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Comparison of solvent mixtures for pressurized solvent extraction of soil fatty acid biomarkers

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ABSTRACT

The extraction and transesterification of soil lipids into fatty acid methyl esters (FAMEs) is a useful technique for studying soil microbial communities. The objective of this study was to find the best solvent mixture to extract soil lipids with a pressurized solvent extractor system. Four solvent mixtures were selected for testing: chloroform:methanol:phosphate buffer (1:2:0.8, v/v/v), chloroform:methanol (1:2, v/v), hexane:2-propanol (3:2, v/v) and acetone. Soils were from agricultural fields and had a wide range of clay, organic matter and microbial biomass contents. Total lipid fatty acid methyl esters (TL-FAMEs) were the extractable soil lipids identified and quantified with gas chromatography and flame ionization detection. Concentrations of TL-FAMEs ranged from 57.3 to 542.2 n mole g^{-1} soil (dry weight basis). The highest concentrations of TL-FAMEs were extracted with chloroform:methanol:buffer or chloroform:methanol mixtures than with the hexane:2-propanol or acetone solvents. The concentrations of TL-FAMEs in chemical groups, including saturated, branched, mono- and poly-unsaturated and hydroxy fatty acids were assessed, and biological groups (soil bacteria, mycorrhizal fungi, saprophytic fungi and higher plants) was distinguished. The extraction efficiency for the chemical and biological groups followed the general trend of: chloroform:methanol:buffer ≥ chloroform:methanol > hexane:2-propanol = acetone. Discriminant analysis revealed differences in TL-FAME profiles based on the solvent mixture and the soil type. Although solvent mixtures containing chloroform and methanol were the most efficient for extracting lipids from the agricultural soils in this study, soil properties and the lipid groups to be studied should be considered when selecting a solvent mixture. According to our knowledge, this is the first report of soil lipid extraction with hexane:2-propanol or acetone in a pressurized solvent extraction system.

1. Introduction

Soil lipids are chemically and biologically diverse, since they come from plant, animal and microbial cells. The characterization of fatty acid biomarkers from the total lipids, phospholipids and neutral lipids, as well as their stable isotope composition [1], can reveal changes in the structure, nutritional status and living biomass of soil microbial communities [2–4]. Fatty acids that are unique or very abundant in the cell membranes of certain microbial groups (e.g., bacteria, fungi, algae, protozoa) serve as biomarkers for detecting the presence of these groups. Some biomarkers are specific for more narrowly defined groups, such as those that distinguish arbuscular mycorrhizal fungi from heterotrophic fungi or biomarkers that can discriminate microorganisms at the genus and species levels [5].

Fatty acid biomarkers are measured following the extraction and transesterification of soil lipids. The classical method of extracting soil lipids uses a solvent mixture containing a citrate or phosphate buffer, chloroform and methanol [6–8]. However, Tunlid and White [9] mentioned that the efficiency of chloroform:methanol:buffer mixture in extracting soil lipids was never fully investigated. Chloroform:methanol mixtures are efficient at extracting lipids from many biological media [10,11], but it is not known if these solvents are the best for extracting soil lipids, considering that clay particles and organic matter could interfere with lipid extraction [7,12,13]. Pressurized solvent extraction (PSE) is a technology that accelerates soil lipid extraction, reduces human contact with solvents, and reduces the volume of solvents used [14–17]. A comparative study of soil lipid extraction efficiency with different solvent mixtures in a PSE system remains to be performed.

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Three solvent mixtures may be good alternatives to the chloroform:methanol:buffer mixture commonly used to extract soil lipids, namely chloroform:methanol, hexane:2-propanol and acetone. Lipids are extracted more rapidly with a chloroform:methanol mixture than with chloroform:methanol:buffer, which requires a lengthy post-extraction step of phase separation to remove non-lipid molecules such as amino acids and carbohydrates extracted by the buffer solution. Hexane:2-propanol is effective in extracting lipids from biological tissues and is considered to have the same extraction efficiency as chloroform:methanol, but with lower toxicity [6,18–20]. However, the performance of hexane:2-propanol varies among biological tissues. For example, Schäfer [21] showed that more fatty acids contained in cereal and yolk lipids were extracted using hexane:2-propanol than chloroform:methanol, but muscle lipids were extracted more efficiently with chloroform:methanol than other solvent mixtures. Acetone has lower toxicity than the other mixtures mentioned above, and is capable of extracting lipids from *Eucalyptus globulus* wood [22] and wheat grains [23]. Despite these findings, there are no reports describing the application of hexane:2-propanol or acetone to extract soil lipids, so this remains to be tested.

The objective of this research was to compare solvent mixtures for extracting lipids from soil with a PSE system and to characterize the extracts based on the chemical structure (e.g., saturated, mono unsaturated and poly unsaturated fatty acids) as well as the biological origin (e.g., fatty acid biomarkers of bacteria, mycorrhizal fungi, saprophytic fungi, higher plants/faunal biota) of their TL-FAMEs.

2. Experimental

2.1. Soil collection and handling

The soils used in this study (mixed, frigid Typic Endoaquents) were collected from the top 15 cm of agricultural fields in southwestern Québec, Canada in August 1999 (soil 4) and September 1999 (soils 1, 2 and 3), prior to crop harvest. Each soil sample was a composite of seven cores (15 cm long, 3 cm intenal diameter) collected randomly from the field. After collection, half of each soil sample was air-dried, passed through a 2 mm mesh sieve, stored at room temperature and used for soil physical and chemical analysis, while the other half was frozen immediately and stored at −20 °C until microbial biomass carbon and lipid analysis was conducted. Agricultural practices at the collection sites and selected soil characteristics are reported in Table 1.

2.2. Reagents and glassware

All organic solvents used in this study were high pressure liquid chromatography (HPLC) grade. Glassware was rinsed with methanol and chloroform or placed in a furnace at 360 °C for at least 2 h before use. Laboratory equipment that did not tolerate heating was rinsed with methanol and then chloroform, and allowed to dry at room temperature (20 °C) before use.

2.3. Pressurized solvent extraction (PSE) system

Soil lipids were extracted with an ASE 200 accelerated solvent extractor (Dionex Corporation, Sunnyvale, CA, USA). Operating conditions consisted of one heating cycle at 80 °C and 8280 kPa during 5 min, three static cycles of 15 min each at the same temperature and pressure, rinsing of the transfer lines and sample cell with the solvent and purging with N_2 for 180 s between each sample [14]. Triplicate samples of each composite soil (6–8 g of freeze-dried soil) were packed into separate 11 mL stainless steel ASE vessels, sealed at both ends with circular cellulose filters to prevent soil particles from entering the extractor. The following solvent mixtures were used: (a) chloroform:methanol:phosphate buffer (1:2:0.8, v/v/v), (b) chloroform:methanol (1:2, v/v), (c) hexane:2-propanol (3:2, v/v) and (d) acetone. Additional chloroform and buffer were added to the extracts from solvent mixture (a) to facilitate separation of the aqueous and organic phases, so the final composition of the chloroform:methanol:phosphate buffer was 2:2:1.8 (v/v/v). This procedure produced 48 extracts (four soils × three replicates × four solvent mixtures), each containing 20–25 mL of soil lipids and solvents. The organic phase was evaporated under N_2 gas.

2.4. Quantification and identification of TL-FAMEs from extractable soil lipids

TL-FAMEs were prepared by mild alkaline methanolysis of total soil lipid extracts [8]. After drying extracts completely under N_2, they were redissolved with 1 mL of iso-octane containing 25 ng μL^{-1} of methyl-nonadecanoate (C19:0) internal standard. The resulting mixture of TL-FAMEs (5 μL injected) were analyzed in split mode (50:1) with a gas chromatograph (Hewlett Packard 6890) equipped with a Simplicity Wax capillary column (cross-linked polyethylene glycol; length, 30 m; film thickness, 0.33 μm; Supelco 2-4326), He as carrier gas (constant at 9.5 psi) and a flame ionization detector. The oven temperature was initially set at 60 °C, then raised to 150 °C (10 °C min^{-1}) and held for 5 min, after which it was raised by 3 °C min^{-1} to a final temperature of 230 °C and held for 20 min. Inlet and detector temperatures were 200 °C and 250 °C, respectively. The linear flow velocity was at 32 cm s^{-1}.

Identification of peaks was based on comparison of retention times to known standards (Supelco 37 Component FAME Mix cat.#47885-U; Supelco Bacterial Acid Methyl Esters cat.#47080-U; Matreya PUFA-2 cat.#1081; Matreya Bacterial Acid Methyl Esters CP Mix cat.#1114; Matreya cis-11-Hexadecenoic Acid cat.#1208 and Matreya 10-Methyloctadecanoate cat.#1763), used directly or derivatized if needed, containing FAMEs with chain length ranging from 8 to 24 carbon atoms. These standards permitted the identification and quantification of 53 different FAMEs from the total

Table 1
Selected properties of the soils (Typic Endoquents, 0–15 cm depth) used in the experiment

Soil	Tillage system[a]	Crop	pH[b]	OM[c] (g kg^{-1})	Sand[d] (g kg^{-1})	Silt[d] (g kg^{-1})	Clay[d] (g kg^{-1})	Textural class	SMB-C[e] (mg C kg^{-1})
1	CT	Soybean	8.0	23.5	211	339	450	Clay	153.7 ± 10.3
2	NT	Soybean	6.8	45.1	543	283	174	Sandy loam	215.3 ± 18.4
3	NT	Soybean	7.0	69.1	0	205	795	Clay	384.8 ± 11.1
4	CT	Bean	6.1	263.3	149	418	433	Silty clay	452.3 ± 23.2

[a] CT: conventional tillage, NT: no-tillage.
[b] Soil:water extracts (1:2 soil:solution ratio) [43].
[c] Organic matter (OM) determined by loss on ignition (360 °C for 4 h).
[d] Particle-size analysis [44].
[e] SMB-C is soil microbial biomass C, mean (±standard error of mean) of three replicate measures. SMB-C = chloroform labile C/K_{EC}, using a K_{EC} of 0.45 [45].

soil lipid extract, hereafter defined as a TL-FAME (single FAME) or TL-FAMEs (groups of FAMEs with similar chemical or biological characteristics).

The concentration of each identified TL-FAME (nmoles per gram dry soil (DS)) was calculated relative to the C19:0 internal standard, which was present in each sample at 25 ng μL^{-1} (0.080 nmol C19:0 μL^{-1}). The contribution of each identified TL-FAME to the total concentration of TL-FAMEs (summed concentration of all extracted soil lipids that were identified with the standards listed above) in a sample was expressed as mole fraction (relative richness, % mole) and used in the multivariate analysis.

2.5. FAME nomenclature, chemical and biological groups

We used the standard ω-nomenclature (A:BωC) for designating the fatty acids [24] where "A" represents the number of carbon in the fatty acid, "B" the number of unsaturation and "C" the position of the double bond closest to the omega (distal) carbon. Identified FAMEs were grouped according to their chemical group (straight and branched saturated, mono- and poly-unsaturated, and hydroxy substituted fatty acids) and biological origin (biomarkers of bacteria, mycorrhizal fungi, saprophytic fungi, higher plant/faunal biota, general biota). Biological groups were distinguished with the following TL-FAMEs: bacteria (i-15:0, a-15:0, 15:0, i-16:0, 16:1ω7, i-17:0, 3-OH-12:0, 17:0, 17:1ω7, 17:0cy, 18:1ω7, 10Me18:0), mycorrhizal fungi (16:1ω5c), saprophytic fungi (18:1ω9c/t, 18:2ω6c/t, 18:3ω6, 18:3ω3) and a general biotic marker (16:0) [2,4,25–32]. All TL-FAMEs with 20 and more carbons (20:0, 21:0, 22:0, 23:0, 24:0, 20:1ω9, 20:2ω6, 20:3ω3, 20:3ω6, 20:4ω6, 20:5ω3, 22:1ω9, 22:2ω6, 22:4ω6, 22:5ω3, 22:6ω3, 24:1ω9) were categorized as TL-FAMEs ≥20 C because of the diverse origins (bacterial and fungal cells, plants, protozoa and other animals) of soil lipids in this group [2,4,33–36].

2.6. Statistical analysis

The effect of solvent mixtures on the quantity of TL-FAMEs (nmol g^{-1} DS, %mole) in the chemical and biological groups was determined with one-way analysis of variance using CoStat, version 6.003 (CoHort Software, Monterey, CA, USA). A post-hoc least significant difference test at $\alpha = 0.05$ was used to compare treatment means. Discriminant analysis (DA) were performed with the TL-FAMEs dataset (in %mol, transformed with $\log_{10}(x+1)$ to achieve normality in the dataset) to test the discrimination of TL-FAMEs due to solvent mixture and soil type using SYSTAT software, version 10 (Systat Software Inc., Richmond, CA, USA). Values presented in graphs and tables are untransformed means followed by the standard errors of the mean ($n = 3$) for each soil.

3. Results and discussion

Soils had diverse characteristics (Table 1), but are representative of agricultural soils in southwestern Québec. The TL-FAME concentrations ranged from 57.3 to 542.2 nmol g^{-1} DS (Table 2). This range is similar to the 160.8–341.2 nmol g^{-1} DS of TL-FAMEs reported for agricultural soils from the Central and San Joaquin Valleys of California using the Microbial Identification System (MIS, Microbial ID Inc., Newark, DE, USA), another method for characterizing soil lipids [37]. In soils 1 and 2, the TL-FAME concentration of the soil lipids was more efficiently extracted with chloroform:methanol:buffer than other used solvents (Table 2). Chloroform:methanol:buffer and chloroform:methanol mixtures gave higher TL-FAME concentrations than hexane:2-propanol or acetone in soil 3, whereas in soil 4, chloroform:methanol:buffer, chloroform:methanol and acetone extracted soil lipids more efficiently, when compared to hexane:2-propanol (Table 2). These results are consistent with other studies showing chloroform:methanol mixtures to be the most efficient for extracting lipids from biological materials [10,38,39]. Regardless of the solvent used, the TL-FAME concentration followed the order soil 4 > soil 3 > soil 2 > soil 1, which may be related to the organic matter and soil microbial biomass carbon content of these soils.

3.1. Chemical and biological groups of TL-FAMEs

Lipids can be selectively solubilized by organic solvents, depending on structural features such as the proportion of nonpolar hydrocarbons in the fatty acids or other aliphatic moieties and the presence of polar functional groups, such as phosphate and sugar moieties [10]. Soil TL-FAMEs contained between 26.8 and 342.0 nmol g^{-1} DS of total saturated fatty acids (SAFAs), the sum

Table 2
Summed concentration (total TL-FAMEs) and chemical groups (SAFAs, saturated fatty acids; UFAs, unsaturated fatty acids; HYFA, hydroxyl fatty acid) of soil lipids extracted with various solvents using a PSE system (All values are in nmol g^{-1} DS)

Solvent mixture	Total TL-FAMEs	StraightSAFAs	BranchedSAFAs	MonoUFAs	PolyUFAs	HYFAs
Soil 1						
Acetone	59.7 c	25.0 c	1.8 c	11.0 c	21.6 c	0.3 c
Chloroform:methanol	101.0 b	38.2 b	5.6 b	21.5 b	34.7 b	0.9 b
Chloroform:methanol:buffer	167.1 a	49.4 a	16.7 a	51.1 a	47.0 a	2.8 a
Hexane:2-propanol	57.3 c	26.4 c	1.8 c	9.7 c	19.1 c	0.4 c
Soil 2						
Acetone	136.1 c	66.4 b	4.4 c	21.5 c	42.9 c	0.9 c
Chloroform:methanol	216.2 b	91.7 a	13.2 b	41.9 b	66.7 b	2.6 b
Chloroform:methanol:buffer	282.1 a	93.4 a	31.5 a	73.0 a	79.4 a	4.8 a
Hexane:2-propanol	137.2 c	63.4 b	4.1 c	20.2 c	48.9 c	0.7 c
Soil 3						
Acetone	242.9 b	85.7 b	4.0 c	28.9 b	124.3b c	0.0 c
Chloroform:methanol	360.6 a	122. 2a	13.5 b	69.8 a	153.3 a	1.9 b
Chloroform:methanol:buffer	361.7 a	121.8 a	30.6 a	70.1 a	135.6 b	3.6 a
Hexane:2-propanol	216.8 b	77.6 b	4.8 c	20.2 b	114.3 c	0.0 c
Soil 4						
Acetone	493.5 ab	297.8 a	6.4 c	24.1 b	164.8 a	0.7 b
Chloroform:methanol	542.2 a	321.7 a	20.3 b	43.7 a	153.2 a	3.2 a
Chloroform:methanol:buffer	438.8 b	233.9 b	27.7 a	54.7 a	119.8 b	2.6 a
Hexane:2-propanol	346.2 c	215.3 b	6.0 c	18.0 b	107.0 b	0.0 c

For each soil (Typic Endoquents), mean values in a column with the same letters are not significantly different (LSD Test, $p < 0.05$, $n = 3$).

Table 3
Summed concentration (total TL-FAMEs) and biological groups (described in the Experimental section) of soil lipids extracted with various solvents using a PSE system (All values are in nmol g^{-1} DS)

Solvent mixture	Total TL-FAMEs	Bacteria	Mycorrhizae	Fungi	FAMEs ≥ 20C	General biomass marker 16:0
Soil 1						
Acetone	59.7 c	5.8 c	1.4 c	12.4 c	30.6 c	4.5 c
Chloroform:methanol	101.0 b	15.3 b	3.7 b	16.5 b	49.8 b	8.1 b
Chloroform:methanol:buffer	167.1 a	44.7 a	10.6 a	25.2 a	61.0 a	13.7 a
Hexane:2-propanol	57.3 c	6.2 c	1.3 c	9.9 d	31.0 c	4.2 c
Soil 2						
Acetone	136.1 c	13.0 c	3.7 c	19.1 c	82.1 b	8.4 c
Chloroform:methanol	216.2 b	31.7 b	7.8 b	28.5 b	117.1 a	16.1 b
Chloroform:methanol:buffer	282.1 a	72.9 a	15.4 a	37.1 a	118.2 a	22.5 a
Hexane:2-propanol	137.2 c	11.6 c	3.5 c	14.4 d	91.8 b	7.3 d
Soil 3						
Acetone	242.9 b	9.6 c	3.6 c	42.7	160.1 c	13.8 c
Chloroform:methanol	360.6 a	26.1 b	8.5 b	46.9	236.1 a	22.2 b
Chloroform:methanol:buffer	361.7 a	68.3 a	17.1 a	47.7	178.8 b	28.1 a
Hexane:2-propanol	216.8 b	9.6 c	3.7 c	36.2	143.8 d	12.8 c
Soil 4						
Acetone	493.5 ab	15.8 c	2.4 c	39.5 a	321.3 a	37.1 bc
Chloroform:methanol	542.2 a	45.5 b	5.7 b	49.4 a	283.9 b	59.1 a
Chloroform:methanol:buffer	438.8 b	61.0 a	9.6 a	39.8 a	216.3 c	46.5 b
Hexane:2-propanol	346.2 c	14.1 c	2.0 c	27.1 b	204.8 c	30.8 c

For each soil (Typic Endoquents), mean values in a column with the same letters are not significantly different (LSD Test, $p < 0.05$, $n = 3$).

of straight SAFAs and branched SAFAs (Table 2). Straight SAFAs were the most common, accounting for 29.6–62.2% of the total soil TL-FAMEs. The concentration of total unsaturated fatty acids (UFAs), which are composed of monoUFAs (monounsaturated fatty acids) and polyUFAs (polyunsaturated fatty acids), ranged from 28.8 to 223.1 nmol g^{-1} DS (Table 2). MonoUFAs accounted for 4.9–30.6% of the soil TL-FAMEs, but the polyUFAs were more common (27.3—52.7% of the soil TL-FAMEs). The 3-OH-12:0 hydroxy fatty acid (HYFA) was less than 2% of the soil TL-FAMEs and concentrations ranged from 0.0 to 4.8 nmol g^{-1} DS (Table 2). Soils extracted with chloroform:methanol:buffer or chloroform:methanol mixtures had higher straight SAFA, branched SAFA, monoUFA, polyUFA and HYFA concentrations than soils extracted with hexane:2-propanol or acetone, except in soil 4, where chloroform:methanol and acetone yielded the highest concentrations (Table 2).

The TL-FAMEs were used to distinguish the biological origin of soil lipids. We found that bacteria contributed 3.2—26.8% of TL-FAMEs, mycorrhizal fungi 0.5—6.3% of TL-FAMEs and saprophytic fungi 7.8—20.8% of TL-FAMEs (Table 3). The general microbial biomass marker, 16:0, represented 5.3—10.9% of the TL-FAMEs (Table 3). We also found that 36.5—66.9% of the TL-FAMEs were ≥20C, which suggests these lipids were derived from higher plants and animals (Table 3). Data on TL-FAMEs ≥20C are not often presented in papers oriented towards soil microbial community dynamics because these fatty acids are not typically biomarkers of bacteria and fungi. However, when they are monitored, TL-FAMEs ≥20C normally represent a larger pool of fatty acids than those of microbial origin [35,40]. Jandl et al. [35] reported that these long chain TL-FAMEs came from above- and below-ground crop residues, animal manure and a variety of soil organisms (protozoa, nematodes, etc.). Soils extracted with chloroform:methanol:buffer or chloroform:methanol mixtures had higher concentrations of all investigated biomarkers than soils extracted with hexane:2-propanol or acetone (Table 3). An exception was found for soil 4, where acetone extracted more efficiently fungal biomarkers and TL-FAMEs ≥20C (Table 3).

The chloroform:methanol:buffer mixture contains an aqueous buffer as well as polar solvents (chloroform and methanol) and it has been suggested that this type of monophasic solution should have a greater ability to break polar bonds and extract lipids from biological materials than the chloroform:methanol mixture alone [6]. Certainly, the chloroform:methanol:buffer and chloroform:methanol mixtures were generally more efficient at extracting TL-FAMEs from soil than hexane:2-propanol and acetone. Hexane (a hydrocarbon solvent) was expected to extract nonpolar lipids efficiently, while polar lipids should be soluble in 2-propanol (a polar sol-

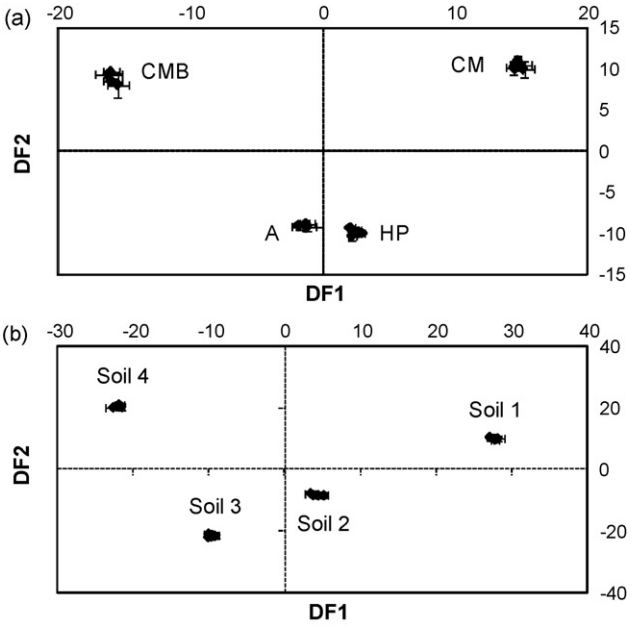

Fig. 1. Discriminant analysis of the TL-FAMEs from soil lipids extracted with four solvent mixtures using a PSE system. Scores plots of discriminant analysis were grouped according to (a) the solvent mixture, and (b) the soil type. Solvent mixtures are identified as A (acetone), CM (chloroform:methanol), CMB (chloroform:methanol:buffer) and HP (hexane:2-propanol). Soil types are described in Table 1. Data are mean scores and the standard errors of the mean ($n = 3$).

vent). However, 2-propanol has lower polarity than methanol, which explains the lower recovery of polar lipid classes and total TL-FAMEs with the hexane:2-propanol mixture than with the chloroform:methanol:buffer and chloroform:methanol mixtures. Acetone is a solvent of medium polarity often used to extract simple lipids and glycolipids or precipitate phospholipids [11], but it was not generally efficient at extracting soil TL-FAME from different chemical groups and diverse biological origins.

Discriminant analysis, based on the individual TL-FAMEs identified from soil lipid extracts, provided significant discrimination between solvent mixtures and soil types (Wilks' lambda = 0.000 at $p < 0.00005$ for all discriminant analysis tests) (Fig. 1a and b). The best discriminating variables selected by the automatic backward stepwise procedure allowed us to correctly classify 98% of the solvent mixtures and 100% of soils (Fig. 1a and b). There is a clear difference in the chemical and biological groups of TL-FAMEs extracted by the four solvents tested in this study. Discriminate analysis also confirms that lipid profiles, revealed by TL-FAME analysis, can distinguish soils that are known to have diverse chemical, physical and biological properties. Whether more subtle changes in the chemical and biological groups of TL-FAMEs within a particular soil (i.e., as a result of experimental treatments or environmental changes) can be detected with this technique is beyond the scope of this study, but has been demonstrated by other researchers [9,16,32,41].

4. Conclusions

Fatty acids are major building blocks for many classes of lipids, including acylglycerols and phospholipids, and are widely used as biomarkers in microbial ecology and to characterize soil microbial communities. We found that chloroform:methanol:buffer or chloroform:methanol extracted TL-FAMEs from soil more efficiently than the hexane:2-propanol and acetone solvent mixtures. The TL-FAME concentration attributed to relevant soil chemical and biological groups was affected by the type of solvent used, with improved extraction efficiency for chloroform:methanol:buffer ≥ chloroform:methanol > hexane:2-propanol = acetone. It is challenging to determine whether solvent mixtures permit quantitative extraction of soil lipids. Spiking a soil with a known concentration of a particular lipid may seem like a plausible option to evaluate the extraction efficiency of solvent mixtures, except that soils are biologically active and there is a high probability that newly-added lipids would be rapidly metabolized by soil microorganisms. Real soil lipids are probably stabilized physically or chemically and thus resist microbial breakdown. Some reports [7,12,13] indicate that solvent mixtures can become saturated in soils with high soil organic matter content, due to the presence of readily available organic molecules (not necessarily lipids). In such cases, longer extraction times, a higher solvent mixture:sample ratio, or a solvent mixture containing more chloroform would be required to generate a representative lipid sample. For example, Iverson et al. [42] showed that marine tissues containing > 2% lipids were extracted more efficiently with a chloroform:methanol (2:1, v/v) mixture than a chloroform:methanol (1:2, v/v) mixture. These considerations deserve further investigation in soils.

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