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FORENSIC ANALYSIS OF SOIL BASED ON
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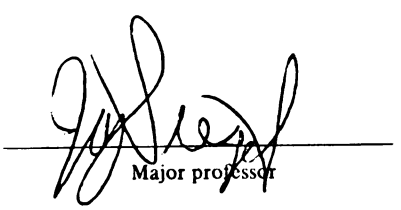

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FORENSIC ANALYSIS OF SOIL BASED ON ITS ORGANIC CONTENT

By

John V. Goodpaster

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ABSTRACT

FORENSIC ANALYSIS OF SOIL BASED ON ITS ORGANIC CONTENT

By

John V. Goodpaster

Trace evidence can be a powerful component in a criminal investigation that may help identify a perpetrator, assist in their prosecution, or influence their sentencing. However, the probative value of trace evidence is highly dependent on the discriminatory power and reliability of the analysis to which the evidence is subjected. In the case of soils, traditional tests have been able to compare and contrast bulk properties such as color, density, mineralogy and so on. The organic portion of soil, which is generally formed from the decay of plant and animal matter, has been largely overlooked. Only recently have researchers begun to focus on the analysis of this portion of soil, and methods have remained largely unexamined and unoptimized. Areas which require investigation and improvement include the methodology for extracting organic matter from soils, the analysis and identification of the extracted components, and the optimum conditions for their chemical separation.

In this work, a systematic study of the types and conditions of analyses for soil organic matter was completed. First, sequential Soxhlet extractions of various soil samples were carried out with five solvents of decreasing polarity: water, methanol, acetone, hexane, and toluene. In general, the majority of

extractable matter was recovered with the more polar solvents. However, some patterns were evident. For example, pristine soils which had little to no human intervention showed a systematic decrease in the amount of extractables as solvent polarity decreased. Perturbed soils, however, had more random distributions of organic matter. Such patterns may be useful in distinguishing natural versus disturbed soil layers.

Spectroscopic analysis of the extracts demonstrated that the polar solvents contained humic substances, which are widely known but poorly understood components of soil organic matter. Both UV-visible absorbance and fluorescence spectroscopy gave results similar to literature spectra for humic species. Also, it appears that different subpopulations of humic substances were isolated in the water and methanol fractions respectively. However, despite the information gained about the nature of the material in the extracts, the spectroscopic results were very similar for all soils and therefore showed little promise as a discriminatory tool. One exception was a cultivated corn field which showed distinctive features in both its absorbance and fluorescence spectra, most likely due to agricultural chemicals.

Finally, the extracts were separated using reversed-phase liquid chromatography. The water extracts did not show any interaction with the non-polar stationary phase and so only a size-exclusion mechanism was operative during their separation. Despite this, clear differences were seen between soils that seemed to reflect the basic soil type. Even more success was obtained with the reversed-phase separation of the methanol extracts which yielded complex

chromatograms that were more easily discriminated. Analysis of the acetone extracts yielded no separation, and these samples (along with the hexane and toluene extracts) would be best suited for a normal-phase separation.

Overall, it appears that analysis of soil organic matter can be a successful means to discriminate soil, including samples of the same basic soil type which differ only in vegetative ground cover. In turn, the ability of these instrumental methods to more uniquely characterize soil assigns more probative value than traditional methods. Given the ubiquitous nature of soil, the further development of powerful and reliable techniques for soil comparisons would be of great benefit to criminal investigations.

To God and my Family, with Love

ACKNOWLEDGMENTS

"The most tangible way in which science, especially chemistry, can be concerned with the well-being of society is its use in the maintenance of the fabric of society as expressed in the constant vigil against crime."

Editor, Chemistry in Britain

Only now is it clear to me how many people have positively influenced my path in the forensic sciences. To begin at the beginning, my thanks go to Mr. John Geroux of St. Thomas Academy in Mendota Heights, Minnesota for his kind and open minded supervision during my senior year independent study into "forensics" (as I called it then). His encouragement to pursue science with vigor was a wonderful gift. As an undergraduate, my experience as an Honors Intern at the FBI Academy in Quantico, Virginia under the supervision of Dr. Edward Bartick was invaluable. My time there quickly solidified my desire to pursue a career in criminal justice and law enforcement. My undergraduate advisor, Dr. Larry Potts of Gustavus Adolphus College in St. Peter, Minnesota, was an extraordinary mentor who provided me with many and varied opportunities to develop my interests in forensic science. My four years spent under Larry's tutelage helped to form me as an analytical scientist and researcher. His concern for his students and teaching has earned my utmost admiration and respect.

The project upon which this thesis is based would not have been successful nor have even occurred without the dedicated and generous effort of a number of people at Michigan State University. My advisor Dr. Jay Siegel,

whose previous research and ongoing enthusiasm about soil analysis has spurred this effort, has been a continual source of advice, encouragement, and vision. I also deeply appreciate his full support of my status as the first dual-degree candidate in forensic science and analytical chemistry at Michigan State. That status has given me the extreme fortune of working with Dr. Victoria McGuffin, who provided invaluable assistance and guidance for this project. Her insights into the chemical problems of soil extractions, spectroscopy and separations are directly reflected in the content and structure of this study. I would also like to acknowledge the thoughtful and constructive feedback of the other members of my thesis committee: Dr. Merry Morash and Dr. Frank Horvath of the School of Criminal Justice, and Chris Bommarito of the Michigan State Police Laboratory.

Furthermore, I am very grateful for the assistance of Dr. Steven Boyd and Dr. Delbert Mokma of the Department of Crop and Soil Sciences. Dr. Boyd provided valuable expertise at the beginning of the project on soil chemistry. Dr. Mokma was an energetic and selfless guide during our sampling scheme, including accompanying us as we tromped through corn fields, climbed fences, and ducked barbed wire where necessary. Dr. Mokma also provided essential equipment and information for obtaining, profiling and processing our samples. Dr. Kathy Severin of the Department of Chemistry generously allowed us the use of departmental instrumentation and supplies, as well as patiently working with us as needed.

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1. INTRODUCTION

Despite previous research, instrumental methods are typically not used to analyze and compare soils in forensic laboratories. This is due in large part to a lack of knowledge and understanding of the best sample preparation conditions for soils, an effective means of separating soil components, or detailed characterization of those components. Hence, this project has sought to address these deficiencies in a number of ways. First, a sampling scheme was devised and carried out to acquire soils of differing base soil type as well as vegetative cover. A systematic study of extraction solvent was then conducted to determine which solvent yielded the greatest quantity and/or most discriminating soil components. UV-visible absorbance and fluorescence spectroscopy were then used to characterize the soil extracts and compare the results to humic substances. Finally, chromatographic analysis of the water, methanol, and acetone soil extracts was completed and separation conditions were optimized.

1.1 Soil

Soil, in its broadest definition, is a loose, heterogeneous, living mixture of inorganic and organic material formed on the earth's surface.[1] This mixture may contain solid, liquid and gaseous phases and is constantly exchanging both energy and matter with its environment. Many materials of natural and anthropogenic origin may be found in a given sample of soil, including but not limited to minerals, microorganisms, ash, decomposed plant and animal tissues,

glass, paint, building materials, plastic, fertilizer, pesticides, and hydrocarbons in varying concentrations.[2]

The naturally occurring portion of soil is formed in one of two ways. First, residual soil is formed in place via the weathering of exposed rock and the decay of organic matter. Second, transported soil is material moved via wind, rain, or glacial action into place. Overall, the natural soil layer can vary in thickness from 10^{-3} to 10^{2} m.[1]

There are many different types of soil, as well as local variations in both the horizontal and vertical dimensions. Recent surveys of the United States have revealed some 20,000 soil types. These types are generally differentiated based on their particle size distribution, i.e., sand, silt and clay in decreasing order. Natural variations in these types can be caused by changes in lithology, which describes geologic induced changes such as streams or glacial action. Alternatively, large variations can be caused by changes in pedology, which describes the thickness and moistness of the soil layer.[1]

1.2 The Value of Soil as Physical Evidence

To the forensic scientist, soil is more specifically defined as collected earth material that is relevant to the matter under investigation.[1] As evidence, soil possesses a number of characteristics which make it valuable. First, soil is widespread. Hence, it is likely to be found at the scenes of serious crimes, provided its value is recognized and it is properly collected. Soil is also diverse. Many different types of soils are known, and they vary considerably from location

to location. In addition, soils have been extensively surveyed and classified for agencies such as the U.S. Department of Agriculture and the U.S. Geological Survey. This can aid the forensic scientist in assessing the frequency of a particular soil type or in locating a region where that type may be found. Finally, soil evidence is differentiable, in that known natural variations can be distinguished through microscopic or chemical analysis even to the point of providing individual characteristics to some samples.[1]

In criminal investigations, soil comparisons can form circumstantial links between a suspect and a crime scene. In addition, the composition of soil tends to reflect its surroundings. Hence, soil identification can reveal information about a perpetrator's environment. Lastly, soil analysis may reveal temporal information such as a sequence of events if there are discernible seasonal changes in a soil sample, or if stratigraphy (layering) has been preserved.[1]

1.3 Established Methodologies

Usually, forensic soil comparisons begin with a microscopic examination of the sample after it has been suitably dried. Note that a drying procedure can change some characteristics of a soil such as salt content, color, mineral oxidation state, nitrate content, or microbial content and activity. Nevertheless, such microscopic examinations can add value to a particular comparison by identifying the more unusual particles in a sample. These comparisons almost always include examination of the sample under normal and polarized light to determine the distribution of minerals in the soil.[3,4] More recently, the highly

discriminatory technique of scanning electron microscopy/energy dispersive x-ray spectroscopy has been used to distinguish and chemically analyze individual grains of soil samples.[5] In this case, it becomes crucial to obtain representative samples from unknown and control soils. If samples are not representative, there could be discernible differences between samples originating from the same source.

Measurement of bulk properties of a soil can also be used for comparison. Towards that end, a host of techniques have been brought to bear in forensic soil analysis.[1] These include examination and quantitation of soil color,[6,7,8] moisture, density gradient, particle size distribution,[9,10] acidity,[11] conductivity, and refractive index. Instrumental techniques such as x-ray diffraction,[12,13] x-ray fluorescence,[14] differential thermal analysis,[13] atomic spectroscopy, and infrared spectroscopy[13] are also popular analysis methods. Overall, for a soil comparison to fully achieve its potential, the methodologies used should provide an appropriate amount of discriminating power. Furthermore, it must be realized that no one technique is sufficient in and of itself to provide a complete comparison.[1]

1.4 Chromatographic Analysis of Soil

In all of the above instrumental techniques, the focus lies with the inorganic/mineral content of soils and their subsequent classification by soil type. Any organic comparisons conducted are simple measurements of the total organic content of a soil or its weight loss upon ignition. However, the potential

of using organic compounds found in soils for their comparison has been increasingly recognized. There are many organic molecules present in soil, including enzymes, carbohydrates, ketones, lipids, amino acids, organic phosphorous, organic sulfur, and aromatic hydrocarbons.[1] Of these potential analytes, biochemical assays of soil enzymes[15] and chromatographic analysis of extractable aromatic hydrocarbons from soil have received the most attention from the forensic community.

The first example of the latter was reported by Andrasko,[16] where polycyclic aromatic hydrocarbons (PAHs) were extracted from urban and rural soil samples with methanol and then analyzed using high performance liquid chromatography (HPLC). PAHs consist of two or more fused aromatic rings, and are widespread and frequently carcinogenic environmental contaminants. PAHs are formed whenever organic matter is exposed to heat, such as in combustion or geological processes. Andrasko identified numerous PAHs in the soil samples based on their retention times and UV-visible absorbance spectra. Examples included fluoranthene, pyrene, chrysene, benzo(*e*)pyrene and benzo(*a*)pyrene, with all of the PAHs found at higher concentrations in the urban samples.

Overall, the method showed good reproducibility and sensitivity but lacked chromatographic resolution. Andrasko's approach was able to discriminate between samples taken 100-150 m apart, whereas samples taken 5-10 m apart were largely indistinguishable. No major changes in PAH composition with depth, time, exposure to UV light, or heating were found. In general, changes in

PAH composition were gradual and tended to reflect the geographic position of a sample rather than the type of soil.

Reuland and Trinler[17] further developed this technique via the analysis of acetonitrile extracts of soils. Samples from seven different locations were compared and were found to be distinguishable either qualitatively (in their number and retention time of peaks) or quantitatively (in their relative peak heights). In addition, the authors noticed some discernible differences over short distances within a cultivated field (1-3 m). This illustrates that this technique can discriminate local variations within the same soil type. However, while all of the samples could be differentiated quantitatively, not all could be differentiated qualitatively and chromatographic resolution was not significantly improved from Andrasko's results.

Later, Siegel and Precord[18] used a similar extraction methodology on samples from varying locations. They modified the method of detection to include the absorbance ratio of the soil components at 254 and 280 nm. All the samples could be differentiated quantitatively, but only half could be differentiated qualitatively. Unfortunately, the authors found that the use of absorbance ratios did not significantly contribute to the discriminating power of the technique.

Finally, subsequent work by Reuland et al.[19] revisited the possibility of using absorbance ratios in the chromatographic analysis of methanol extracts from soil samples. In this study, three different absorbance ratios were used together with single wavelength detection. Out of 91 possible comparisons, 82

were classified as both qualitatively and quantitatively different. Five comparisons yielded quantitative differences only and four comparisons indicated chromatographically equivalent samples. The use of ratiograms in this work tended to support the qualitative or quantitative similarity or dissimilarity of the samples. Of the four comparisons classified as chromatographically equivalent in the above work, three were based on samples taken from specific locations within the same sampling area. In the last case both samples were taken from heavily traveled roadways, which would further support the hypothesis that this method strongly reflects the geographic location of a sample rather than its soil type. Overall, only one out of 91 comparisons showed chromatographic equivalence between two samples known to be different. This gives some measure of the rate with which this technique would yield false positive results.

1.5 Humic Substances

Despite the above developments, there remains a lack of understanding of the chemical nature of the extracted components from soils. In particular, only certain PAHs have been identified in soil extracts, whereas a large amount of other material that may be of value is also present. For example, components of the naturally occurring humus layer have shown promise for discriminating soil samples. Humus is the fraction of the organic content of soil remaining after most animal and plant residues have decomposed. In forested areas, humus generally lies beneath a 1-2 inch layer of undecomposed leaves and twigs and

above the major soil layer of the area. Humic acid is one of the substances that is derived from this soil layer and is defined as a mixture of dark-colored organic matter that can be precipitated by acidifying a dilute-alkali extract of soil. In contrast, fulvic acid is also derived from the humus layer but tends to have a smaller molecular weight and is soluble in water at all pH values.[1]

The explicit chemical structures of humic and fulvic acid are unknown. However, elemental characterization of a humic acid from highly weathered coal found the elements C, O, H, N, and S in order of decreasing concentration. In addition, ^{1}H NMR results revealed a high concentration of aromatic protons as well as polycyclic aromatic and quinone functionalities, which reflect the high degree of humification, or degradation of the substance. However, only trace amounts of long aliphatic chains, saccharides, methoxyl groups, amines or olefins were found.[20] Subsequent NMR studies also showed that in general, humic acids tend to have more aromatic carbons, fewer carbohydrate carbons, and fewer aliphatic protons than fulvic acids.[21]

Not surprisingly given the aromatic character of the material, humic substances are fluorescent. The humic acid discussed above absorbed in the region 300-450 nm and had a featureless emission band centered at 535 nm. It was noted that this emission maximum was shifted to longer wavelength versus other humic acids, presumably due to the high degree of aromaticity in the sample.[20] Both the distribution of fluorescence lifetimes[22] and phase-resolved excitation-emission matrices[23] of humic acids have also been used to study their properties. These studies indicated systematic changes in the fluorescence of

humic substances with pH and the ability to compare humic acids from various sources based on their fluorescence lifetimes.

Vibrational spectroscopy has long been used to determine functional group information about humic substances. For example, IR spectra of the weathered coal humic acid discussed above gave evidence for amide, aromatic, ketone, ester, and carboxylic acid functional groups.[20] Early attempts to acquire Raman spectra of humic substances suffered from a large fluorescence background but did give results similar to disordered graphite-like materials.[24] More recent use of Fourier transform and surface enhanced methods has allowed information about the carbon backbone of humic substances to be acquired and has shown that various humic substances from different sources had very similar backbones.[25]

Pyrolysis gas chromatography-mass spectrometry has also been used to characterize humic substances.[26,27] While the majority of pyrolysis products formed were light gases such as CO, CO_2, or CH_4, other major pyrolysis products were aromatic in nature. These included alkylbenzene derivatives and heterocyclic compounds. However, it is difficult to discriminate between aromatic building blocks of the original humic substance and those that were formed during pyrolysis. Independent measures of the aromaticity of the substances using ^{13}C NMR seem to indicate that aromatic rings with varying lengths of alkyl chains were important constituents in the humic substance. A comparison of pyrolysis to other chemical means of digestion showed that it is a valuable tool with greater ease of use and fewer experimental variables.[28]

Finally, traditional separation techniques have been used to resolve the complex mixtures of humic substances found in soils. The most common method is size-exclusion chromatography, which fractionates samples on the basis of molecular weight. Studies of this kind are generally designed to determine the characteristics of different size fractions of naturally occurring humic acids. However, and of relevance to this work, more recent studies have indicated that the molecular weight distributions of humic acids may vary systematically between different soils.[29-34] Reversed-phase chromatography using gradient elution has also been successfully applied to humic and fulvic acids by separating components based on their relative polarity.[35-37] As soil samples contain many unidentified components of uncertain structure, systematically varying the strength of the mobile phase using a gradient becomes important for achieving high resolution.

2. EXPERIMENTAL METHODS

2.1 Chemicals

A commercially available standard reference soil was obtained for initial studies (Resource Technology Corporation, CLN SOIL-3). Water used for Soxhlet extractions was distilled and deionized (Corning Glass Works, Model MP-3A). Toluene (Aldrich), methanol, acetone, and hexane (Baxter Healthcare, Burdick and Jackson Division) were high purity, spectroscopic-grade solvents. These solvents were also used as mobile phases, in addition to HPLC grade water and 1,4-dioxane (Aldrich).

2.2 Sampling

Three soil samples were acquired from a cultivated cornfield, a nearby grass lawn adjacent to a rural road, and a wood lot, all on the lands of Michigan State University in East Lansing, MI. The cornfield and grass lawn sample were classified as Marlette loam soils while the wood lot sample was classified as a Colwood-Brookston loam soil.[38] Samples were acquired using a solid core sampler. The upper 10 cm of the core was isolated, air-dried, crushed using a mortar and pestle, passed through a 2 mm sieve, and stored under refrigeration (see Figure 1 for a flow chart of the entire experimental procedure).

2.3 Sequential Soxhlet Extractions

Samples of the standard, grass lawn, corn field, and forested area soils weighing ~20 g each were extracted using a Soxhlet apparatus. The extractions

lasted for 24 hours with a sequential series of the solvents (water, methanol, acetone, hexane, and toluene). The samples were placed in double thickness cellulose thimbles (Whatman, 80 x 20 mm) to prevent silt particulates from leeching into the extracts. Despite this precaution, the water extracts required centrifugation (International Equipment Company, Model CL) and the methanol extracts were filtered using 0.45 μm disk filters (Alltech) in order to remove insoluble material. The solvent was then removed from the extracts by rotary evaporation in pre-weighed round bottom flasks. Finally, the residue was reconstituted into the original extraction solvent and transferred to volumetric flasks.

2.4 UV-visible Absorbance Spectroscopy

UV-visible absorbance spectra of the soil extracts were acquired with a commercially available spectrometer (ATI, Unicam UV2) in a 1-cm cuvette cell. The spectrometer contained a deuterium and a tungsten lamp, a photodiode detector, and was operated with a 2 nm bandpass.

2.5 Fluorescence Spectroscopy

Fluorescence excitation and emission spectra, as well as excitation-emission matrices (EEM) of the soil extracts were acquired with a commercially available spectrometer (Hitachi, Model F-4500) in a 1-cm cuvette cell. The fluorimeter contained a 150 W xenon lamp, a photomultiplier detector, and was operated with a 2.5 nm bandpass.

2.6 High Performance Liquid Chromatography

Samples were filtered before analysis using 0.45 μm disk filters (Alltech). Chromatograms were obtained with a commercially available chromatograph (Hitachi). The system consisted of a gradient pump (L-6200A), autosampler (AS-4000), and diode-array absorbance detector (L-4500A). The system used a reversed-phase octadecylsilica column (Supelco, Hypersil ODS, 250 x 4.6 mm, 5 μm particles, 120 Å pore size) with attached guard column (Alltech, Opti-guard C_{18}).

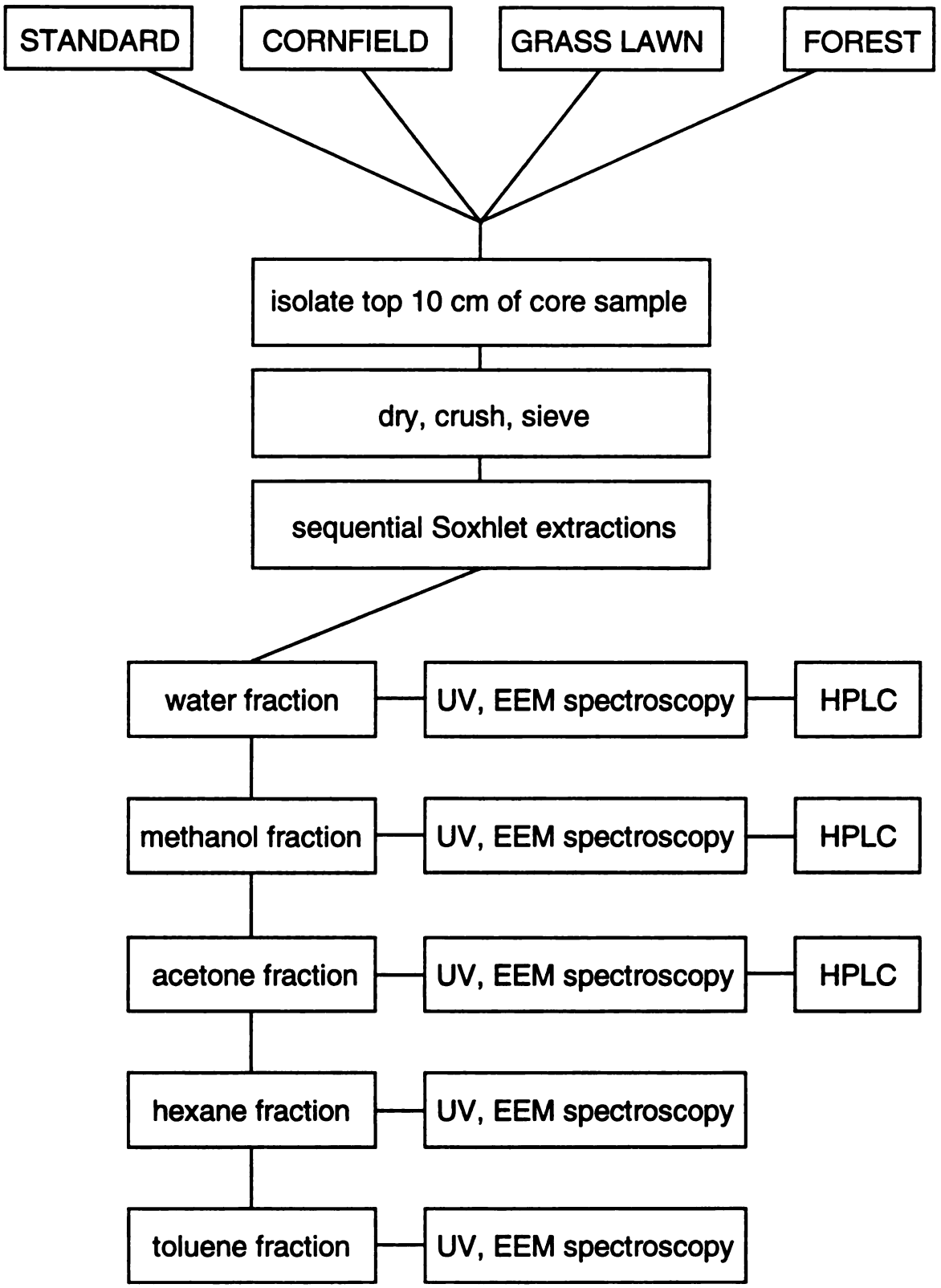

Figure 1: Flow chart for the soil analysis procedure

3. RESULTS AND DISCUSSION

3.1 Sequential Soxhlet Extractions

The results of the sequential extractions are summarized in Figure 2 in the form of weight fraction extracted as a function of solvent and soil sample. Based on these data a number of conclusions can be made. In general, the amount of extractable material decreased as the polarity of the solvent decreased. This reinforces what is known about humic substances, which are widely thought of as large molecular weight polyelectrolytes which would preferentially dissolve in aqueous and/or polar solvents. Furthermore, any inorganic salts would also be extracted in these solvents. The latter, less polar solvents would tend to extract non-humic substances such as carbohydrates, lipids or large fatty acids that are also found in the organic portion of soil. The profile or pattern formed by these data seems to indicate that more pristine soils that are largely free from human activity (the standard and forest soils) show similar profiles, while the grass lawn and cornfield do not. In particular, the soil isolated from the grass lawn showed a large amount of material in its acetone extract, which may reflect a larger proportion of lipid-like material relative to humic substances in the soil. Finally, the cornfield was largely depleted of organic matter relative to the nearby grass lawn, which is perhaps indicative of the time of sample collection (after the fall harvest). Overall, polar solvents were the most successful at extracting more organic material, and each soil gave a fairly distinctive pattern of extractables in the various solvents; although less perturbed soils tended to show similar

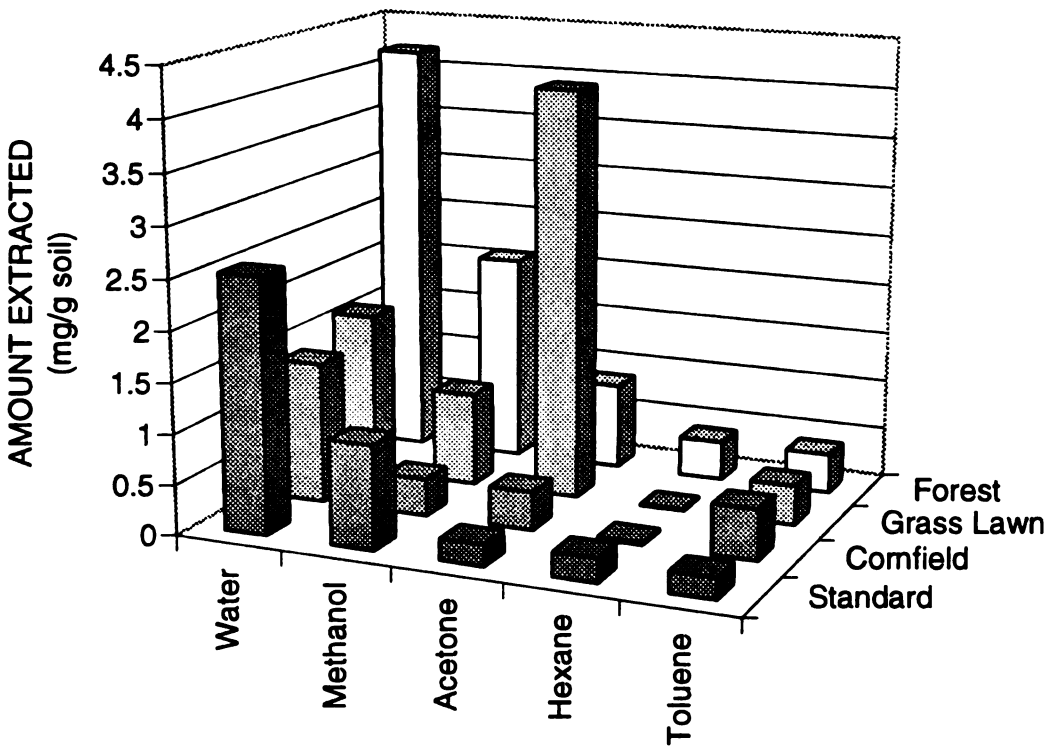

Figure 2: Results of sequential Soxhlet extractions

shapes. However, this type of pattern could be used as a marker for soils that are free of human activity.

3.2 UV-visible Absorbance Spectroscopy

As the concentration of the extracts varied with both solvent and sample, UV-visible spectra are shown in Figures 3-6 in the form of absorptivity as a function of wavelength. In general, the material in the water, methanol and hexane fractions showed the greatest absorptivity. Unfortunately, the strong absorption of acetone and toluene below 328 and 286 nm, respectively, makes comparisons of the material in these solvents difficult. The largely featureless shape of the absorptivity curves in water and methanol are typical for humic substances as reported in the literature.[39] Interestingly, the spectra of the hexane extracts also have this similar shape, with one important exception. The hexane extract from the cultivated cornfield absorbed only weakly, but showed distinctive structure that may be due to aromatic compounds present in the soil (see Figure 3, the data has been multiplied by a factor of 10 for comparison). These compounds could be present as the result of fertilization, or the use of herbicides or pesticides. Such a result may indicate a potential means to discriminate cultivated soils from other, non-agricultural samples.

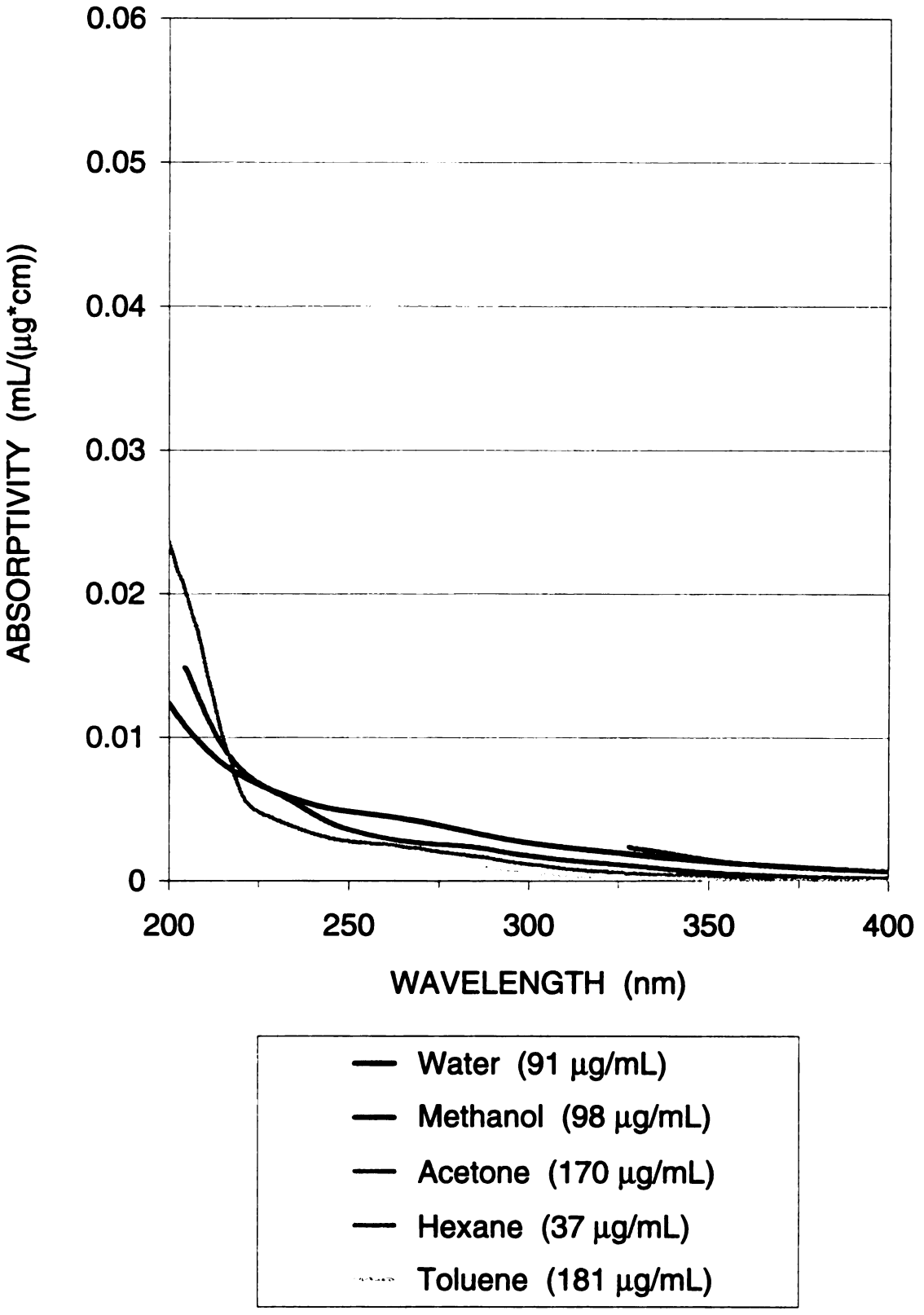

Figure 3: UV-visible absorbance spectra of standard soil extracts

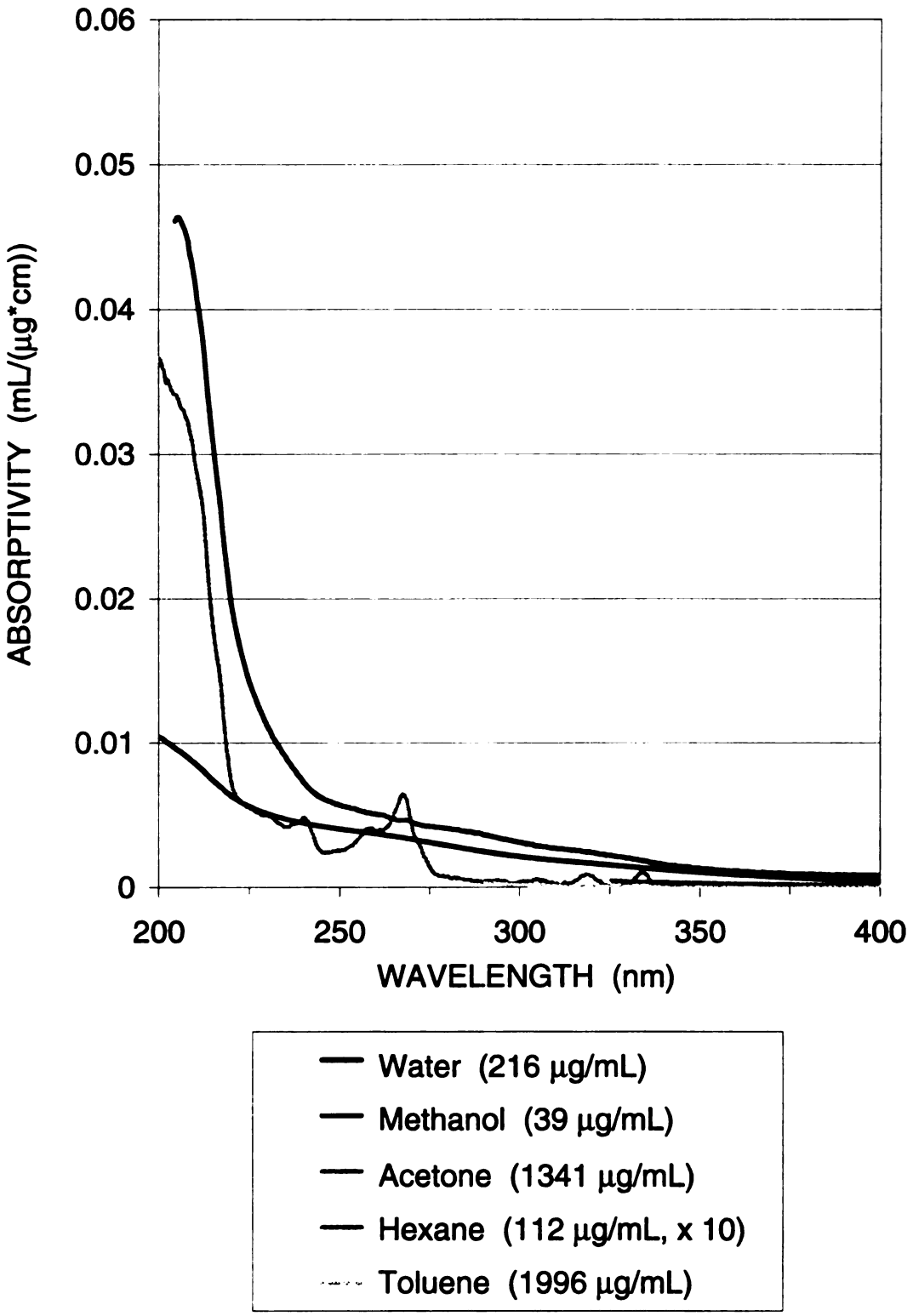

Figure 4: UV-visible absorbance spectra of cornfield soil extracts

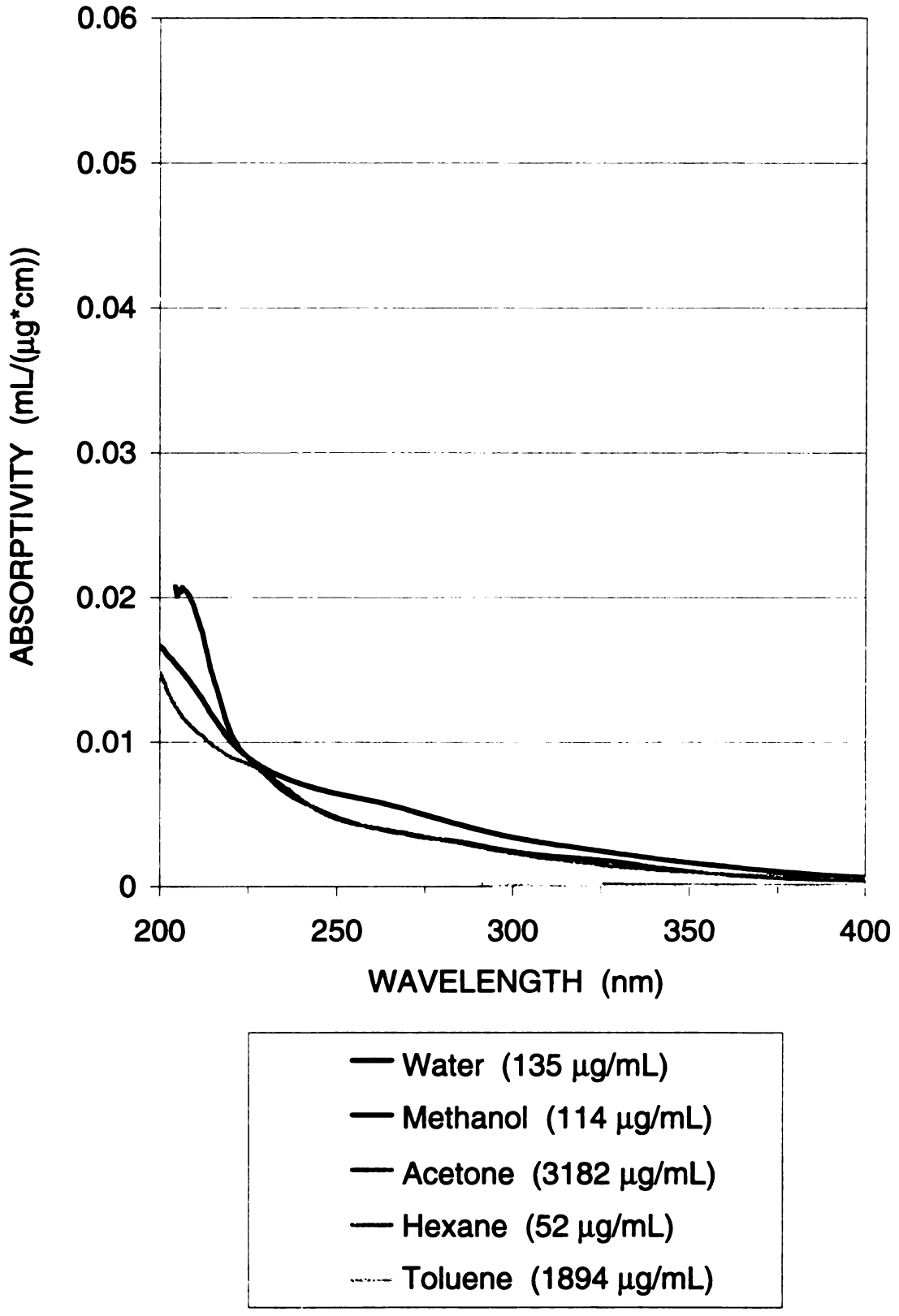

Figure 5: UV-visible absorbance spectra of grass lawn soil extracts

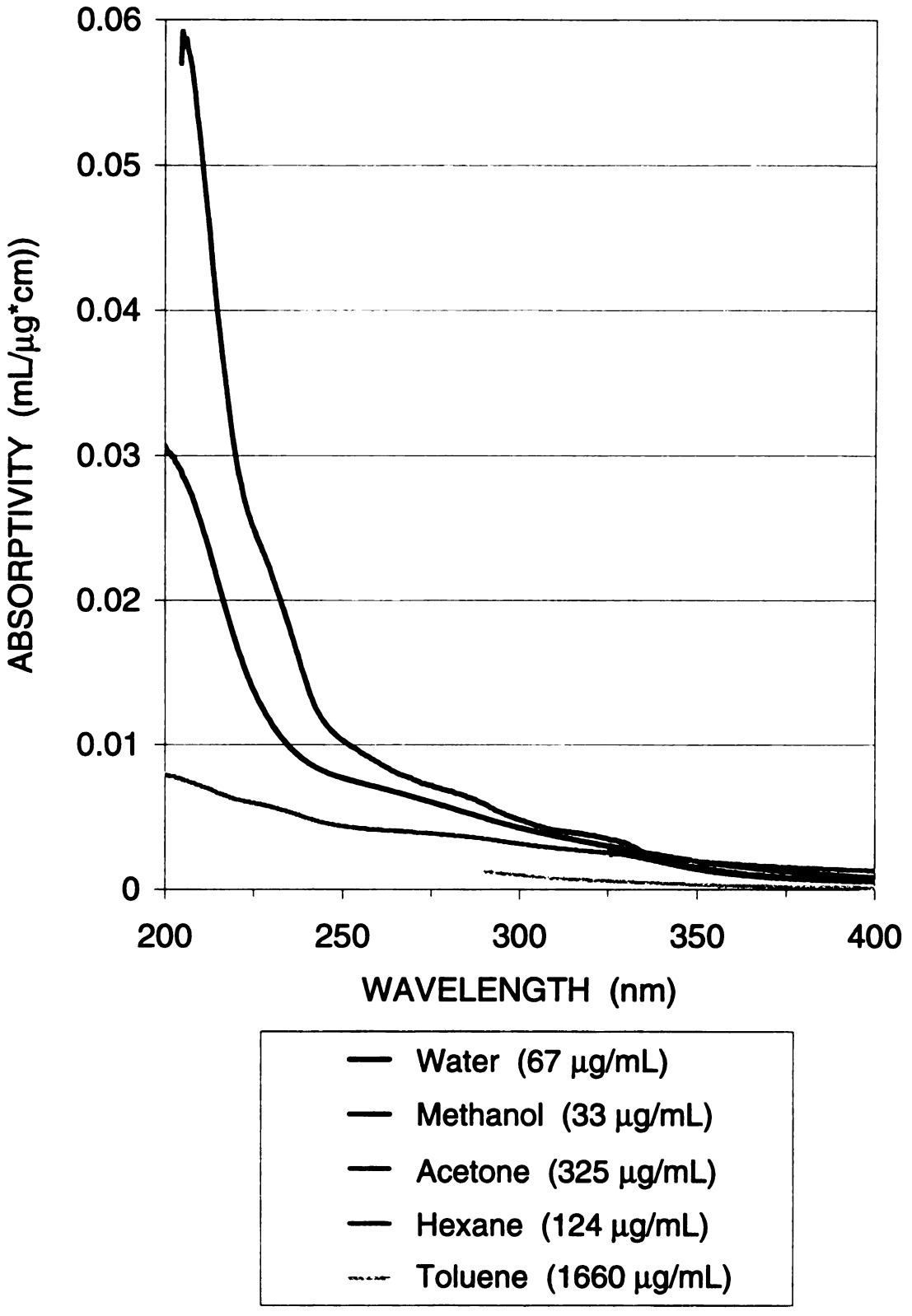

Figure 6: UV-visible absorbance spectra of forest soil extracts

3.3 Fluorescence Spectroscopy

Fluorescence excitation-emission matrix (EEM) spectra for each extract are shown in Figures 7-11. These figures show contour plots of the fluorescence of the sample with excitation wavelength shown along the vertical axis and emission wavelength shown on the horizontal axis. The contour lines represent steps of 1.0 units in fluorescence intensity. In addition, two cross sections taken at the excitation and emission maxima of the contour plot are shown on the lower right and upper right, respectively.

In general, the excitation and emission maxima for all samples fell within the ranges of 290 - 340 nm and 350 - 440 nm, respectively. Also, with few exceptions, emission maxima tended to decrease with the polarity of the extraction solvent. It should be noted that EEM data are particularly sensitive to the concentration of the extract. This wavelength dependence is due to inner and outer filtering effects, whereby strong absorption of the excitation and/or emission beam tends to shift the measured excitation and emission maxima to longer wavelengths where such absorption is less prevalent. This makes comparing and/or discriminating samples more difficult, but some chemical insight can be gained from the results obtained in this study.

For the water and methanol extracts, a broad and weak emission band is seen which is similar to published fluorescence spectra of humic substances.[39] Furthermore, the emission maxima of the methanol extracts were at shorter wavelengths than the corresponding water extracts. This may indicate that the

material in the water fraction has a higher degree of humification. Chemically, this implies that the humic components possess a greater degree of aromatic conjugation (therefore emitting fluorescence at a longer wavelength) in addition to a greater number of polar groups (therefore increasing their water solubility).

All the acetone extracts showed a large, intense emission band around 420-440 nm which did not vary significantly between samples. Subsequent EEM spectra of pure acetone showed weak, intrinsic fluorescence in this region, although not of an intensity to explain the large fluorescence signal seen in the soil extracts. It is more likely that this signal is due to fluorescence from condensation products of acetone formed during the 24 hour extraction.

No discernible fluorescence was seen in the hexane extracts, with the important exception of the cornfield soil. In that case, a structured fluorescence emission was seen around 380 nm. This further supports the UV-visible absorbance data by indicating the presence of aromatic compounds in this cultivated soil.

The toluene extracts, as in acetone, showed a relatively intense emission band that was largely invariable between samples. The single exception was the forest soil extract, which had excitation and emission maxima at longer wavelengths than other soils. This may indicate an increased amount of conjugated aromatic functionalities in this soil. However, it must be noted that pure toluene shows weak fluorescence in approximately the same region. Furthermore, it is possible that a large increase in this signal could occur upon extraction due to reactions of the solvent to form fluorescent products.

Therefore, in the case of both acetone and toluene subtracting a solvent background which has been refluxed over 24 hours may be useful in compensating for any inferences.

Overall, the ability of fluorescence spectroscopy to differentiate these samples is rather limited as the spectra obtained were quite broad and featureless. The notable exception is the cornfield soil, where trace amounts of aromatic compounds yielded a clear spectrum with distinct structure.

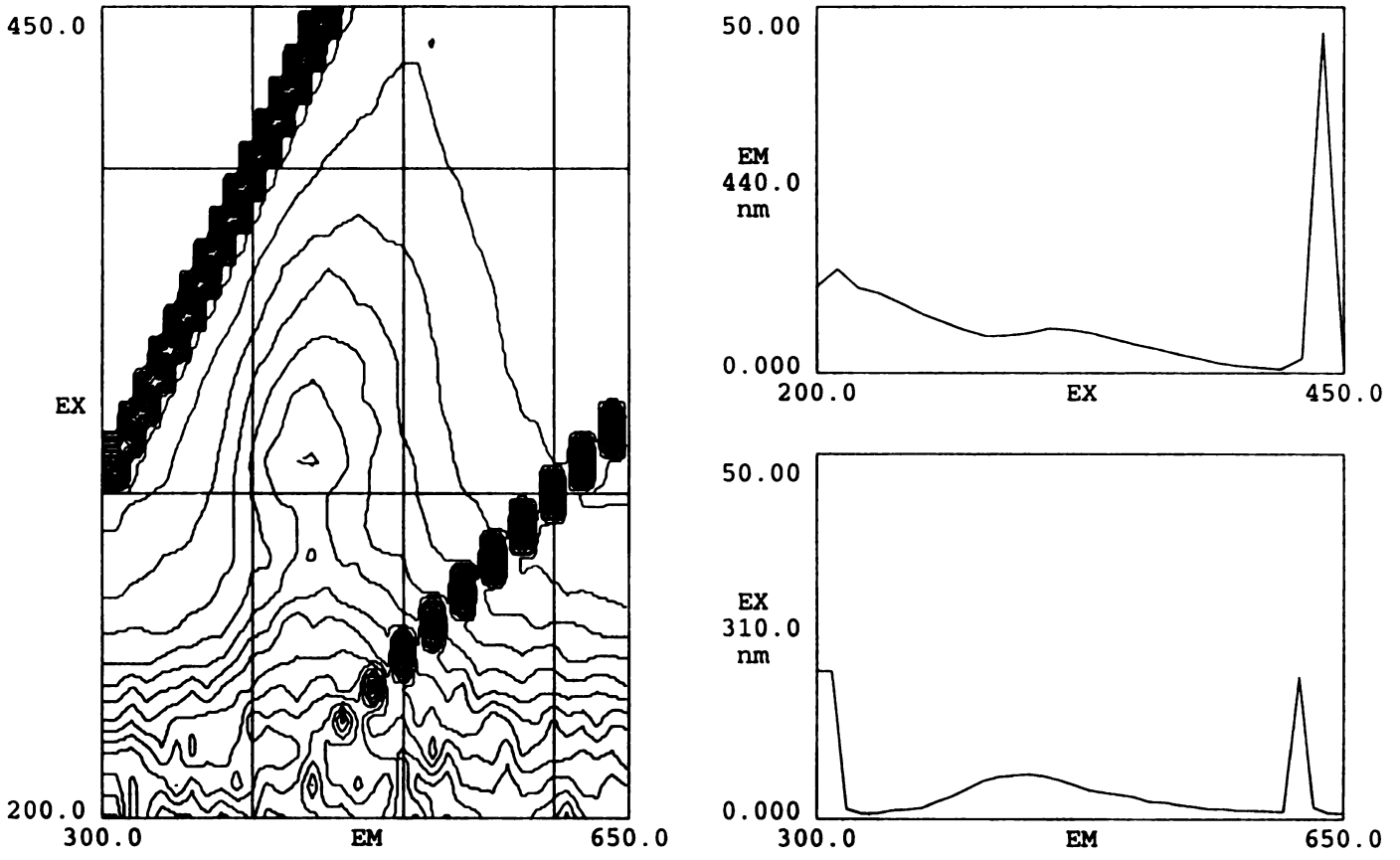

Figure 7A: Fluorescence excitation-emission matrix of the standard soil water extract (91 µg/mL)

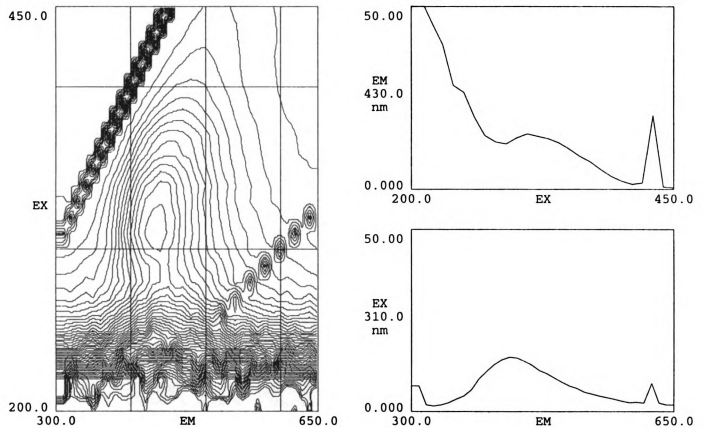

Figure 7B: Fluorescence excitation-emission matrix of the cornfield soil water extract (216 μg/mL)

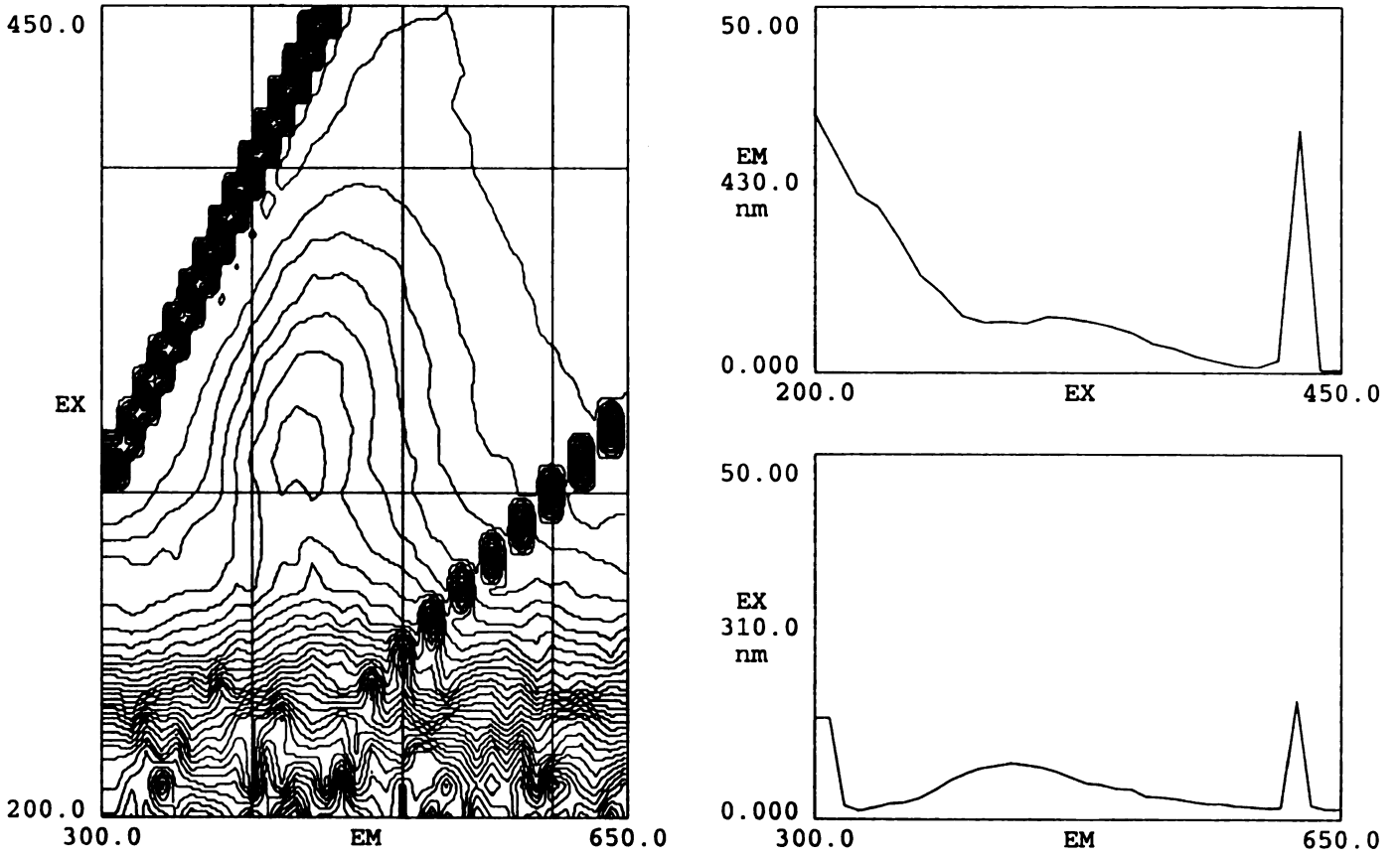

Figure 7C: Fluorescence excitation-emission matrix of the grass lawn soil water extract (135 µg/mL)

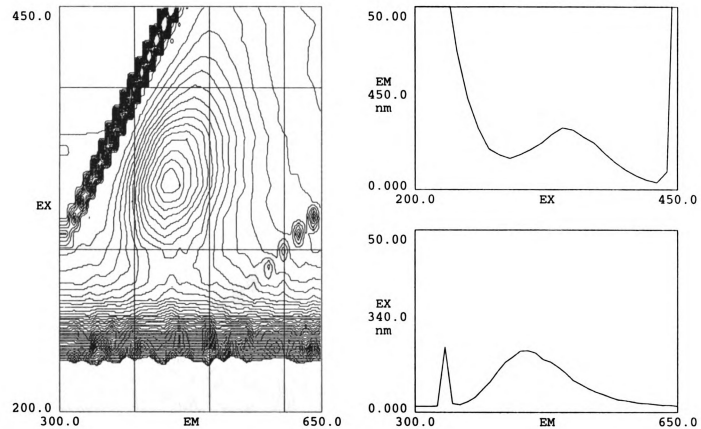

Figure 7D: Fluorescence excitation-emission matrix of the forest soil water extract (337 µg/mL)

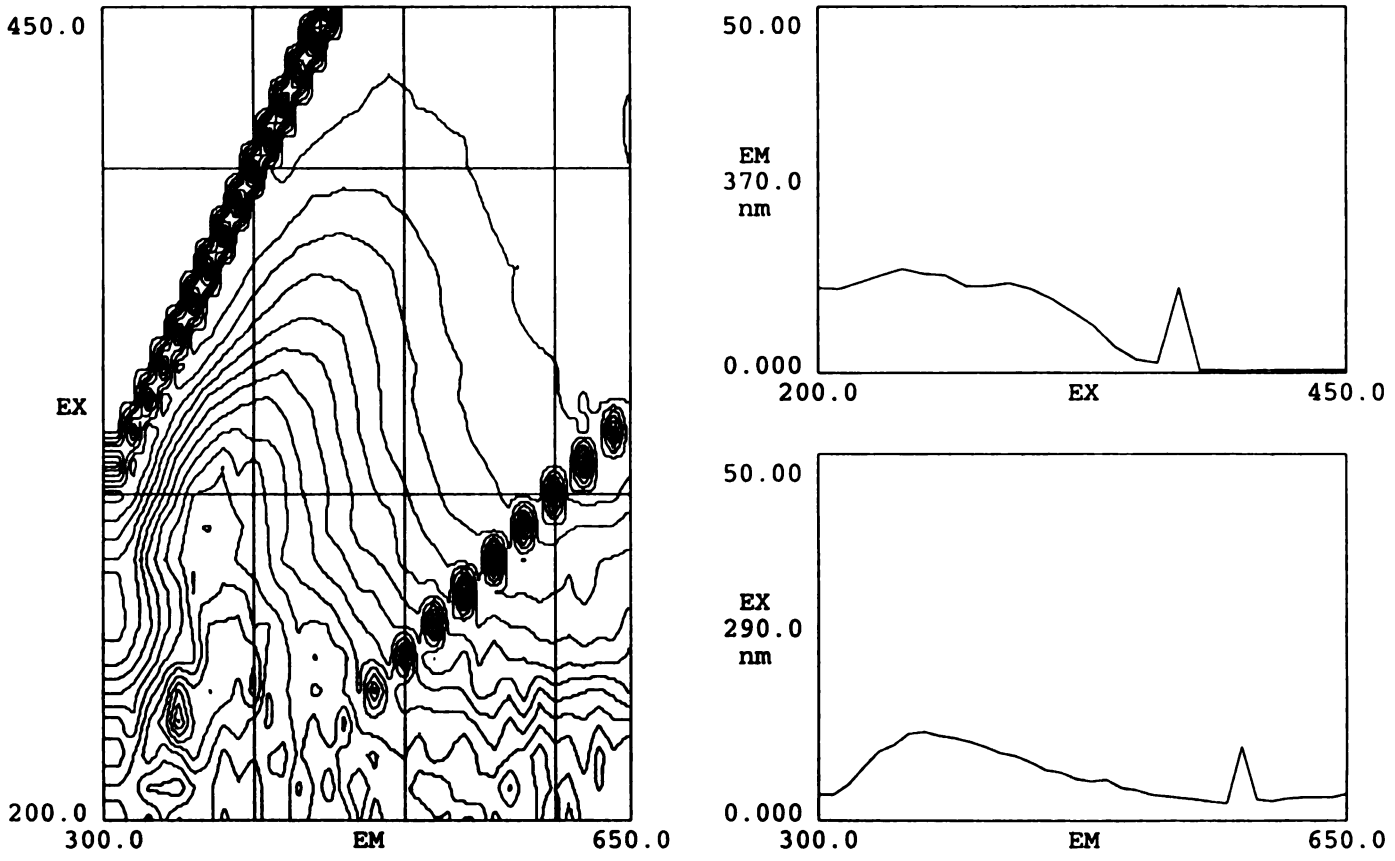

Figure 8A: Fluorescence excitation-emission matrix of the standard soil methanol extract (98 µg/mL)

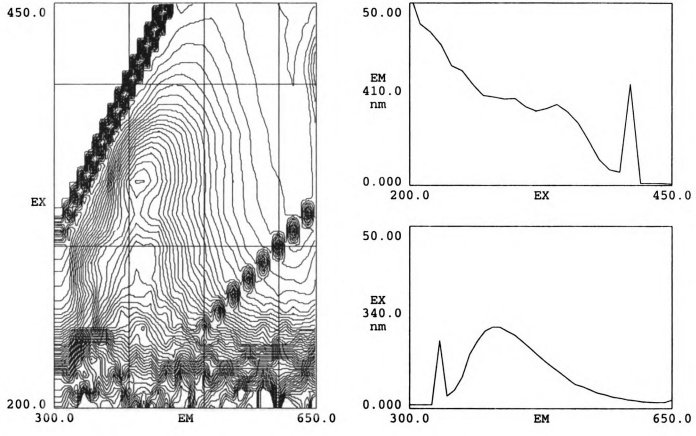

Figure 8B: Fluorescence excitation-emission matrix of the cornfield soil methanol extract (195 µg/mL)

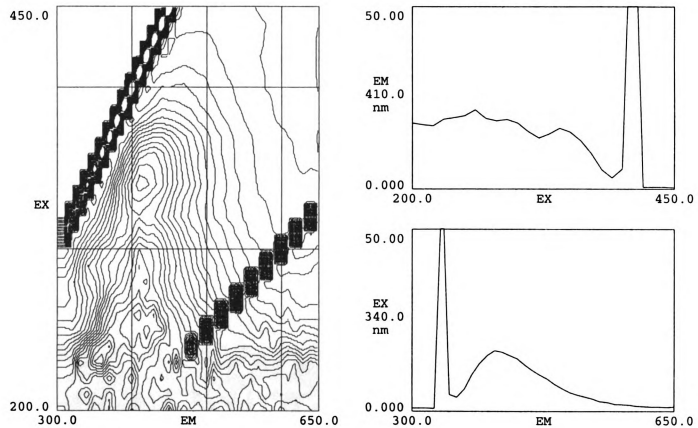

Figure 8C: Fluorescence excitation-emission matrix of the grass lawn soil methanol extract (114 μg/mL)

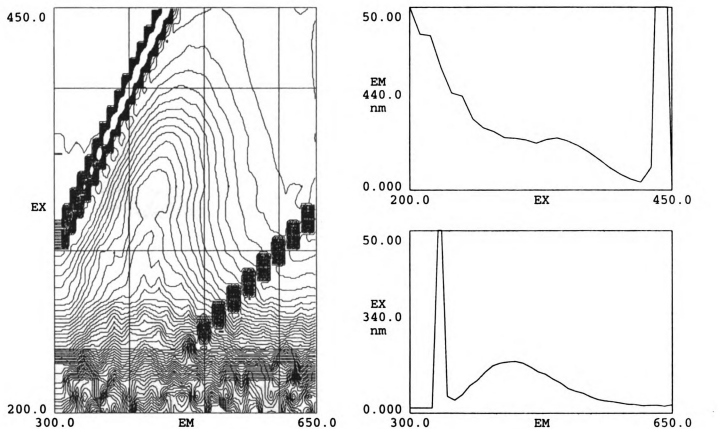

Figure 8D: Fluorescence excitation-emission matrix of the forest soil methanol extract (164 µg/mL)

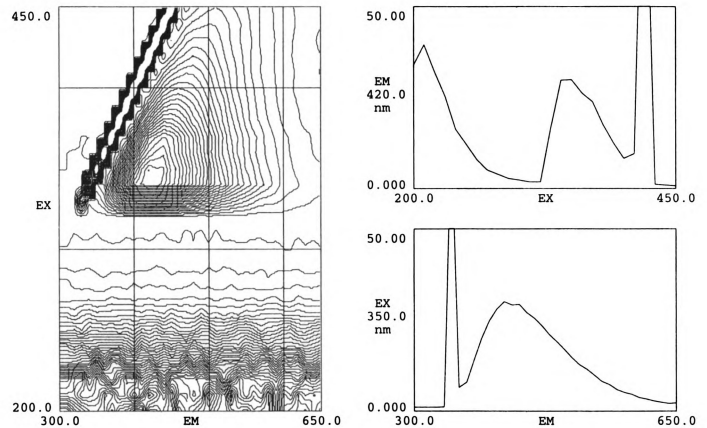

Figure 9A: Fluorescence excitation-emission matrix of the standard soil acetone extract (170 µg/mL)

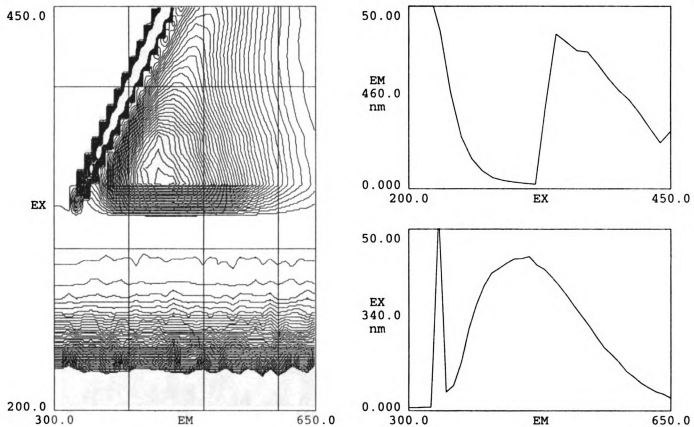

Figure 9B: Fluorescence excitation-emission matrix of the cornfield soil acetone extract (1341 µg/mL)

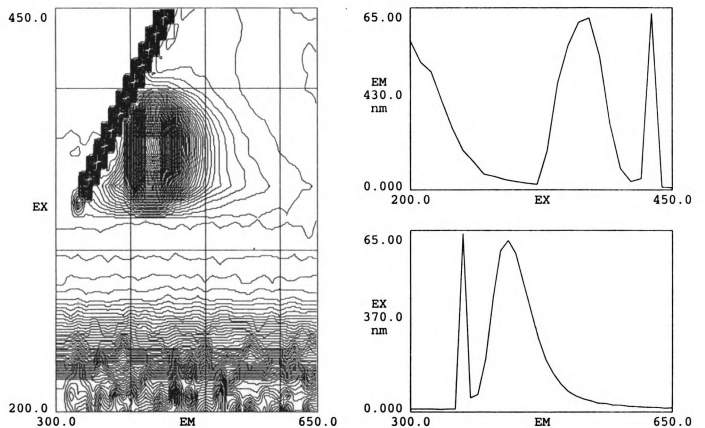

Figure 9C: Fluorescence excitation-emission matrix of the grass lawn soil acetone extract (636 μg/mL)

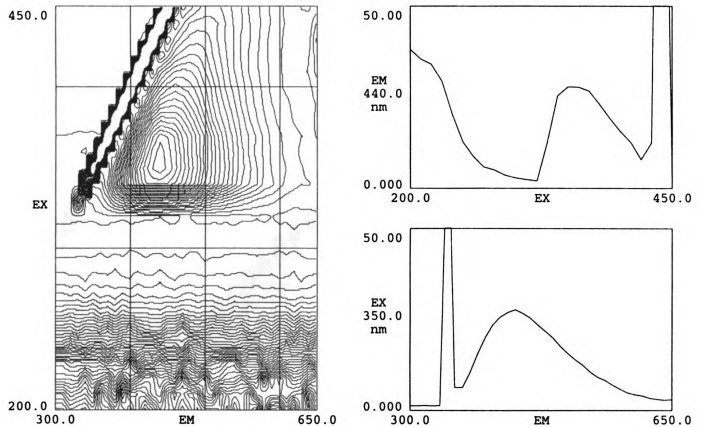

Figure 9D: Fluorescence excitation-emission matrix of the forest soil acetone extract (325 µg/mL)

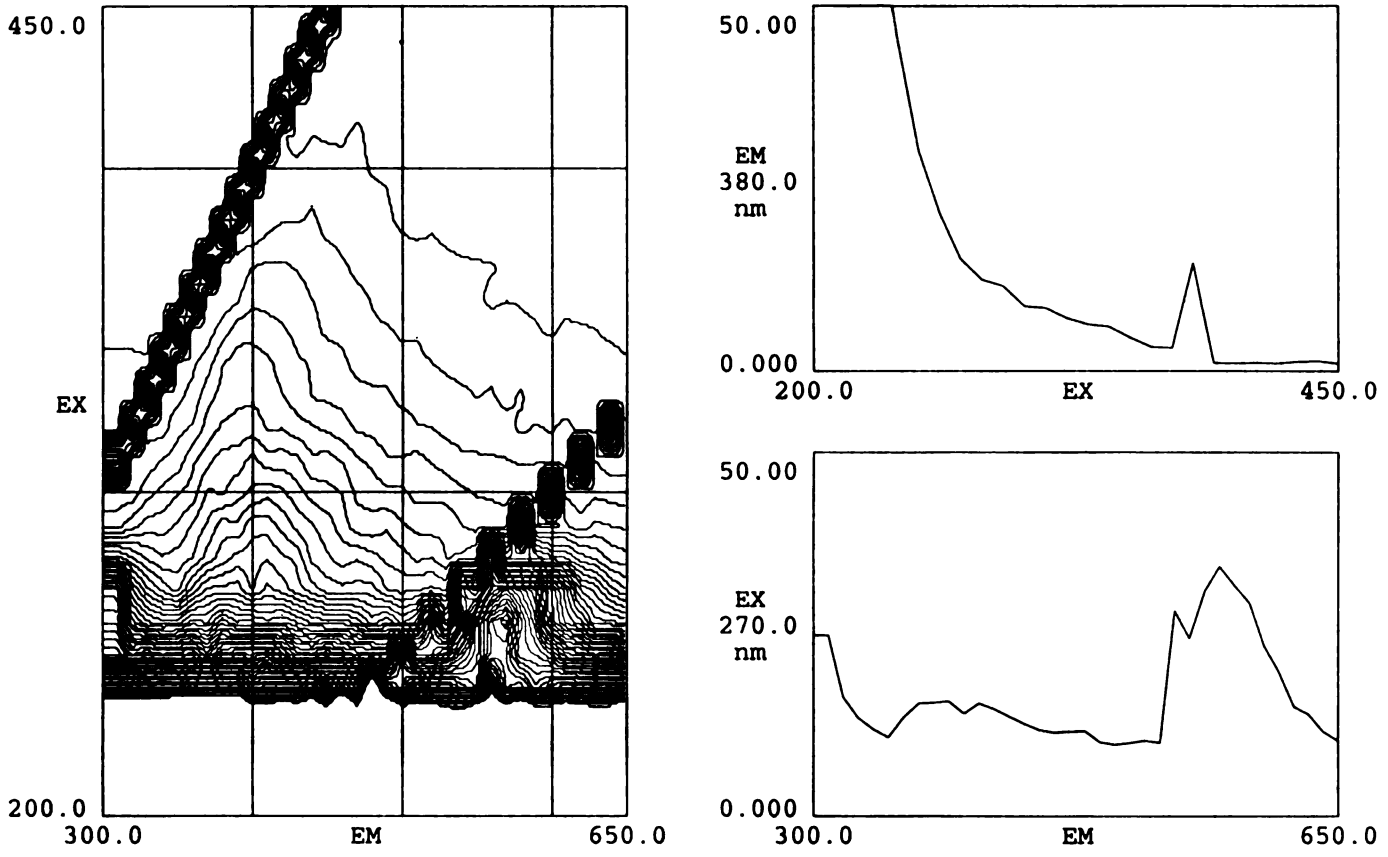

Figure 10A: Fluorescence excitation-emission matrix of the standard soil hexane extract (37 µg/mL)

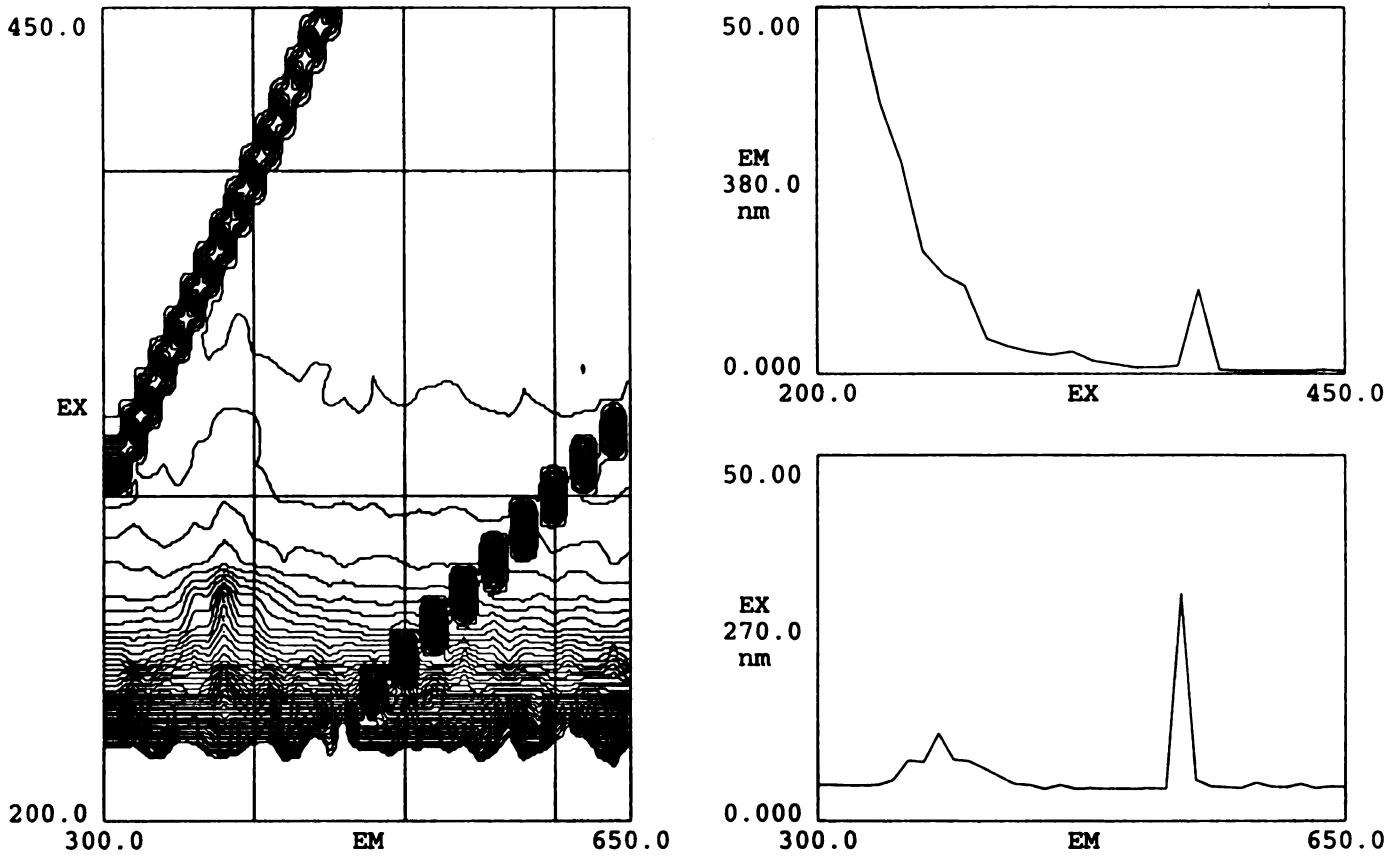

Figure 10B: Fluorescence excitation-emission matrix of the cornfield soil hexane extract (112 µg/mL)

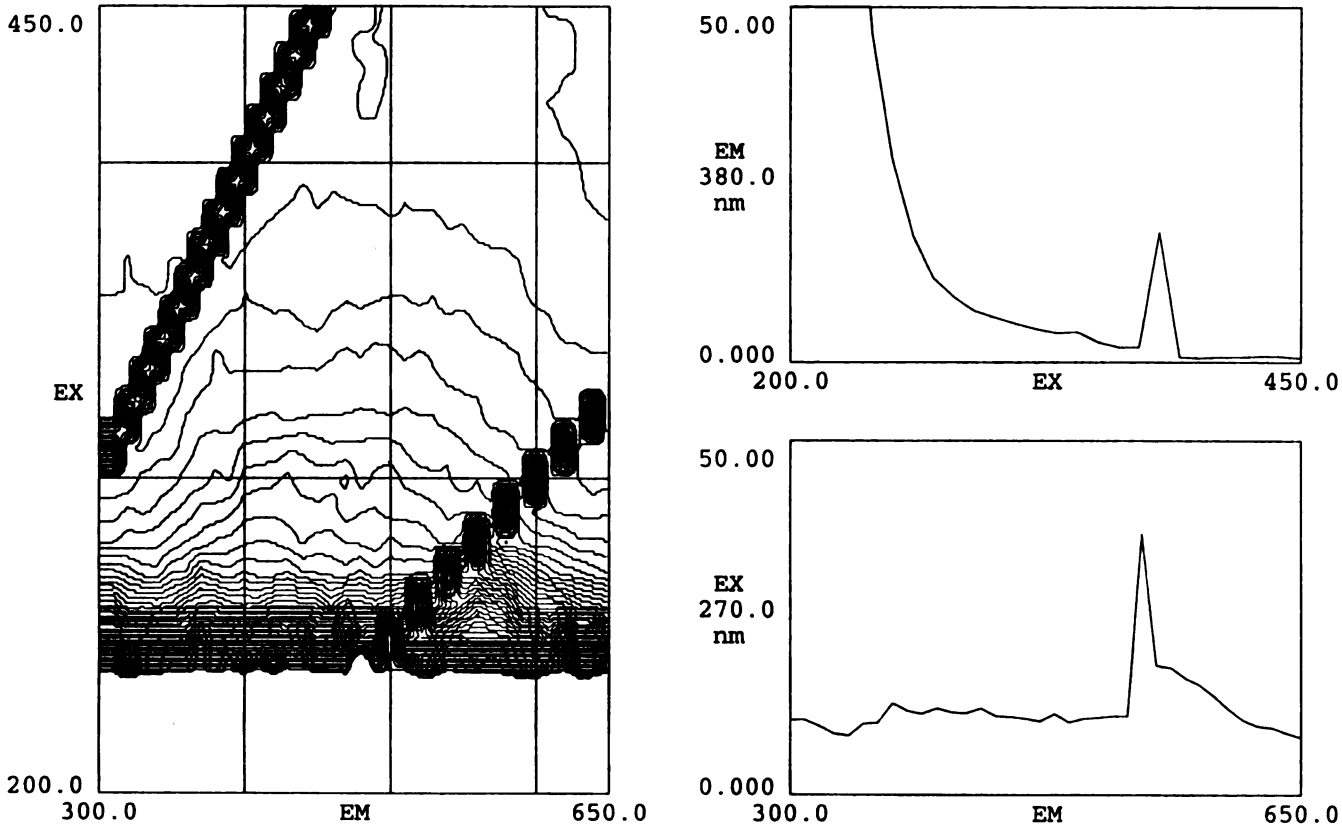

Figure 10C: Fluorescence excitation-emission matrix of the grass lawn soil hexane extract (52 µg/mL)

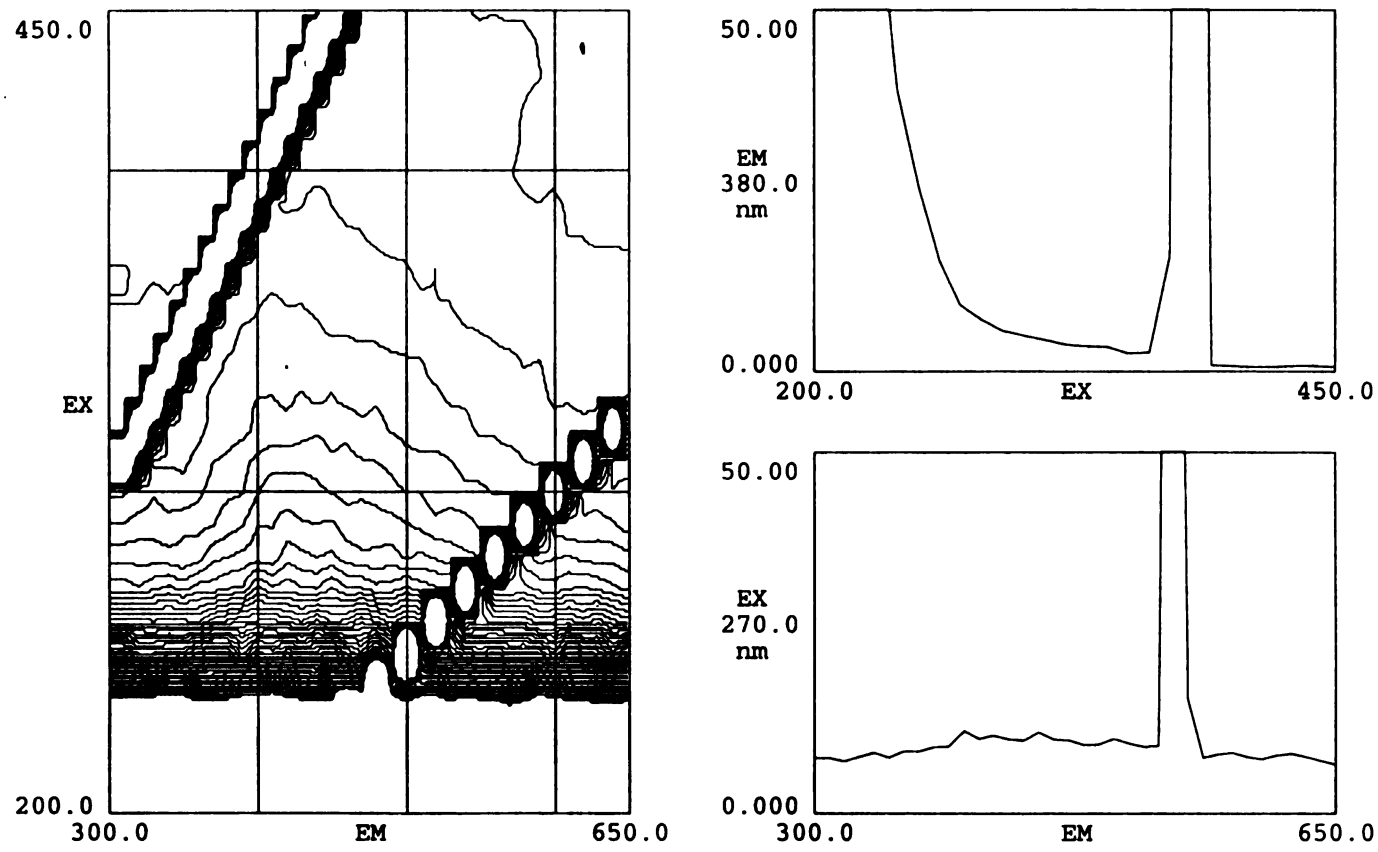

Figure 10D: Fluorescence excitation-emission matrix of the forest soil hexane extract (124 µg/mL)

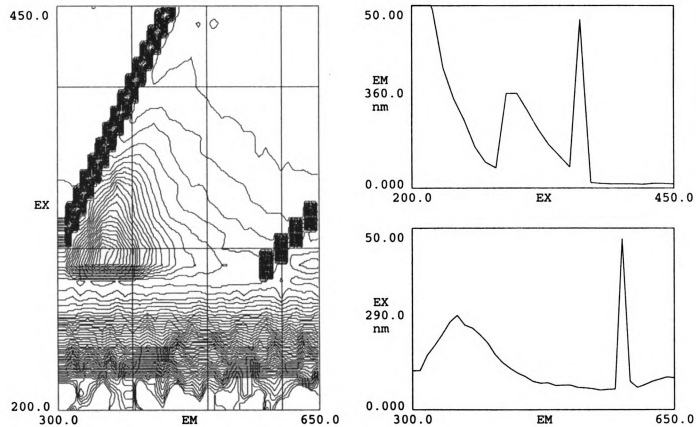

Figure 11A: Fluorescence excitation-emission matrix of the standard soil toluene extract (181 μg/mL)

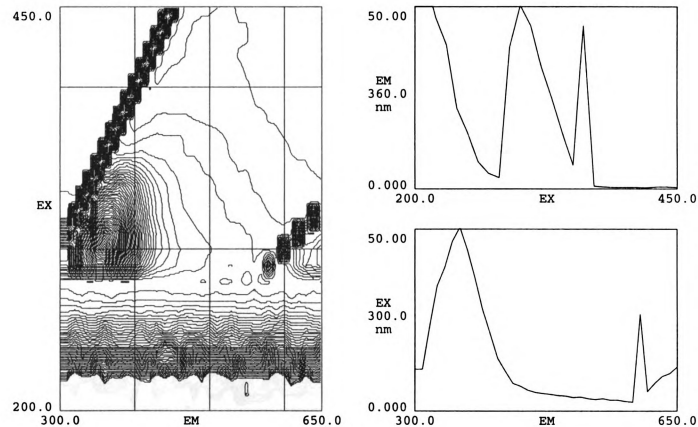

Figure 11B: Fluorescence excitation-emission matrix of the cornfield soil toluene extract (1996 μg/mL)

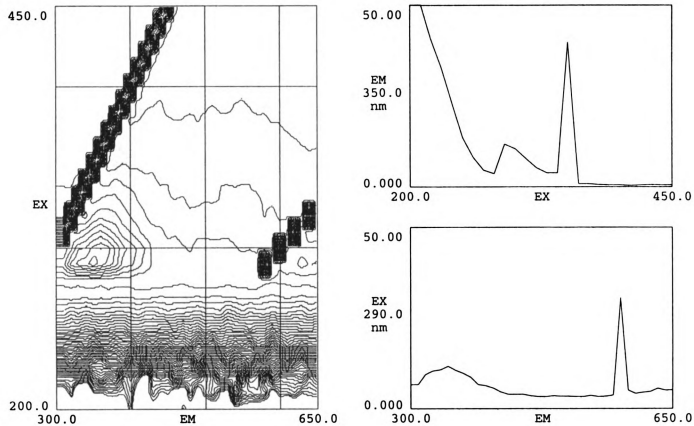

Figure 11C: Fluorescence excitation-emission matrix of the grass lawn soil toluene extract (1894 µg/mL)

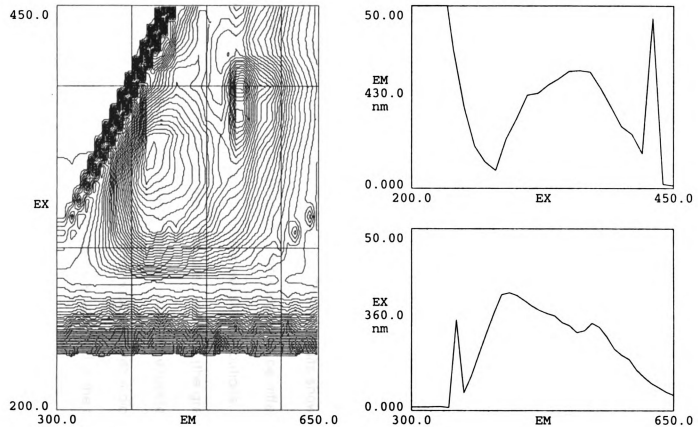

Figure 11D: Fluorescence excitation-emission matrix of the forest soil toluene extract (1660 µg/mL)

3.4 High Performance Liquid Chromatography

The results of chromatographic analysis for the water and methanol extracts are shown in Figures 12 - 18. The separation of the water extracts was particularly difficult and a number of mobile phases were tried without success, including isocratic methanol:water mixtures, methanol:water gradients, and methanol:0.5% (v/v) acetic acid in water gradients. Eventually, it became clear that the only detectable components were eluting prior to the void volume of the system. This indicates that the components of the water extracts were not interacting significantly with the stationary phase of the column and were being excluded from the 120 Å pores of the silica particles, thereby creating a size exclusion separation mechanism. Such a mechanism was successfully exploited by using a 98% water/2% methanol mobile phase at a flow rate of 0.1 mL/min and a 20 µL injection volume (see Figure 12). These results showed that the water-soluble humic substances present in the soils could be differentiated based on their size and, therefore, molecular weight distributions. Furthermore, the two soils that were located on the same base soil type (the grass lawn and corn field) both show similar chromatograms, as would be expected.

Since the chromatographic system was equipped with a diode array detector, full UV-visible absorbance spectra were acquired of the column effluent. The spectra for the chromatographic peaks discussed above are shown in Figure 13. These largely featureless spectra are similar for all extracts. Furthermore, they are characteristic for humic species and help confirm that the

water-soluble organic matter is primarily made up of large molecular weight humic substances that possess many polar and/or ionizable functional groups. In turn, this results in no significant interaction with a C_{18} stationary phase and high water solubility.

The chromatograms for the methanol extracts are shown in Figure 14. Optimization of the separation conditions for these samples indicated that a mobile phase gradient gave the best results. The data shown uses a linear methanol:water gradient from 60 to 100% methanol over 30 minutes with a 15 minute hold at 100% methanol. A 0.85 mL/min flow rate was used with a 20 µL injection volume. As with the water extracts, the major component in these extracts is a non-retained peak that is being excluded from pores in the stationary phase particles. The UV absorbance spectra of these peaks are shown in Figure 15 and are also very similar between extracts. The presence of a more defined peak at 230 nm differentiates these samples from the water extracts. Together with the fluorescence data discussed above, it is apparent that two separate fractions of humic substances are being isolated in the water and methanol fractions respectively.

Of greatest importance to this work, however, is the presence of a number of small, well-defined peaks eluting after 10 minutes in these chromatograms. These peaks, shown at 210, 220 and 230 nm in Figures 16-18 show high potential for discriminating the soil samples. The wavelength that produced the most complex, and hence the most easily differentiable, chromatograms was 230 nm. These peaks were found to be reproducible as well as not being

present in a blank injection of solvent. It also should be noted that the samples had to be concentrated above 1 mg/mL in order to achieve sufficient signal-to-noise ratios for these components.

As a final experimental note, the acetone extracts for the soil samples were also analyzed. Unfortunately, while a number of mobile phases were used (consisting of methanol:1,4-dioxane), none produced any discernible retained peaks, nor was any size-exclusion mechanism noted.

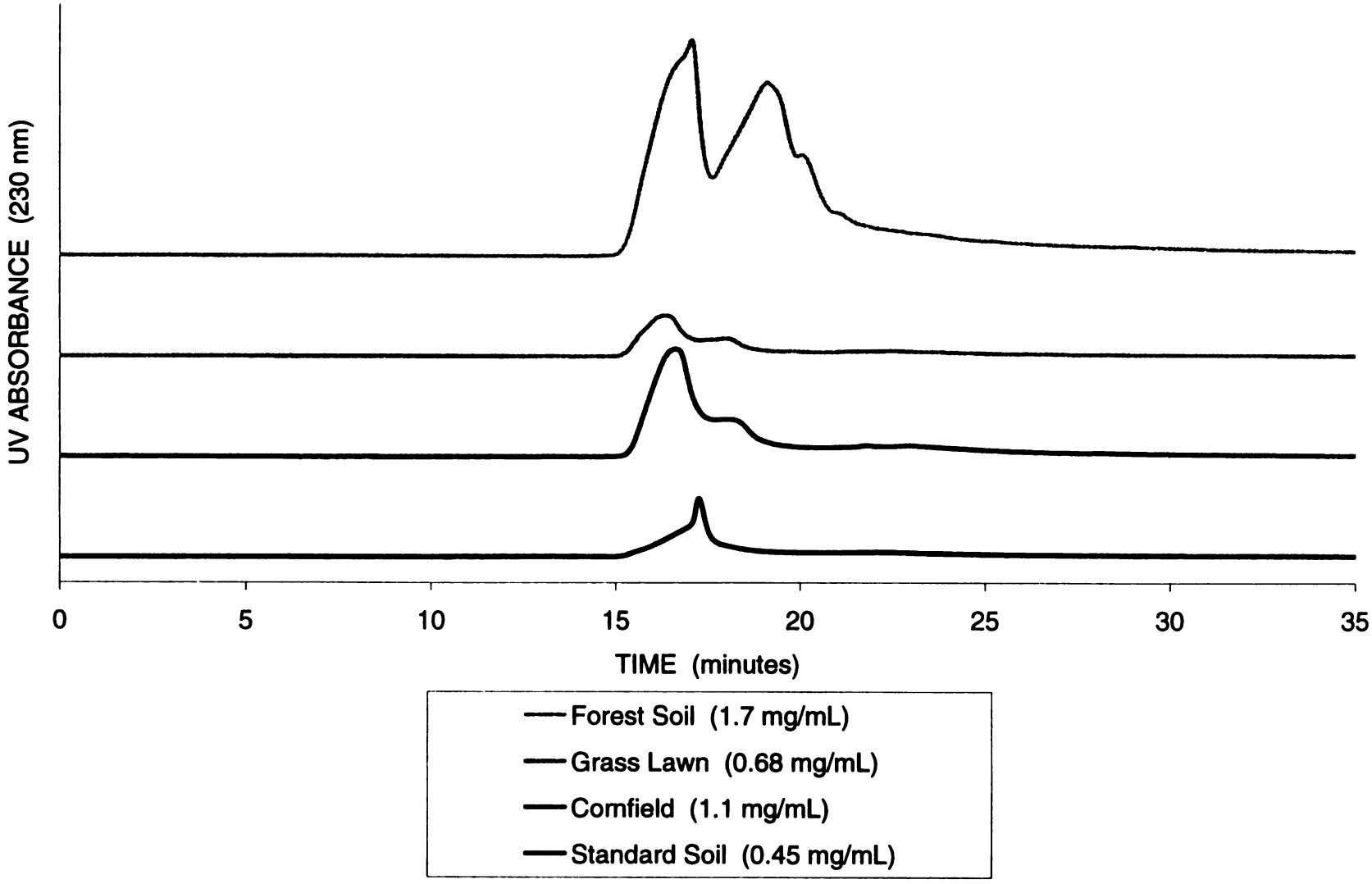

Figure 12: Comparison of water extract chromatograms

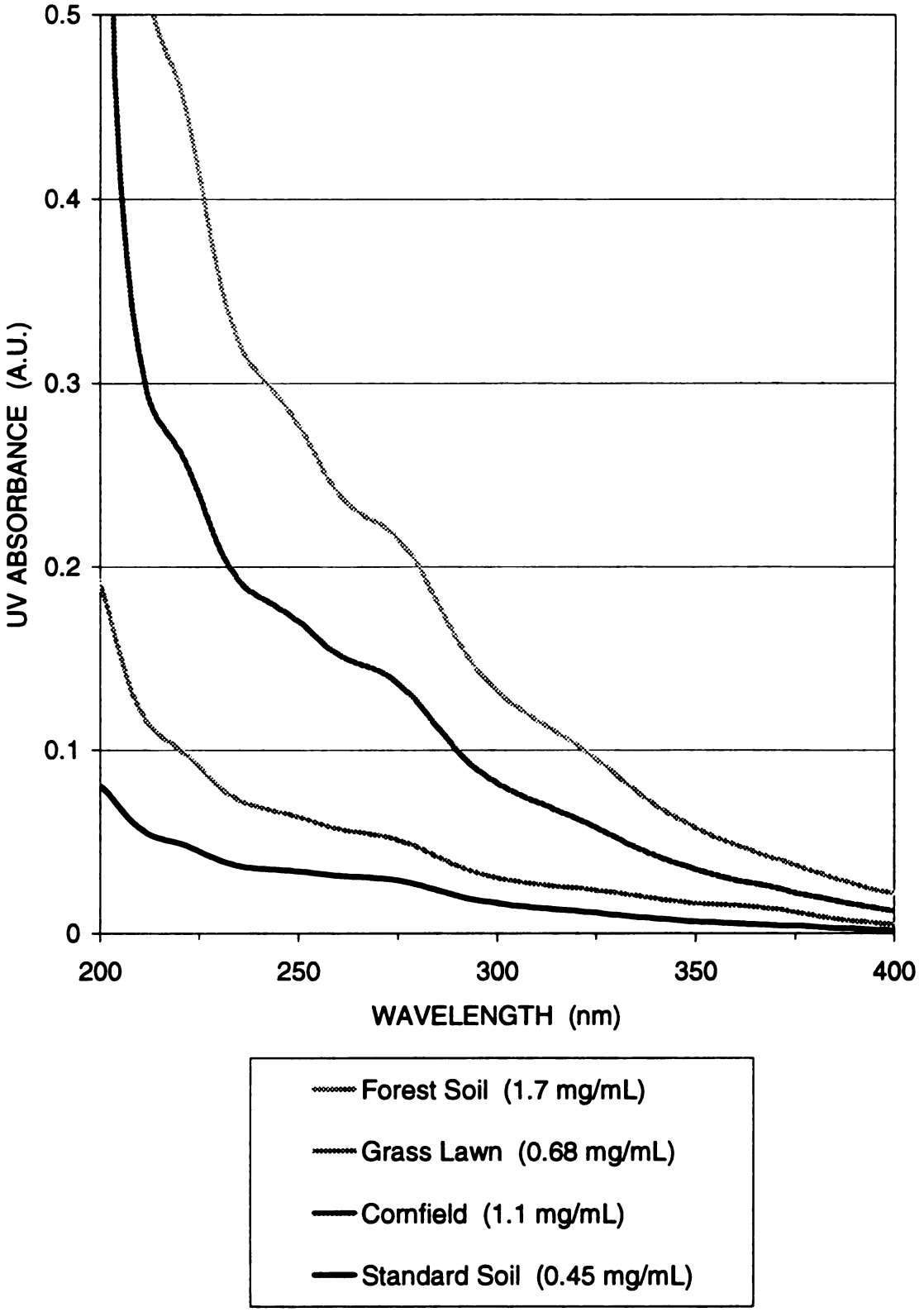

Figure 13: UV-visible absorbance spectra of soil water extracts at retention time 16.5 min

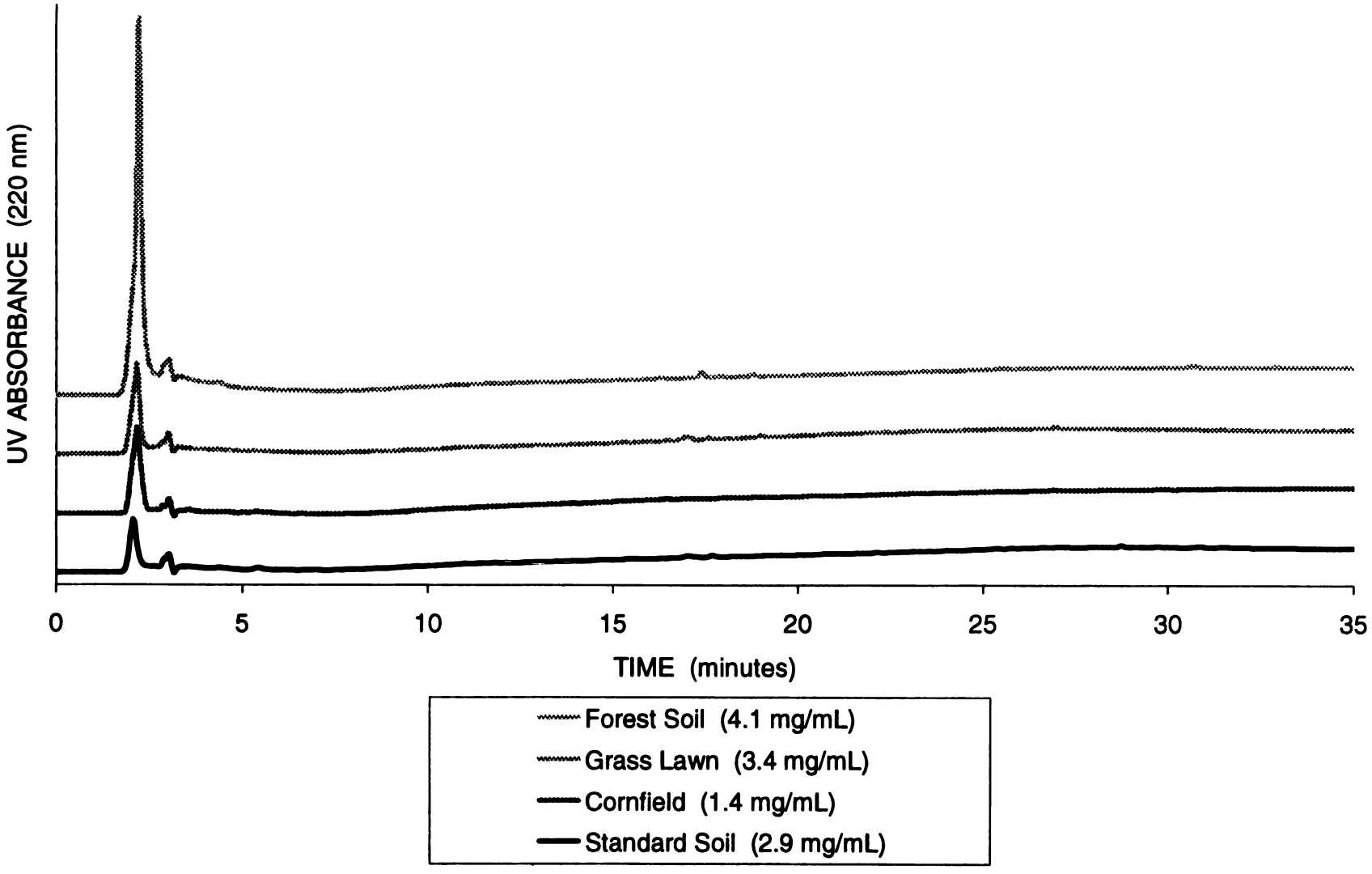

Figure 14: Comparison of Methanol Extract Chromatograms

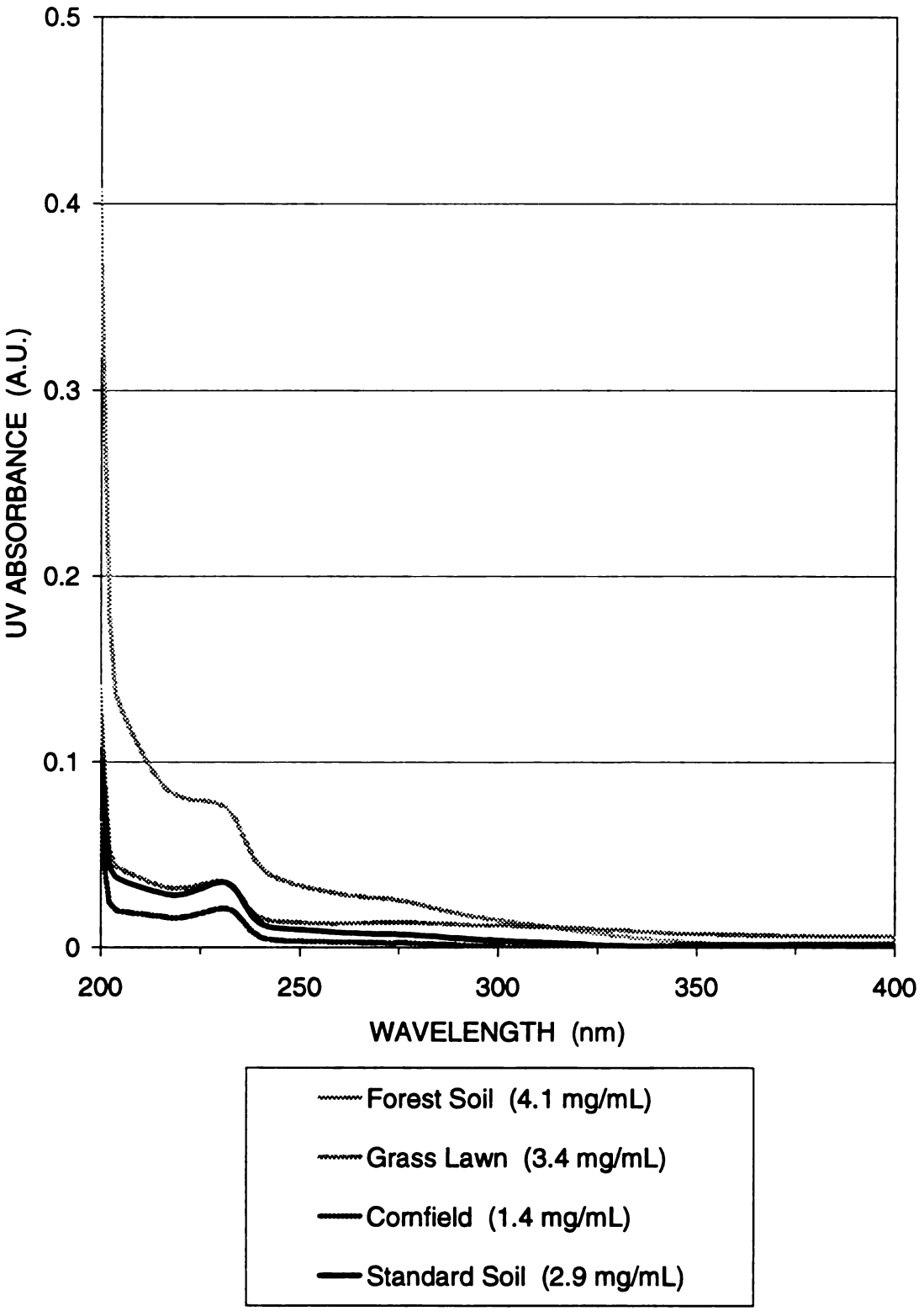

Figure 15: UV-visible absorbance spectra of soil methanol extracts at retention time 2.0 min

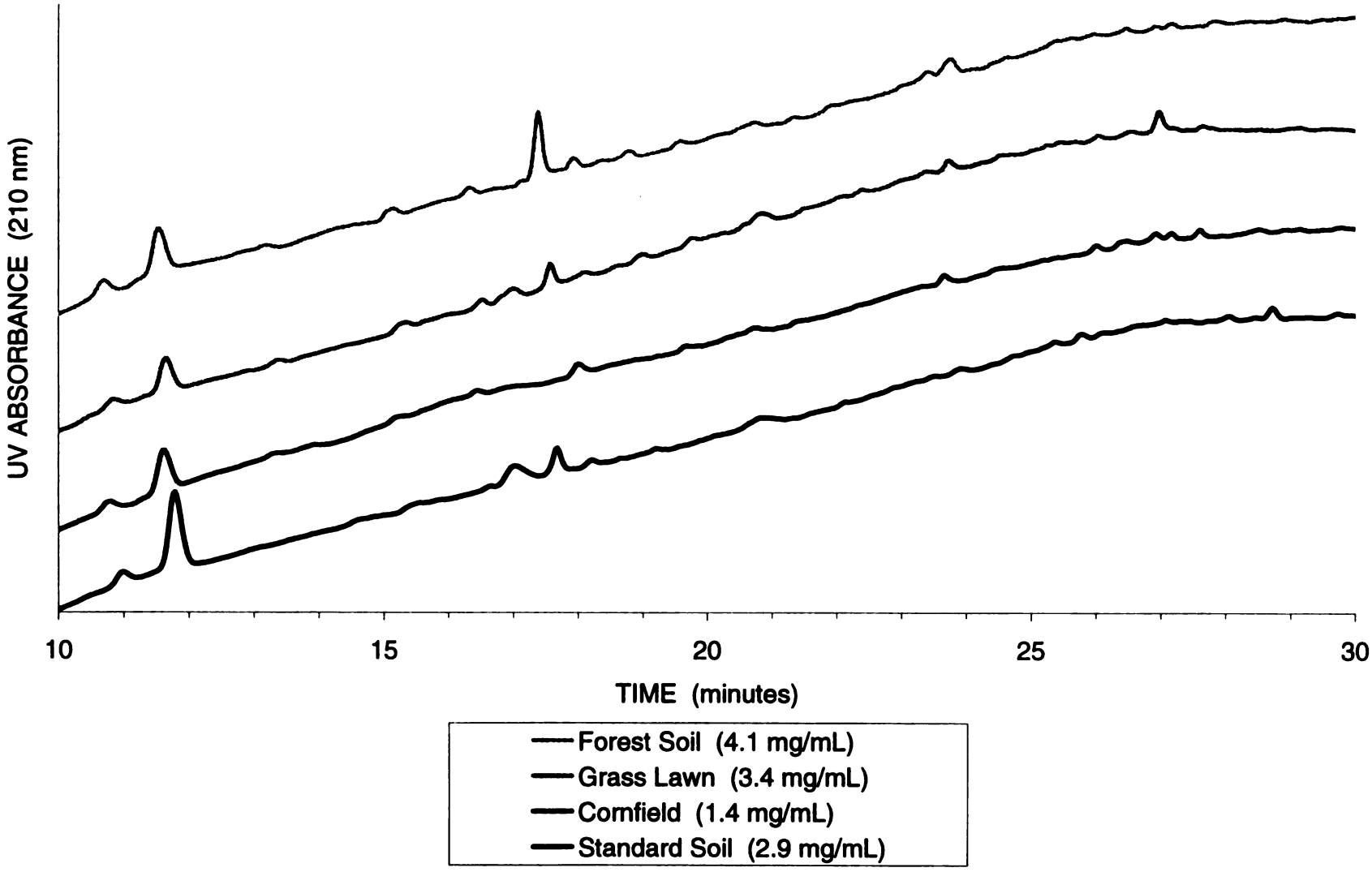

Figure 16: Comparison of methanol extract chromatograms at 210 nm

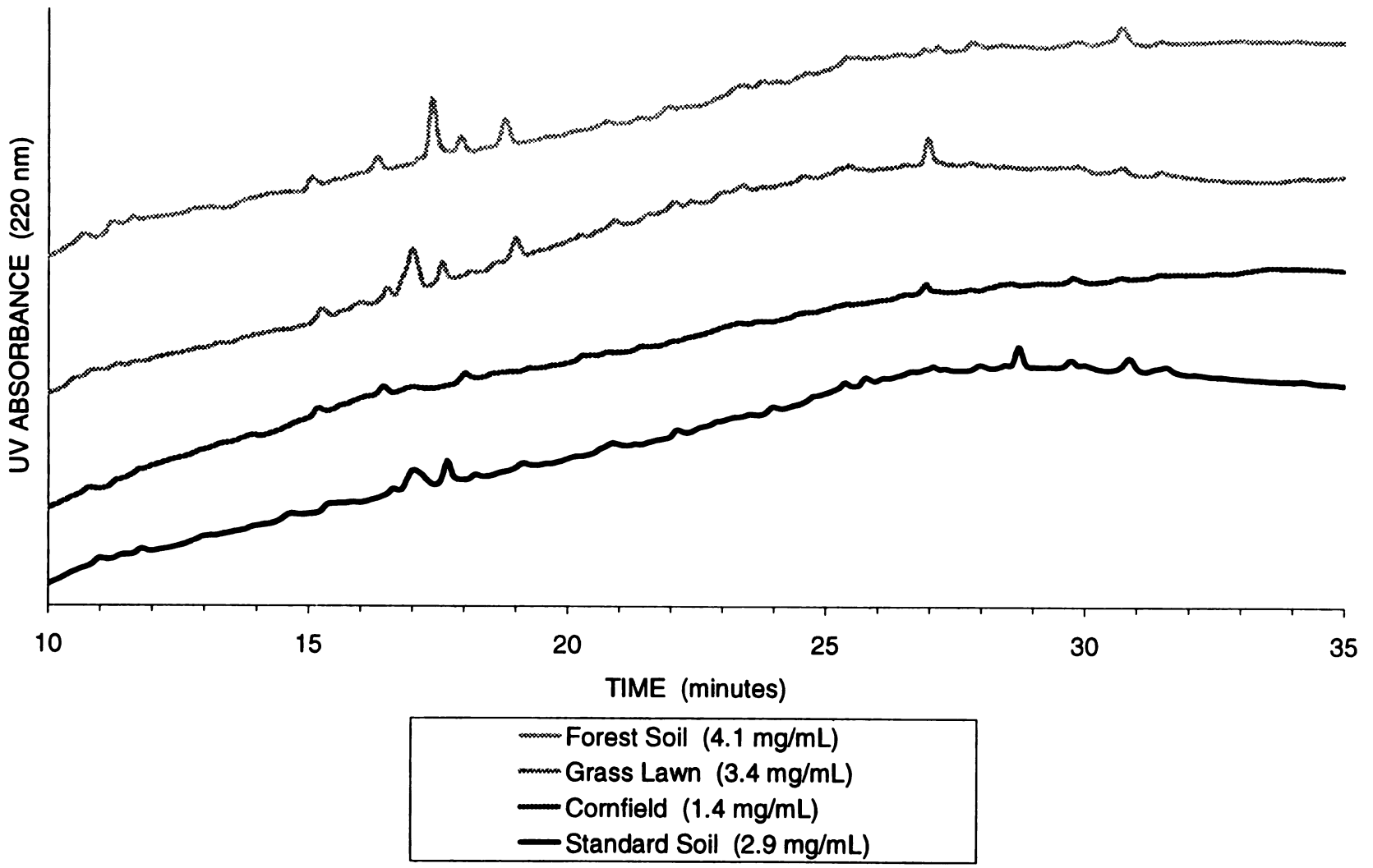

Figure 17: Comparison of methanol extract chromatograms at 220 nm

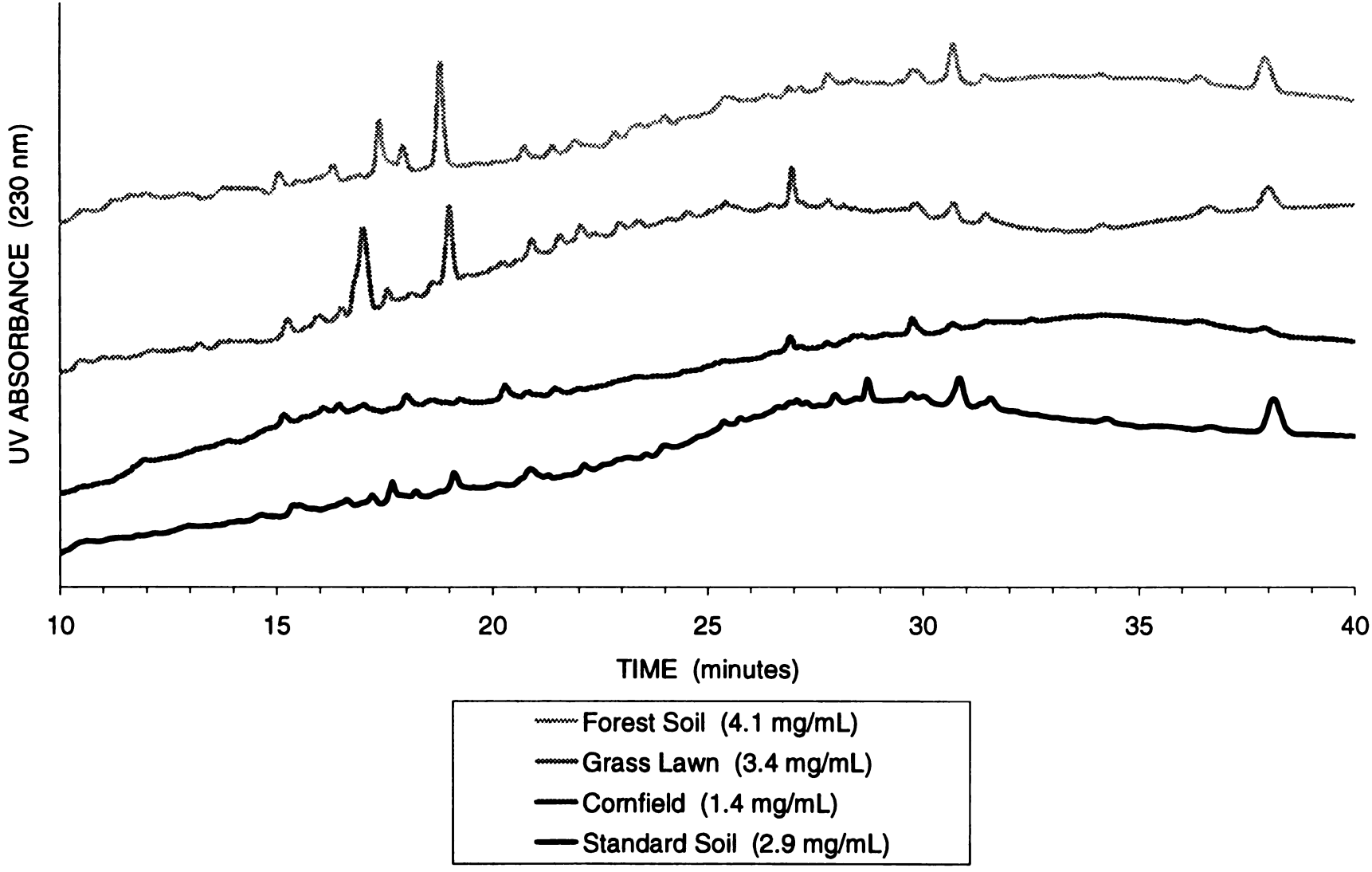

Figure 18: Comparison of methanol extract chromatograms at 230 nm

4. CONCLUSIONS AND FUTURE DIRECTIONS

The use of soil as evidence in criminal investigations will surely increase if techniques are developed which more fully characterize them. Currently, the analytical focus has remained on the inorganic portion of soil while the use of the organic component of soil as a source of information has been limited. In this work, a systematic study of the extraction and analysis of soil organic matter has been undertaken.

Sequential extraction of soils using various solvents has shown that the fractionation patterns of soils may be a useful characteristic of comparison. Furthermore, Soxhlet extractions have proven to be a convenient, exhaustive, and quantitative method for isolating fractions of a soil sample. However, it appears that all pristine soils may show similar patterns. This method also has the disadvantages of being highly time consuming, amenable only to neutral solvents and requiring lengthy filtering and concentration of extracts for further analysis. Some alternative approaches to extraction may be more applicable to soil samples as well as the context of a forensic laboratory. For example, the use of ultrasound could greatly accelerate extraction times, or supercritical fluid extraction could also efficiently isolate fractions of soil samples.

The analysis of soil extracts using UV-visible absorbance and fluorescence spectroscopy gave informative results but unfortunately they were not highly discriminating. For example, the results showed that the nature of the humic substances in the water and methanol extracts differed, but little

difference was seen between soil types. Only in cases where a soil contains a unique component (e.g. potential agricultural chemicals in the cultivated corn field) would absorbance and fluorescence analysis discriminate one sample from others. Future researchers would be well advised to examine other spectroscopic techniques such as FTIR spectroscopy, surface-enhanced Raman spectroscopy, or NMR spectroscopy. These techniques have been successfully applied to the analysis of humic substances and may be useful for distinguishing soil extracts after samples have been suitably prepared.[40]

Lastly, the separation of soil extracts using HPLC showed the greatest potential for discriminating soils. The analysis of water extracts was based on a size exclusion mechanism and was able to differentiate soils based on their underlying soil type. A more efficient size-exclusion separation with a column whose pore size is optimized for the molecular weight range of humic substances would likely improve these results greatly. In addition, the use of FTIR as a detection method could yield more informative results than UV absorbance. However it must be remembered that such an analysis separates only on the basis of molecular weight distribution rather than chemical composition.

The most promising results were generated using a reversed-phase separation of the methanol extracts. It was clear that there are a number of methanol soluble components in soils that can be successfully separated using a gradient elution approach. As long as care is taken to sufficiently concentrate the samples, this method should be a powerful innovation to soil comparisons.

Furthermore, there may be even more discriminatory compounds in the non-polar extracts (acetone, hexane and toluene). In these cases, the use of a normal-phase separation may generate the necessary chromatographic resolution. Finally, another separation technique that has demonstrated potential for humic substances is capillary electrophoresis, which would be an efficient and rapid method well suited for aqueous soil extracts.[41]

Despite these accomplishments and reccomendations, it is important to note that a thorough and quantitative assessment must be made of the discriminatory power of this technique. Such an assessment is needed in order to determine the ability of this approach to successfully compare soil evidence in criminal investigations. Two main issues to be addressed are discerning differences between soil samples which originate from slightly different locations and discerning changes that may occur at a single location over time. Furthermore, some quantitative criteria must be established for how much similarity must exist between two samples for them to be deemed as likely originating from the same source. By conducting a larger and more detailed sampling study over a suitable period of time, these questions could be resolved and the practical application of this technique encouraged.

Overall, this study examined the types and conditions of analysis that are required for forensic soil analysis. Not all methods demonstrated the ability to differentiate soils, but it has been shown that some do show that potential. Specifically, both the relative amounts of extractables as a function of solvent polarity and the separation of these extracts according to molecular weight or

solubility yield distinctive patterns. These developments, as well as future work introducing other spectroscopic or separation methods and developing quantitative means of comparing data, can only continue to improve the analysis of soil.

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