J. Trop. Agric. and Fd. Sc. 34(1)(2006): 165–172

Characterization of humic acid from humification of oil palm empty fruit bunch fibre using *Trichoderma viride*

(Pencirian asid humik daripada humifikasi tandan kosong kelapa sawit menggunakan *Trichoderma viride)*

M.S. Umi Kalsom*, H. Nur**, A.A. Norlea* and S. Ngaspan*

Key words: humic acid, *Trichoderma viride*, fourier transform infrared and nuclear magnetic resonance, oil palm empty fruit bunch

Abstract

Humic acid (HA) extracted from fermentation of EFB using *Trichoderma viride* was characterized by using three methods: i) elemental analysis performed by Scanning Electron Microscopy (SEM) with integrated Energy Disperse Spectroscopy (EDS) detector and element-analyzer, ii) fourier transform infrared (FTIR), and iii) solid state cross polarization magic angle spinning carbon 13-nuclear magnetic resonance (CP/MAS ^{13}C NMR) spectroscopy. The elemental composition and functional groups of the HA extracted from EFB resembled those reported for HA of peat and incompletely humified materials. The CP/MAS ^{13}C NMR data correlated with results obtained by FTIR spectroscopy, both indicating that the HA were composed of partially degraded constituents of plant tissue, which still retain their chemical structures. The major plant components identified in the HA were lignin, carbohydrates and long-chain aliphatic structural groups.

Introduction

Humification is a decay process involving the transformation of biomolecules, originating from dead organisms, and microbial activity where humic substances are formed and nonhumic substances are decomposed (Bernal et al. 1993; Schnitzer et al. 1998). Lignin as well as polyphenols and derived polymers from lower plants and microorganisms are the starting materials in this process. Humic substances comprise relatively high molecular mass compounds with mixed aliphatic and aromatic characteristics (Stevenson 1994). Based on solubility in acids and alkalis, they can be divided into three main fractions: humic acid (HA), fulvic acid and humin (Senesi and Loffredo 2001). The structure of HA consists of a heterogenous association of molecules or small humic sub-units of different chemical nature and origin (Schnitzer 1978). The molecular weight of HA depends on these associations which varies from 2,000–300,000 Da.

The importance of HA in agriculture has been acknowledged for over 150 years. The extension of organic farming and sustainable agriculture has led to increasing applications of organic fertilizers. Composts, as organic fertilizers, contain a substantial

*Strategic Resources Research Centre, MARDI Headquarters, Serdang, P.O. Box 12301, 50774 Kuala Lumpur, Malaysia

**Ibnu Sina Institute for Fundamental Science Studies, Universiti Teknologi Malaysia, 81310 UTM Skudai, Johor, Malaysia

Authors' full names: Umi Kalsom Md. Shah, Hadi Nur, Norlea Abdul Aziz and Ngaspan Sardan

E-mail: umishah@mardi.my

amount of organic matter, with a significant amount of humic substances (Deiana et al. 1990). Humic acid constitutes stable fraction of carbon, thus regulating the carbon cycle and the release of nutrients, including nitrogen, phosphorus and sulphur in the soil. Additionally, the presence of HA improves water holding capacity, pH buffering and thermal insulation (Stevenson 1994). Humic acid contains a variety of functional groups, including carboxylic acid (COOH), phenolic hydroxy (OH), enolic (OH), alcoholic (OH), quinone, hydroxylquinone, lactone, and ether (Sposito 1986).

Microbial degradation of HA is an important part of humus turnover, therefore essential for maintaining the global carbon cycle (Haider and Martin 1988). With respect to contribution to the global carbon cycle, humification is the second important process in turnover after photosynthesis (Hedges and Oades 1997). The potential of *Trichoderma* species as lignocellulose degrading agents has been recognized since early 1960 (Selby and Maitland 1967). This fungus occurs worldwide and is easily isolated from soil, decaying wood, and other forms of plant organic matter. It grows rapidly in versatile conditions and utilizes various kinds of substrates (Eveleigh 1985). Rapid growth rate and production of numerous spores with varying shades of green characterize the species in this genus (Gams and Bisset 1998).

Oil palm is one of the most important crops in Malaysia. However, the empty fruit bunches (EFB) that remain after oil production are regarded as wastes and have not been utilized effectively. The aim of this study was to characterize the humic acid extracted from solid fermentation of EFB fibre using *Trichoderma viride*.

Materials and methods

Microorganism

Trichoderma viride was isolated from EFB compost. The fungus was sub-cultured every month on potato dextrose agar (PDA) and stored at 4 °C.

Solid fermentation

A sample of 100 g of the EFB fibre (1–2 cm size) was amended with 0.1% $(NH_4)_2SO_4$, 0.5% yeast extract, 0.2% K_2HPO_4 and 65% (v/w) distilled water in 2 litre Erlenmeyer flask and sterilized by autoclaving at 121 °C, 15 p.s.i. for 30 min. After cooling at room temperature, the substrate mixture was then inoculated with 10^8 spores of *T. viride.* Three replicates of flask cultures were incubated stationary at 30 °C. Samples were harvested after 5 weeks of incubation.

Extraction of humic acid

HA was extracted following the method described by Olk et al. (1996). Briefly, 45 g of the fermentation substrate was added with 450 ml 0.2 M KOH under an atmosphere of N_2. The mixture was then shaken for overnight at room temperature and left to stand for 18 h. The extract was then separated by centrifugation at 10,000 rpm for 20 min. The supernatant was collected and the pH adjusted with 6 M HCl to pH 1–2 to precipitate out humic acid. The precipitate was allowed to stand for 12 h, centrifuged and the HA was recovered by dissolving in a small volume of 0.1 N KOH. The HA was then purified with a mixture of hydrochloric and hydrofluoric acid (0.1 M HCl/0.3 M HF) and finally dialysed using dialysis Visking tubing against distilled water for more than 24 h. The HA gel was then air dried and ground to pass a 0.25 mm sieve.

Elemental analysis

The solid HA was analysed for its elemental composition. The percentage of H was analysed using an auto element-analyser (CHNS-932 Model Lecco, ST Joseph, MI, USA). Other elements such as C, N, O, and S were analysed using Various Pressure SEM, Model EELS attached to LEO 912AB energy filter transmission EM 120kv integrated with EDS.

Fourier transform infrared (FTIR) analysis
FTIR analysis was performed on a Perkin Elmer FTIR spectrophotometer. KBr pellets were made by accurately weighing 2 mg of dried HA and 300 mg of dried KBr and pressing the mixture under vacuum at 10 t for 10 min. Measured wavelength range of 4000–400 cm^{-1}. Data collection and processing was performed by GRAMS/386 version 3.02 software.

Solid state cross polarization magic angle spinning carbon-13 nuclear magnetic resonance spectroscopy (CP/MAS ^{13}C NMR)
Solid-state ^{13}C CP/MAS NMR spectrum was recorded at room temperature on a Bruker Avance 400 MHz NMR operating at a static magnetic field of 9.4 T. Magic angle spinning was performed at 5.0 kHz, making use of 4 mm ceramic Si_3N_4 rotor. The ^{13}C CP/MAS experiment was performed using a 3.8 microsecond 90° pulse with a delay time of 5 s, a contact time of 1 ms and spinning rate of 5 kHz and 2000 transients. Chemical shifts were measured with respect to tetramethylsilane. The Bruker Win NMR software was used to measure peak areas of the following chemical shift regions: 0 to 47 ppm (aliphatic alkyl C), 47–90 (alkyl-O or C-O, C-N bonds as in carbohydrates, alcohols), 90–102 ppm (acetal), 102–145 ppm (aromatic C), 145–167 ppm (phenolic C), 167–187 ppm, (carboxyl, ester and amide), 187–220 ppm (carbonyl C).

Results and discussion
Solid state fermentation
The fungal mycelium was observed to grow on the EFB substrate after 3 days of incubation. Green spores of *T. viride* was observed after 1 week incubation. The green spores continued to colonise the EFB substrate until the fourth week when the fungus started to change to whitish culture. The yield of humic acid from solid fermentation of EFB (13.7 g per 100 g EFB) has been reported in our previous work (Umi Kalsom et al. 2003). As solid fermentation of EFB progresses, the yield of HA increased, until at week 5 when the yield of humic acid became stable.

Elemental analysis using SEM
The elemental analysis revealed that the solid HA was primarily composed of carbon (53.5%), oxygen (34.4%), nitrogen (4.0%) sulfur (1.0%) and silica (0.8%). Since EDS could not detect hydrogen, the hydrogen content (6.3%) was analysed using auto-analyzer. The elemental composition of the HA obtained in this study was almost similar to HA extracted from refuse compost as reported by Chien et al. (2003) which consisted of carbon (55.2%), oxygen (32.4%), nitrogen (6.5%), hydrogen (4.8%) and sulfur (1.0%). The carbon and nitrogen content of the EFB was 80.9% and 1.2% respectively.

FTIR spectra
The IR spectra of HA extracted from solid fermentation of EFB using *T. viride* is shown in *Figure 1*. Interpretation of the spectra was based on Pavia et al. (1996). Major characteristic bands recorded included: a broad O-H and N-H stretching vibration band was obtained around 3413 cm^{-1} for intermolecular hydrogen bonding (H-bonded OH groups) attributed to phenolic groups. Methylene hydrogens ($-CH_2-$) gave rise to two aliphatic C-H stretching bands; a sharp peak at 2925 cm^{-1} for asymmetric stretching and a shoulder at 2852 cm^{-1} for symmetric stretching.

A pronounced peak at 1652 cm^{-1} could be attributed to aromatic C=C, C=O and/or C=O of conjugated ketones or to C=N amide stretching. The methyl asymmetric C-H bending was observed at 1458 and 1443 cm^{-1}. Peaks at 1529 and 1326 cm^{-1} could be attributed to the presence of nitro groups. A peak at 1271 cm^{-1} could be attributed to C-O stretching of aryl ethers. A shoulder peak at 1123 cm^{-1} could be attributed to aromatic ring bends, symmetric bending of aliphatic CH2, OH or C-O stretching of various groups. Peaks at 1160,

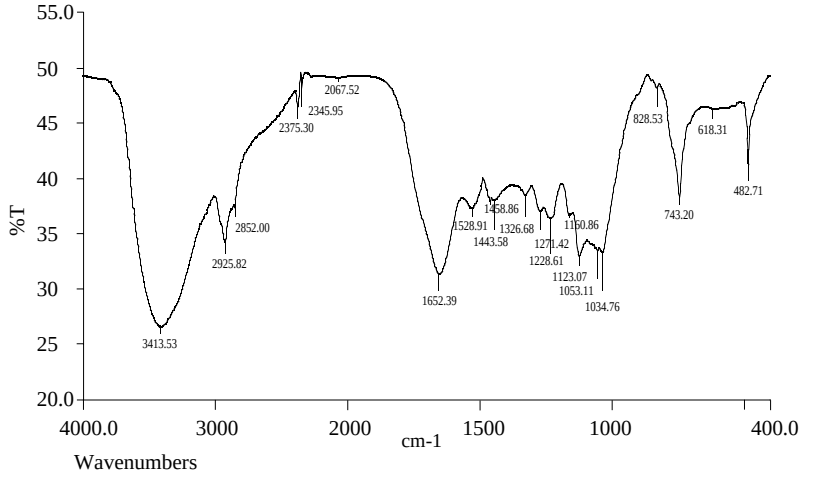

Figure 1. FTIR spectrum of humic acid extracted from solid-state fermentation of EFB fibre using ***Trichoderma viride***

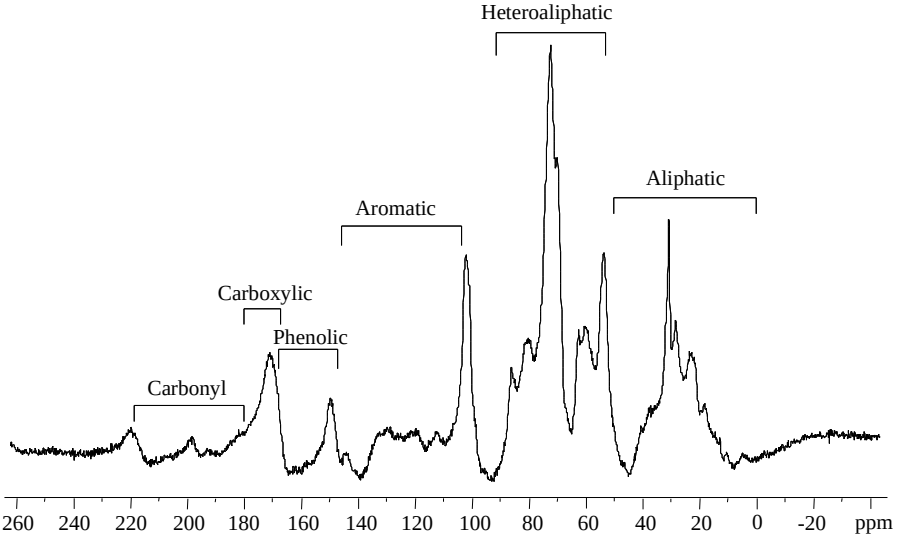

Figure 2. The [13]C CP/MAS NMR spectrum of the humic acid extracted from solid fermentation of EFB fibre by ***Trichoderma viride***

1053 and 1034 cm^{-1} could be attributed to C-O stretching of alcohol, sulfoxides, carbohydrates or polysaccharides-like substances, or Si-O of silicates.

A strong band obtained at 743 cm^{-1} could be attributed to an ortho disubstituted out-of-plane = C-H bending vibration. This band is probably due to lignin absorption arising from the stretching of ortho disubstituted rings (1, 2-disubstituted rings) (Amalfitano et al. 1994). This absorbance due to lignin existence is not surprising because EFB consisted of vegetative matter, much of which only partly decomposed. In general, the FTIR spectrum of HA extracted from EFB obtained in this work was almost similar to the spectrum of humic acid extracted from composted farmyard manure as reported by Ouatmane et al. (2000).

Table 1. Distribution percentage of the characteristic carbons in ^{13}C nuclear magnetic resonance spectra with cross-polarization and magic-angle spinning (CP/MAS ^{13}C NMR) of humic acid extracted from fermented EFB

Chemical shift (ppm)	C-containing group	Proportion of carbon in structural groups (%)
220–187	Carbonyl	1.6
187–167	Carboxylic	9.9
167–145	Phenolic	1.9
145–102	Aromatic	6.6
102–90	Acetal	–
90–47	Heteroaliphatic	58.6
47–0	Aliphatic	21.4

Solid state cross polarization magic angle spinning carbon-13 nuclear magnetic resonance spectroscopy (CP/MAS ^{13}C NMR)

NMR is useful in characterizing humic acid especially in determining the proportion of stable (aromatic) and labile (aliphatic) structural components. Solid state cross polarization magic angle spinning (CP/MAS) carbon-13 NMR spectroscopy has made possible acquisition of high resolution ^{13}C spectra from the solid substance (Wilson 1989). The CP/MAS ^{13}C NMR spectra of HA extracted from EFB is presented in *Figure 2.* The CP/MAS ^{13}C NMR spectra exhibited major peaks at δ 19, 23, 29 and 32 ppm (aliphatic C), δ 55 and 60 ppm (N-alkyl C) δ 74 and 82 ppm (carbohydrates or aliphatic alcohols) δ 104 ppm (C-substituted aromatic C), δ 153 ppm (O-substituted aromatic or phenolic), δ 174 ppm (carboxyl and amide C), δ 202 and 224 ppm (carbonyl C).

The resonance integrals of seven common spectral regions are shown in *Table 1* which shows that 21.4% of the total component was made up of aliphatic groups and 6.6% of the total component was made up of aromatic groups. The other components were heteroaliphatic (58.6%), phenolic (1.9%), carboxylic (9.9%) and carbonyl (1.6%). The substantial amount of alkyl-C in the HA extracted from EFB was very similar to those of HA extracted from refuse compost (Chien et al. 2003). The HA extracted from EFB exhibited a higher level of C-substituted aromatic C and a lower content of phenolic C. This is consistent with the characteristic of the source EFB sample which contained about 50.4% cellulose, 21.9% hemicellulose and 10.0% lignin (Umi Kalsom et al. 1997). The 153 ppm peak could be assigned to C-3 and C-5 of syringyl units (Chefetz et al. 2002). The relationship between the chemical nature of the polymethylenic domains in humic acid and the degree of humification is not yet well understood and should be further investigated.

According to Fan et al. (2000), humic acid consisted of an aromatic core with some distinct features resembling lignin structure. Both carbohydrates and peptides are part of this structure, possibly enriched at the surface and therefore more mobile and more easily detected by NMR spectroscopy. As has been mentioned earlier, humification is the process whereby plant tissue is converted into natural organic matter. In general, the molecular species of plant tissue can be divided into two categories: rigid and mobile components. Lignin and cellulose normally exist in the rigid network in which they can undergo only very limited molecular motion, whereas the relatively low molecular weight molecules are soluble in cellular fluids and are thus able to move more freely. During fermentation of EFB by *T. viride*, the cell wall ruptures and the component molecules from one organelle

are mixed with molecules from other organelles. The reaction that takes place during this mixing process constitutes the first step in humification process. Since the CP/MAS ^{13}C NMR spectra measure the mobile components of the EFB tissue after fermentation, the spectra could therefore provide an insight into the first reactions that the mobile components underwent during humification.

Interpretation of results requires a clear understanding of the actual information obtained with the different methods and their respective limitations. Spectroscopic methods are severely limited when applied to HA because of the complicated nature of the molecule being studied. IR spectra for HA are the result of numerous components in the mixture that transmit, reflect and absorb at any particular frequency. All signals are superimposed upon each other, and the result is a deceptively simple spectra with broad bands (MacCarthy and Rice 1985). Despite their complexity, the IR spectra do contain a few diagnostic bands which have unambiguous assignments that demonstrate the functionalities present in the humic substances. The IR spectra do not contradict the result obtained from characterization using CP/MAS ^{13}C NMR.

Conclusion

Elemental analysis using SEM integrated with EDS detector, FT-IR and CP/MAS ^{13}C NMR spectroscopy were used to characterize the humic acid fraction extracted from solid fermentation of EFB fibre by cellulolytic fungi *T. viride*. The resulting proportions of the functional group were found to be related to humic properties associated with humification. Integration of the CP/MAS ^{13}C NMR spectra of the humic acid showed that, about 21.4% of the total component was made up of labile aliphatic group and about 6.6% of the total component was made up of stable aromatic group. The other components were heteroaliphatic (58.6%), phenolic (1.9%), carboxylic (9.9%) and carbonyl (1.6%). Thus, humic acid extracted from solid fermentation of EFB fibre using *T. viride* can be regarded as an association of humic sub-units which are heterogenous and appear to contain both highly humified components and higher level of low or non-humified components which are probably of proteinaceous nature.

Acknowledgement

The authors would like to thank Mr Salahuddin for the FTIR analysis and Ms Surya Azura for the SEM analysis. This work was funded by IRPA (Research Grant No. 09-03-03-0072 AE001).

References

Amalfitano, C., Pignalosa, V. and Ramunni, A. (1994). Chemical and physical properties of humic acids extracted from an andisol manured with green horse bean. In: *Humic substances in the global environment and implications on human health* (Senesi, N. and Miano, T.M., ed.) p. 216. Amsterdam: Elsevier

Bernal, M.P., Paredes, C., Sanchez-Monedero, M.A. and Cegarra, J. (1998). Maturity and stability parameters of composts prepared with a wide range of organic wastes. *Bioresource Technology 63:* 91–9

Chefetz, B., Salloum, M.J., Deshmukh, A.P. and Hatcher, P.G. (2002). Structural Components of humic acids as determined by chemical modifications and carbon-13 NMR, pyrolysis, and thermochemolysis-Gas Chromatography/ Mass spectrometry. *Soil Science Society of American Journal 66:* 1159–71

Chien, S.W.C., Huang, C.C. and Wang, M.C. (2003). Analytical and spectroscopic characteristics of refuse compost-derived humic substances. *International Journal of Applied Science and Engineering 1:* 62–71

Deiana, S., Gressa, Manunza, C., Rausa, R. and Seeber, R. (1990). Analytical and spectroscopic characterization of humic acids extracted from sewage sludge, manure, and worm compost. *Soil Science 50:* 419–24

Eveleigh, D.E. (1985). *Trichoderma.* In: *Biology of Industrial Microorganisms* (Demainand, A.L. and Solomon, N.A., ed.) p. 487–509. Menlo Park, California: Benjamin Cummings Publishing Co.

Fan, T.W.M., Higashi, R.M. and Lane, A.N. (2000). Chemical characterization of a chelator-treated soil humate by solution-state multi-nuclear two-dimensional NMR with FTIR and pyrolysis-GCMS. *Environ. Sci. Technol*. *34:* 1636 – 46

Gams, W. and Bisset, J. (1998). Morphology and identification of *Trichoderma*. In: *Trichoderma & Gliocladium* (Hannan, G.E. and Kubicek, C.P., ed.) Vol. 1, p. 3–34. London: Taylor and Francis

Haider, K. and Martin, J.P. (1988). Mineralization of ^{14}C-labelled humic acids and humic Acid bound ^{14}C-xenobiotics by *Phanerocheate chrysosporium. Soil. Biol. Biochem*. *20:* 425–9

Hedges, I.J. and Oades, J.M. (1997). Comparative organic geochemistries of soils and Marine sediments. *Org. Geochem*. *27:* 319–61

MacCarthy, P. and Rice, J.A. (1985). Spectroscopic methods (other than NMR) for determining functionality in humic substances. In: *Humic substances in soil, sediment and water* (Aiken, G.R., ed.) p. 527–59. New York: John Wiley & Sons

Olk, D.C., Cassman, K.G., Mahieu, N., Randall, E.W., Kinchesh, P., Sanger, L.J. and Anderson, J.M. (1996). Changes in chemical properties of organic matter with intensified rice cropping in tropical lowland soil. *Eur. J. Soil Sci*. *47:* 293–303

Ouatmane, A., Dorazio, V., Hafidi, M., Revel, J.C. and Senesi, N. (2000). Elemental and spectroscopic characterization of humic acids fractionated by gel permeation chromatography. *Agronomie 20:* 491–504

Pavia, D.L., Lampman, G.M. and Kriz, G. (1996). Infrared spectroscopy. In*: Introduction to spectroscopy: A guide for students of organic chemistry* (Pavia, D.L., Lampman, G.M. and Kriz, G., ed.) p. 14–145. Washington: Saunders College Publishing

Schnitzer, M. (1978). Humic substance: chemistry and reactions. In: *Soil organic matter* (Schnitzer, M. and Khan, S.U., ed.) p. 1–64. Amsterdam: Elsevier

Schnitzer, M., Dinel, H., Mathur, S.P., Schulten, H.R. and Owen, G. (1993). Determination of compost biomaturity. *Biol. Agric. Hortic*. *10:* 109 – 23

Selby, K. and Maitland, C.C. (1967). The cellulase of *Trichoderma viride*, separation of the components involved in the solubilization of cotton. *Biochemistry J*. *104:* 716

Senesi, N. and Loffredo, E. (2001). Soil humic substances, In: *Biopolymers. Lignin, humic substances and coal* Vol 1. (Hofrichter, M. and Steinbuchel, A., ed.) p. 247–99. Germany: Wiley-VCH,Weineim

Sposito, G. (1986). Sorption of trace metals by humic materials in soils and natural waters. *CRC Critical Reviews in Environmental Control 16:* 193–229

Stevenson, F.J. (1994). *Humus chemistry. Genesis, composition, reactions.* 2nd ed. New York: John Wiley & Sons

Umi Kalsom, M.S., Emmy, A.K.R., Sashikala, M.P. and Norlea, A. (2003). Potential of *Trichoderma viride* in degradation of lignocellulose for humic acid production. *Transactions of the Malaysian Society of Plant Physiology 12:* 122 – 7

Umi Kalsom, M.S., Ariff, A.B., Shamsuddin, Z.H., Tong, C.C., Hassan, M.A. and Karim, M.I.A. (1997). Production of cellulase by a wild strain of *Chaetomium globosum* using delignified oil palm empty-fruit-bunch fibre as substrate. *Appl. Microbiol. Biotechnol 47:* 590 – 5

Wilson, M.A. (1989). Solid-state nuclear magnetic resonance spectroscopy of humic substances: Basic concepts and techniques. In: *Humic substances II: In search of structure* (Hayes, M.H.B., ed.) p. 309 – 38. New York: John Wiley & Sons

Abstrak

Asid humik (HA) yang diekstrak daripada fermentasi tandan kosong kelapa sawit (EFB) oleh kulat *Trichoderma viride* telah dicirikan melalui 3 kaedah; i) analisis unsur menggunakan mikroskop elektron yang mempunyai pengesan spektroskopi tenaga berselerak (EDS), ii) spektroskopi penukar gelombang infra merah fourier (FTIR), dan iii) kekutuban campuran magik sudut berpusing karbon 13-gema magnetik nuklear (CP/MAS ^{13}C NMR) secara pepejal. Komposisi unsur dan kumpulan berfungsi terkandung di dalam HA yang diekstrak daripada EFB menyerupai HA yang terdapat di dalam tanah gambut dan bahan humus yang tidak reput sepenuhnya. Analisis menggunakan CP/MAS ^{13}C NMR menunjukkan data yang selari dengan data yang diperoleh daripada analisis spektroskopi FTIR, dengan kedua-duanya menunjukkan kandungan HA terdiri daripada juzuk tisu tumbuhan yang separuh reput. Komponen utama HA yang dikenal pasti ialah lignin, karbohidrat dan kumpulan alifatik.

Accepted for publication on 29 August 2005