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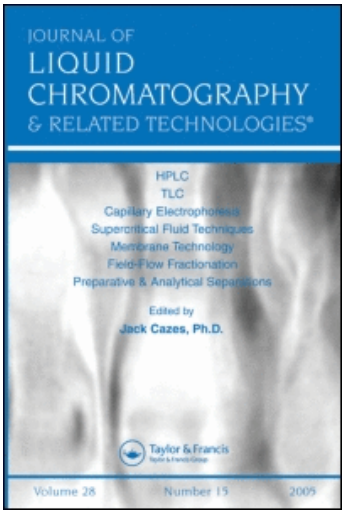

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
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FINGERPRINTING OF THE DEVELOPMENT OF AEROBIC COMPOSTING PROCESSES OF AGRICULTURAL WASTES BY ON-LINE COMBINATION OF LIQUID CHROMATOGRAPHY AND MASS SPECTROMETRY

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Online publication date: 31 May 2001

To cite this Article Corradini, Danilo , Mezzetti, Maurizio , Grego, Stefano , Spiteller, Michael and Mincione, Enrico(2001) 'FINGERPRINTING OF THE DEVELOPMENT OF AEROBIC COMPOSTING PROCESSES OF AGRICULTURAL WASTES BY ON-LINE COMBINATION OF LIQUID CHROMATOGRAPHY AND MASS SPECTROMETRY', Journal of Liquid Chromatography & Related Technologies, 24: 9, 1229 — 1244
To link to this Article: DOI: 10.1081/JLC-100103444
URL: http://dx.doi.org/10.1081/JLC-100103444

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J. LIQ. CHROM. & REL. TECHNOL., 24(9), 1229–1244 (2001)

FINGERPRINTING OF THE DEVELOPMENT OF AEROBIC COMPOSTING PROCESSES OF AGRICULTURAL WASTES BY ON-LINE COMBINATION OF LIQUID CHROMATOGRAPHY AND MASS SPECTROMETRY

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ABSTRACT

Liquid chromatography (LC) was employed in conjunction to mass spectrometry (MS) to develop a straightforward LC-MS procedure for monitoring the variation of functionalized nonpolar organic compounds during the development of an aerobic composting process of agricultural wastes. The LC separation was

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performed by a reversed-phase microbore HPLC octadecyl-silica column, which was eluted by a linear acetonitrile gradient in water. The reversed-phase HPLC column was connected on-line to a triploquadrupole mass spectrometer by an atmospheric pressure ionization interface.

Weighed amounts of agricultural wastes sampled at different time intervals during the aerobic composting process were extracted by dichloromethane and analyzed by LC-MS. The resulting fingerprinting offered a means to monitor the variations of functionalized nonpolar compounds during the aerobic process and indicate the presence of compounds that are not degraded by the biotranformation, such as ursolic acid and β-sitosterol.

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INTRODUCTION

The combination of liquid chromatography (LC) and mass spectrometry (MS) has become a technique, which can be routinely used, in a wide variety of research laboratories and application areas. This can be attributed to the major progress of atmospheric pressure ionization (API) interfaces that accept liquid flow rate ranging from 1 μL/min to hundreds μL/min. Thus, the API ion source can be coupled to HPLC columns of different sizes, ranging from standard-bore columns (4.6 mm I.D.) to narrow-bore (2.0 mm I.D.), micro-bore (1.0 mm I.D.) and packed capillary columns (0.320 mm I.D.).[1,2]

The most frequently applied chromatographic mode employed in LC-MS is reversed-phase chromatography (RPC),[3] mainly because besides its versatility and high resolving power it uses volatile hydroorganic eluents that are compatible with API-MS detection. Moreover, RPC is one of the most frequently applied HPLC mode for the analysis of organic compounds in complex matrices.

This study has been performed to develop a straightforward LC-MS procedure for determining characteristic fingerprints of the different stage of the development of the composting process of agricultural wastes. This is a useful process for the recycling of nutrients and to maintain or restore organic levels and important soil physical characteristics of agricultural lands. The composting process is generally considered the most efficient aerobic biotreatment of organic wastes in producing, under controlled conditions, a hygienic and agronomically advantageous soil organic admentement (compost) at acceptable operational cost.[4]

The biotransformation process involves all organic fractions present in the composting material and leading to a final product, the compost, with a variety of chemical-structural compositions. During composting, most of the easily degradable molecules are transformed by microbial biomass with a high emission

of CO_2 and synthesis of humic substances at maturity phase. The knowledge of the dynamic of biotransformation of the organic component during the composting process is of primary importance for the evaluation of the agronomic efficacy, environmental safety, and economic value of compost which, at the end, will be applied to soil. Immature compost poses problems of malodorous during storage, flies, and bag bursting during marketing, and phytotossicity and pollution during use.[5]

Several different methods have been used to characterize this process, including carbon to nitrogen ratio (C/N),[6] parameters of the water soluble fraction,[7] and humification indexes based on the relation between humic and nonhumic substances.[8] These methods include both microbiological and biochemical tests.[9]

Although all these methods may contribute to shed light on the dynamic of biotransformation during the composting process, they are very specific and are not suitable to be used to determine a general index of quality. This limitation is mainly due to the complexity and wide chemical nature of substrate matrix present in the raw material. Such limitations are much more evident when raw wastes or compost with a low degree of maturity are investigated.[10]

Our research is focused on the study of the organic compounds comprising the fraction soluble in nonpolar solvents with the aim of establishing a new quality index. This requires the development of advanced methods to monitor the dynamic of the biotransformation process from the raw material to the stable final mature product.

In a previous study,[11] the ^{1}H NMR analysis of samples of the nonpolar fractions extracted with hexane from the plant material at the beginning and at the end of the composting process has permitted us to evidence a general variation of nonpolar compounds with the formation of long chain hydrocarbons. The present investigation is aimed at developing an HPLC-MS method capable of monitoring the variation of nonpolar fraction and to isolating and identifying specific nonpolar functionalized compounds useful for the characterization of the biotransformation process. We have been allowed, therefore, to examine the samples extracted by dichloromethane, a solvent much more efficient than hexane, for the extraction of many differently functionalized nonpolar compounds.

EXPERIMENTAL

Chemicals

Reagent-grade hexane, dichloromethane, ethyl acetate, methanol, and HPLC-grade water, methanol, and acetonitrile, were obtained from either Carlo Erba (Milan, Italy) or Merck (Darmstadt, Germany).

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HPLC

Optimization of chromatographic separations was performed using a Beckman (Fullerton, CA, USA) Model 342 liquid chromatograph, equipped with two Model 114 M solvent delivery pumps, a Model 420 system controller, a Model 340 dynamically stirred high pressure mixer, a Model 160 variable wavelength UV-VIS detector, and a Rheodyne (Cotati, CA, USA) Model 7125 injection valve with a 5-μL sample loop. Chromatograms were obtained with a Spectra-Physics (San Jose, CA, USA) Model SP 4400 Integrator. The LC-MS experiments were carried out using a Gynkotek (Germering, Germany) Model 580 HDG HPLC unit connected on-line by an APCI interface to a Finnigan MAT (San Jose, CA, USA) Model TS 7000 triplequadrupole mass spectrometer. The chromatographic separations were obtained using a Macherey-Nagel (Düren, Germany) CC 250/2 Nucleosil 100-5 C_{18} column (250 x 2.0 mm, I.D.), equipped with a CC 8/3 Nucleosil 50-3 precolumn.

This column was eluted by a two segments linear gradient starting from 20% (v/v) acetonitrile in water, by increasing, linearly, the content of acetonitrile in the water-acetonitrile mobile phase from 20 to 60% (v/v) acetonitrile in 30 minutes, followed by a second linear gradient from 60 to 100% (v/v) acetonitrile in 40 min. Peak identification of β-sitosterol and ursolic acid were accomplished by the determination of the molecular mass and confirmed by coelution with authentic standards.

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Composting Process and Extraction

Five cubic meters of grass clippings were piled on an open windrow and turned every two or three days for a total composting period of one hundred days (compost 1). Compost 2 consisted of an 8:2 (w/w) mixture of grass clippings and wood wastes, which was subjected to a composting process of ten months in a composting factory. Representative samples from both composts were collected at different composting times.

During the composting process, weighed amounts of the agricultural wastes were sequentially extracted in a Soxhlet apparatus using five different solvents of increasing polarity (Hexane, dichloromethane, ethyl acetate, methanol, and water). The carbon and nitrogen content, the values of their relative moisture (U) and humification index (HI), and the degree of humification rate of all samples were determined by the standard methods previously reported.[11]

RESULTS AND DISCUSSION

Reversed-phase HPLC coupled to MS was investigated to develop a straightforward and rapid screening method for monitoring the dynamic of bio-

transformation during the composting process of agricultural wastes and testing the quality of the final product. The experiments were performed on plant material composted in our experimental farm (compost 1) and in a composting factory (compost 2). The organic material extracted by dichloromethane at different time intervals during the aerobic composting process was subjected to the investigation by HPLC using both UV and APCI-MS detection.

Initial phases of this investigation were centered on the optimization of the resolution of organic compounds contained in the samples extracted by dichloromethane. During these initial phases of the study, UV detection at 205 nm was utilized to expedite the optimization procedure avoiding the complexity of on-line APCI-MS detection. A number of factors such as gradient shape, flow rate, type, and concentration of the organic modifier were optimized before testing the utility of on-line LC-MS for monitoring the dynamic of biotransformation during the composting process and testing the quality of the final product.

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The sample extracted by dichloromethane is expected to contain a large number of analytes, mainly terpenoids, hydrocarbons, lipids, and steroids of different grade of oxygenation, which are believed to vary in composition and concentration with the type of the agricultural wastes and the development of the aerobic composting process. The peak capacity, measured under the different experimental conditions, was used to compare their effect on the separation of the analytes in the dichloromethane extracts from the two composts. The peak capacity is calculated by dividing the net retention time of the last peak in the chromatogram by the average peak width. It expresses the maximum number of peaks that can theoretically be resolved in the chromatogram and it is a function of the intrinsic column efficiency, gradient time, and flow rate.[12]

In order to evaluate the influence of different operational parameters on the resolution of the analyte extracted by dichloromethane, peak capacity for each investigated column was measured using different organic modifiers, gradient times, and flow rates. The chromatographic performance and the resolution of the analytes in the dichloromethane extracts were greatly affected by the type of organic modifier employed in the hydroorganic mobile phase, as well as by gradient shape and flow rate. These parameters were optimized according to the approach described by Snyder *et al.*[13] with the purpose of maximizing peak capacity. Highest peak capacity, to which optimum peak resolution and chromatographic performance corresponded, were obtained using the microbore CC 250/2 Nucleosil 100-5 C_{18} column (250 x 2.0 mm, I.D.), eluted at constant flow rate of 250 μL/min by a two segments acetonitrile linear gradient in water, consisting of a first linear gradient from 20.0 to 60.0% (v/v) acetonitrile in 30 min, followed by a second linear gradient segment from 60.0 to 100% (v/v) acetonitrile in 40 min. The chromatograms of the organic fraction extracted by dichloromethane from representative samples collected at different time intervals during the composting processes of compost 1 and compost 2, are displayed in Figures 1 and 2, respectively.

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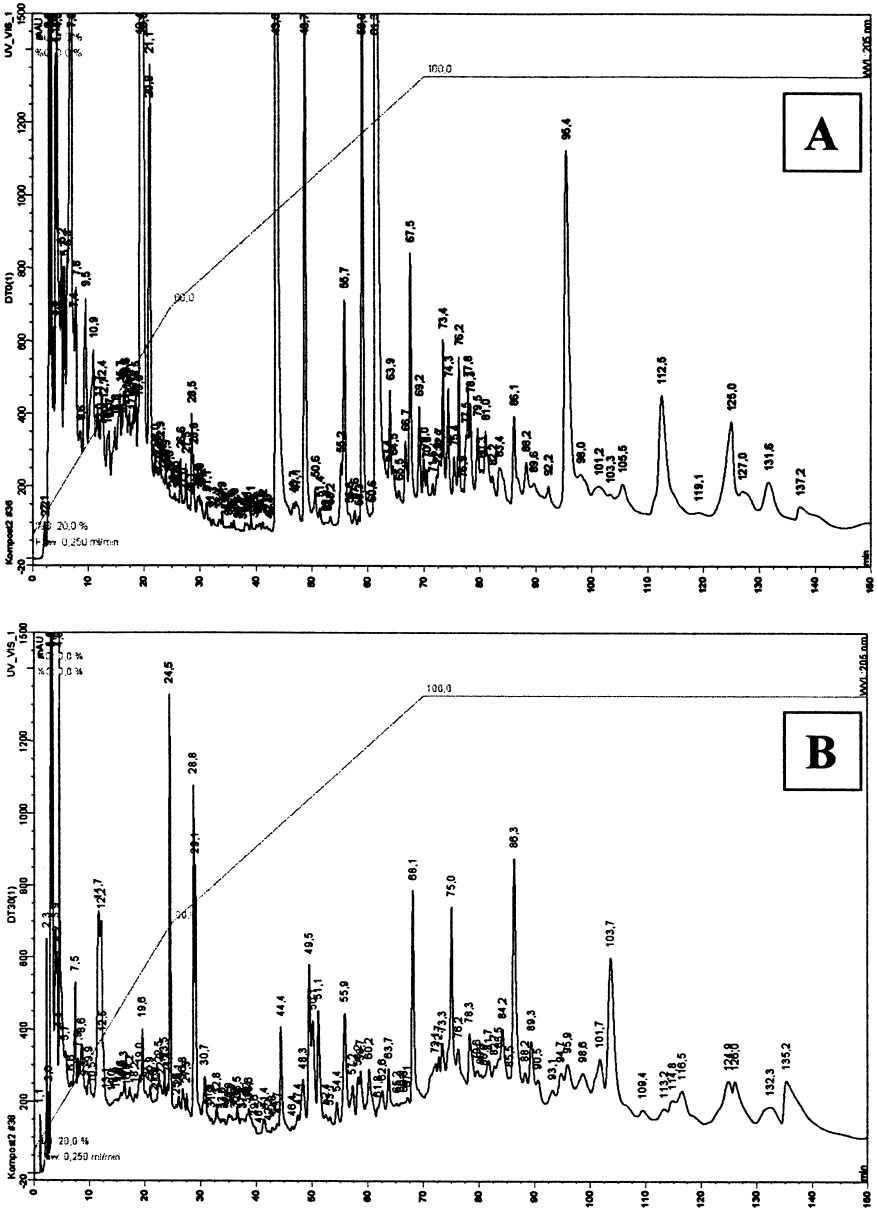

Figure 1. Elution profiles of the organic material extracted by dichloromethane from representative samples of compost 1 collected at the initial time (panel A), after 30 days (panel B), and after 100 days (panel C) of the aerobic composting process. Column, CC 250/2 Nucleosil 100-5 C_{18}, equipped with a CC 8/3 Nucleosil 50-3 precolumn; elution, two segment acetonitrile linear gradient in water, consisting of a first linear gradient from 20.0 to 60.0% (v/v) acetonitrile in 30 min, followed by a second linear gradient segment from 60.0 to 100% (v/v) acetonitrile in 40 min; flow rate of 250 µL/min; detection, UV at 205 nm; temperature, 30°C.

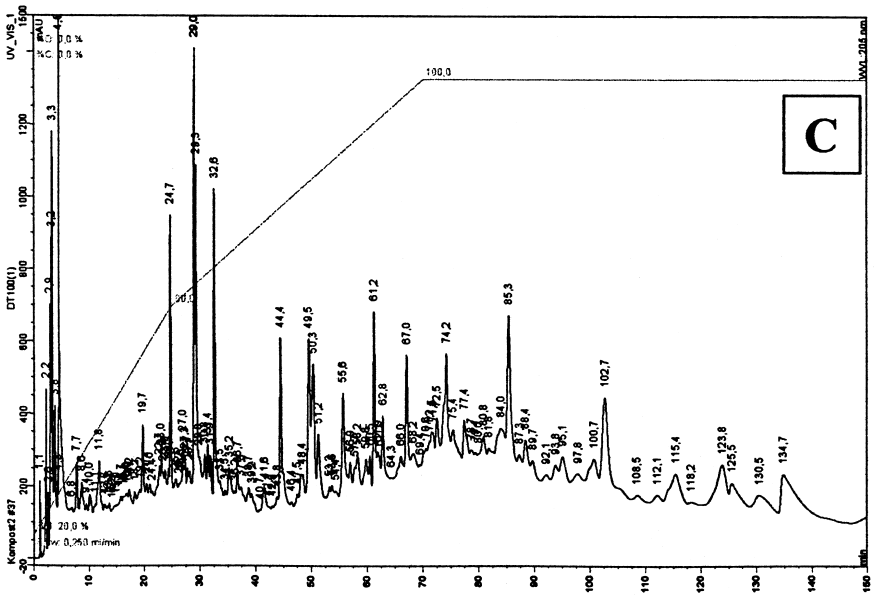

Figure 1. Continued.

The optimized chromatographic method was employed for on-line LC-APCI-MS analysis of the samples extracted by dichloromethane during the composting process. The LC-APCI-MS analysis of these samples revealed that the dichloromethane extracts contained a large number of organic compounds with molecular mass ranging from 200 to 800 mass units. The less nonpolar compounds of molecular mass in the range 200-800 mass units, which eluted the reversed-phase column at the beginning of the gradient, decreased in number with increasing the composting time. Few of these compounds were not revealed in the samples collected at the end of the composting process, indicative of their complete degradation by the aerobic biotreatment. This is in agreement with the expectation that oxygenated polar compounds are more easily transformed in humic substances, which are not contained in the samples extracted by dichloromethane. Minor changes were observed for the more nonpolar compounds, that were more retained by the reversed-phase column, which appeared to be effected to a minor extent by the composting process. On the other hand, few compounds of molecular mass values in the range 445-715 amu, which were not present in the starting material, were detected in the samples extracted at the end of the aerobic bioprocess. These compounds eluted from the RP columns at retention times ranging from 24 to 32 minutes. The content of acetonitrile in the

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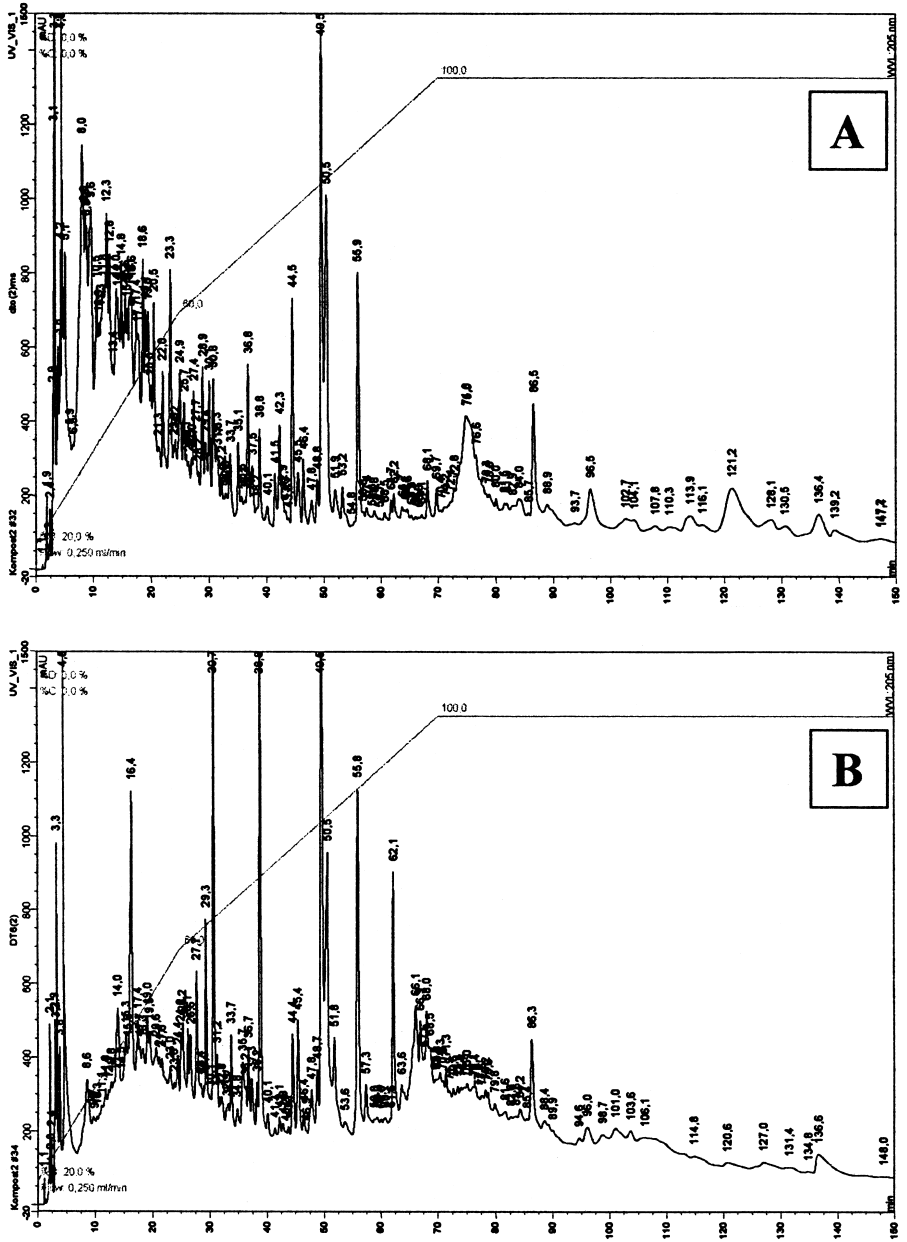

Figure 2. Elution profiles of the organic material extracted by dichloromethane from representative samples of compost 2 collected at the initial time (panel A), after 6 months (panel B), and after 10 months (panel C) of the aerobic composting process. Experimental conditions as in Figure 1.

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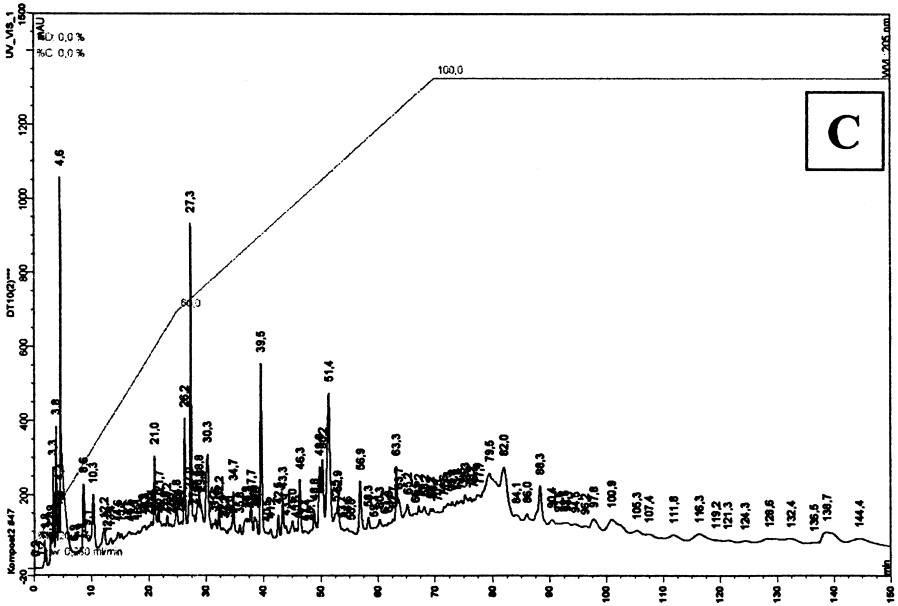

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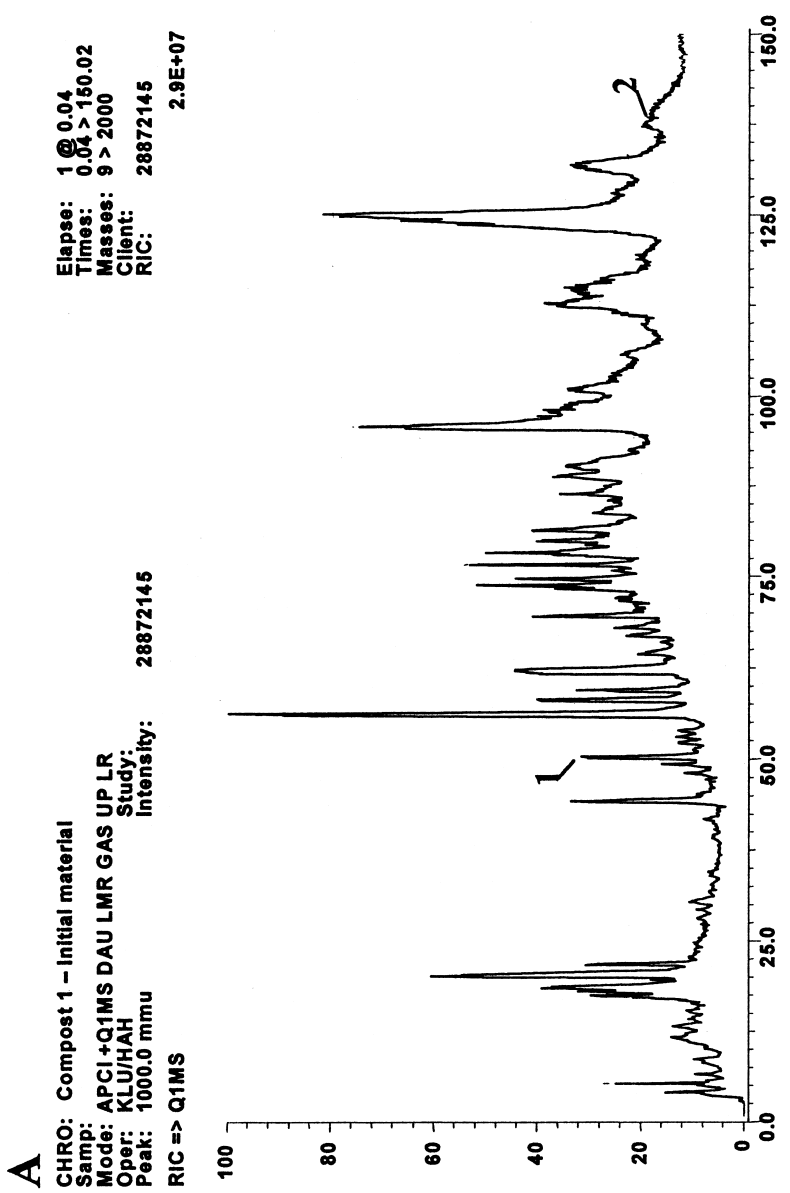
mobile phase within this time range is varying from 60 to 72% (v/v). This indicates a medium-high hydrofobicity for the formed compounds.

Major differences in the general trend displayed by the two composts were related to the decrease in number of the less nonpolar compounds, which was much more evident for compost 2. In addition, for compost 2, the more nonpolar compounds decreased in number at the end of the composting process.

It is worth noting that both composts revealed to contain two characteristic compounds that were detected both in the starting material and in the final product by LC-APCI-MS (see Figures 3 and 4). These molecules that appeared not to be degraded by the biotransformation were identified by tandem mass spectrometry as ursolic acid and β-sitosterol.

The differences in the chromatographic fingerprinting of the samples extracted by dichloromethane from the two different composts were compared to the data obtained by the chemical-physical tests traditionally employed to characterize the composting process. The values of the relative moisture (U), carbon to nitrogen ratio (C/N), humification index (HI), and the degree of humification rate (HR) determined for the two composts before, during, and at the end of the composting process are reported in Table 1. For compost 1, the carbon to nitrogen ratio drops from the initial value of 10.4 to the value of 8.5 after one hundred



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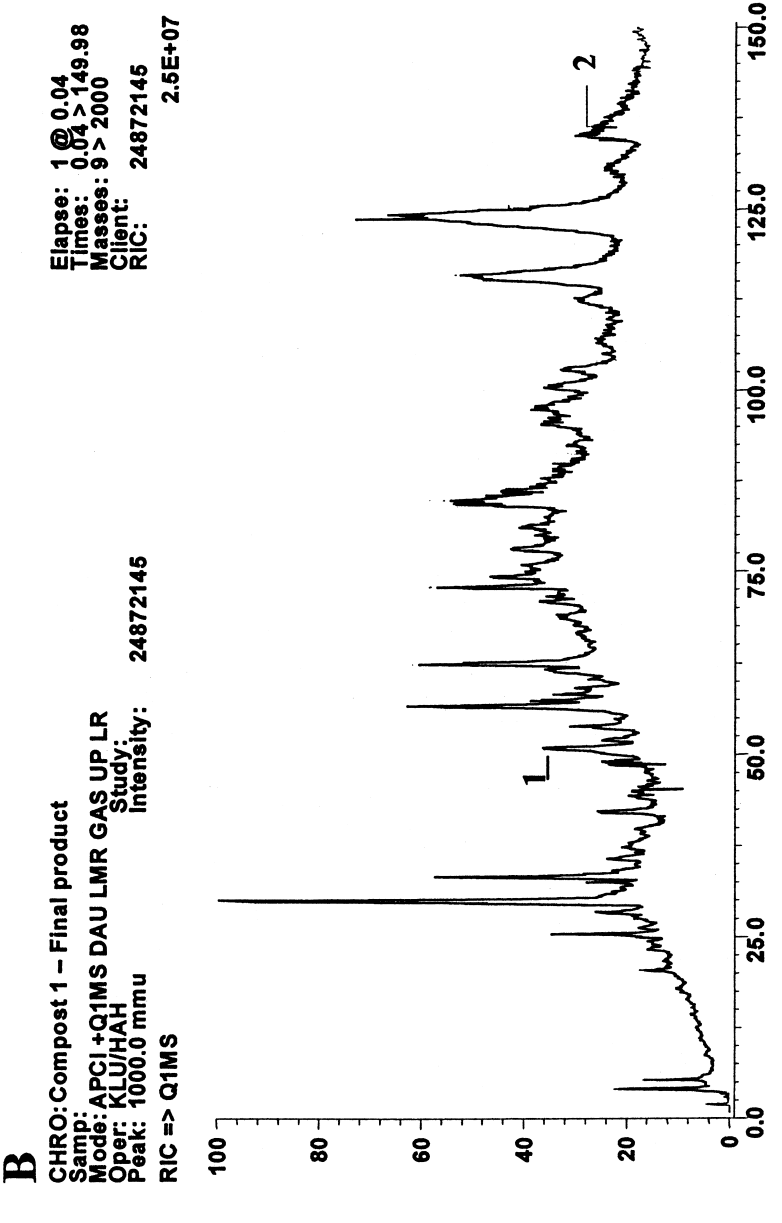
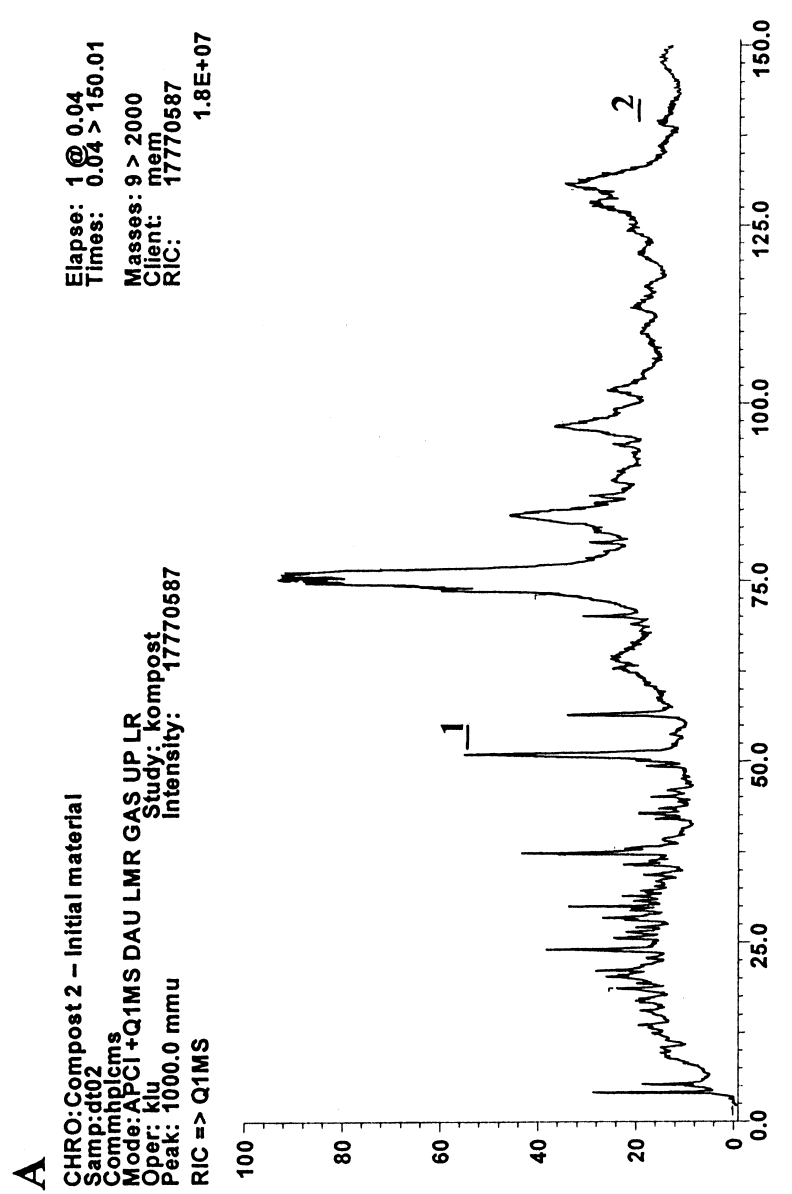


Figure 3. Elution profiles of the organic material extracted by dichloromethane from representative samples of compost 1 collected at the initial time (panel A) and after 100 days (panel B) of the aerobic composting process. Experimental conditions as in Figure 1, except detection by MS. Ursolic acid (peak 1) and β-sitosterol (peak 2) have been identified by tandem mass spectrometry.



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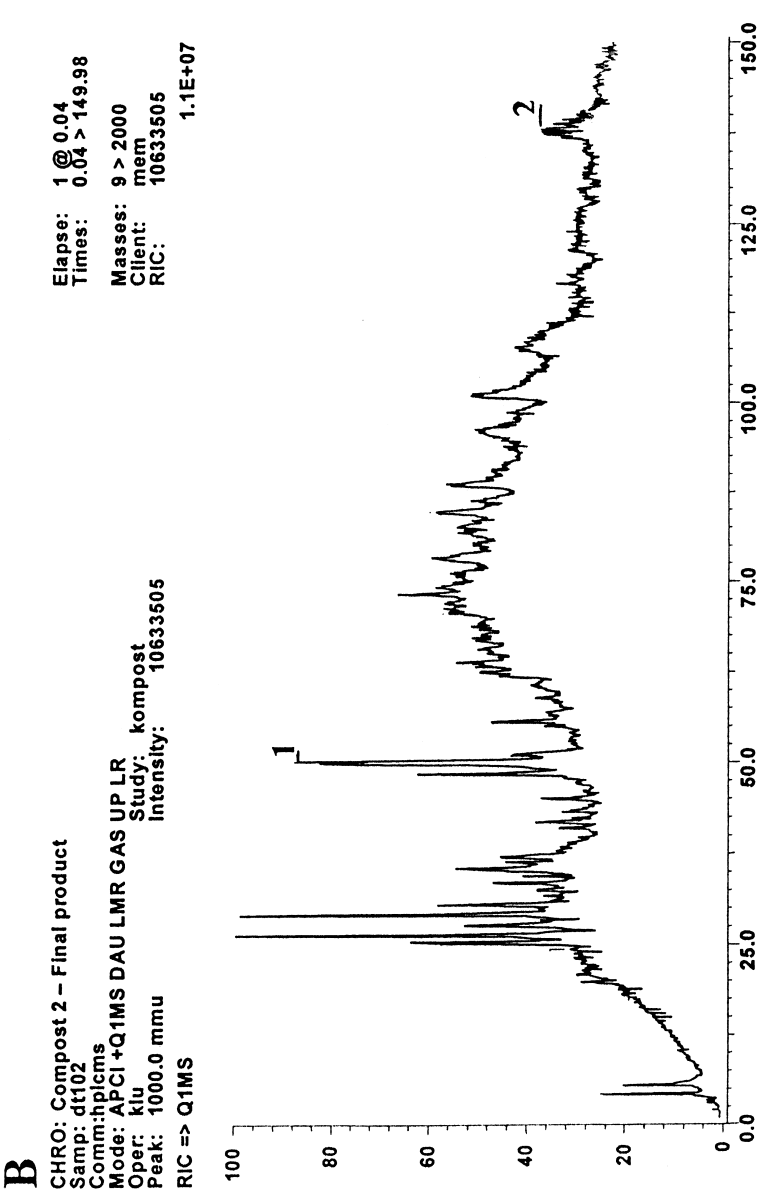

Figure 4. Elution profiles of the organic material extracted by dichloromethane from representative samples of compost 2 collected at the initial time (panel A) and after 10 months (panel B) of the aerobic composting process. Experimental conditions as in Figure 1, except detection by MS and peak identification as in Figure 2.

Table 1. Values of Relative Humidity (RH), Carbon to Nitrogen Ratio (C\N), Humification Index (HI), Humification Rate (HR), and Degree of Humification (DH)

	Compost 1				
Time (Days)	RH %	C\N	HI	HR %	DH %
0	84.5	10.4	—	—	—
16	73.7	12.8	—	—	—
30	59.6	8.8	—	—	—
100	39.3	8.5	1.4	37.1	40.3

	Compost 2				
Time (Months)	RH %	C\N	HI	HR %	DH %
0	53.1	39.3	—	—	—
6	47.7	28.7	—	—	—
30	59.6	19.9	0.29	74.6	77.1

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days of composting process. For compost 2, the C/N ratio falls from the value of 39.27 at the beginning of the composting process to the value of 19.98 after ten months of the biotransformation process.

These parameters reveal that for both composts the aerobic biotreatment causes the degradation of organic matter, as it is inferred by the increase of the C/N ratio, the loss of humidity, and the production of humic substances. On the other hand, the observed differences in the values of these parameters for the two composts are indicative of distinct dynamics of biotransformation of the organic components in the two processes. For example, the humification index (HI), which expresses the ratio between the nonhumic and the humic fraction, reported for compost 2 is about one third of that obtained for compost 1, indicating a more complete maturity phase reached by compost 2. Accordingly, it can be inferred that compost 2 is more mature and of better quality than compost 1.

The HPLC fingerprinting offers a means to monitor the variations of the nonpolar fraction during the aerobic composting process and indicates the presence of bioresistent molecules that are not degraded by the biotransformation, such as ursolic acid and β-sitosterol, which for this reason could be used as indexes of quality of the compost. In addition, the further structural characterization of other compounds in the starting material and in the final products, which is in progress, are believed to shed light on the dynamic of the composting

process and to be used for testing botanical origin and quality of the final product.

CONCLUSION

The LC-APCI-MS analysis of the dichloroethane extracts of agriculture wastes subjected to the composting process can be used to monitoring the development of this process. Less nonpolar compounds of molecular mass in the range 200-800 mass units decrease in number with increasing the composting time. Minor changes are observed for the more nonpolar compounds of comparable molecular mass that seem to be affected, to a minor extent, by the aerobic biotransformation.

However, in the higher mature compost (compost 2), the more nonpolar compounds decrease in number at the end of the composting process, with the exception of β-sitosterol, which is also determined in the ten months processed compost. In this mature compost, the LC-APCI-MS analysis reveals that the fraction extracted by dichloromethane is mainly composed of organic compounds of higher molecular mass (500-800 mass units) and medium polarity.

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Received November 20, 2000 Manuscript 5442
Accepted December 5, 2000

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