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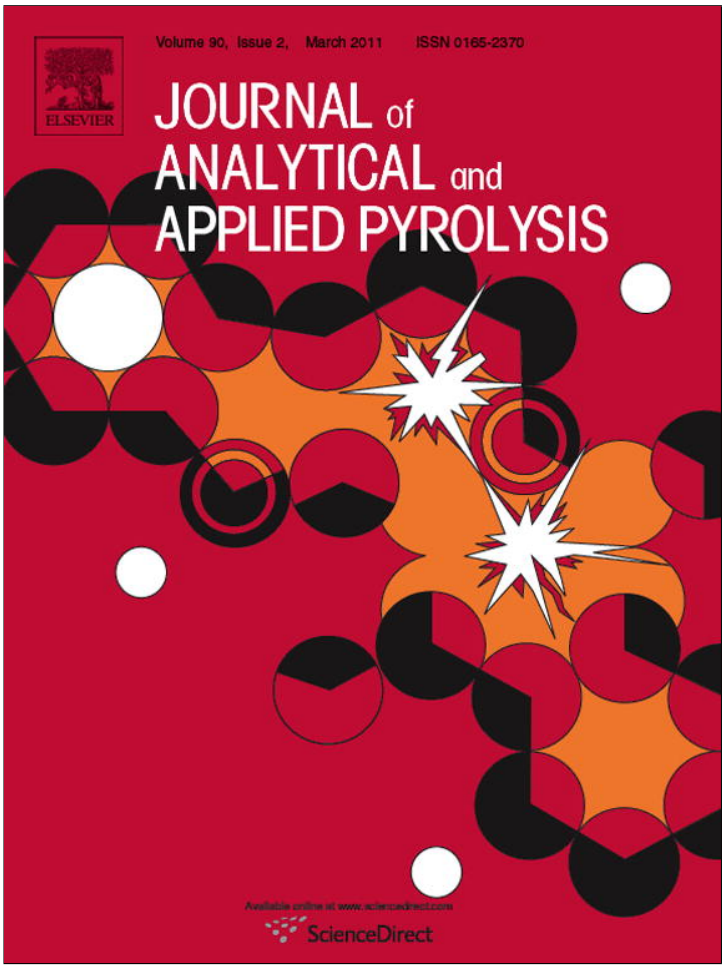

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Pyrolysis-mass spectrometry and gas chromatography-flame ionization detection as complementary tools for soil lipid characterization

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ABSTRACT

Lipid biomarker profiles are a powerful tool for assessing soil microbial community structure, but intensive laboratory work and data analysis are needed to construct profiles from phospholipid fatty acids and other common biomarkers. Pyrolysis mass spectrometry (Py-MS) is a alternative method that provides a rapid and sensitive 'fingerprint' of soil lipids and may be sufficient to characterize lipids from various sites. The objective of this work was to evaluate the capacity of pyrolysis metastable atom bombardment time-of-flight mass spectrometry (Py-MAB-TOF-MS) to provide replicable analysis of soil lipids, compared to a routine gas chromatography-flame ionization detection (GC-FID) method. Soils were collected from six agricultural fields under soybean, corn and asparagus production. Soil lipids extracted with 1:2 chloroform:methanol solvent were analyzed with Py-MAB-TOF-MS or transesterified into fatty acids and then analyzed by GC-FID. The two methods were complementary, but distinct: lipid fingerprints, generated from Py-MAB-TOF-MS spectra, included extractable soil lipids from microbial, animal and plant origins plus non-living organic matter in the samples, whereas fatty acid profiles generally represented lipids from soil bacteria and fungi. We conclude that the soil lipid fingerprints generated from Py-MAB-TOF-MS present more variability than lipid biomarker profiles from the GC-FID method because they include a broader group of extractable soil lipids. Further work is needed to identify the molecular fragment masses in Py-MAB-TOF-MS spectra that could precisely identify soil lipids of microbial origin.
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1. Introduction

Characterization of soil lipid biomarkers is a powerful tool for studying the structure of living biotic communities as well as the source, turnover and stabilization of non-living organic matter [1–3]. A wide array of lipid molecules could be used as biomarkers, including fatty acids, sterols, respiratory quinones and alkanes. In living biota, fatty acids are the major building blocks of phospholipids, glycolipids and neutral lipids such as sterol esters, monoacylglycerols, diacylglycerols and triacylglycerols [4]. For example, the lipid biomarker profile obtained from analysis of total lipid fatty acids is an appropriate tool for assessing how agricultural practices and environmental stresses affect the soil microbial community [5,6].

Generating lipid biomarker profiles for assessing soil microbial communities or other studies on the chemical structure and biological origin of soil lipids demands intensive laboratory work (lipid extraction, fractionation, derivatization, chromatography) and data analysis. Simpler 'fingerprinting' techniques might be adequate for the characterization of soil lipids extracted from living organisms and other soil organic matter fractions. Available fingerprinting techniques include: Raman spectroscopy, Fourier transform infrared spectroscopy, direct infusion mass spectrometry and Py-MS [7–10]. Fingerprinting techniques have the potential to be more rapid and have a higher throughput than profiling methods because they do not require chromatographic separation or derivatization of the target compounds, however, they require chemometric interpretation of the complex analytical data generated from simultaneous acquisition of hundreds of metabolites [8].

Among fingerprinting techniques, Py-MS has several advantages, such as speed of analysis, sensitivity and high sample throughput. In most Py-MS systems, the chemical compounds in a sample are desorbed and volatilized during a rapid heating phase, followed by ionization with electron impact, and detection by mass spectrometry [7]. This technique was successfully used, sometimes with a thermally assisted hydrolysis and methylation step, to discriminate and classify bacteria [11], fungi [12], higher

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plants [13] and soil organic matter [14]. The modified Py-MS system in this study (Py-MAB-TOF-MS) has a metastable atom bombardment capacity that permits better control of the ionization energy and reduces chemical fragmentation during ionization, compared to electron impact ionization [15,27]. Recently, Py-MAB-TOF-MS systems have been used to fingerprint lipids of plant, animal and microbial origins [16–18,27]. We are not aware of any studies that have used a Py-MAB-TOF-MS system to characterize soil lipids, but hypothesize that a Py-MAB-TOF-MS system will produce a replicable lipid fingerprint that will complement the fatty acid biomarker profile generated by GC-FID for the same set of soil samples.

The objective of this work was to evaluate the reproducibility and pattern of the lipid fingerprint produced by the Py-MAB-TOF-MS system, compared to the lipid biomarker profile generated by the conventional GC-FID method, for six agricultural soils with diverse cropping histories.

2. Experimental

2.1. Soil collection and handling

The soils used in this study were mixed, frigid Typic Endoaquents collected from the top 15 cm of agricultural fields in southwestern Québec, Canada that were under soybean (S1, S2, S3 and S4), corn (CORN) and asparagus (ASP) production. Each soil sample was a composite of 18–25 cores (15 cm long, 3 cm internal diameter) collected from random locations in each field and mixed together. Immediately after collection, half of each soil sample was frozen at −20 °C to preserve soil lipids. The other half of the sample was air-dried, sieved through a 2 mm mesh sieve and sent for soil physical and chemical analysis. Agricultural practices and selected soil characteristics for each field are reported in Table 1.

2.2. Soil lipid extraction

All organic solvents used in this study were HPLC grade. Glassware and laboratory equipment were prepared following the recommendations of White and Ringelberg [19]. Soil lipids were extracted with an ASE 200 Accelerated Solvent Extractor (Dionex Corporation, Sunnyvale, CA, USA) according to the operating conditions described by Macnaughton et al. [20], which 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. Between 6 and 8 g of freeze-dried soil was packed into a 11-mL stainless steel ASE vessel that had been rinsed with 1:2 chloroform:methanol solvent. Soil lipids were extracted with 1:2 chloroform:methanol solvent, dried under N_2 gas and quantitatively transferred to a vial using 1:2 chloroform:methanol, dried under N_2 gas and re-dissolved in 1 mL of 1:2 chloroform–methanol prior to direct analysis with the Py-MAB-TOF-MS. Soil lipid extracts were transformed to fatty acid methyl esters (FAMEs) before analysis by GC-FID (see below).

2.3. Analysis of soil lipid extracts using the Py-MAB-TOF-MS system

Soil lipid extracts were analyzed with a Py-MAB-TOF-MS (Dephy Technologies, Montreal, Canada) as described elsewhere [18,21]. One microliter sample was applied to the pyroprobe (Pyroprobe 2000 pyrolyzer; CDS Analytical, Oxford, PA, USA). Pyrolysis was achieved by ramping the probe temperature by 20 °C ms^{-1} from ambient to 1100–1200 °C, with a final hold time of at least 50 s. The probe was specially modified to enable helium flow (1–2 mL/min) through the quartz capillary, thus enhancing transfer of pyrolysis products into the MAB source. The ionization gas was N_2, which has an ionization energy of 8.67 eV (85%) and 11.88 eV (15%). The mass range was scanned between 40 and 1000 m/z. Each soil lipid extract (one per composite sample) was analyzed five times.

2.4. Preparation of the fatty acid methyl esters and analysis with the GC-FID system

Soil lipid extracts were transformed to FAMEs before analysis by GC-FID. Total lipid fatty acid methyl esters (TL-FAMEs) were prepared by mild alkaline transesterification of the soil lipid extract [19]. Ester-linked fatty acid methyl esters (EL-FAMEs) were also generated by subjecting unmodified soil samples to direct mild alkaline transesterification [5]. The EL-FAMEs were extracted after the transesterification step. In both preparations, the fatty acid methyl esters were not fractionated from the rest of the lipid biomarkers. The TL-FAMEs are most like to contain mainly extractable free lipids. Using an alkaline reagent for the transesterification, only ester-linked fatty acids could be targeted and not the free fatty acids, that are most likely coming from plants [20]. The EL-FAME method by directly transmethylating the fatty acids could also recovered fatty acids that were ester-linked to insoluble (in organic solvents) minerals and organic macromolecules such as cutin and suberin. After drying under N_2, the TL-FAMEs and EL-FAMEs were dissolved with 1-mL of iso-octane containing 25 ng μL^{-1} of methyl-nonadecanoate (C19:0) internal standard. Each TL-FAME mixture (one per composite sample) was analyzed five times, and each EL-FAME mixture (one per composite sample) was also analyzed five times. This involved injecting 5 μL of each analytical replicate in split mode (50:1) with a gas chromatograph (Hewlett Packard 6890) equipped with an Ultra-2 capillary column (cross-linked 5% diphenyl-95% di-methylpolysiloxane; length, 25 m; internal diameter, 0.20 mm; film thickness, 0.33 μm; Agilent J&W 19091B-102). Hydrogen was the carrier gas (68.9 kPa), nitrogen was the "makeup" gas (30 mL min^{-1}), and air was used to support the FID flame. The temperature program ramped from 170 °C to 270 °C at 5 °C min^{-1} and was held at 270 °C for 2 min. Inlet and detector temperatures were 250 °C and 300 °C, respectively. The retention times of the peaks were converted to equivalent chain length (ECL) values [22]. Identification of peaks was based on comparison of retention times (ECLs) to commercial FAMEs 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), and led to the identification of 70 TL-FAMEs and 78 EL-FAMEs. The settings were the same as those used in the MIDI protocol (MIDI, Inc., Newark, Delaware, USA, www.midi-inc.com) [5,22], thus, peak identity could be validated by sending a subset of all samples to a certified laboratory (Laboratoire de Santé Publique du Québec, Sainte-Anne-de-Bellevue, QC) using the MIDI system. The MIDI library was built/designed to allow the identification of fatty acids from microbial origin. However, microbes and plants share some common fatty acids.

2.5. Calculations and statistical analysis

The Py-MAB-TOF-MS analysis of the total lipid extracts gave ion masses ranging from 40 to 1000 m/z (m/z, mass-to-charge ratio). The ion intensity for each mass was normalized to percent total ion counts. From the GC-FID data, the percent area of each FAME was calculated as: (peak area of each identified FAME/total peak area of all identified FAMEs) × 100%.

This work was considered exploratory, since we are not aware of other studies that attempted to characterize soil lipids with Py-MAB-TOF-MS. Analysis of variance was used to distinguish the

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Table 1
Selected properties of the soils (Typic Endoquents) used in the experiment.

	Soil identification					
	S1	S2	S3	S4	CORN	ASP
Location	Saint-Césaire	Saint-Césaire	Sainte-Brigide-d'Iberville	La Présentation	Ste-Anne-de-Bellevue	Ste-Anne-de-Bellevue
	45°25′ N	45°25′ N	45°19′ N	45°40′ N	45°30′ N	45°30′ N
	73°00′ W	73°00′ W	73°04′ W	73°03′ W	73°35′ W	73°35′ W
Crop	Soybean	Soybean	Soybean	Soybean	Corn	Asparagus
Tillage[a]	CT	CT	NT	NT	CT	NT
Sampling year	2000	2000	2000	2000	2001	2001
Sampling depth	0–15	0–15	0–15	0–15	0–20	0–15
pH[b]	6.7	6.3	6.2	6.3	6.1	6.0
OM ($g\,kg^{-1}$)[c]	42	36	51	40	45	48
Sand ($g\,kg^{-1}$)	118	130	140	384	815	530
Clay ($g\,kg^{-1}$)	321	353	367	246	96	170
Silt ($g\,kg^{-1}$)	561	517	493	370	89	300
Textural class	Silty clay loam	Silty clay loam	Silty clay loam	Loam	Sandy loam	Loamy sand

[a] CT = conventional tillage (field was plowed and harrowed before planting); NT = no-tillage (no cultivation, crop residues left on soil surface).
[b] Soil:water (1:2 (w/v)).
[c] Organic matter (OM) determined by loss on ignition (360 °C for 4 h).

molecular fragments differing between the six soils, using $\alpha = 0.05$ as a threshold to reject the null hypothesis. Principal components analysis (PCA) of data from the Py-MAB-TOF-MS system revealed that seventeen components explained more than 99% of the total variance. There were six components that explained more than 90% of the total variance in the TL-FAME and EL-FAME datasets. All statistical analyses were performed using SYSTAT software, version 10 (Systat Software Inc., Richmond, CA, USA).

3. Results and discussion

3.1. Soil lipid fingerprints from the Py-MAB-TOF-MS system

Visual examination of the mass ionization spectra of pyrolyzed soil lipid extracts revealed a distinctive lipid fingerprint for each soil (Fig. 1) and was supported by the statistical analysis of Py-MAB-TOF-MS spectra. There were 523 molecular fragments from the soil lipid extracts that differed significantly ($\alpha \leq 0.05$) among soils. To reduce the dimensionality of the dataset, these molecular fragments were used as input in a PCA, generating 17 components that explained more than 99% of the total variance. The first principal component (PC1) explained 43.3% of the variance and was plotted against the second principal component (PC2), which explained 19.7% of the variance (Fig. 2). There was overlap in the standard error bars associated with the scores for S2, S3 and S4, and among the standard error bars of S3, S4 and CORN (Fig. 2).

Due to the exploratory nature of this work, the identity of extractable soil lipids detected with the Py-MAB-TOF-MS system is unknown. However, some characteristics of extractable soil lipids deserve mention. Examination of PCA loadings of the molecular fragments (Table 2) revealed that fragments with masses from 250 to 292 m/z were negatively correlated with PC1 (with loadings > −0.900) and fragments between 605 and 667 m/z were positively correlated with PC1 (with loadings > 0.900). Fragments from 354 to 482 m/z were negatively correlated with PC2 (with loadings > −0.725), while those from 113 to 201 m/z were positively correlated with PC2 (with loadings > 0.800).

Moreover, peaks in the spectra could be annotated by peaks identified in other studies [3,28–30]. However these identifications are tentative because we are only using the mass of the molecular ion of a given compound as identifying criterium. Each soil lipid spectra contains peaks that could correspond to aliphatic lipids homologous series (odd and even) of n-alkanes (C12–C36, m/z 170–506), n-alkanoic acids (C12–C36, m/z 200–536), n-alkanols (from C12 to C30, m/z 186–438). We found also an acyclic isoprenoid (phytol at m/z 410), ω-hydroxyalkanoic acids (ω-hydroxydocosanoic acid at m/z 356 and ω-hydroxytetracosanoic acid at m/z 384), carbohydrates (glucose/mannose at m/z 180 and sucrose at m/z 342), a phenolic compound (ferulic acid at m/z 194), monoacylglycerides (C16:1 monoacylglyceride at m/z 328, C16:0 monoacylglyceride at 330 and C18:0 monoacylglyceride at m/z 358). Finally, some peaks could be assigned to sterols such as squalene (at m/z 410), biosynthetic precursor of sterols, β-amyrin (at m/z 426), cholesterol (at m/z 386), campesterol (at m/z 400), β-sitosterol (at m/z 414), stigmasterol (at m/z 412) and ergosterol (at m/z 396). All annotated peaks have ion intensities above 0.1% of the total ion intensity for a given spectrum. More than one compound could be assigned for a given peak. More in-depth analysis by gas chromatography–mass spectrometry will be required to confirm these interpretations. However, it demonstrates the capacity of the Py-MAB-TOF-MS system to detect meaningful lipid biomarkers.

Further work is needed to determine which molecular fragments represent soil lipids that are broadly distributed (e.g., present in all Typic Endoaquents due to soil formation processes) and distinguish soil lipids whose presence is related to site-specific

Table 2
Loading values from PCA of molecular fragment masses (m/z, mass-to-charge ratio) detected in lipid extracts from 6 different soils by a Py-MAB-TOF-MS system. Values are the 10 highest and 10 lowest loading values of for each principal component (PC) axis.

PC1 loading	Fragment mass (m/z)	PC2 loading	Fragment mass (m/z)
−0.967	266	−0.865	354
−0.958	268	−0.830	364
−0.954	290	−0.826	366
−0.954	282	−0.817	394
−0.948	292	−0.803	424
−0.943	250	−0.781	468
−0.934	270	−0.777	370
−0.932	278	−0.769	395
−0.930	284	−0.766	434
−0.927	288	−0.753	482
0.925	666	0.807	137
0.931	663	0.807	113
0.932	639	0.812	123
0.932	633	0.851	149
0.936	605	0.876	153
0.937	635	0.883	173
0.940	621	0.888	181
0.944	637	0.902	201
0.951	667	0.913	159
0.956	665	0.930	160

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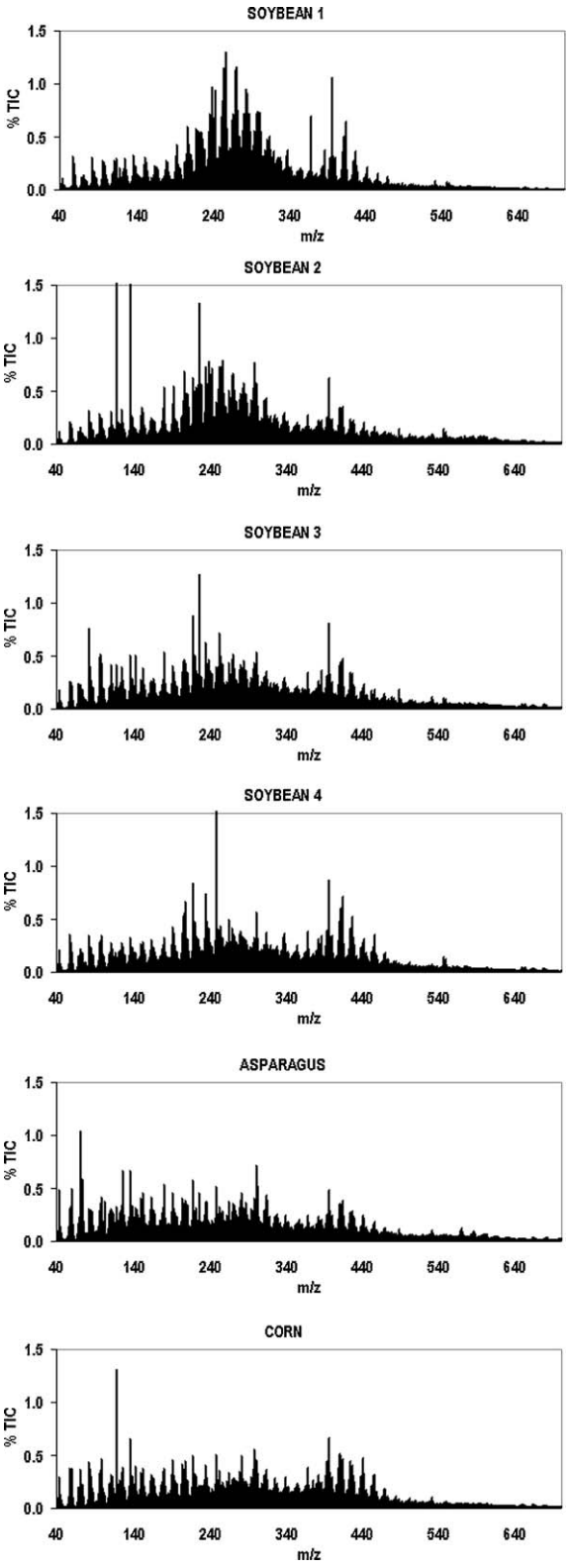

Fig. 1. Examples of mass spectra, showing the % total ion count plotted against the mass-to-charge ratio (m/z) of each molecular fragment in pyrolyzed total lipid extracts of soils cropped with soybean, corn and asparagus using a Py-MAB-TOF-MS system. Each mass spectrum is a mean of 5 analytical replicates.

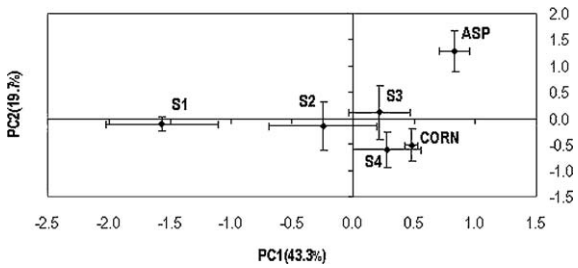

Fig. 2. Plots of the scores ± standard error from the PCA of molecular fragment masses detected in lipid extracts from 6 soils by Py-MAB-TOF-MS system. The percentage of variance explained by each principal component is in parenthesis: PC1 vs. PC2. See Table 1 for soil description.

differences in land use history, crops, organic amendments, soil microbial communities, or other factors.

3.2. Fatty acid biomarker profiles from TL-FAMEs and EL-FAMEs analyzed by GC-FID

Analysis of variance of the 70 TL-FAMEs identified from soil lipid extracts showed that 59 TL-FAMEs differed significantly ($\alpha \leq 0.05$) among soils. These TL-FAMEs were analyzed by PCA and produced 6 principal components that explained 92.7% of the total variance. A plot of scores from the two first principal components, which accounted for 57.2% of the variance, showed the scores and variability (standard error) in the lipid profiles of the six soil samples (Fig. 3a). Other researchers have used EL-FAME analysis to characterize microbial communities that evolved during 25 years of wheat-fallow cropping after land conversion from a native mixed prairie [23], to study the effect of soil type and agricultural management practices on microbial communities [24] and to monitor microbial changes during composting [25]. We identified 78 EL-FAMEs, and 62 of these differed significantly ($\alpha \leq 0.05$) among soils. The PCA of these 62 EL-FAMEs generated 6 principal components explaining 93.3% of the variance. The first two principal components, which explained 83.4% of the variance, are shown in Fig. 3b. In contrast to the TL-FAMEs, there was greater variation associated with the replicates from each soil sample, and a notable overlap in

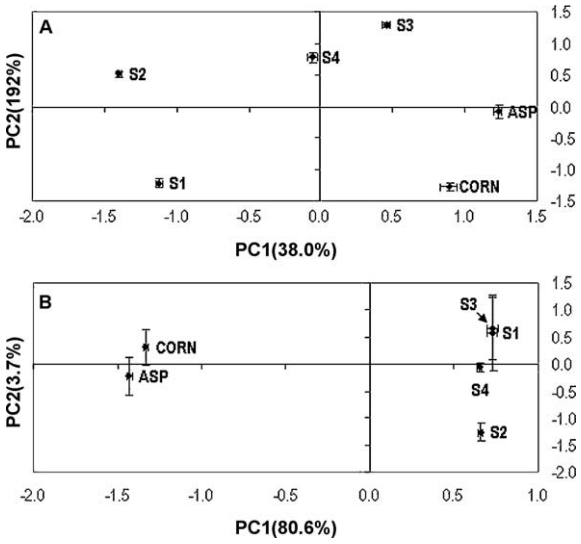

Fig. 3. Plot of scores ± standard error from (A) the PCA of TL-FAMEs from six soils by GC-FID, with the percentage of variance explained by each principal component in parentheses: PC1 vs. PC2, and (B) the PCA of EL-FAMEs from six soils by GC-FID, with the percentage of variance explained by each principal component in parentheses: PC1 vs. PC2. See Table 1 for soil description.

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the error bars of samples S1 and S3 (Fig. 3b). The variation along PC2 was similar for the EL-FAMEs of ASP and CORN samples (Fig. 3b).

3.3. Comparison of Py-MAB-TOF-MS and GC-FID analyses

The pyrolysis of soil lipids with the Py-MAB-TOF-MS system generates many molecular fragments (from 40 to 1000 m/z), but we did not have enough information to differentiate and categorize those obtained from living cells from those obtained from non-living organic matter. Therefore, we consider that the lipid fingerprints, generated from analysis of Py-MAB-TOF-MS spectra, reflect the composition of extractable soil lipids. In contrast, the analysis of (TL- and EL-) FAMEs with GC-FID produces fatty acids biomarkers, mostly from living organisms. Generally, fatty acid biomarker profiles from FAME analysis reflect the microbial community structure in a particular soil [5,6,23,26]. This may partially explain why the PCA of the lipid fingerprint produced by Py-MAB-TOF-MS showed more variability between analytical replicates, as seen from the standard error bars in Fig. 2, than the fatty acid profiles generated with GC-FID (Fig. 3). Mathematically, the maximum number of principal components is constrained by the number of data points considered in a PCA, which explains why the variance:covariance structure of the larger Py-MAB-TOF-MS dataset was represented with 17 principal components whereas the TL- and EL-FAMEs generated 6 principal components each. Thus, some of the variability associated with mean scores in the PC1 and PC2 plots of the Py-MAB-TOF-MS lipid fingerprint was related to the larger number of variables included in this PCA, compared to the lipid profiles of TL- and EL-FAMEs.

While soil samples could be distinguished from lipid fingerprints (Fig. 2), the overlap in standard errors of lipid fingerprint scores for samples S2, S3 and S4 may reflect common soil properties and soybean production in these agricultural fields. Samples S3, S4 and CORN also showed overlapping variability (Fig. 2), which could be related to the fact that the soybean fields are under a two-year rotation (corn-soybean) with no-tillage practices, meaning that corn residues from the previous cropping season were left on the soil surface. Lipids originating from partially decomposed corn residues such as waxes, sterols, steryl esters and glycerolipids, which are among the molecular fragments generated by Py-MAB-TOF-MS, may have been present in all these samples, but this remains to be confirmed. With the GC-FID system, the TL-FAME method provided the most clear discrimination and lowest variability among replicate analyses. The EL-FAME analysis showed overlap in the error bars for samples S1 and S3, as well as samples CORN and ASP (Fig. 3b). Otto et al. [3] noted that treating soils with an alkaline reagent and solvents, the EL-FAME procedure, would probably extract fatty acids from bound soil lipids, such as undecomposed plant residues and stable soil organic matter. The similarity in EL-FAME profiles from S1 and S3 suggests a high representation of fatty acids originating from the soybean crop, whereas the EL-FAME profiles of CORN and ASP could include fatty acids from the native soil organic matter, which is expected to be comparable at these nearby sites (<2 km between fields with closely related soil texture and soil organic matter contents). Visually, the PCA plots produced from the Py-MAB-TOF-MS lipid fingerprint showed a pattern that was similar to the TL-FAME profile, but quite different from the EL-FAME profile.

4. Conclusions

For analytical replicates, the lipid fingerprint produced by the Py-MAB-TOF-MS system presented more variability than the fatty acid biomarker profile generated by the conventional GC-FID method. This was attributed to the fact that the Py-MAB-TOF-MS system provides information on a broader group of extractable soil lipids, including those of plant and animal origin, compared to those included in the (TL- and EL-) FAME profiles. The TL-FAME method generated a fatty acid profile with the smallest variation between replicates and distinct profiles for soils from six agricultural fields. The EL-FAME method probably included some fatty acids originating from plants and native soil organic matter, as there was moderate and overlapping variation associated with the PC scores of some soil samples. In terms of reproducibility, these methods can be ranked as: TL-FAMEs by GC-FID > EL-FAMEs by GC-FID > soil lipids by Py-MAB-TOF-MS. This ranking is affected by the fact that the Py-MAB-TOF-MS system generates more data and requires more principal components to explain the variance:covariance structure of the dataset.

The advantage of the Py-MAB-TOF-MS system is that it offers the possibility of examining a broader array of soil lipids, including those from soil microbial communities, than the conventional GC-FID method. While GC coupled to a MS system could be a powerful technique to precisely identify soil lipids, it has certain drawbacks (extensive preparation and long analysis time – around 60 min per sample). The Py-MAB-TOF-MS system, in contrast, provides fast analysis and reliably classifies of lipids from distinct sources (soils, plants). Future work should focus on the development of standard libraries for soil lipid identification and quantification. This would permit the user to identify soil lipids of microbial origin for direct and unbiased comparisons between the GC-FID methods and the Py-MAB-TOF-MS system. This would also offer the possibility of using the Py-MAB-TOF-MS system as complementary tool for assessing soil microbial community structure in natural and managed ecosystems. In conclusion, the Py-MAB-TOF-MS system has the capacity to provide a snapshot of the whole lipid composition of a soil extract, providing a unique fingerprint for a given soil that reflects the characteristics and diversity of lipids within the soil organic matter pool.

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