GF/D; an effective pore size in air of <1.0 μm; [18]). Filters were cut into 0.5 inch squares prior to any lipid extraction. Approximately 1500 m^3 were collected for each sample. To provide homogenous samples for comparison of extraction efficiency, sample filters were divided equally for use with the different extraction methodologies.

2.3. Lipid biomarker analysis

All solvents were of GC grade and were obtained from Baxter Scientific Products (McGaw Park, IL). All glassware was washed in a 10% (v/v) Micro cleaner solution (Baxter Diagnostics, Deerfield, IL), rinsed five times in tap water and then five times in deionized water. The glassware was then heated overnight in a muffle furnace at 450°C.

A number of different extraction methodologies were investigated using the ASE 200. Initial studies were carried out on the pure biomass (*E. coli*, *S. aureus*, *M. fortuitum*, *A. niger*, *B. subtilis*) loaded filters and the bulk *S. cerevisiae*. Parameters investigated included solvent system, temperature, extraction time, and static cycle number. The accelerated solvent extractions were carried out at 1200 PSI as recommended by the manufacturer. For each study, the conventional modified Bligh and Dyer extraction was used to provide a baseline extraction efficiency.

2.3.1. Modified Bligh and Dyer lipid extraction

Using the modified Bligh and Dyer extraction procedure [9] pure biomass loaded sample filters, and controls (glass fiber filter onto which no biomass had been deposited and a buffer blank) were extracted for lipids. All filter loaded environmental samples were processed using the same methodology. Briefly, filter loaded samples were extracted in separatory funnels containing a single phase (chloroform:methanol:phosphate buffer (50 mM, pH 7.4); 1:2:0.8 v/v/v) for a minimum of 4 h before adding chloroform and deionized water (final solvent ratios, chloroform:methanol:phosphate buffer/water; 1:1:0.9 v/v/v) to form two phases. The first stage of the Bligh and Dyer was different for the soil samples. Soil (5 g) was transferred into glass centrifuge bottles, to which the first phase of the modified Bligh and Dyer solvent was added. The volume of the chloroform was at least seven times greater than the weight of the soil [11]. After a 4-h extraction the bottles were centrifuged (30 min at $1000\times g$) to separate the solvent from the soil, and this first phase was decanted into a separatory funnel. The appropriate volumes of chloroform and deionized water were added to give the correct final ratio (as above). Lastly, for all the above samples the two phases were allowed separate for 16 h, and the lipid phase was dried by rotary evaporation prior to storage in chloroform at −20°C.

2.3.2. Accelerated solvent extractor (ASE 200)

Independent of changing the extraction parameters, extraction using the ASE 200 was as is detailed below. The pure biomass studies were performed to determine the most efficient extraction procedure for lipids from environmental samples. The specific extraction conditions are described below. Excepting the *B. subtilis* spores, two solvent systems (chloroform:methanol 1:2 v/v, and methanol:chloroform:phosphate buffer, 50 mM, pH 7.4, 2:1:0.8 v/v/v) were investigated using the ASE 200. Lipid extractions for *B. subtilis* were performed following those for the other pure biomass samples, therefore, only the most efficient solvent system was used for this biomass (methanol:chloroform:phosphate buffer, 2:1:0.8 v/v/v). For each type of pure biomass two temperatures were investigated (80 and 120°C), with three different static cycle times (5, 10 and 15 min). Excepting *E. coli*, each biomass type was extracted using either one or two static heat cycles. Initially, *E. coli* was extracted using one, two or three static cycles, however, the most efficient extractions were achieved with one or two static cycles. Consequently, the investigation of static cycle number was limited to one or two cycles for the remainder of the biomass. Only the most efficient extraction parameters determined from these studies were investigated with the environmental samples. As a result, only one solvent system was investigated (methanol:chloroform:phosphate buffer, 2:1:0.8 v/v/v), at two different temperatures (80 and 120°C).

Due to the greater complexity of the environmental sample matrices compared to the pure biomass, the difference between two and three 15-min static extraction cycles was investigated.

Fig. 1 is a schematic diagram of the accelerated solvent extraction system used in this study. For safety purposes, sensors for temperature, pressure and solvent leaks are built into the ASE 200 and will alert the operator as well as, if necessary, shutting down the system. Solvent gases from the unit were vented into a fume hood. All extraction vessels (11-ml volume) were solvent-rinsed in acetone prior to use to remove any lipid contaminants. The outlet end of the cell was then lined with a chloroform-rinsed cellulose filter, stopping particulates from entering the system. Using chloroform-rinsed tweezers, filter samples were loaded into the cells and, where necessary, excess space was filled with muffle furnace-sterilized sand (Ottawa sand, 20–30 mesh, Fisher Scientific, Atlanta, GA), minimizing the solvent volume required. The diatomaceous earth dried soil samples were poured into the cells. After screwing the top of the cell into place (hand tight) cells were loaded into the ASE 200. After loading the extraction cell into the ASE system, the cell was filled with extraction solvent by opening the pump valve, following which the cell was pressurized achieving a high pressure seal at both ends of the cell. Sample cell heating (under constant pressure) followed and when the appropriate temperature was reached the static extraction occurred. The extract was then transferred into the sealed sample vial (40-ml volume) following displacement with fresh solvent. Following this, further static cycles were performed where necessary. After the final static cycle extractions, the cell was purged with nitrogen for 3 min finishing the extraction process. The solvent system was then rinsed before the ASE 200 automatically loaded the next sample and collection vial. Where phosphate buffer was used in the first phase extraction solvent, an appropriate volume of chloroform and deionized water was added to give the correct final ratio (chloroform:methanol:phosphate buffer/water; 1:1:0.9 v/v/v) to form the two phases. The chloroform layer was transferred into a new test tube and evaporated under nitrogen at 37°C. Where no phosphate buffer was used, the methanol:chloroform (2:1 v/v) was evaporated directly under nitrogen prior to fractionation.

2.3.3. Lipid fractionation, derivatization and analysis

The total lipid fractions obtained following both the modified Bligh and Dyer and/or the accelerated solvent extractions were fractionated into neutral-, glyco-, and polar lipids on silicic acid columns as described in [19]. The phospholipid-containing polar lipid fraction was subjected to a mild alkaline methanolysis, transesterifying the fatty acids into methyl esters [19].

Fatty acid methyl esters were separated and quantified by gas chromatography-mass spectrometry (GC/MS). Samples were dissolved in hexane containing nonadecanoic acid methyl ester (C 19:0; 50 pmol/μl). Samples were injected into a Hewlett-Packard HP5890 series II gas chromatograph interfaced with a HP5972 series mass selective detector (Hewlett Packard, Wilmington, DE). The gas chromatograph was equipped with a non-polar column (HP1; 50 m, 0.25 mm internal diameter, 0.25

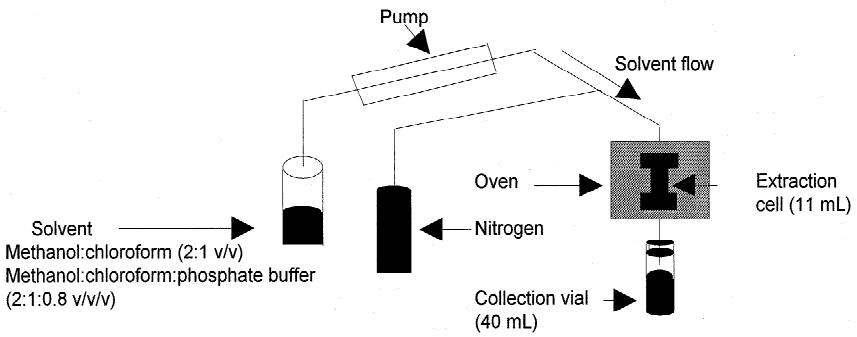

Fig. 1. A schematic diagram of the accelerated solvent extractor (ASE 200).

df; Hewlett Packard). Splitless injections were made using a Hewlett-Packard model 7673 autosampler. The carrier gas was helium. The column was programmed from an initial temperature of 100°C to 150°C at 10°C per min, held at this temperature for 1 min, and then raised at 3°C per min to 282°C where it was held for 5 min. The injector and source housing temperatures were maintained at 270°C and 290°C, respectively. Mass spectra were collected at an electron energy of 70 eV. Fatty acids were identified both by relative retention times compared with authentic standards (Matreya Inc., PA) and by the mass spectra. Fatty acids are designated as described by Ringelberg et al. [10].

2.4. Statistical analysis

Lipid extractions were carried out in triplicate with error bars on graphs representing standard deviation. The Student *t*-test was used to determine significant differences between extraction efficiencies using the different procedures.

3. Results and discussion

3.1. Pure biomass samples

Using the ASE 200, maximum extraction of PLFA from pure biomass was achieved with the methanol:chloroform:phosphate buffer (2:1:0.8 v/v/v) solvent system with two 15-min static cycles. Each extraction used ~25 ml of solvent, comparing favorably with the conventional Bligh and Dyer extraction which required at least 50 ml solvent. The maximum sample extraction time was 35–45 min, again comparing well with the Bligh and Dyer extraction which could take up to 24 h. Generally, there was no significant difference between extraction efficiencies at 80 or 120°C ($P>0.05$). Fig. 2 shows the maximum recovery of PLFA using the ASE 200 as a percentage of the PLFA extracted using the modified Bligh and Dyer (100%). Although there was no significant difference between extraction efficiencies obtained using the modified Bligh and Dyer or the ASE 200 for the vegetative biomass, PLFA was extracted with significantly higher efficiency from the spore forms analyzed (fungal and bacterial; $P<0.05$). *Bacillus* spp. spores can contain higher relative proportions of certain terminally branched saturate PLFA than do the corresponding vegetative cells [14] and the ASE 200 enabled recovery of higher relative proportions of terminally branched saturate PLFA (specifically i15:0 and i17:0) than were extracted using the modified Bligh and Dyer extraction.

Following comparison of different extraction parameters, it was evident that solvent system, time and static cycle number had the greatest influence on extraction efficiency (data not shown). Generally, the methanol:chloroform:phosphate buffer (2:1:0.8 v/v/v) solvent system gave more consistent and higher recoveries of PLFA than did the metha-

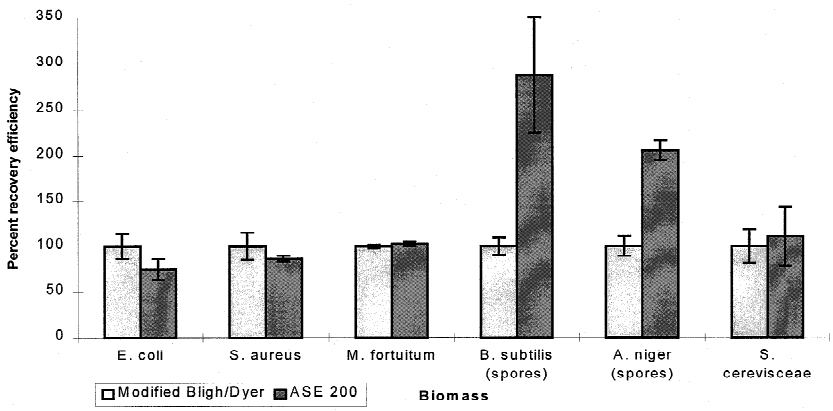

Fig. 2. Efficiencies (as a percentage of the modified Bligh and Dyer extraction (100%)) obtained using the ASE 200 for extraction of PLFA from pure biomass. Error bars represent standard deviation, $n=3$.

nol:chloroform (2:1 v/v) solvent system. Compared to extraction efficiencies obtained using the methanol:chloroform:phosphate buffer system, the extraction efficiencies obtained using methanol:chloroform were significantly lower ($P<0.05$) for the *S. cerevisiae* (4.2%), *M. fortuitum* (38%) and *S. aureus* (72%). Principal components analysis (PCA) enabled comparison of the multivariate PLFA profiles obtained following the different extraction procedures for each type of biomass. Independent of whether the modified Bligh and Dyer or the accelerated solvent extraction was used, there was no specific trend in the extraction efficiencies achieved for specific PLFA when using the methanol:chloroform:phosphate buffer solvent system. However, where the solvent system contained only methanol:chloroform, a decreased relative proportion of the PLFA 18:1ω9c was extracted from the *S. cerevisiae* while an increased relative proportion of the PLFA 10me18:0 was extracted from the *M. fortuitum*.

3.2. Environmental samples

Compared to the modified Bligh and Dyer, a greater amount of PLFA was extracted from soil using the ASE 200, however, the increase was not significant (Fig. 3; $P>0.05$). Also, there was no significant difference between the efficiency of the Bligh and Dyer and the ASE 200 for PLFA extractions from water sample (Fig. 3), however, using the ASE a significantly greater ($P<0.05$) amount of PLFA was extracted from the air biomass sample. Although the percentage recovery of PLFA for both the air and soil samples increased with the extra 15 min static cycle, these increases were not significant (Fig. 3; $P>0.05$). The increase in the recovery of PLFA was probably due to the rinse and purge procedures within the ASE 200, both of which displaced solvent extractant from the samples into the collection vials. During the conventional modified Bligh and Dyer procedure, no such solvent displacement is performed.

Extraction recoveries at 120°C were generally lower, although not significantly so, than those at 80°C. It is likely that some decomposition of the PLFA was occurring at this higher temperature. Due to comparatively low recoveries of PLFA from airborne biomass samples in preliminary studies at 120°C, further studies were not performed at this temperature for this type of biomass.

To further investigate any impact on the types/amounts of PLFA recovered when using the ASE 200 (at either 80 or 120°C with two or three static cycles) compared to the modified Bligh and Dyer, PCA was performed on the PLFA profiles obtained. Although there were some out-lying PLFA profiles for both the soil and water samples (following either Bligh and Dyer or ASE extractions), the relative proportions of PLFA extracted were consistent independent of extraction procedure used (data not

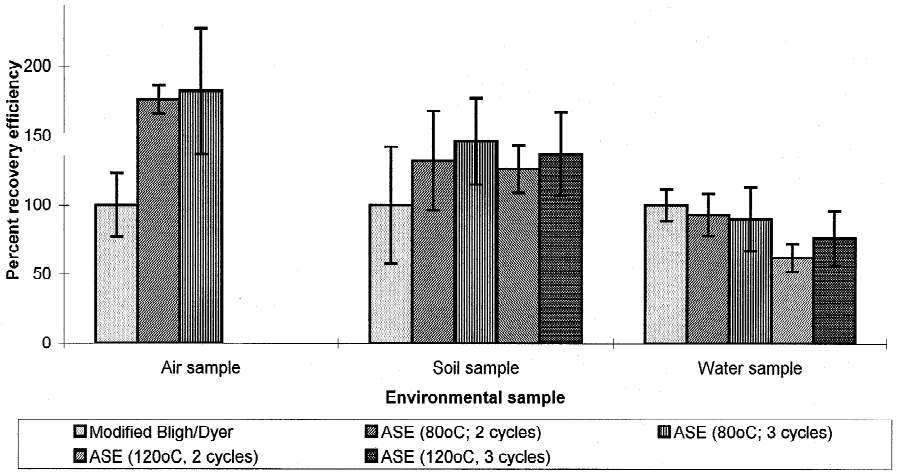

Fig. 3. Efficiencies (as a percentage of the modified Bligh and Dyer extraction (100%)) obtained using the ASE 200 (80 and 120°C) for extraction of PLFA from environmental samples. Error bars represent standard deviation, $n=3$.

shown). Conversely, the PLFA profiles from the air samples exhibited clustering (Fig. 4a). Compared to the profiles obtained using the Bligh and Dyer extraction, the PLFA profiles obtained following extraction using the ASE at 80°C contained larger amounts of typically eukaryote type PLFA (Fig. 4b). The first principal component accounted for 97% of the variance and the second, 1.1%. The first principal component was most heavily influenced by the PLFA 18:3ω3, 18:1ω9c, and 16:0, all of which are most commonly present in eukaryote biomass (Fig. 4b). The second principal component was most heavily influenced by 18:0, 18:1ω7t and 18:1ω7c. For the remainder of the PLFA, there was no difference in the relative proportion extracted using either procedure. We concluded that, compared with

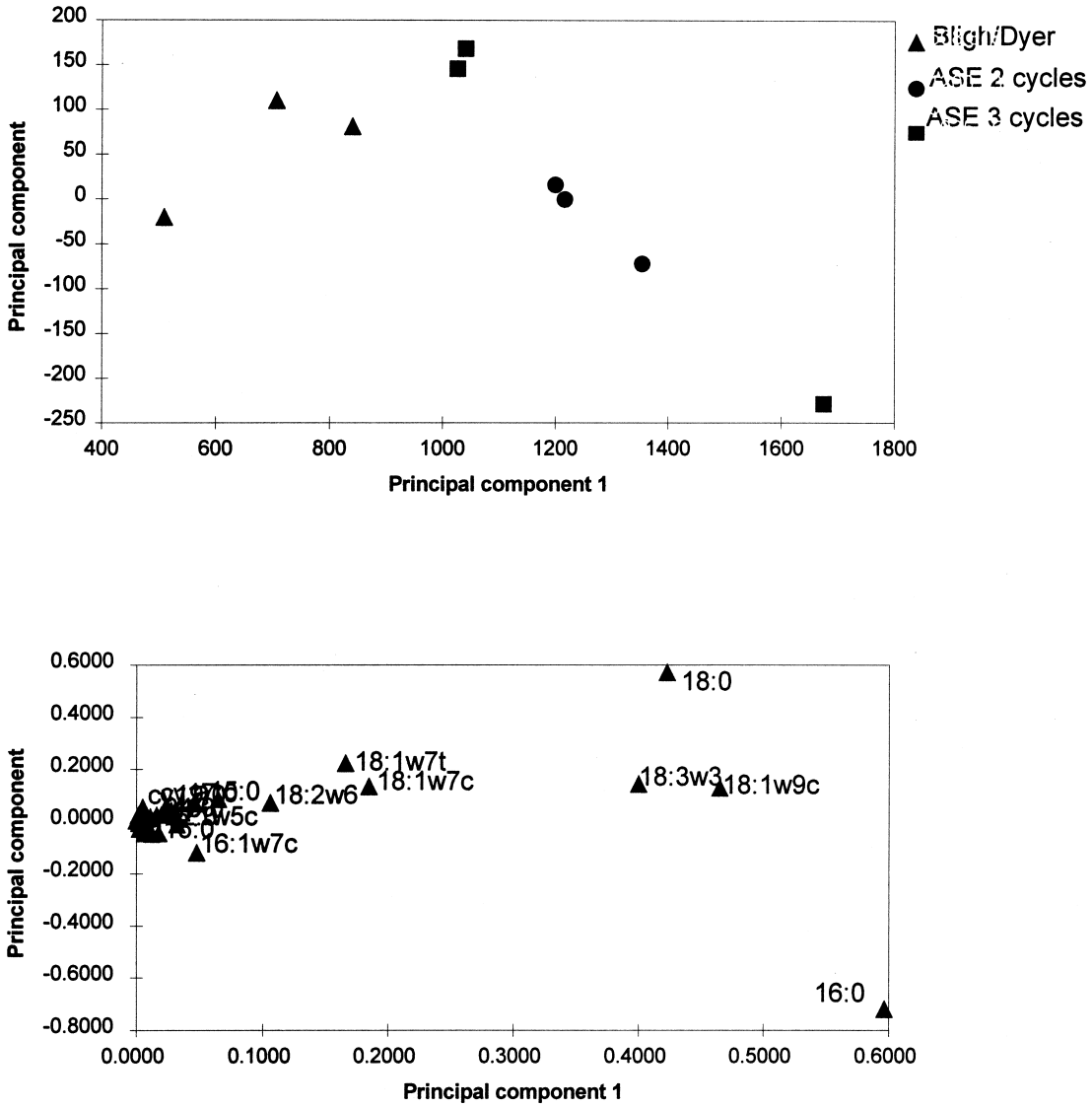

Fig. 4. (A) A scatter plot of the scores from a principal components analysis of the PLFA profiles from Fig. 3 (from airborne biomass). Principal component 1 described 98% of the variance, and principal component 2, 1.1%. (B) A scatter plot of the coefficient of loading derived from the principal components analysis in (A).

the Bligh and Dyer extraction, ASE extraction procedures on predominantly prokaryote samples should have no impact on the extraction efficiencies of the different PLFA. However, the accelerated solvent extraction was substantially more efficient than the Bligh and Dyer for a predominantly eukaryote sample and this will impact upon community structure analysis of such samples.

It is clear that using a methanol:chloroform:phosphate buffer solvent system (1:2:0.8 v/v/v) at 80°C and 1200 PSI, the ASE 200 enabled rapid extraction of phospholipids from both pure biomass and environmental samples. The accelerated solvent extraction required substantially less materials, labor and time than did the conventional modified Bligh and Dyer extraction. Compared to the modified Bligh and Dyer extraction, PLFA yields obtained using the pressurized hot solvent extraction were not significantly different for the bacteria, yeast, or water samples but were significantly higher ($P<0.05$) in the case of the bacterial and fungal spores and the airborne biomass.

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