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Evolution of Composting Process in Maize Biomass Revealed by Analytical Pyrolysis (Py-GC/MS) and Pyrolysis Compound Specific Isotope Analysis (Py-CSIA)

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Abstract: An innovative approach based on the combination of analytical pyrolysis coupled with gas chromatography-mass spectrometry (Py-GC/MS) with compound-specific isotope analysis (Py-CSIA) is used to study the composting process of maize biomass. This multidisciplinary approach aims to elucidate the decomposition rate of the main biogenic materials (lignin, cellulose, proteins, lipids, and waxes) responses to the composting process. According to Py-GC/MS data/structural composition, a noticeable and significant decrease during the first stage of the composting process of carbohydrates and aromatic compounds is found, followed by a gradual increase of all compounds till the end of the experiment. This trend, along with an increase of fatty acids methyl-ester at the first composting stage, sustains the microbial activity and its stabilization over time. Py-CSIA data showed a significant enrichment in ¹³C in all identified compounds over time, supporting the semi-quantitative results and the decomposition of initial biomass throughout the composting process. This trend is also perceptible in lignin moieties, long-chain aliphatic structures, and isoprenoids, as highly recalcitrant compounds, presumably due to depolymerization and carbon translocation of side-chain molecules during the composting process. Compound-specific isotope values showed a good correlation with the bulk isotope data, and this served as validation of the technique. However, bulk values showed higher heterogeneity because those represent an average of all organic compounds in the sample. By combining isotopic and structural information using Py-GC/MS and Py-CSIA, we are able to provide further information and a more detailed approach to the study of the decomposition process of biomass by considering the diverse dynamics of the main biogenic compounds.

Keywords: compost; lignocellulose characterization; stable carbon isotopes; compound-specific $\delta^{13}\text{C}$

1. Introduction

Composting process is the most popular and applied technique for the treatment of organic wastes from varying origin, comprising from animal manures to municipal and industrial wastes, and including agricultural harvesting residues [1,2]. Today, the interest in this practice has been widely applied since it is known to provide multiple benefits to soils, especially in agricultural ones (depleted in organic matter, subjected to severe erosion and water scarcity problems), besides having relevance as carbon sequestration agent [3,4]. Furthermore, compost material has been proven to have a significant fertilizer value, depending on crop requirements, amendment properties, and its production process [5–7].

Composting consists of the decomposition of organic, biodegradable fraction of waste to produce a stable product to later be applied to soil or any organic substrate. Therefore, composting can be seen as an accelerated version of the natural microbial decomposition of lignocellulose. The microbial activity chemically alters the compost; hence, an estimation

of the remaining necromass main components is desirable to understand the composting process [8].

The main characteristics of composted organic matter vary with the nature of the initial wastes and with the changes throughout the composting process. Compost organic matter is highly heterogeneous, with moieties exhibiting diverse humification degrees [9]. Accounting for maize as one of the greatest crops at worldwide scale, the revalorization and transformation of its agricultural waste comprise a valuable, sustainable practice [10]. In addition, amendments with naturally ^{13}C -enriched biomass from C4 photosynthetic pathway plants, such as maize ($\delta^{13}\text{C}$ -9 to -19‰), can be used to trace the fate and study the dynamics of 'native' and 'new' soil organic carbon (SOC) developed under C4-vegetation in agricultural soils [11–14]. Therefore, the detailed study of the chemical changes exerted in maize biomass during the composting process is also relevant for understanding of the whole process and the ulterior transformation of this material in the soil. Knowledge of the chemical composition of initial biomass and compost is also required to explain the possible role of the structural properties of this type of material during the evolution of soil organic matter (SOM).

Pyrolysis coupled with gas chromatography and mass spectrometry (Py-GC/MS), commonly known as analytical pyrolysis, is a powerful tool to analyze and elucidate information on the molecular structure of complex organic materials. The technique has been successfully applied in previous studies for studying the transformations and evolution of composting biomass, as well as the potential products it can generate upon decomposition processing [10,15–18]. The technique has been also used to understand the mechanisms and kinetics associated with the thermal decomposition of biomass [19,20]. Analytical pyrolysis also poses several analytical advantages over other techniques for OM characterization: minimal or no sample preparation needed, small sample size required, and the ability to detect and characterize in a single analysis a wide variety of organic materials, including recalcitrant and low solubility organic forms [21–23].

It is known that, after fixation by primary producers, the stable isotope composition of light major elements C and N in organic matter varies with decay or decomposition, and this feature can be used to study biomass evolution during composting [24,25]. Normally, N is progressively enriched in the heavy isotope, and $\delta^{15}\text{N}$ values increase with the composting process [26], whereas a $\delta^{13}\text{C}$ depletion has been usually observed during composting [14]. Although a slighter ^{13}C increase is expected due to the heterotrophic activity on the organic matter [27,28], changes in particular OM components during the composting process may predominate in the observed $\delta^{13}\text{C}$ shifts, i.e., preferential losses in the naturally ^{13}C enriched celluloses (labile polysaccharides) components during the first stages of the composting process, as well as the accumulation of the more recalcitrant lignin and waxes, which are comparatively ^{13}C depleted [29]. These effects may result in a net even or slight $\delta^{13}\text{C}$ depletion of the final compost.

A best and more accurate approach to the conventional isotope analysis in bulk samples to study the mechanisms of organic matter evolution during the composting process, is the analysis of $\delta^{13}\text{C}$ in separate compounds. To the best of our knowledge, few studies have applied isotopic determinations to elucidate the degradation dynamics of organic amendments [30]. The compound-specific isotope analysis (CSIA) approach has been previously used to measure C stable isotope composition in major organic matter components, e.g., fatty acids [31,32], *n*-alkanes [33], polysaccharides [34], lignin phenols [35], or sterols [36]. Isotope ratio mass spectrometry (IRMS) constitutes a helpful approach to differentiate several biogenic compounds from an organic, complex sample and to authenticate the origin of biomass. Determination of compound-specific isotopic composition can provide a fingerprinting that reflects decomposition process, technical process in food sciences, etc. [32]. The stable carbon isotope composition ($\delta^{13}\text{C}$) is informative of origin, transformation, utilization, and stabilization of carbon amendments in soils.

Pyrolysis-gas chromatography coupled on-line with isotope ratio mass spectrometry (Py-GC-C/IRMS) has been previously used to study the $\delta^{13}\text{C}$ values of the main compo-

nents of wood [37], polysaccharides [38], and essential oils [39], as well as to determine turnover rates of specific carbohydrates, lignin, lipids, and N-containing compounds from arable soils of a wheat C3/maize C4 rotation experiment [40]. These studies demonstrated that the technique did not produce isotopic fractionation and that the pyrolysis products represented the isotope composition of their precursors. This technique, named Py-CSIA [38], is a fast, reproducible, and robust method that inherits the advantages posed by the conventional analytical pyrolysis for the study of complex, poorly soluble organic matrices and is suitable for detailed studies on organic matter dynamics.

In this work, the molecular composition and evolution during the composting process of maize biomass was recorded over a period of two and a half years. We hypothesize that the combination of Py-GC/MS and Py-CSIA can give accurate information of the humification degree of amenable compounds from compost and its evolution over time. Therefore, the objectives of this study were: (i) to characterize the molecular composition of the biomass and compost samples during the experimental trial using Py-GC/MS, (ii) to investigate whether the isotopic composition of specific compounds (Py-CSIA) can provide information on the origin and decay of certain compounds, supported by bulk isotopic analysis, and (iii) to test the suitability of coupling Py-GC/MS and Py-CSIA to monitor complex organic samples, avoiding any sample preparation, extraction, or derivatization.

2. Materials and Methods

2.1. Composting Samples and Preparation

The experiment was set at “La Hampa”, an experimental farm that belongs to the Institute of Natural Resources and Agrobiology of Seville (IRNAS-CSIC). The farm is located in Coria del Río municipality (37°17' N, 6°3' W), 13 km SW of the city of Seville (Spain) under a typical Mediterranean climate. There, the average temperature during the duration of the experiment was 12.2 °C during winter and 25.8 °C during spring-summer season, with mild rainy winters (496 mm mean annual rainfall) (data from the farm's agroclimatic station).

Surpluses of maize from an ongoing experiment set in February 2017 with a continuous monoculture maize crop were collected and formed into a composting pile. The compost was produced from approximately 2 m³ of maize biomass in the composting pile located outdoors. The initial biomass was collected in November 2017, after the harvesting season. The pile included dry leaves, the stalk (sheath and blade), and rest of roots, spare seeds, and husk. No other products were included in the compost, to avoid altering $\delta^{13}\text{C}$ of the maize. The phase of composting consisted of mechanical turning and forced aeration. Monthly turning was carried out with a shovel, specially designed for composting waste. Depending on the season, the pile was watered to maintain moisture above a minimum (40%). At the final stage of the composting experiment, the average moisture was 70%, whilst pH and electrical conductivity values (measured in a 1:5 compost/water extract) show values of 6.5 and 422 $\mu\text{S}/\text{cm}$, respectively.

The experiment lasted from November 2017 (designated as T0), when the maize biomass was chopped and piled, to June 2020. Samples were taken before composting at T0, and at four times during the composting process: November 2018 (T1), February 2019 (T2), November 2019 (T3), and June 2020 (T4). Compost samples consisted of a composite sample formed from eight subsamples randomly collected from the composting pile. In addition, biomass—not composted—samples were taken by randomly selecting different parts of harvested maize plants. The samples were dried at 70 °C for two days, and subsequently at 100 °C for 2 h, to assure humidity removal and crushed in a grinder (RetschMühle, Haan, Germany) to <0.5 mm.

2.2. Pyrolysis Gas Chromatography Mass Spectrometry (Py-GC/MS)

Direct pyrolysis–gas chromatography–mass spectrometry (Py-GC/MS) analysis was performed as described in reference [41], using a double-shot pyrolyzer (model 2020i; Frontier Laboratories Ltd., Fukushima, Japan) attached to a GC Agilent 6890 N (Ag-

ilent Technologies, Santa Clara, CA, USA) and an Agilent 5973 mass selective detector. The mass spectra were acquired at 70 eV ionizing energy. Samples (1.5 mg) were placed in small ultra-alloy deactivated pyrolysis capsules and introduced into a preheated micro-furnace at 400 °C for 1 min. The evolved gases were directly transferred into the GC/MS for analysis. The gas chromatograph was equipped with a low polar-fused silica (5% phenyl-methylpolysiloxane) capillary column Agilent J&W HP-5 ms Ultra Inert, of 30 m × 250 µm × 0.25 µm film thickness. The oven temperature was held at 50 °C for 1 min and then increased to 100 °C at 30 °C min⁻¹, from 100 °C to 300 °C at 10 °C min⁻¹, and stabilized at 400 °C for 10 min using a heating rate of 20 °C min⁻¹ in the scan mode. The carrier gas used was helium at a controlled flow of 1 mL min⁻¹.

The pyrolysis products were identified by comparison with spectra stored in the Wiley and NIST libraries, using single ion monitoring and with published spectra reported in the literature for lignin and plant material [42]. The peak areas of the different identified compounds were integrated and calculated as total abundances. For the comparison among Py-GC/MS data, the relative abundance of every compound was calculated as a percentage of the total chromatographic area.

2.3. Pyrolysis Compound Specific Isotopic Analysis (Py-GC-C/IRMS)

The carbon isotope composition of individual compounds was determined by direct pyrolysis compound specific isotopic analysis ($\delta^{13}\text{C}$ Py-CSIA). This was performed using a double-shot pyrolyzer (model 3030D; Frontier Laboratories Ltd., Fukushima, Japan) attached to a GC IsoLink™ IRMS System (Thermo Fisher Scientific, Bremen, Germany). The system consists of a trace GC Ultra system, fitted with the same chromatography column type, and configured with the same chromatographic conditions as previously described, connected to a GC-Isolink System equipped with a micro-reactor for combustion (C) set at 1020 °C and coupled with a Delta V Advantage isotope ratio mass spectrometer via a ConFlo IV universal interface.

The samples were weighted (1.5 mg) and pyrolyzed under the same conditions as previously described and the evolved gases directly injected into the GC-C/IRMS system for analysis. All samples were analyzed in triplicate ($n = 3$). Each chromatographic compound was gasified in the Isolink System, and pure CO₂ gas was mixed into the He carrier flow as pulses of reference gas.

Structural features of specific chromatographic compounds (peaks) were inferred by comparing and matching the mass spectra obtained with the Py-GC/MS analysis with the Py-CSIA chromatograms obtained using the same pyrolysis and chromatographic conditions. Background subtractions and isotope abundances were calculated using the ISODAT 3.0 software (Thermo Scientific, Bremen, Germany).

The precision of $\delta^{13}\text{C}$ Py-CSIA and accurate of the measurements was evaluated and corrected against the reference material *n*-alkane mixture C4 (5 dissolved *n*-alkanes C-17 to C-25 in equal concentrations: Indiana University, Bloomington, IN, USA). The linear correlation between standard and measured (IRMS) $\delta^{13}\text{C}$ values from the C4 mixture was used to derive sample $\delta^{13}\text{C}$ values relative to the VPDB scale [43]. The reference *n*-alkane mixture was analyzed in a daily routine, before and after each analytical run. Standard and measured isotopic values fitted well along a straight line, with a linear regression R² no lower than 0.99 in all cases.

2.4. Elemental Analyzer (EA-Isolink)

For $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ bulk isotopic determination, biomass and compost samples were weighted (c. 0.7 mg) and enveloped into tin capsules. The samples were analyzed in duplicate ($n = 2$). The analysis was performed using an EA IsoLink™ IRMS System (Thermo Fisher Scientific, Bremen, Germany). The EA microanalyzer was equipped with a combustion furnace set up at 1020 °C and coupled via a ConFlo IV Interface unit to a continuous flow Delta V Advantage isotope ratio mass spectrometer (IRMS) for measuring $\delta^{13}\text{C}$ and

$\delta^{15}\text{N}$. Isotopic values were corrected by using the appropriate standards recognized by the International Atomic Energy Agency (IAEA).

The stable isotope abundances are reported in the delta (δ) notation (e.g., $\delta^{13}\text{C}$) in variations relative to an international measurement standard. The isotope value is defined in [44], according to the Equation (1).

$$\delta^i E_{\text{sample}} = \frac{R \left(\frac{iE}{jE} \right)_{\text{sample}}}{\left(\frac{iE}{jE} \right)_{\text{standard}}} - 1, \quad (1)$$

where “ R ” is the molar ratio of the heavy (iE) to light (jE) most abundant isotope of chemical element “ E ” ($^{13}\text{C}/^{12}\text{C}$ for $\delta^{13}\text{C}$ values; $^{15}\text{N}/^{14}\text{N}$ for $\delta^{15}\text{N}$ values). The “ δ ” values are reported in per mil (‰). The stable isotope standard for reporting carbon measurements is the Vienna Pee Dee Belemnite limestone (VPDB scale) and for nitrogen measurements atmospheric nitrogen (Air). The analytical precision and accuracy of bulk $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ was typically less than ± 0.15 and 0.70 ‰, respectively.

The total C and total N contents were determined by the dry combustion method using the same instrument specified above (EA IsoLink) in the CN analyzer mode. The C/N ratio was calculated and used as a supplemental information to study the evolution of the decomposition process of biomass into composting material.

2.5. Statistical Analysis

Normality and homoscedasticity were checked prior to all the analyses using the Kolmogorov–Smirnov test and the Levene test, respectively. When data did not meet the normality or homogeneity requirements, a log transformation was applied before further analysis. In the cases where the normality and homoscedasticity requirements were met, an analysis of variance (ANOVA) and a multiple comparison test (Tukey) was made. In cases these requirements were not met, non-parametric tests were completed, i.e., the Kruskal–Wallis test, along with a Dunn post-hoc test for multiple non-parametric comparisons, and the Mann–Whitney U test. These statistical analyses were made with a 95% confidence level using the statistical package SPSS 20.0 (SPSS Inc., Chicago, IL, USA).

3. Results and Discussion

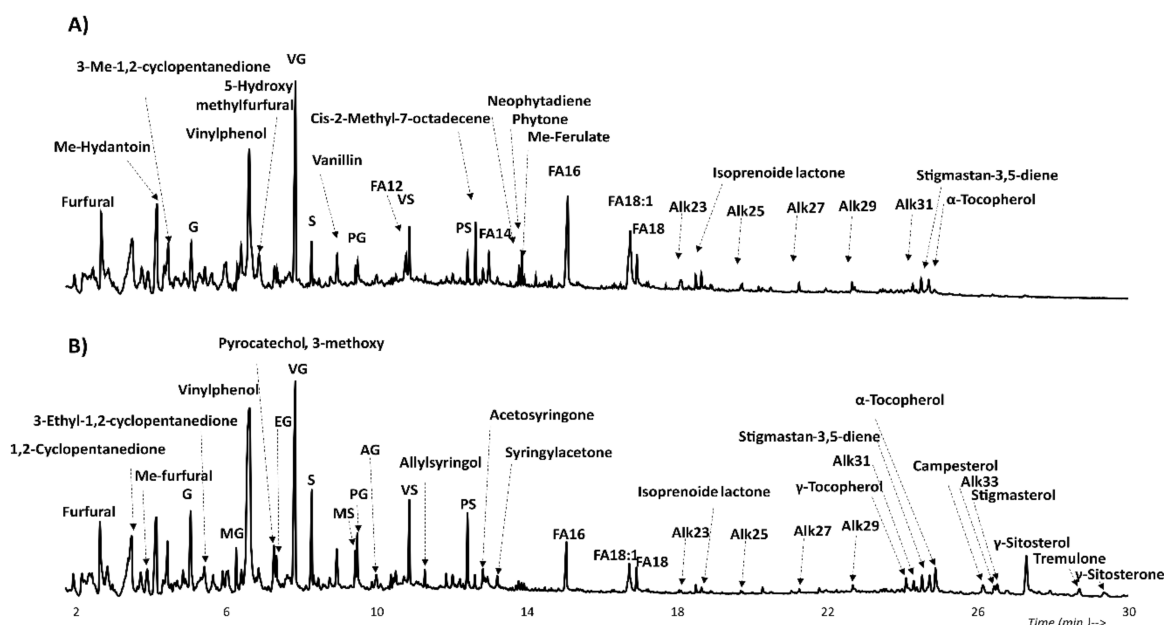
3.1. Pyrolysis Gas Chromatography Mass Spectrometry (Py-GC/MS)

A total of 97 distinct compounds were identified by analytical pyrolysis (Table S1) that can be grouped attending to their biogenic origin (Table 1). Representative pyrograms of biomass and compost material are shown in Figure 1. The pyrolyzates from all samples were found dominated by lignocellulose derived compounds; polysaccharides (PS) and lignin-derived phenols that included the main three methoxyphenol types: *p*-hydroxyphenyl (LH), guaiacyl (LG), and syringyl units (LS). Other compounds were also present in relative minor proportion, such as nitrogen-derived compounds (N) derived from proteins and polypeptides, aromatic compounds with unspecific origin (ARO), and some unidentified compounds (UNK). It is also remarkable the presence of mid-chain lipids (LIP), alkanolic acids (fatty acids, FA), fatty acid methyl esters (FAME), isoprenoids (ISO), series of long-chain *n*-alkanes (ALK), and sterols (EST). Their relative abundance, although relevant for the analysis, was low compared with that of the two major pyrolysis components (polysaccharide and lignin derived).

Table 1. Relative average abundances (%) of the main pyrolysis compounds grouped according to their chemical nature and probable biogenic origin.

Origin ¹	T0	T1	T2	T3	T4
PS	27.3 ^{b,*}	14.8 ^b	19.3 ^b	19.7 ^b	23.0 ^c
ARO	5.3 ^{a,b,c}	3.8 ^b	5.1 ^b	5.6 ^b	6.5 ^c
N	4.6 ^{b,c}	4.2 ^b	4.1 ^b	3.8 ^{a,b}	5.4 ^c
LH	9.9 ^d	9.3 ^c	12.0 ^c	11.9 ^b	17.5 ^d
LG	15.2 ^{b,c}	16.2 ^b	18.02 ^b	17.6 ^b	20.6 ^c
LS	11.5 ^c	16.1 ^b	18.8 ^{b,*}	14.0 ^b	15.2 ^c
LIP	0.68 ^a	0.54 ^{a,b}	0.46 ^a	0.37 ^a	0.12 ^a
FA	12.6 ^c	9.3 ^b	9.0 ^b	7.8 ^{a,b}	5.6 ^c
FAME	0.9 ^a	2.0 ^{a,*}	1.6 ^a	1.1 ^a	0.3 ^a
ISO	3.0 ^{a,b}	2.8 ^{a,b}	1.3 ^{a,b}	1.7 ^{a,b}	1.3 ^b
UNK	1.1 ^{a,b}	2.07 ^{a,b}	1.1 ^{a,b}	1.5 ^{a,b}	0.5 ^a
ALK	4.0 ^{a,b}	7.4 ^{a,b,*}	4.3 ^{a,b}	4.7 ^a	2.0 ^a
TOC	0.8 ^{a,b,c}	2.5 ^{a,b,*}	1.1 ^{a,b}	2.6 ^a	0.3 ^a
EST	0.69 ^{a,b}	6.4 ^{a,*}	1.8 ^a	5.9 ^a	0.7 ^a

¹ PS: polysaccharides, LH: hydroxyphenyl-units from lignin, LG: guaiacyl-units from lignin, LS: syringyl-units from lignin, N: nitrogen-derived compounds, ARO: aromatics, UNK: unknown compounds, LIP: mid-chain lipids, FA: fatty acids, FAME: fatty acid-methyl ester, ISO: isoprenoids, ALK: *n*-alkanes, TOC: tocopherols, EST: sterols. Treatments with the same letters indicate no significant differences (Kruskal–Wallis test, $p < 0.05$, Dunn post-hoc) among compound families within the same sampling time. “*” indicates significant differences between different composting times.

**Figure 1.** Example of Py-GC/MS chromatograms comparing the original biomass (T0; A) and the compost material (T3; B), with an indication of the most relevant compounds.

3.1.1. Lignocellulosic Compounds

Main pyrolysis products from polysaccharides (PS) were 2,3-dihydro-5-methylfuran-2-one, 5-methy-2-furfuraldehyde, 4-hydroxy-5,6-dihydro-(2H)-pyran-2-one, and 2-hydroxy-3-methyl-2-cyclopenten-1-one. Those compounds based on cyclopentenone and cyclopenten-

tenedione structures (furancarboxaldehyde, furanmethanol) are known pyrolysis products of polysaccharides [45]. Other relevant compounds derived from polysaccharides and known as the most specific pyrolysis product from cellulose [46], such as furfural and anhydrosugars (levoglucosane), were also abundant. Differences were found in their relative abundances, with a significant decrease noticeable from T0 to T1 (Table 1), that gradually increases along with the maturation of the compost pile. The initial decrease observed indicates a fast metabolization by the action of different cellulolytic and hemicellulolytic enzymatic activities of decomposers fungi and bacteria, corresponding to the first stages of the composting process [47,48]. The following increase from T1 to T4 is probably originated from new syntetizates from the microbial biomass with slower turnover rates that may indicate an ongoing humification process [49].

The lignin-derived pyrolysates observed in all samples were mainly derived from the guaiacyl-type (LG), followed by syringyl-type, compounds (LS). LG compounds are considered specific biomarkers; hence, it helps to comprehend the specific effect of decomposition processes on lignin [50]. The relative abundance of LG and LS units (Table 1; both summarized as LIG in Figure 2) significantly increases during the composting process, which reflects its resilience to degradation and the occurrence of a selective preservation of lignins during composting. On the other hand, it can be observed that there is a higher proportion of LG units in comparison to LS units, which points to increasing humification processes [51], typical from the composting process. In addition, although few hydroxyphenyl-type compounds were found, mainly 4-vinylphenol was found at high proportion. This can be originated from the pyrolysis of other plant compounds, such as hydroxycinnamic acids, i.e., *p*-coumaric acid [52]. The presence and high relative abundance of 4-vinylphenol found in maize biomass is in line with previous studies about lignin composition in herbaceous species [53].

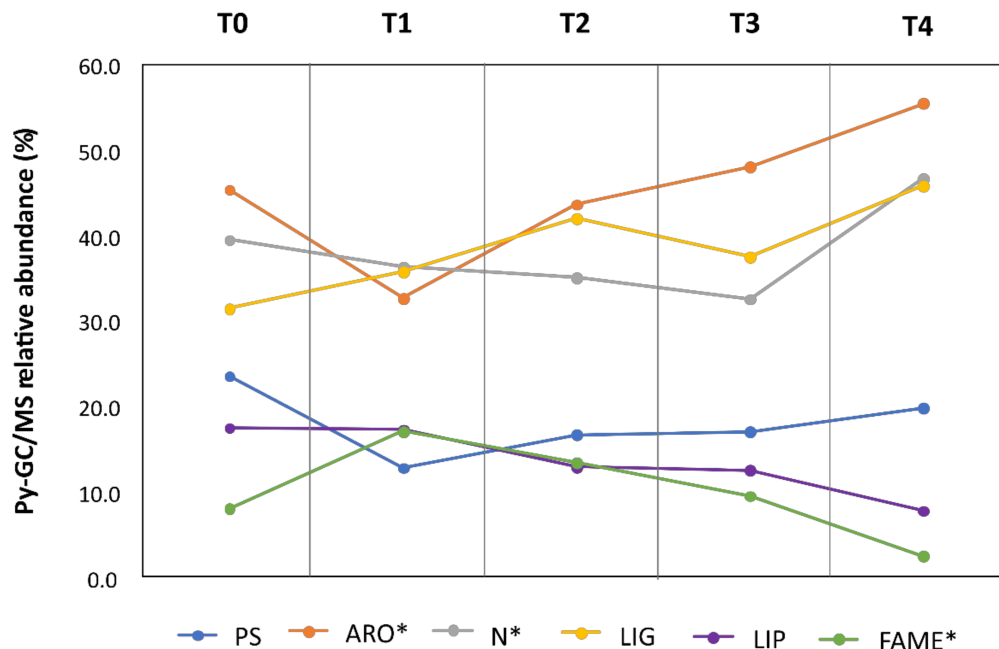


Figure 2. Evolution of the relative abundance (average; %) of main biogenic compounds released by analytical pyrolysis (Py-GC/MS) during the composting process. Compounds families with (*) indicate that values are 10-fold represented ($\times 10$).

To better study lignocellulose dynamics during composting, relevant indexes were calculated from the pyrolysis results (Table 2). The total lignin/polysaccharides and total LG/polysaccharides ratios confirmed what has been found before: a conspicuous decrease of polysaccharides is observed at the beginning of the composting process from T0 to T1, and, later, a steadily increase is observed, showing a progressive slow

decrease in the indexes but always retaining higher values than at T0. The increase in the LG/polysaccharides ratio at T1 may correspond to the microbial activity producing a preferential degradation of carbohydrates and less condensed lignin units. On the other hand, the total lignin peaks increase during the first year of composting (T0–T2), and a reduction of LG units is observed at expenses of the LS with a decrease in the LG/LS ratio. These trends corroborate that the celluloses in maize residues are preferentially degraded, leaving a residue enriched in lignin.

Table 2. Semiquantitative analysis of polysaccharides and lignin compounds released after Py-GC/MS.

	T0	T1	T2	T3	T4
Polysaccharide peaks (%)	27.3	14.8	19.3	19.7	23.0
Total lignin peaks (%)	37.2	42.2	49.5	44.0	53.5
Total lignin/polysaccharides	1.4	2.9	2.6	2.2	2.3
LG ^a /polysaccharides	0.79	1.1	1.0	0.92	0.9
LG/LS ^b	1.9	1.1	1.0	1.3	1.4
Ph-C1 ^c	1.5	2.4	2.6	2.0	3.6
Ph-C2 ^d	8.5	8.7	9.2	9.7	11.2
Ph-C3 ^e	5.0	7.8	7.8	7.2	5.6
Ph-C1 + C2/Ph-C3 ^f	2.0	1.4	1.5	1.6	2.6
Ketones	1.7	2.6	2.7	1.7	2.1
Acids	2.2	3.0	4.2	1.9	2.6
Aldehydes	2.4	2.1	3.2	3.1	2.3
Al/K + Ac ^g	0.62	0.38	0.47	0.86	0.48

^a guaiacyl-units from lignin; ^b syringyl-units from lignin; ^c phenylmethane-type compounds; ^d phenylethane-type compounds; ^e phenylpropane-type compounds; ^f ratio phenylmethane + phenylethane/phenylpropane; ^g ratio aldehyde/ketone + acid.

Non-oxidized alkyl-phenols, defined as Ph-C1, Ph-C2, and Ph-C3, are identified as 4-methylguaiacol, 4-ethylguaiacol, 4-vinylguaiacol, and 4-propylguaiacol. An increase in the sum of the relative abundance of non-oxidized alkyl phenols points to the selective preservation of some constituents of the lignin macromolecule in the compost pile. An increase in the relative abundance of higher oxidation degree G-compounds (vanillin, acetovanillone, and vanillic acid methyl ester; guaiacylacetone, propiovanillone) suggest an oxidative action resulting from the decomposition process of compost [54]. This may come from either oxidation or oxidation followed by cleavage of the C3 alkyl chain from G units at the C α and C β position. An indicative of this oxidative attack could be the observed decrease in the (aldehyde/ketone + acid) (Al/K + Ac) ratio. In this case, the Al/K+Ac ratio decreases, except for a rise during T3. An increase in the (Ph-C1 + Ph-C2/Ph-C3) ratio, along with an increase in Ph-C2 compounds and a decrease in Ph-C3 compounds, may indicate the ability of decomposition process in compost not only to oxidize lignin but also to breakdown the C3 side alkyl chain linkages. There is a final increase in this ratio, followed by a constant increase in Ph-C2 compounds, which may indicate this decomposition process in compost.

3.1.2. Other Compounds

Regarding other compounds present in minor proportions in the pyrolysates, noteworthy is the increasing abundance of non-specific aromatics (ARO) with composting time. This is a consequence of the humification process, where lignin methoxyphenols lost their methyl group to more condensed units. This condensation process leads to the increase of phenolic units, such as phenol. Other aromatic compounds (2,3-dihydroxybenzaldehyde

and 1,2-benzenediol, 3-methoxy-) may also derive from the pyrolysis of lignins but also from tannins [55].

Nitrogen-containing compounds are commonly produced from the pyrolysis of proteins, polypeptides, alkaloids, and amino acids [56]. These compounds have also been used, along with the carbohydrates, for tracing microbial origin of the pyrolysate [57]. Three N-derived compounds were found: indole, methyl indole, and 3-Methyl hydantoin, which are known to be a pyrolysis product of proteins [58,59]. Its abundance increases at the end of the composting process (T3–T4), suggesting a higher abundance of microbial exoenzymes or a higher contribution from microbial biomass [60].

Short- and mid-chain fatty acids (FA C14, C16, C18, C22, C23) were present in relatively large amounts. The relative abundance of fatty acids (FA) decreased with composting time. The even C mid-chain fatty acids methyl esters (FAME C14–C18) are produced as metabolites at T1, that may correspond to early stages of composting by anaerobic microorganisms [61].

Series of odd C numbered long-chain *n*-alkanes (ALK) are well known plant-derived markers, usually attributed to specific plant biopolymers input (waxes, cutin, suberin) [62]. A unimodal long-chain *n*-alkanes series was detected in the range C21–C33 with odd-over-even C number and a maximum at C29, typical of herbaceous biomass [63]. During the humification process, the aliphatic structures contained in plant waxes are easily biodegraded, usually resulting in a decrease of the total relative abundance of *n*-alkanes. This is also the case for maize composting, as can be observed in Figure 2 (LIP).

Compounds with an isoprenoid structure (ISO) can be found, such as phytol, neophytadiene, and 2-pentadecanone, 6,10,14-trimethyl- are typical compounds in composting processes [64]. The origin of these isoprenoids are chlorophyll side chains and the oxidation of tocopherols (Vitamin E). The relative abundance of phytol decreases during composting because of its high biodegradability [65]. Finally, some unknown compounds (*m/z* 83, 280; *m/z* 379, 394) were found in small proportions, probably originated from degraded lignins and tannins.

3.2. Pyrolysis Compound Specific Isotopic Analysis (Py-CSIA)

Major compounds released and identified by conventional analytical pyrolysis (Py-GC/MS) providing chemical structural information were also identified in the chromatograms obtained by Py-GC-C/IRMS (Py-CSIA) containing the $\delta^{13}\text{C}$ information. This was possible by overlaying both pyrograms obtained using the same chromatographic conditions, as illustrated in Figure 3.

It was possible to assign both structural and $\delta^{13}\text{C}$ values for 40 major pyrolysis peaks (labeled as ‘*’ in Table S1). These identified pyrolysis products corresponded to the main biogenic compound classes of carbohydrates (polysaccharides, PS), proteins (N), lignins (LH, LG, and LS), lipids (fatty acids, FA; long-chain alkanes, ALK), isoprenoids (ISO), tocopherols (TOC), and sterols (EST). Average compound-specific values for each compound family are detailed in Table S2, and the $\delta^{13}\text{C}$ evolution during the composting process is depicted in Figure 4.

Compound-specific $\delta^{13}\text{C}$ values obtained by direct maize biomass pyrolysis were found in the range -8 and -25 ‰ (Figure 4 and Table S2). It is noteworthy that our Py-CSIA analyses significantly isotopically discriminated compounds from distinct biogenic origin (Figure 4 and Table S2). Polysaccharide derived (PS) are ^{13}C enriched as it occurs in plant polysaccharides [66], whereas sterols (EST) and, mainly, alkanes (ALK) are significantly ^{13}C depleted [67]. They are well in the range of a C4 photosystem plant, such as maize.

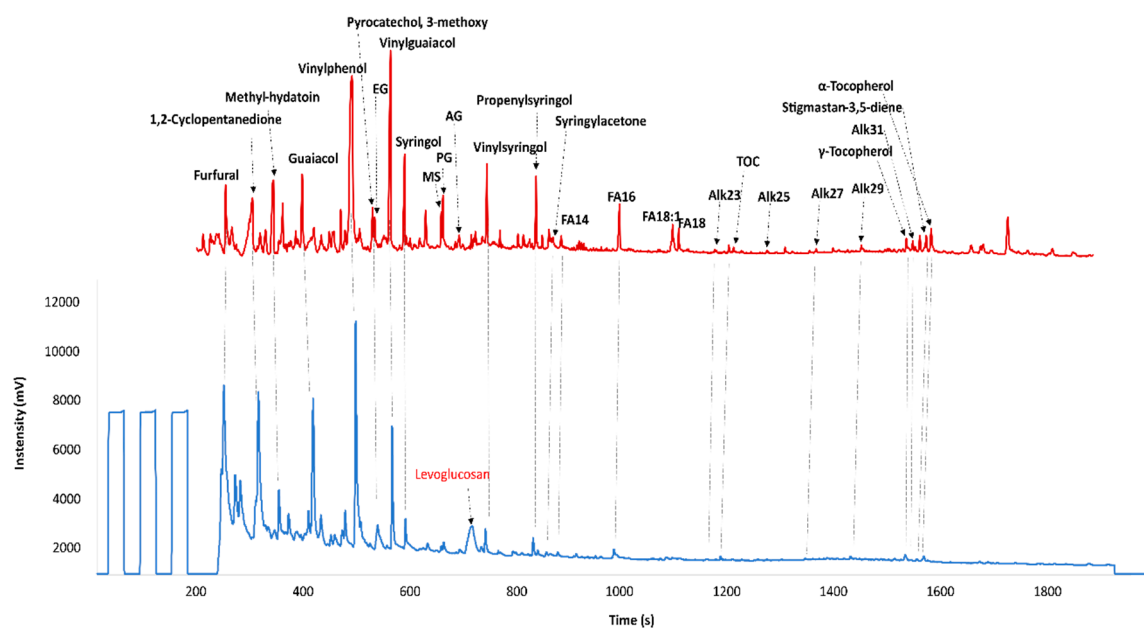


Figure 3. Example of Py-GC/MS (above in red) compound correspondence with a compound specific $\delta^{13}\text{C}$ chromatogram obtained by Py-CSIA (below in blue, mass 44 represented) of a representative sample of compost (T3). The three first large peaks are the reference gas signals.

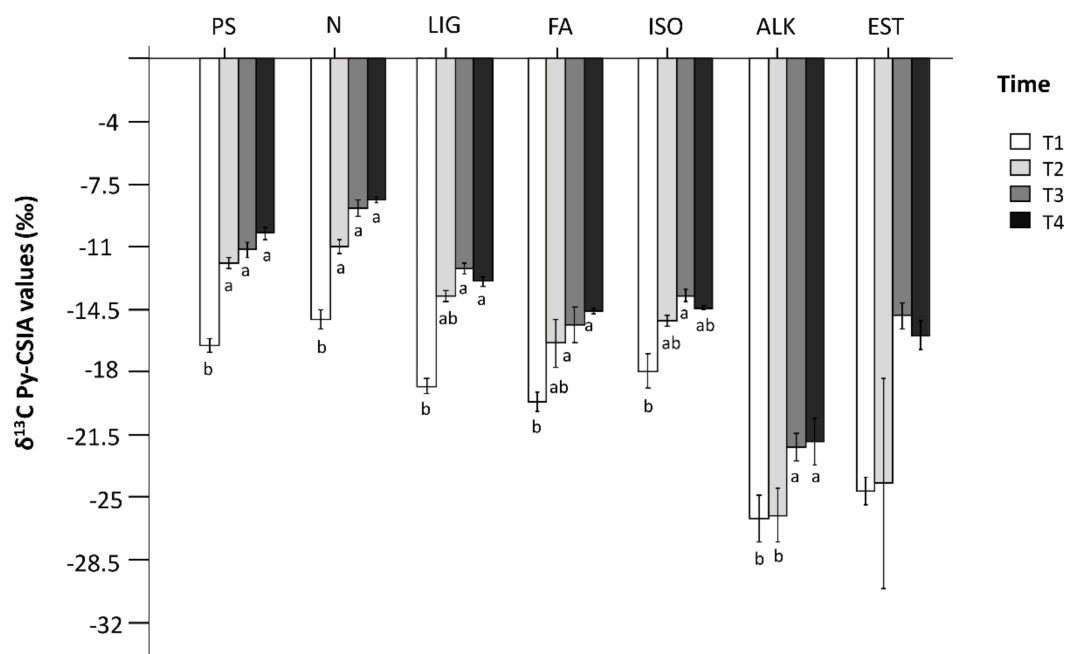


Figure 4. Distribution of $\delta^{13}\text{C}$ Py-CSIA values (average expressed as ‰; $n = 3$) of the different compost samples comprising the time of the experiment. Treatments with the same letters indicate no significant differences (Kruskal–Wallis test, $p < 0.05$, Dunn post-hoc) between different composting times for the same biogenic group.

Although most compounds groups showed a good homogeneity with standard deviation values below 2‰, a few groups (ALK, TOC, and UNK) showed high mean deviations up to 11‰; this may be caused by: (i) a poor reproducibility as in TOC and UNK that included only a few compounds with small peaks, some with coelutions inherent to the relatively complex pyrograms, or (ii) by known $\delta^{13}\text{C}$ shifts related to the molecular structure of a particular chemical group, like ALK, known to decrease $\delta^{13}\text{C}$ values with increasing chain length [68]. Therefore, TOC and UNK groups were excluded from Figure 4 and from further statistical analyses. Finally, some peaks, such as that of the anhydrosugar

levoglucosane (RT: 10.64 min, Figure 3), cannot be mathematically deconvoluted from other peaks to calculate $\delta^{13}\text{C}$.

Generally, $\delta^{13}\text{C}$ values for all organic compounds became increasingly enriched during the composting process, with statistical significance from T1 to T2 for all compound groups, except for TOC and EST (stigmasta-3,5-diene). This low significance level may indicate a preferential preservation of these moieties in the humification process, which would depend on long-term microbial activity and have relatively high resident time [40]. Compounds with greater isotopic shifts can be attributed to the plant and microbial compartments, such as PS, N, FA, and EST, where the rate of isotopic enrichment between identical pyrolysis products along the composting process varied from 4 to 9‰. Most biochemical processes discriminate against the heavier carbon isotope creating products depleted in ^{13}C and leaving reactants enriched in ^{13}C during the composting process. Thus, the remaining compounds would be ^{13}C depleted relative to the original biomass, as reflected in Figure 4.

Focusing on PS compounds, their isotopic composition pointed to a modification of the molecular chains as remaining fragments derived from the initial biomass (differences up to 6–8‰), leaving thus more enriched residues. Most of these identified pyrolysis products may indicate that this organic matter is modified, leading us to believe in a more continuous process of humification during the composting process, and/or their selectively preservation [69,70]. On the other hand, the great isotopic shift of EST from T2 to T3—although not statistically significant—seems to constitute an indicator of the thriving in the composting pile by microorganisms. Consequently, these data point out to a complete degradation of these compounds by the microbiota, showing fast turnover times.

^{13}C values of lignin moieties were up to 6‰-enriched relative to T1, only significantly regarding LG and LS units, which agrees with previous findings [35,69]. However, this isotopic enrichment cannot be fully explained, as lignin moieties are not expected to be degraded by any sort of microbial or bacterial assimilation, aside from specific enzymatic pathways. One possible explanation to this ^{13}C enrichment relies on the modification on the side chains of lignin phenolic units during the composting process: the change on the position of the carbon [35], because of breaking down the C3 side alkyl chain linkages (as supported by the relevant indexes calculated in Table 2). Likewise, this could apply to the enrichment of high molecular weight *n*-alkanes and isoprenoids, defined by their highly recalcitrant structures.

The stable isotope composition of carbon for ALK ranged from −15.43‰ (C-23) to −29.28‰ (C-29). These values conform to the established knowledge of *n*-alkanes from C_4 plants [63]. Short odd-chain alkanes had a higher level of ^{13}C enrichment than larger chains: an enrichment up to 4.3‰ of odd-chain alkanes suggests that, during the biosynthesis of these aliphatic carbon structures, there is a differential carbon isotopic fractionation along the composting process [71]. This may indicate that *n*-alkanes constitute a group of biomarkers based on the disposition of their chains, rather than the compounds itself. For ISO, these compounds showed an intermediate enrichment. The majority of these identified pyrolysis products could indicate a more humified carbon pool, confirming the technical suitability of analytical pyrolysis to monitor the composting process.

3.3. Bulk $\delta^{13}\text{C}$, $\delta^{15}\text{N}$ IRMS, and C, N Content Analysis

In agreement with the general CSIA trend, bulk $\delta^{13}\text{C}$ values of maize biomass expertise a light but significant enrichment during the composting process from −14.64 at the initial stage (T1) to −13.07 at final stage (T4) ($F = 56.835$, $p = 0.000$) (Figure 5, Table S2). Both, average CSIA values and bulk, measurements showed $\delta^{13}\text{C}$ values corresponding to literature reported values for maize compost [72–74]. Moreover, it could be pointed out—as a good signal in analytical terms—that the average $\delta^{13}\text{C}$ Py-CSIA values and $\delta^{13}\text{C}$ bulk values are highly correlated ($R^2 = 0.8059$; Figure 5). However, and accounting for the great heterogeneity of complex samples, such as compost, the variability in bulk data is greater than in compound-specific values, where a larger amount of sample is being weighted

and yielded in a bulk combustion reactor. Nevertheless, that heterogeneity is considerably minimized during the sample preparation sampling and process.

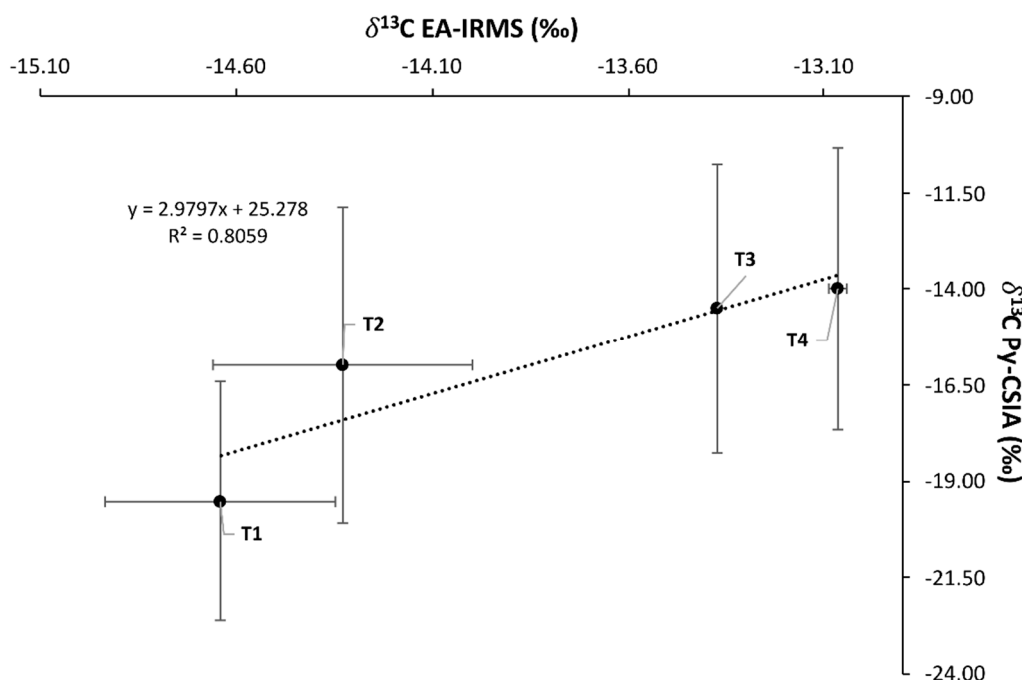


Figure 5. Correlation between $\delta^{13}\text{C}$ mean values obtained from Py-CSIA ($n = 3$) versus EA-IRMS values ($n = 2$). Error bars represent mean standard deviation (horizontal: bulk measurements; vertical: Py-CSIA).

Regarding the total content of organic carbon, a significant increase can be observed till T3, at which point it starts decreasing (Table 3). Although the C content is expected to decrease due to the mineralization mechanism, this variability may be explained by the increase of the aromaticity and further condensation of the newly compounds that are being generated during the composting process, thus compensating the C content.

Finally, the C:N ratio has been frequently used to assess compost maturity [75]. An initial C:N ratio of 30 is considered optimal for ensuring that the composting process takes place satisfactorily [9]. Then, during composting, C:N ratios decrease to 10–15, as previously reported for fully stabilized compost [76], providing additional information of the humification stage of OM of composts. C:N ratios varied from 17.4 to 21.9 at its final stage. As the starting C:N ratio is low, this ratio is increasing during the composting process, indicating a mineralization of nitrogen as supported by its total content. However, there is not a clear trend that may totally support this statement, apart from considering that values around 20 are a good proper indicator of mature composting material.

Table 3. Description of the compost samples: total C and N contents, C to N ratios, $\delta^{13}\text{C}$, and $\delta^{15}\text{N}$ bulk isotopic data. Average values are as ($n = 2$), including standard deviations (in brackets).

Origin ¹	T1	T2	T3	T4
C content (%)	21.4 (4.7) ^a	25.8 (3.8) ^a	33.6 (2.5) ^b	30.1 (0.5) ^b
N content (%)	1.2 (0.2) ^a	1.4 (0.1) ^a	1.7 (0.01) ^b	1.4 (0.01) ^a
C:N ratio	17.4	18.5	19.3	21.9
$\delta^{13}\text{C}$ bulk (‰)	−14.6 (0.3) ^a	−14.3 (0.3) ^a	−13.4 (0.01) ^b	−13.07 (0.02) ^c
$\delta^{15}\text{N}$ bulk (‰)	13.5 (0.3) ^b	14.01 (0.6) ^{bc}	15.0 (0.4) ^c	9.7 (0.4) ^a

Same letters indicate no significant differences (Kruskal–Wallis test, $p < 0.05$, Dunn post-hoc); lowercase letters indicate significant differences comparing different composting times.

4. Conclusions

Pyrolysis gas-chromatography mass-spectrometry (Py-GC/MS) is a suitable technique to investigate the degradation and evolution of diverse biogenic materials. This technique, together with pyrolysis compound-specific isotope analysis (Py-CSIA), showed clear evidences of the humification process of the composting maize material with a general enrichment of ^{13}C for most biogenic compounds over time. Remarkable is the isotopic shift observed for polysaccharides, N-derived compounds, fatty acids, and sterols as plant constituents, adding to a gathering body of evidence about the microbial, preferential degradation of most labile chemical structures. On the other side, isotope shifts observed in more recalcitrant organic materials, such as series of lignin moieties, long-chain aliphatic structures, and isoprenoids, may be indicator of depolymerization and carbon translocation of side-chain molecules occurring during the composting process.

The coupling of the semi-quantitative information provided by conventional Py-GC/MS, complemented with bulk C & N IRMS analysis and the isotopic composition of individual compounds using Py-CSIA helped in gaining understanding about the structure and composition of maize compost at different times, enlightening its decomposition process. Both complementary analytical techniques provide relevant information for the evaluation of the composting process, that is based on the accurate characterization of the organic matter compounds and chemical structure, variations in the relative abundance of the distinct chemical structures, and their isotopic shifts with time. This methodology is convenient in that it avoids the use of sample preparation and the use of derivatizing agents that may interfere the isotope composition of the pyrolysates. The results presented here demonstrate that this method is suitable to investigate structure and isotope composition of pyrolysis products and is applicable, not only to biomass or compost evolution from distinct origins but also to the study of other materials and matrices (soils, sediments, foodstuffs, archaeological artefacts, synthetic polymers, etc.).

Supplementary Materials: The following are available online at <https://www.mdpi.com/article/10.3390/app11156684/s1>, Table S1: Pyrolysis products and relative abundance¹ of biomass and compost samples taken at different times, yielded by Py-GC/MS. Table S2: Compound specific $\delta^{13}\text{C}$ (‰) values of compost pyrolysis products at different sampling time. Values are reported in ‰ (V-PDB). Mean standard deviation is shown in brackets ($n = 3$).

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