UNIVERSITY OF NAPOLI "FEDERICO II"

AGRICULTURAL FACULTY

Soil Chemistry Department

Molecular characterization of soil humic acid after recycled organic biomass addition

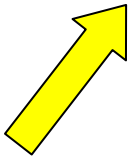
Dr. Spaccini Riccardo

OBJECTIVE

*Molecular characterization of soil **humic acids** extracted after soil treatments with recycled organic biomass*

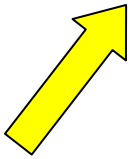
bulk characterization
Spectroscopic analyses:

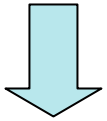
- CPMAS^{13}CNMR (CrossPolarizationMagicAngleSpinning)



molecular characterization

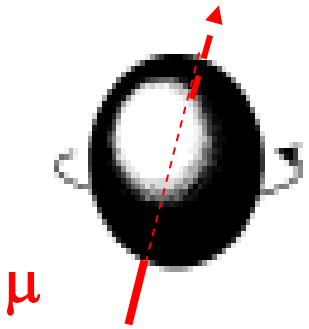
- Sequential extractions





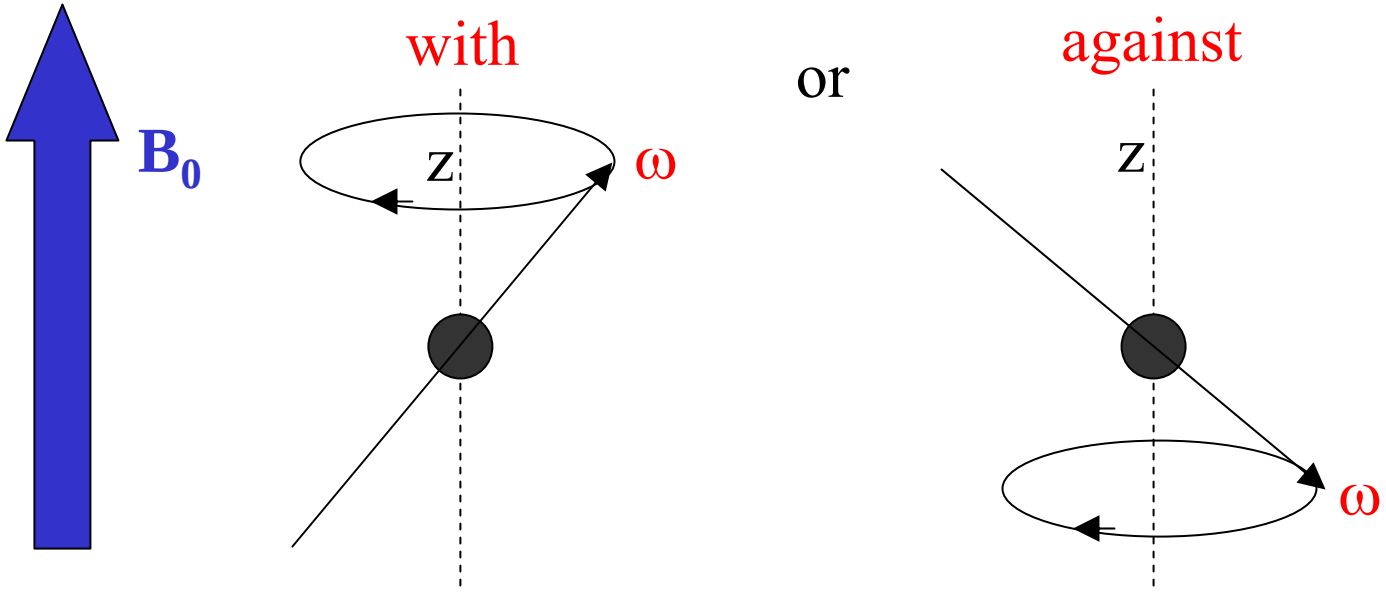
GasCromatografy MassSpectrometry

NMR spectroscopy

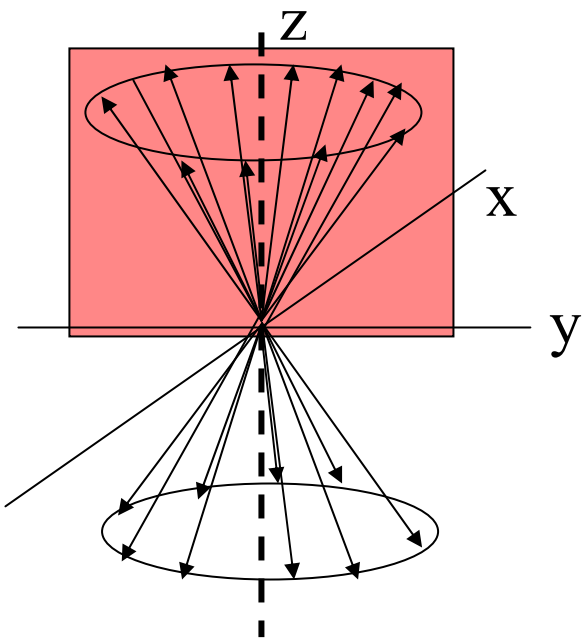


a spinning nucleus, with magnetic properties, (^{1}H, ^{13}C, ^{31}P, ^{15}N) possess a magnetic moment μ

once immersed in external strong field (B_0) the magnetic moment of nuclei with a spin quantum number I = ½ will be lined up to B_0



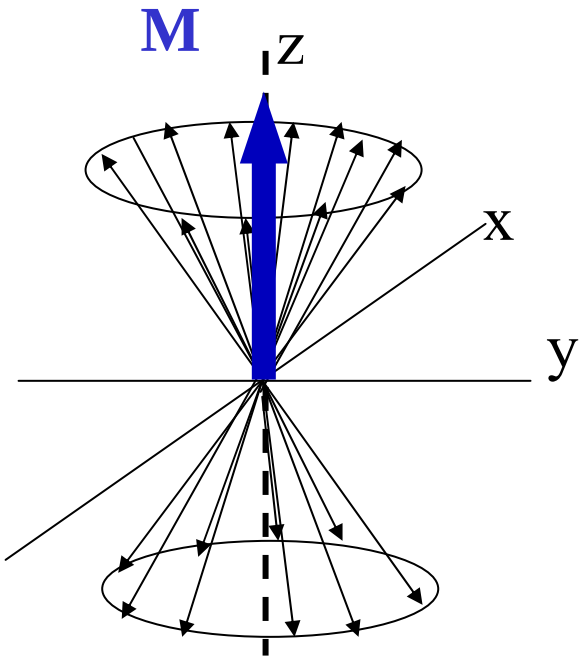
thereby assuming a circular path around the main z axis called *precession* with an angolar frequency ω (larmor frequency) tipical of each nucleus

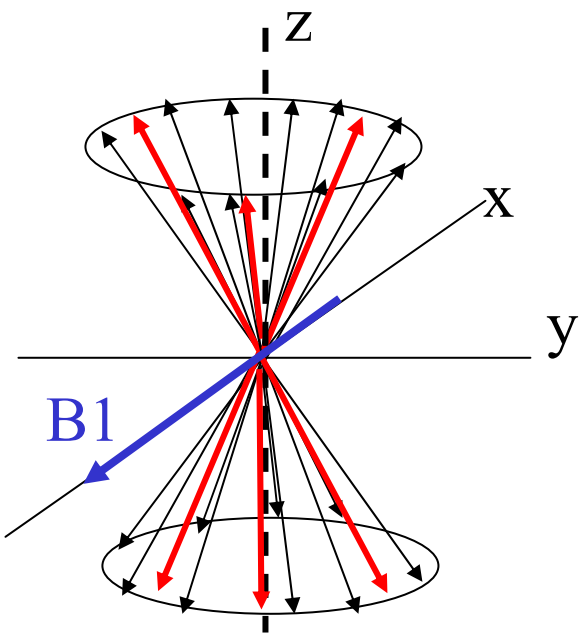


the various nuclei (e.g.^{1}H) will have a random distribution along z axis;

following the Boltzman distribution the nuclei aligned with B0 have lower energy and are in slight excess

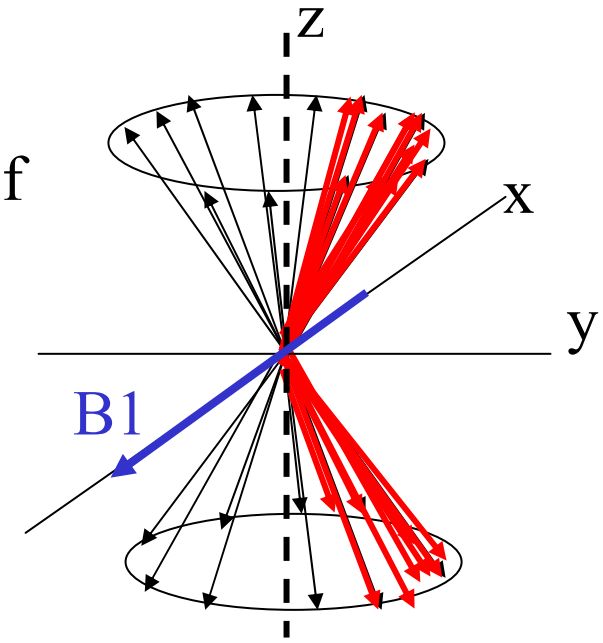
operating a vectorial sum, the trasversal (X and Y) components will cancel out, leaving a resultant vector **M** representing the total magnetic moment of those nuclei

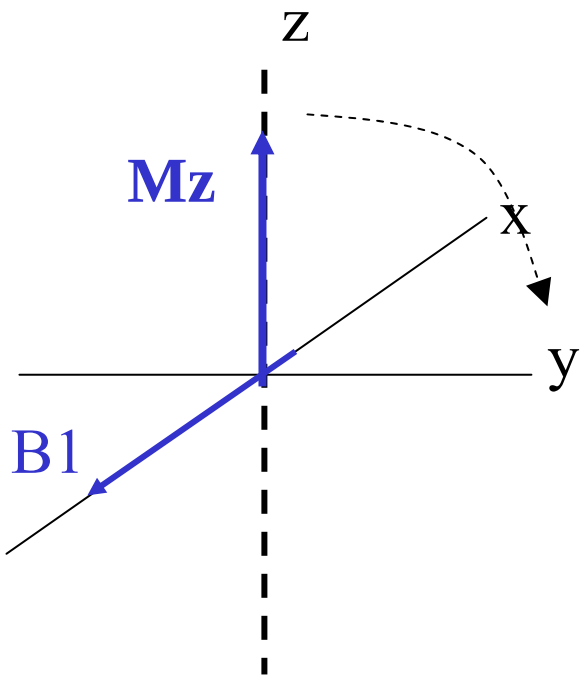




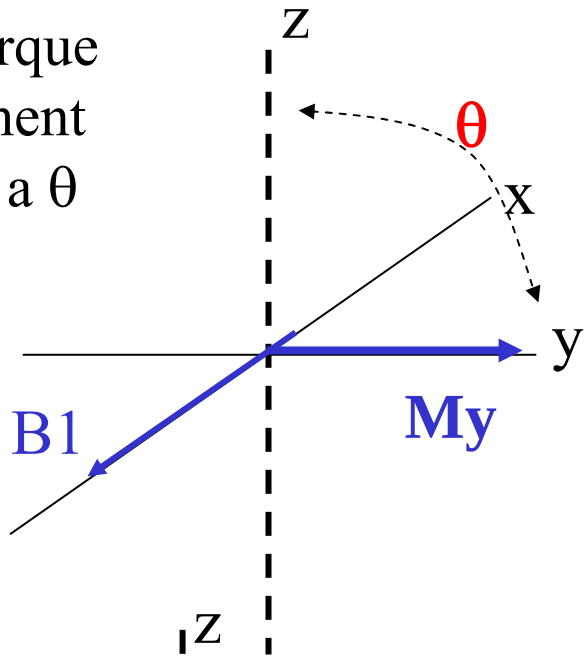
the application of a radiofrequency B1 on the X axis with a frequency = to the Larmor (ω) frequency of the nuclei (resonance) will equalize the nuclei distribution along Z+ and Z- directions……..

….changing at the same time the phase of of sigular spinning nucleus that will be grasped towards the Y- direction (phase coherence)



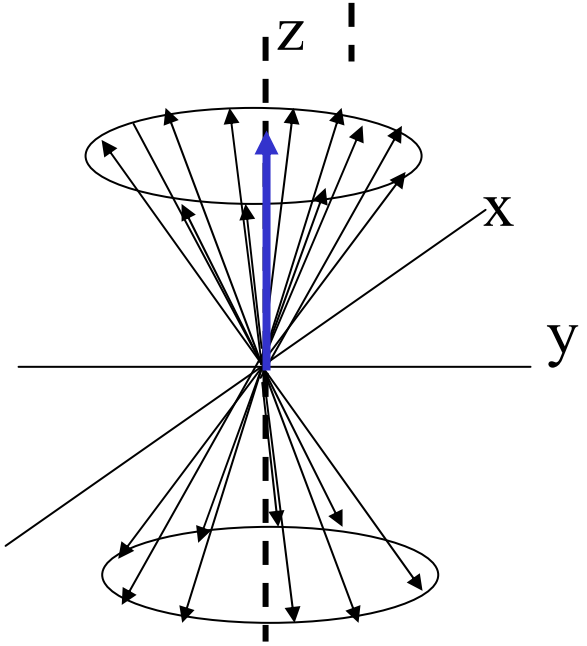


the result will be a torque of the magnetic moment **M** from Z to Y with a θ angle



ending the B1 application will result in a relaxation process

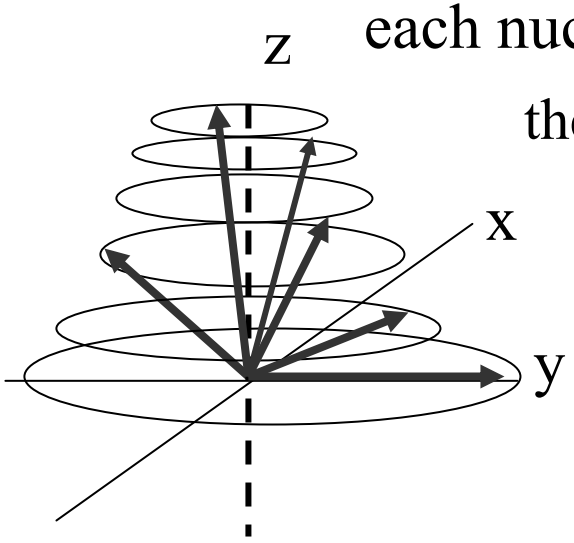
the nuclei return to the equilibrium restoring the initial distribution



this relaxation is detected and produce the NMR signal

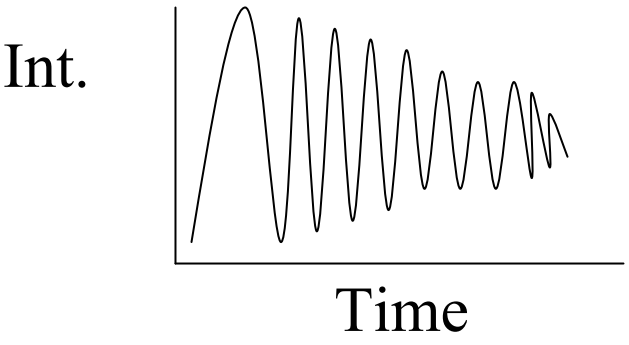
the relaxation is characterized by energy emission and a loss of phase coherence

both processes are detected and produce the NMR signal



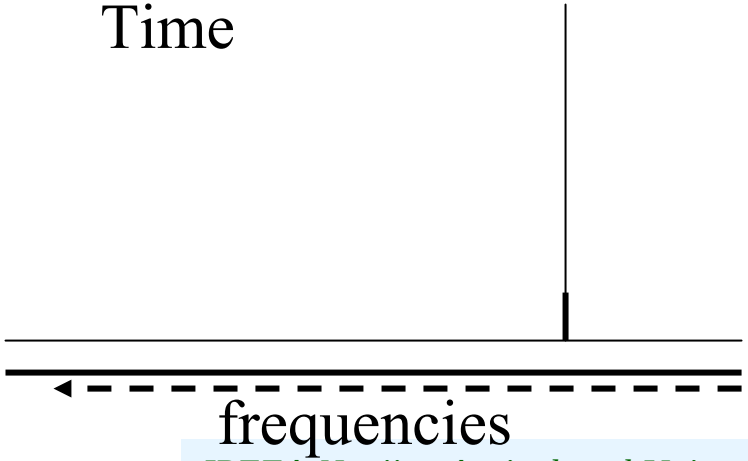
each nucleus will relax with a circular path

thereby producing a sinuoisodal FID (freeinductiondecay)



this signal is characterized by Intensity *versus* Time domain

the mathematical Fourier Transformation will convert the FID in a classical NMR signal Intensity versus Frequency domain



the intensity of radiofrequency B1 (MHz) is related to the intensity of B0 field (Tesla)

B0=9.4 Tesla ⇨ B1= 400 MHz B0=14.1 Tesla ⇨ B1= 600 MHz

this affects the amplitude of resonance frequencies that will change in different instruments

B1=60Mhz ⇨ signal= 90Hz B1=300Mhz ⇨ signal= 450Hz

in order to compare the response of different instruments the frequency resonances are divided for the applied B1 field

Hz/MHz

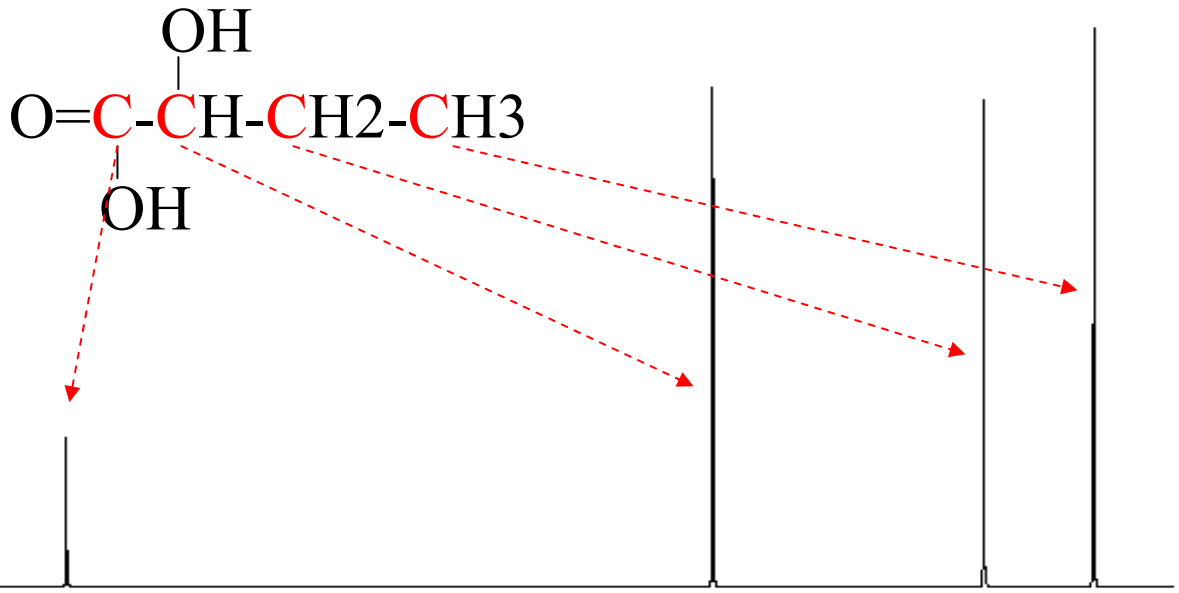
thereby expressing the resonances of the nuclei in Ppm (part per million)

$90Hz/60MHz \times 10^6$ ⇨ 1.5 ppm $450Hz/300MHz \times 10^6$ ⇨ 1.5 ppm

•the electronic environment of each atom (for instance chemical bond) will affects its magnetic properties (***shielding effect***) thereby modifying the frequency resonance of different atoms (***chemical shift***)

•this property allows to distinguish the different nuclei in the organic molecule (aromatic, phenolic, aliphatic etc.)

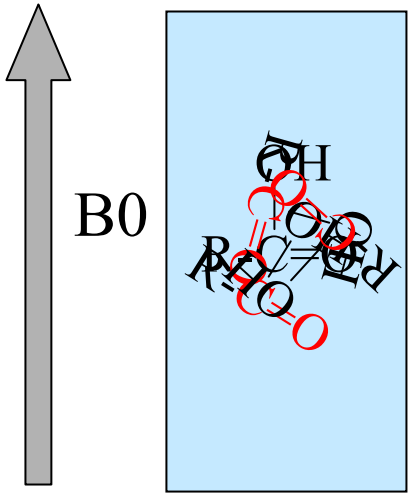
FID of 2-hydroxy-butyric acid



The NMR in **solid state** is characterized by the following drawbacks:

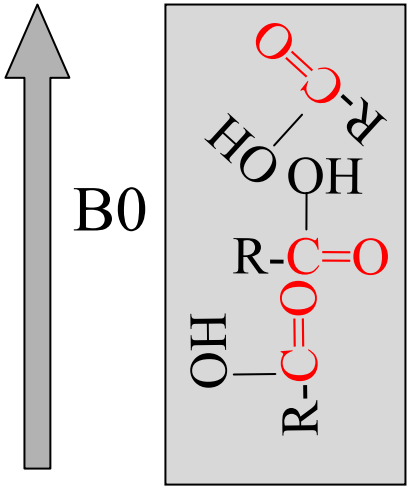
Chemical Shift Anysotropy (CSA):

depends on the orientation of the chemical bond respect to external magnetic field B0 that will increase the range of chemical shift effect



in liquid state the molecules are free to move (tumbling)
each chemical bond will assume each possible orientation respect to B0 thereby averaging to zero the CSA effect

Signal Amplitude < 1Hz



in solid state the chemical bonds have fixed orientation respect to B0
the electronic environments will produce a large CSA effect

Signal Amplitude > 100 Hz

The NMR in **solid state** is characterized by the following drawbacks:

Dipolar coupling between nuclei

depends on the interaction between the dipolar moment of vicinal nuclei through the intra- and inter-molecular space

in liquid state the molecular tumbling average these interactions and cancel out the total dipolar coupling

Signal amplitude < 1Hz

in solid state the strong dipolar coupling between H nuclei do not allow the 1H-NMR experiment

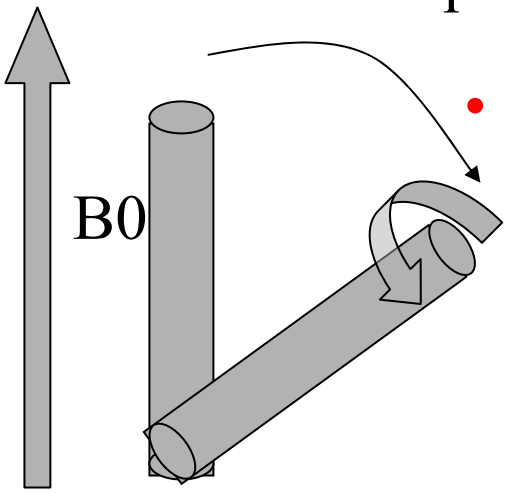
⇩

Solid state ^{13}C NMR experiment

in solid state the dipolar coupling between H and C decrease the resolution and increase the signal amplitude over 100 Hz

The solid state drawbacks are removed or reduced :

- application of high power dipolar decoupling technique
  remove the dipolar coupling between H and C nuclei
- the sample is positioned at 54° respect to Bo (Magic Angle)



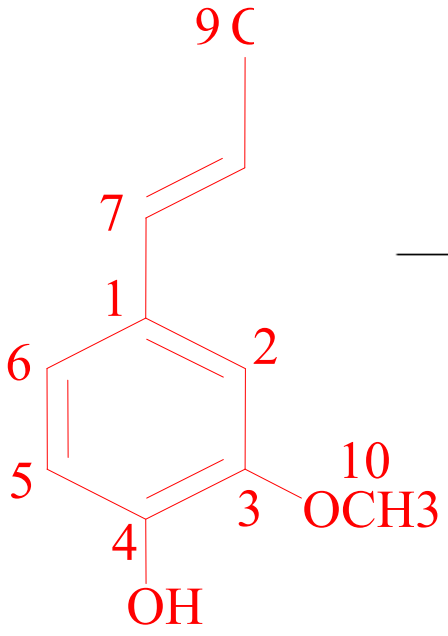
- spinning the sample at higher angular velocity
  5KHz < ÷ >15KHz

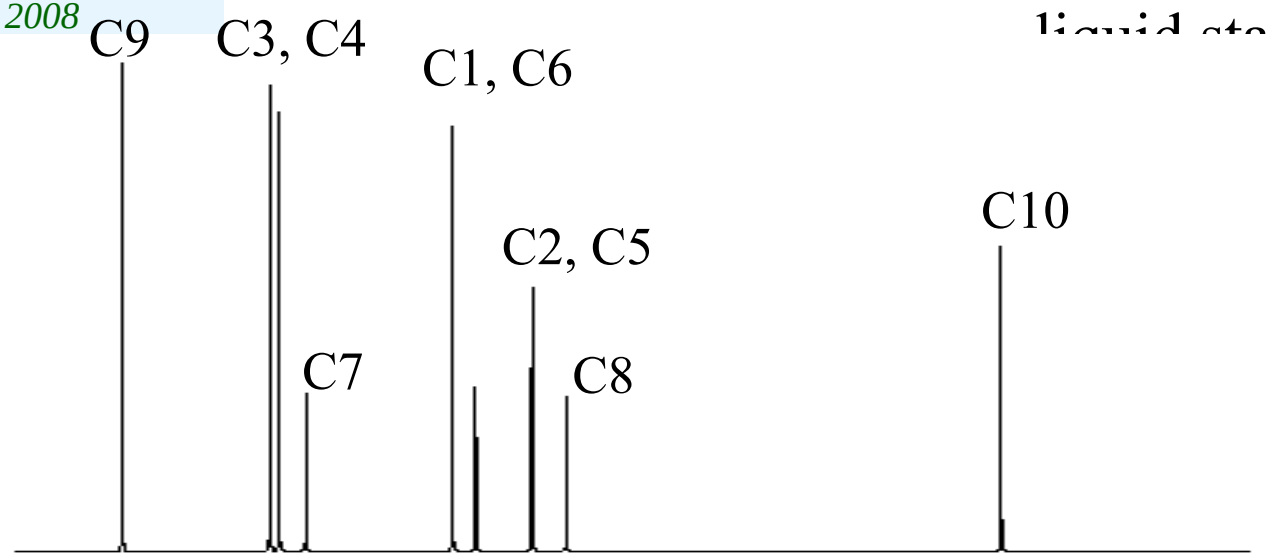
with MagicAngle Spinning (MAS)

solid state resemble to liquid state

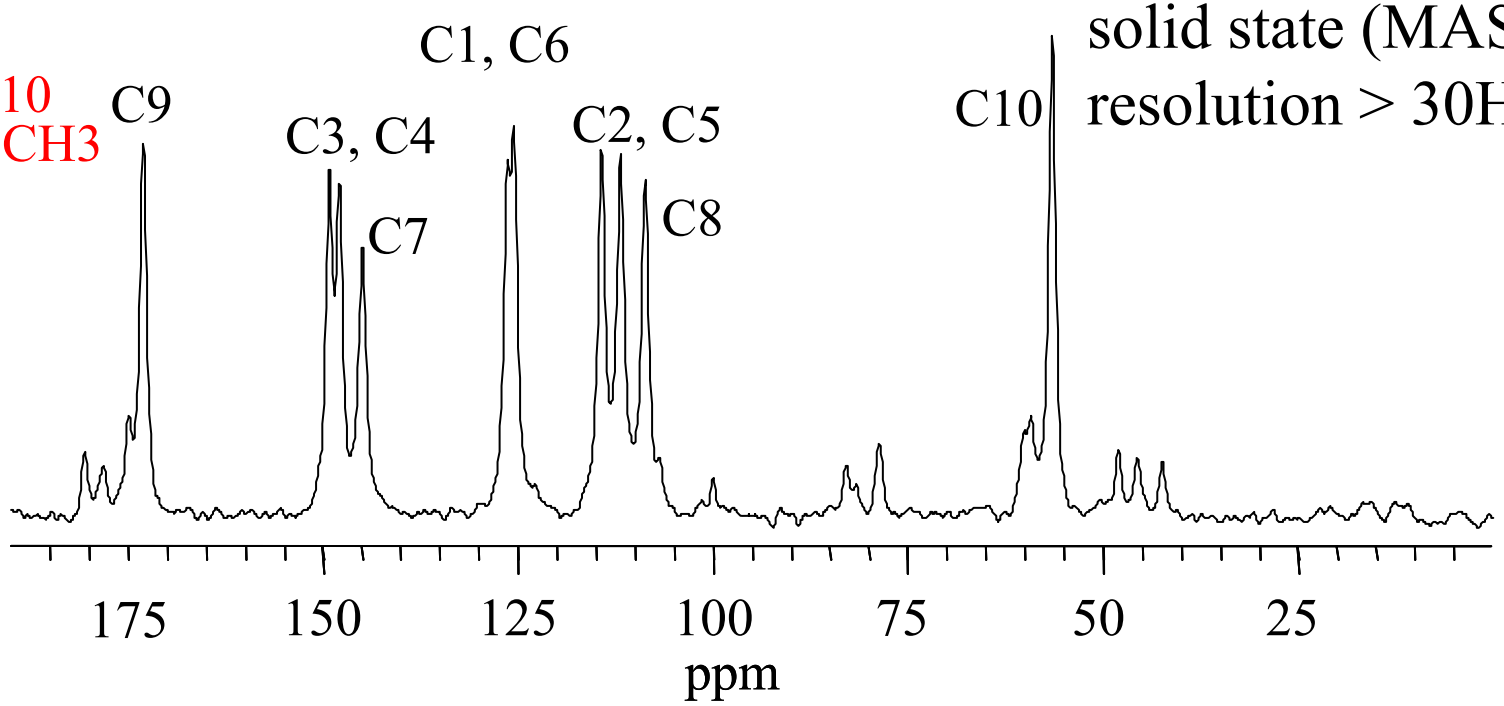
MAS reduce the CSA effect

Ferulic





solid state (MAS)
resolution > 30Hz



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bulk characterization

Spectroscopic analyses:

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  molecular characterization

- Sequential extractions

GasCromatografy MassSpectrometry

The recycled organic biomass (compost) was composed by the following organic residues

45% urban solid waste 40% plant residues (tobacco) 15%plant trimming

the recycled organic biomass was produced through a composting process made of a common oxidation period (*active phase)* of 30 days, followed a stabilization period (*curing phase)* of of 120 days

30 t ha^{-1} of recycled organic biomass were added to soil for 4 years

soil humic acids were then extracted from soil at the following intervals

4^{th} year (HA 0 year) 5^{th} year (HA 1 year) 6^{th} (HA 2 year)

Humic acid extraction

200 g soil

Soxhlet extraction with ethyl ether

1M NaOH + 0.1M $Na_4P_2O_7$ shaken overnight (1 liter)

centrifugation 7000 rpm

supernatant

pellets

filtration quartz filter 0.45 μ

washing with deionized water till pH7

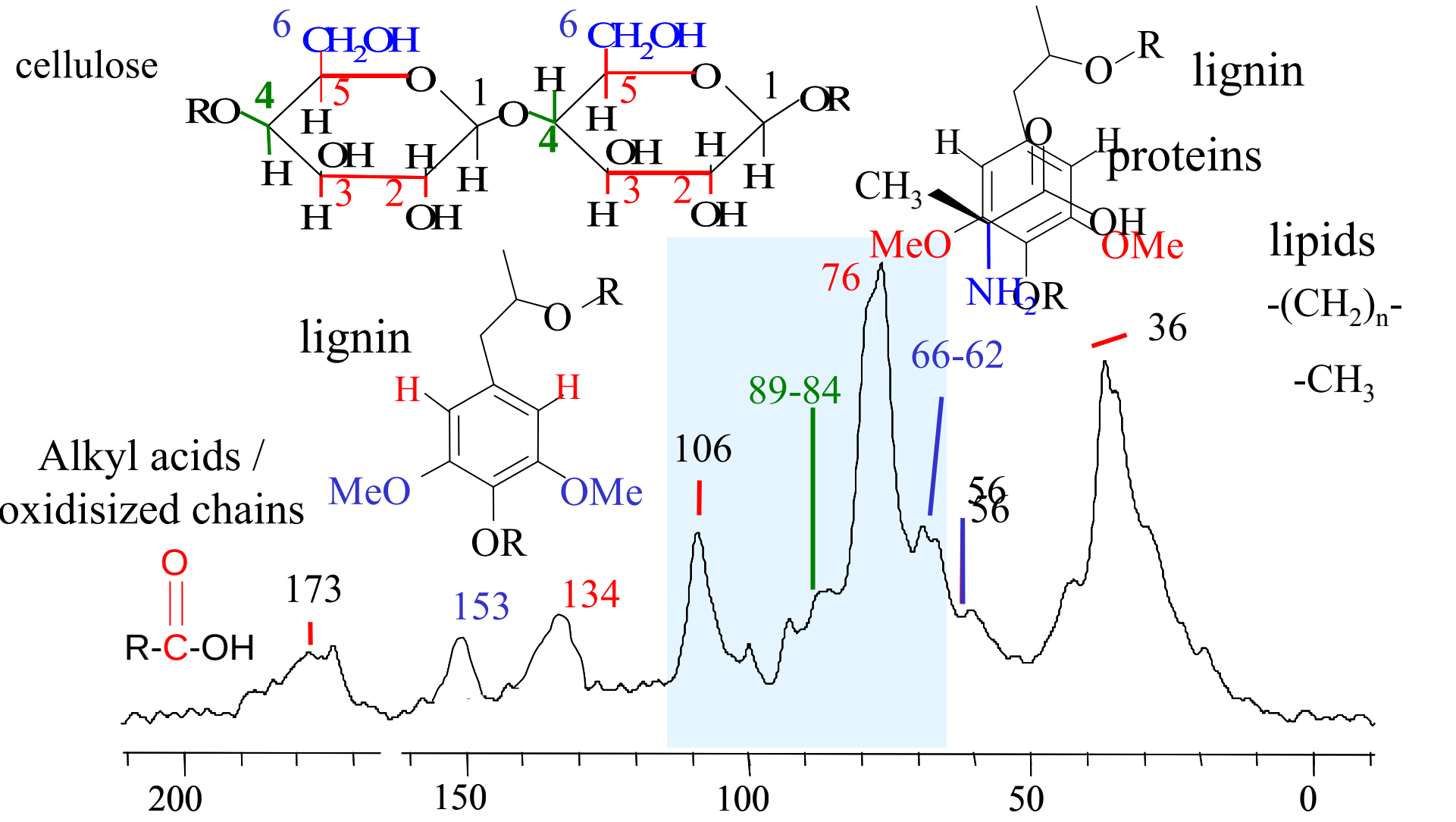
pH 1 with HCl

humic acids

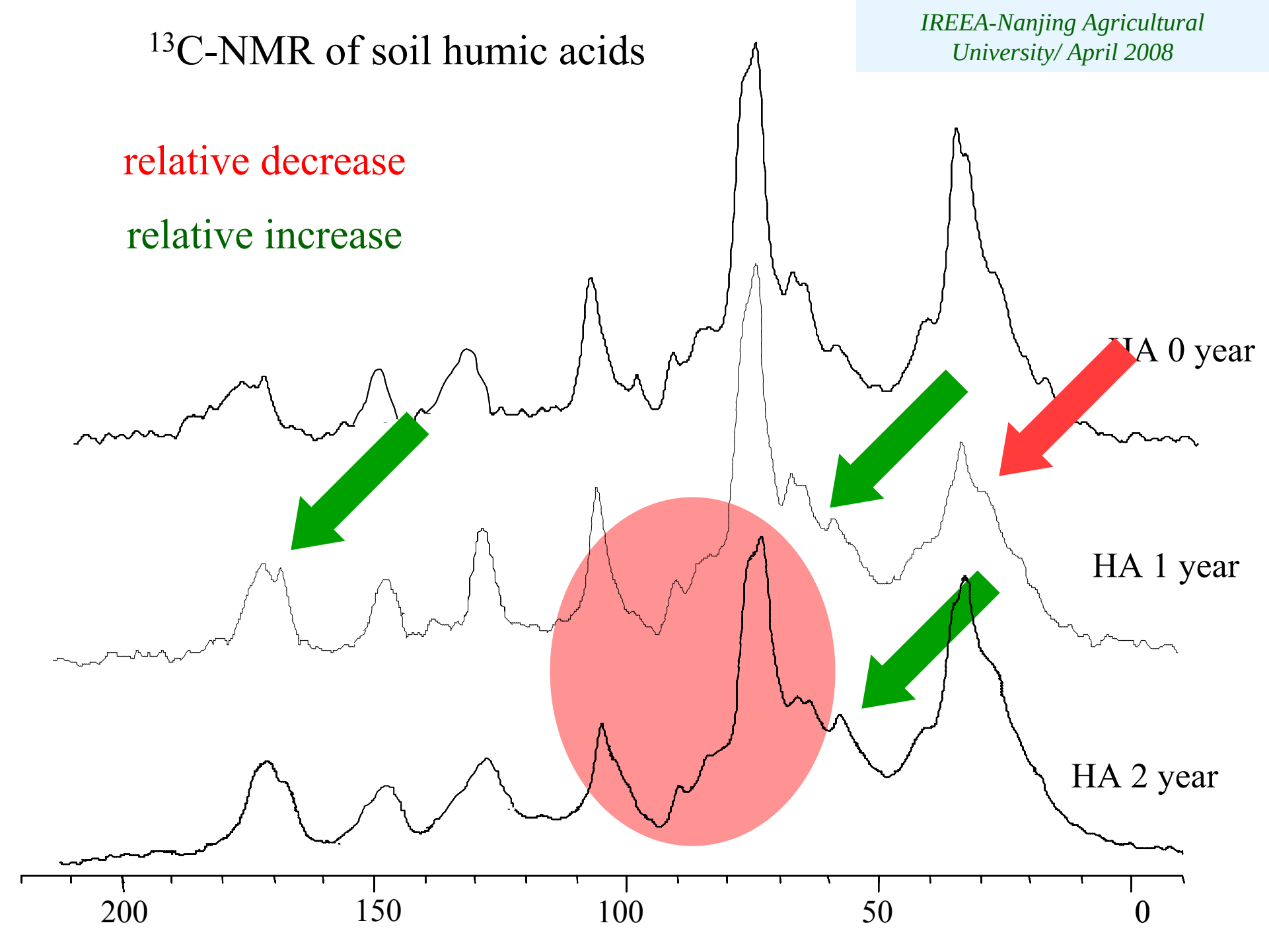
purification with HF/HCl solution

dialysis

Solid state ^{13}C-NMR CPMAS HA 0 year



^{13}C-NMR of soil humic acids



Relative areas (%) of NMR spectra of humic acids

	200-160 (oxidis.)	160-110 (lignin)	110-60 (carbohyd)	60-45 (lignin/prot)	45-0 (lipids)	HB
HA 0year	3.5	10.2	46.7	5.7	33.8	0.89
HA 1year	4.6	9.8	47.9	9.0	28.8	0.77
HA 2year	5.1	13.8	38.3	9.1	33.7	1.06

$$\text{Hydrobhobic index} = \frac{[(0\text{-}45)+(45\text{-}60)+(110\text{-}160)]}{[\,(45\text{-}60)+(60\text{-}100)+\,(160\text{-}200)]}$$

- the initial composition of soil humic acids was characterized by a predominance of alkyl lipid compounds and polysaccharides (cellulose and hemicellulose) with a significative presence of aromatic compounds (lignin)

- after one year the NMR data suggested a relative decrease of aliphatic contents, maybe related to more bioavailable wax components; no significant variation were shown by carbohydrate region whereas a relative increase were shown by both the methoxyl groups of lignin component and the carboxyl/carbonyl signal.

- the NMR spectra of soil humic acids after 2 year showed a significant a decrease in carbohydrates content thereby revealing a predominance of hydrophobic material represented by alkyl lipid components and lignin aromatic compounds

sequential extraction

Initial HA → CP-MAS ^{13}C NMR

↓

CH_2Cl_2 /MeOH 2:1 v/v × 2
(unbound components) → SPE (NH2 aminopropyl)

- $CH_2Cl_2/CH_3CHOHCH_3$(isoP) 50:50 **Neutral fraction** → GC-MS
- isoP/Acetic acid 98:2 **polar fraction**

↓

HA residual → Acid trans-esterification BF_3-MeOH → SPE (NH2 aminopropyl) → GC-MS

↓

HA residual → Alkaline solvolysis KOH/MeOH → SPE (NH2 aminopropyl) → GC-MS

↓

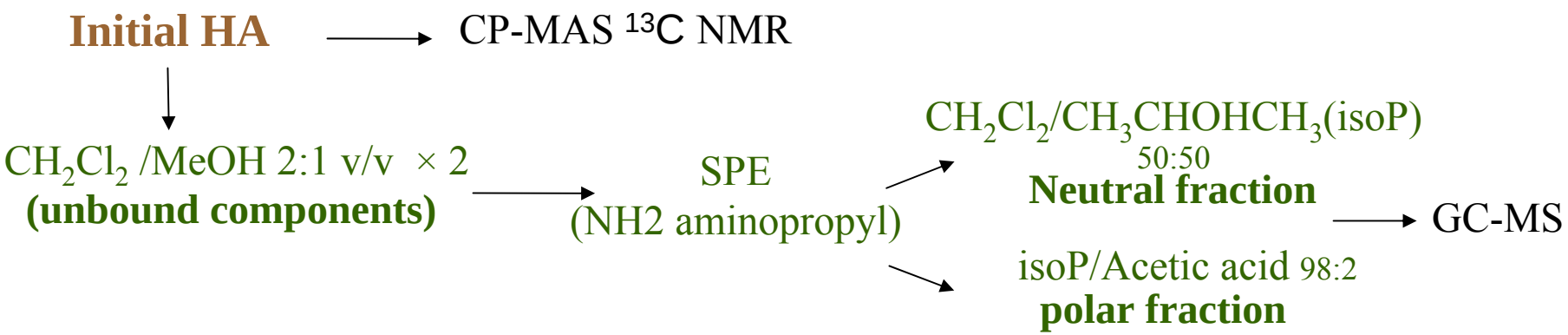
HA residual → Acid hydrolysis of hemicellulose (not cellulosic carbohydrates) (H_2SO_4 2.5 M reflux 2h) → GC-MS

↓

Final HA residue → CP-MAS ^{13}C NMR

bound components

sequential extraction



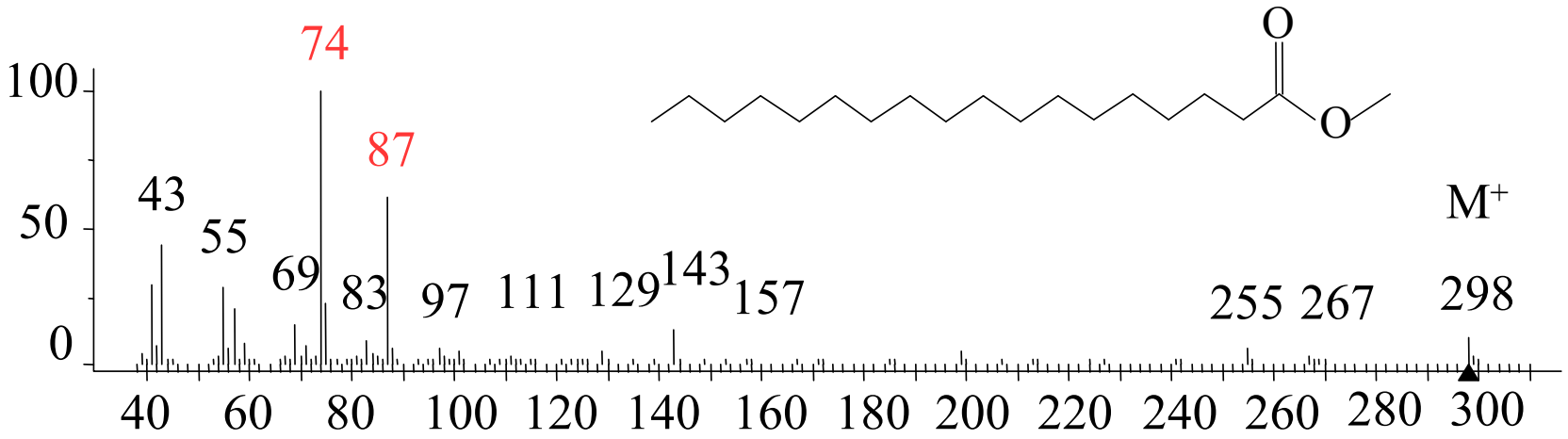
Yield (μg g^{-1} dry weight) and composition of main classes of unbound components extracted from soil humic acids

	HA 60
Fatty acids	24225; $C_{12} \div C_{28}$
unsaturated (%)	51.9
long chain (%)	**5.2**
branched fatty acids	472; $_{15} \div C_{19}$

plant waxes

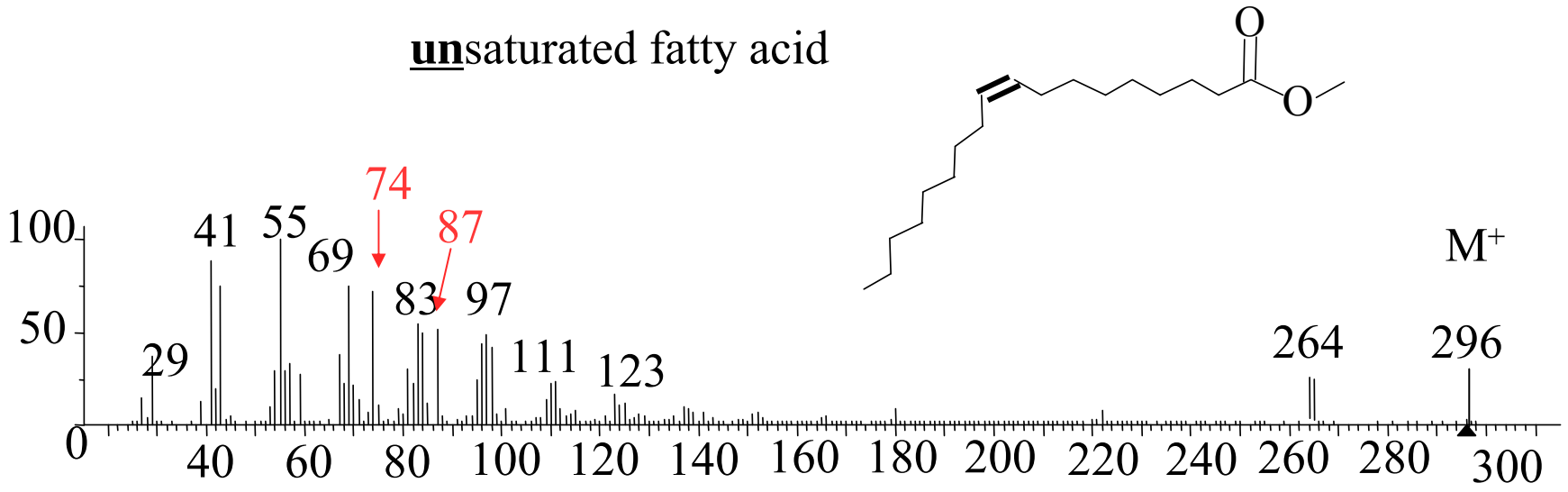
microbial markers

saturated fatty acid



Octadecanoic acid (Stearic acid), methyl ester

unsaturated fatty acid

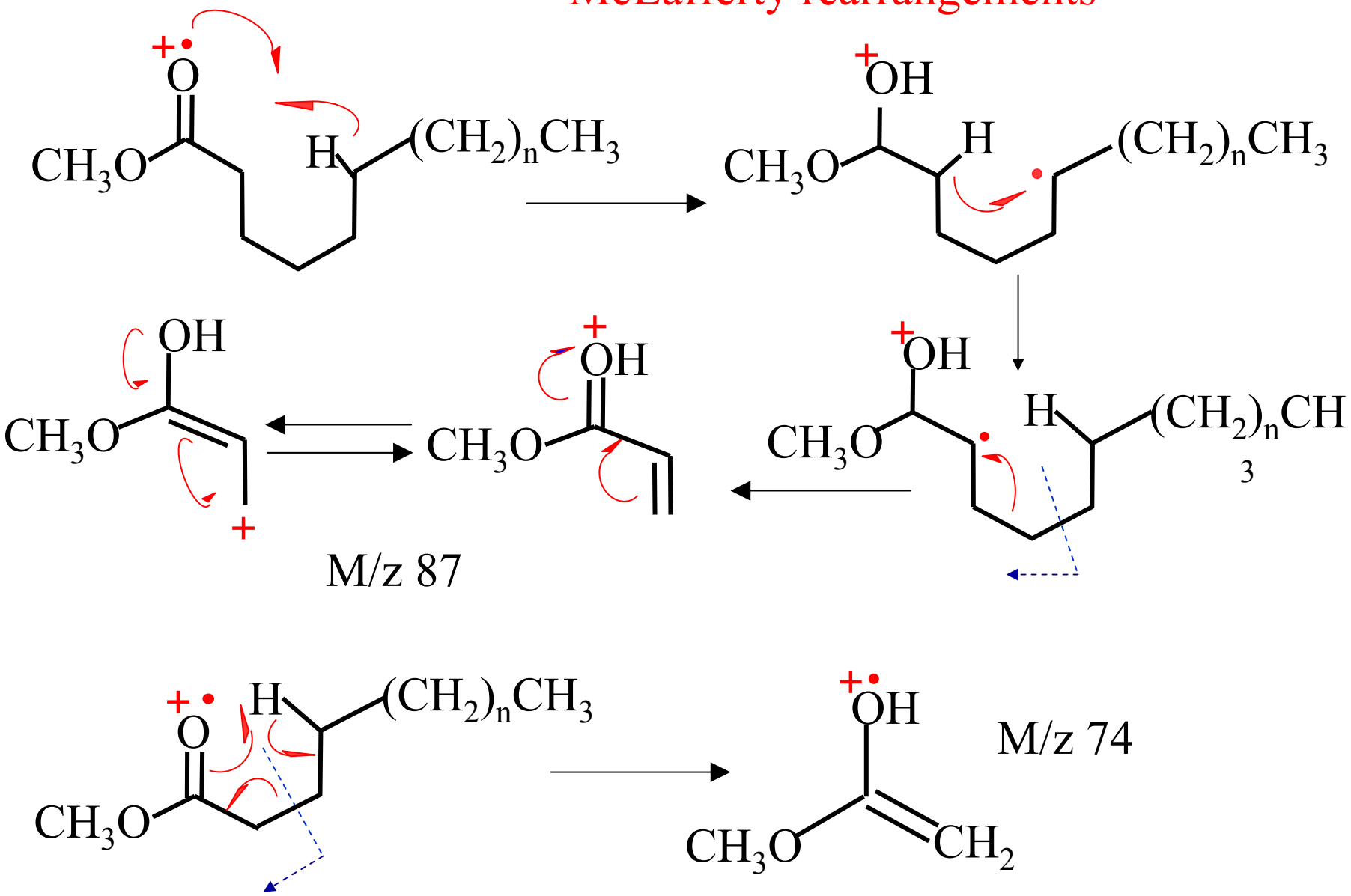


9-Octadecenoic acid (Z) (oleic acid)-, methyl ester

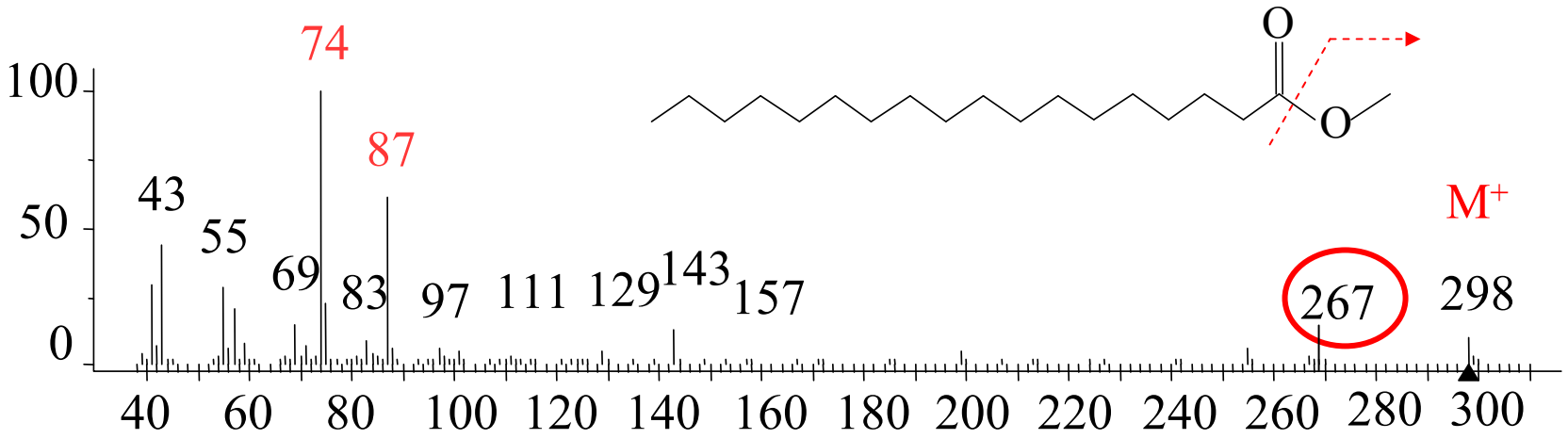
IREEA-Nanjing Agricultural University/ April 2008

Fragmentation of fatty acid methyl esters

McLafferty rearrangements

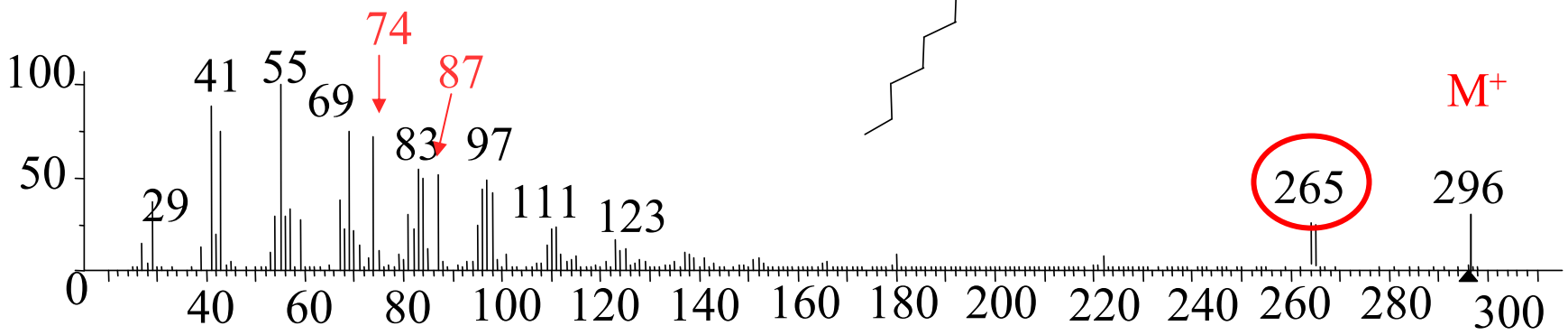


C18 saturated fatty acid



Octadecanoic acid (Stearic acid), methyl ester

C18 **un**saturated fatty acid



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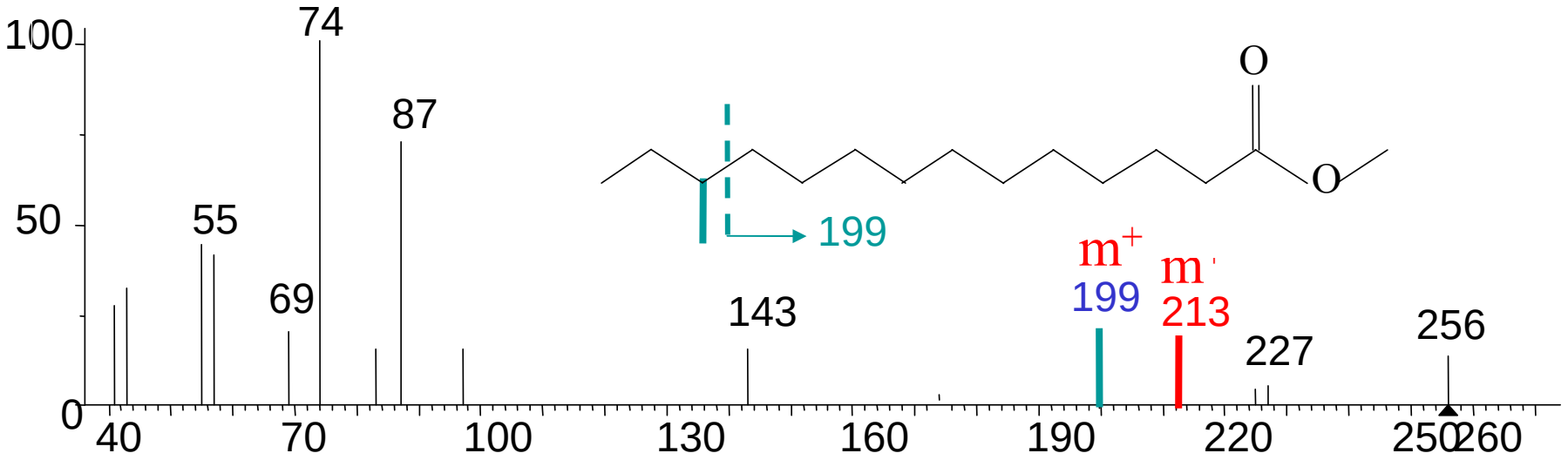
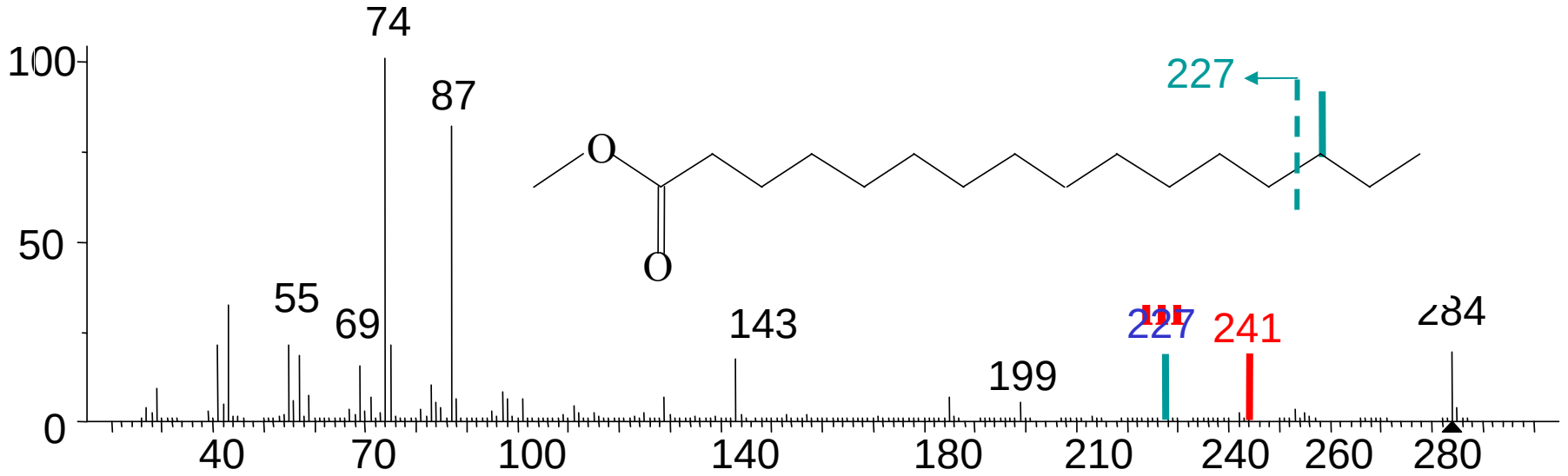


13-methyl-(12-methyl), Tetradecanoic Acid (methyl ester),

Common name ***iso (anteiso)*** Pentadecanoic Acid



15-methyl-(16-methyl), Hexadecanoic Acid, (methyl ester),

Common name ***iso (anteiso)*** Eptadecanoic Acid

IREEA-Nanjing Agricultural University/ April 2008

Yield (μg g^{-1} dry weight) and composition of main classes of unbound components extracted from soil humic acids

	HA 0 year
Fatty acids	24225; $C_{12} \div C_{28}$
unsaturated (%)	51.9
long chain (%)	5.2
Branched fatty acids	472; $C_{15} \div C_{19}$
alkanes	7540; $C_{25} \div C_{33}$ **CPI** = 3.8; **ACL** = 29.8
alcohols	2370; $C_{22} \div C_{28}$
sterol	1810
resin acids	1530

CPI CarbonPreferenceIndex =

$$\frac{(\Sigma \text{ odd } C25 \div C31 + \Sigma \text{ odd } C27 \div C33)}{2 \times (\Sigma \text{even } C24 \div C32)}$$

>1 prevalence of chain with odd C atoms

<1 prevalence of chain with even C toms

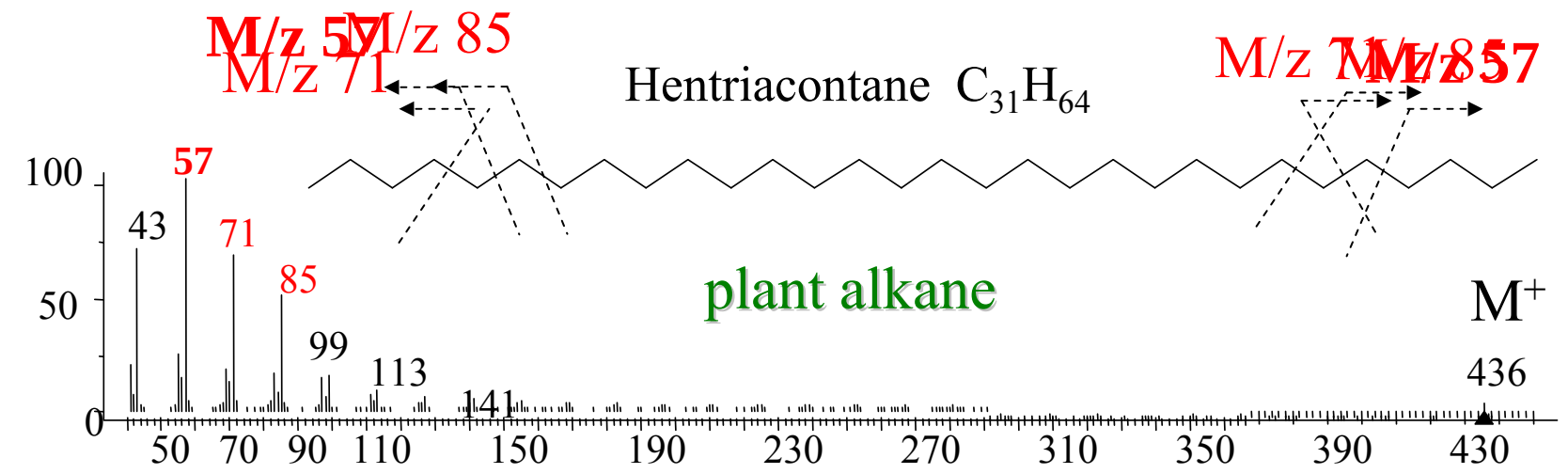
ACL Average Chain Lenght =

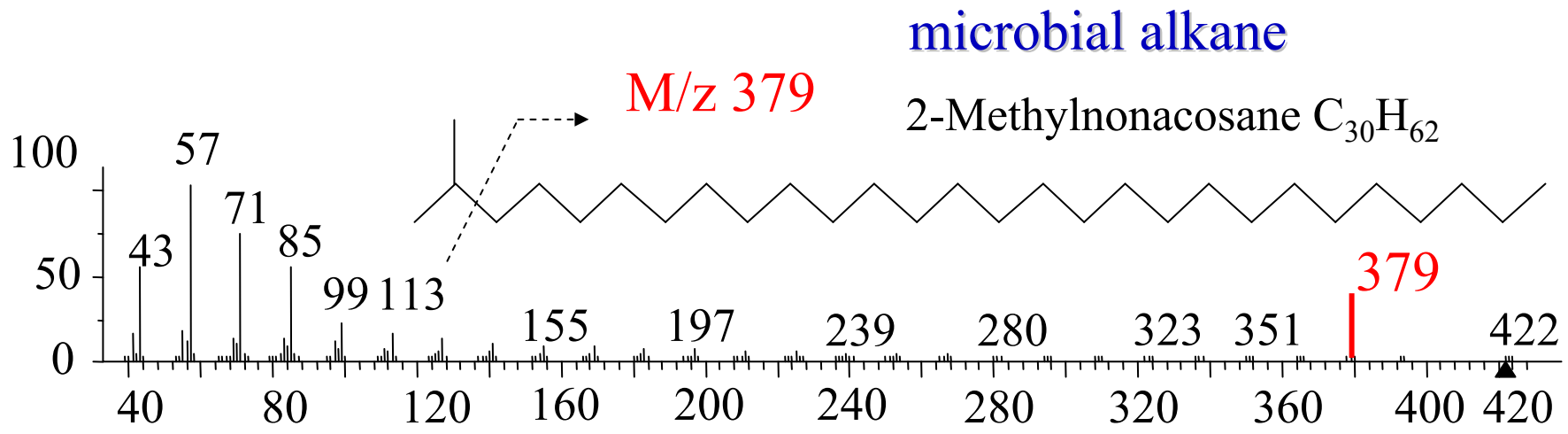
$\Sigma([Ci] \times i) / \Sigma[Ci]$

[Ci]=concentration of *n*-alkane containing i carbon atoms

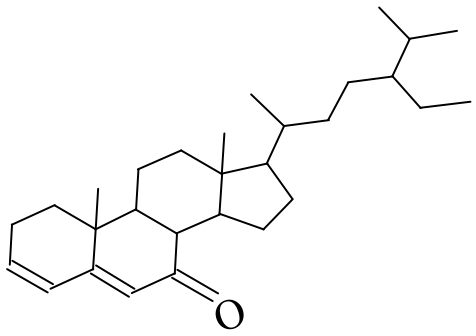
high values indicate the prevalent plant origin of alkanes

plant markers

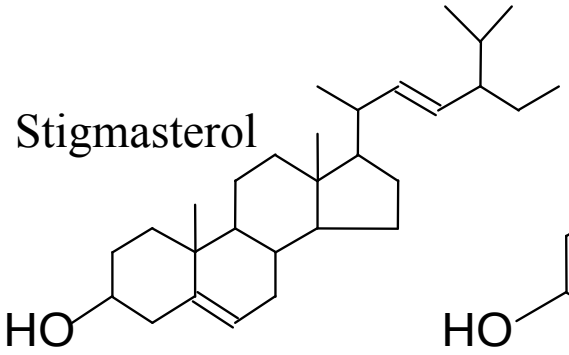

M/z 57
M/z 85
M/z 71
Hentriacontane C31H64
M/z 71
M/z 85
M/z 57
100
50
0
57
43
71
85
99
113
141
plant alkane
M+
436
50 70 90 110 150 190 230 270 310 350 390 430

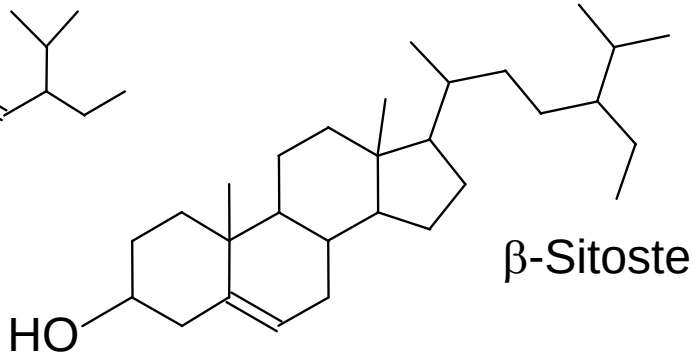

microbial alkane
M/z 379
2-Methylnonacosane C30H62
100
50
0
57
71
85
43
99
113
155
197
239
280
323
351
379
422
40 80 120 160 200 240 280 320 360 400 420

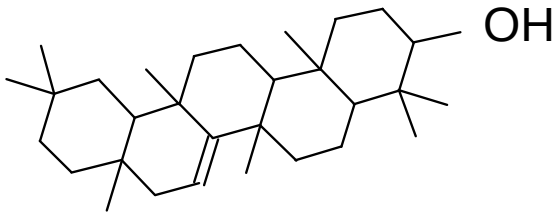
main sterol compounds identified in the unbound components of humic acids



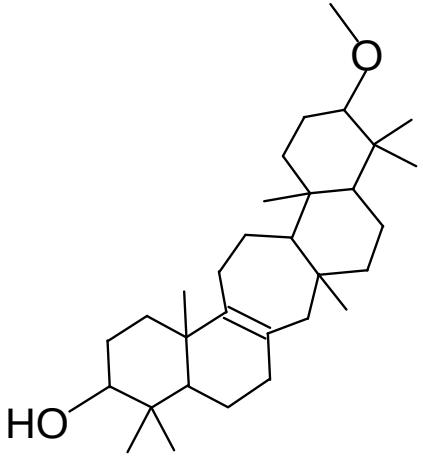
Stigmasta-3,5-dien-7-one



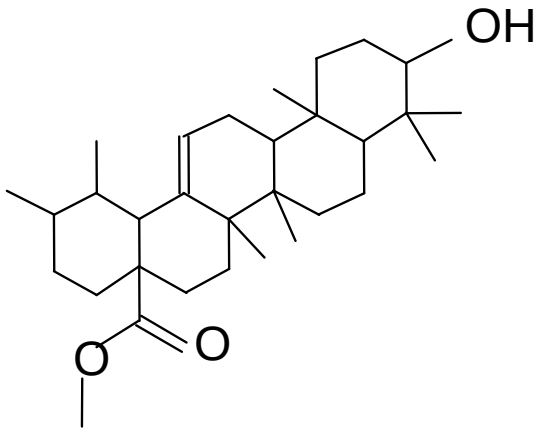




D-Friedoolean-14-ene, 3-ol-,

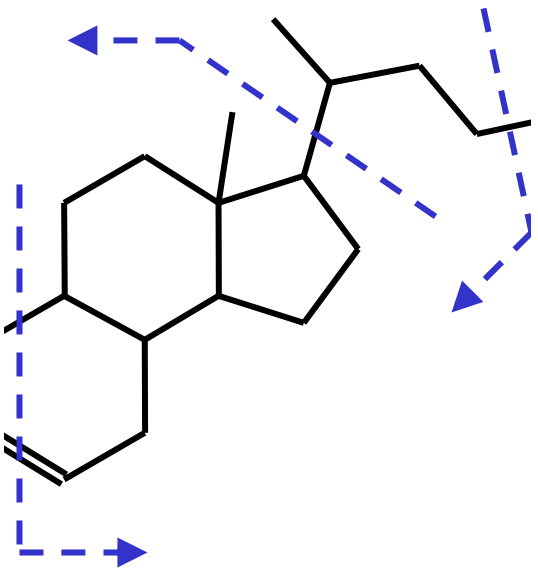


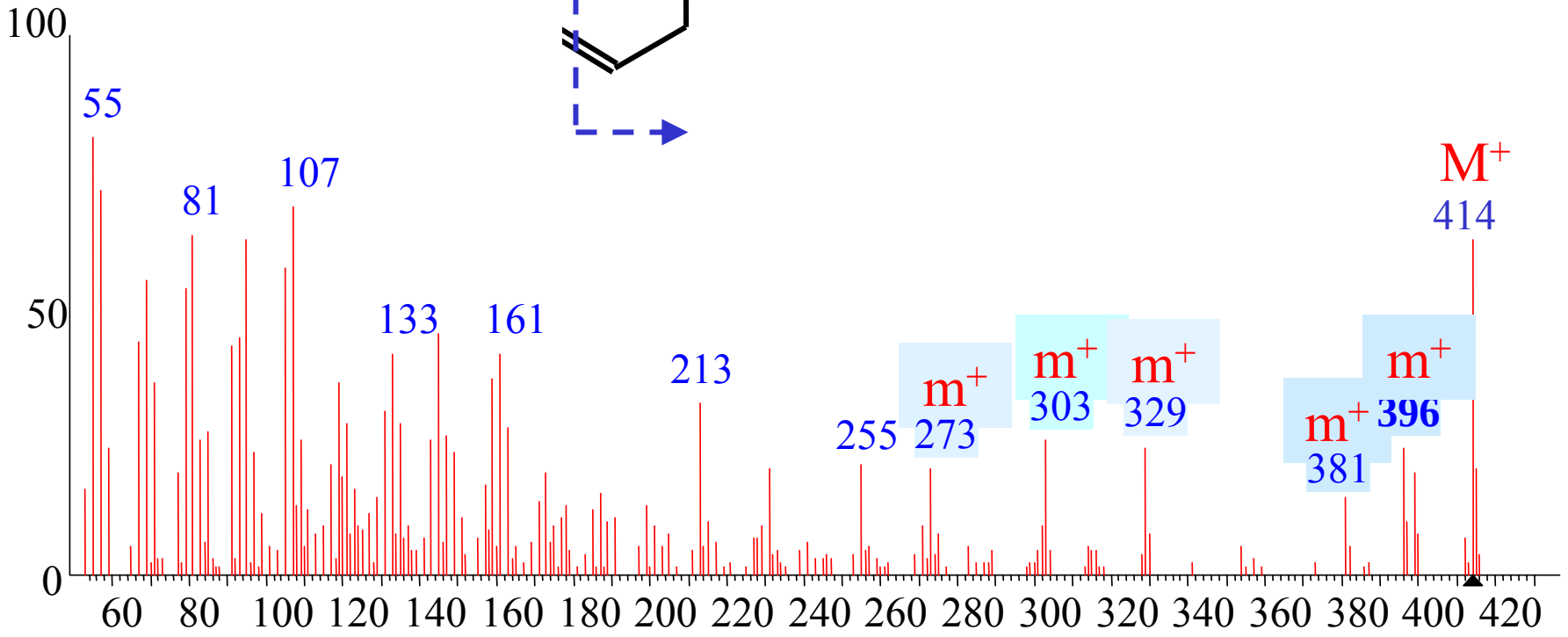
Homo-27-norgammacer-13-en-21-ol, 3-methoxy



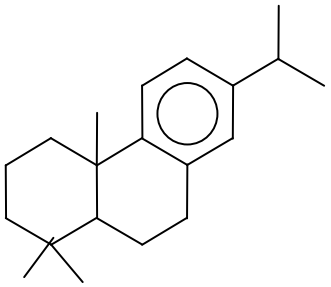
Urs-12-en-28-oic acid, 3-hydroxy-,

sitosterol

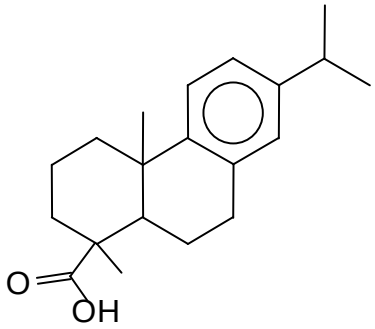



100
50
0
55
81
107
133
161
213
255
m+ 273
m+ 303
m+ 329
m+ 381
m+ 396
M+ 414
60 80 100 120 140 160 180 200 220 240 260 280 300 320 340 360 380 400 420

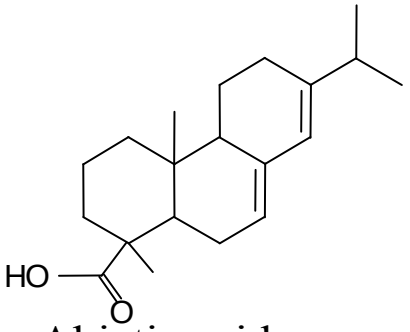
Main resin acids derivatives identified among the unbound components of humic acids



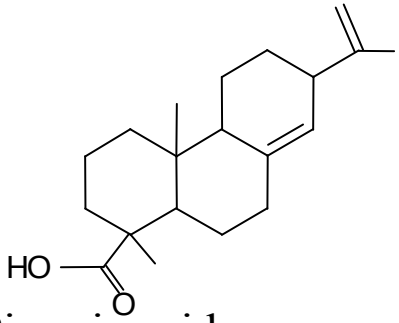
Dehydroabietane



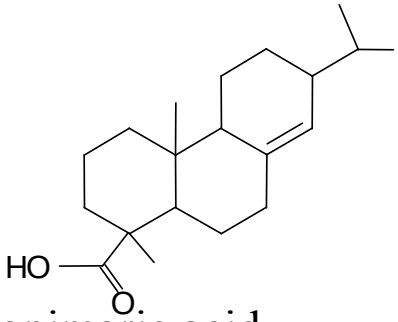
Dehydroabietic acid



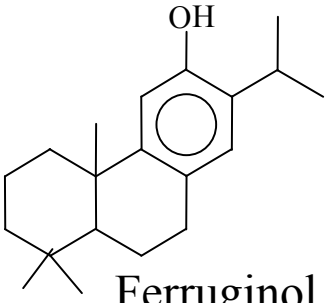
Abietic acid



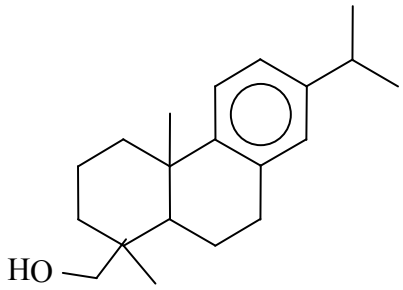
Pimaric acid



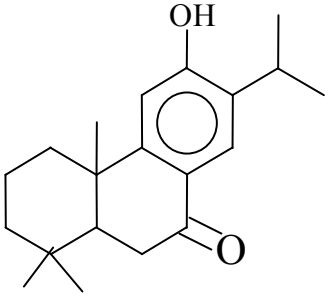
Isopimaric acid



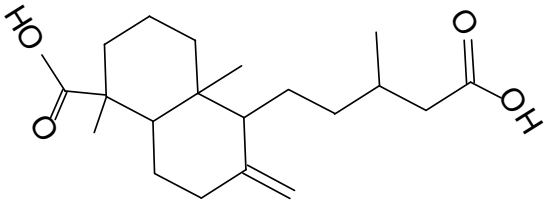
Ferruginol



Dehydroabietol

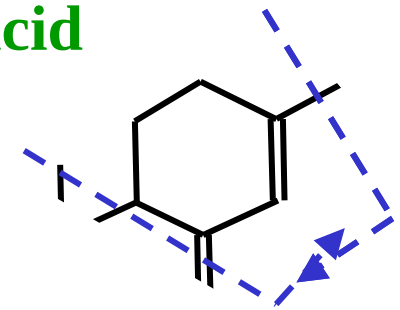


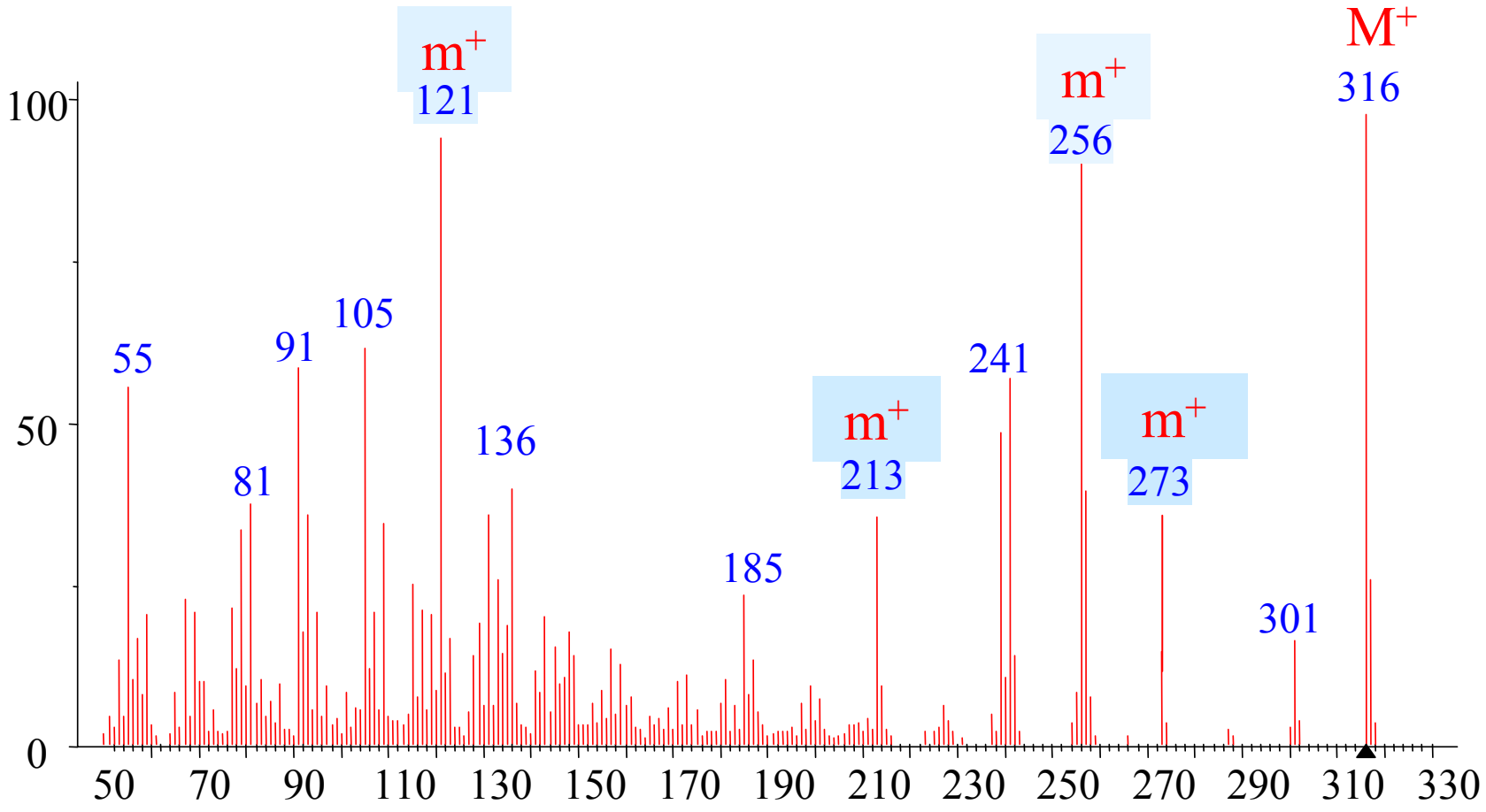
Sugiol



1-Naphthalenepentanoic acid, decahydro-5-(methoxycarbonyl)-.beta.,5,8a-trimethyl-2-methylene-, methyl ester

Abietic acid



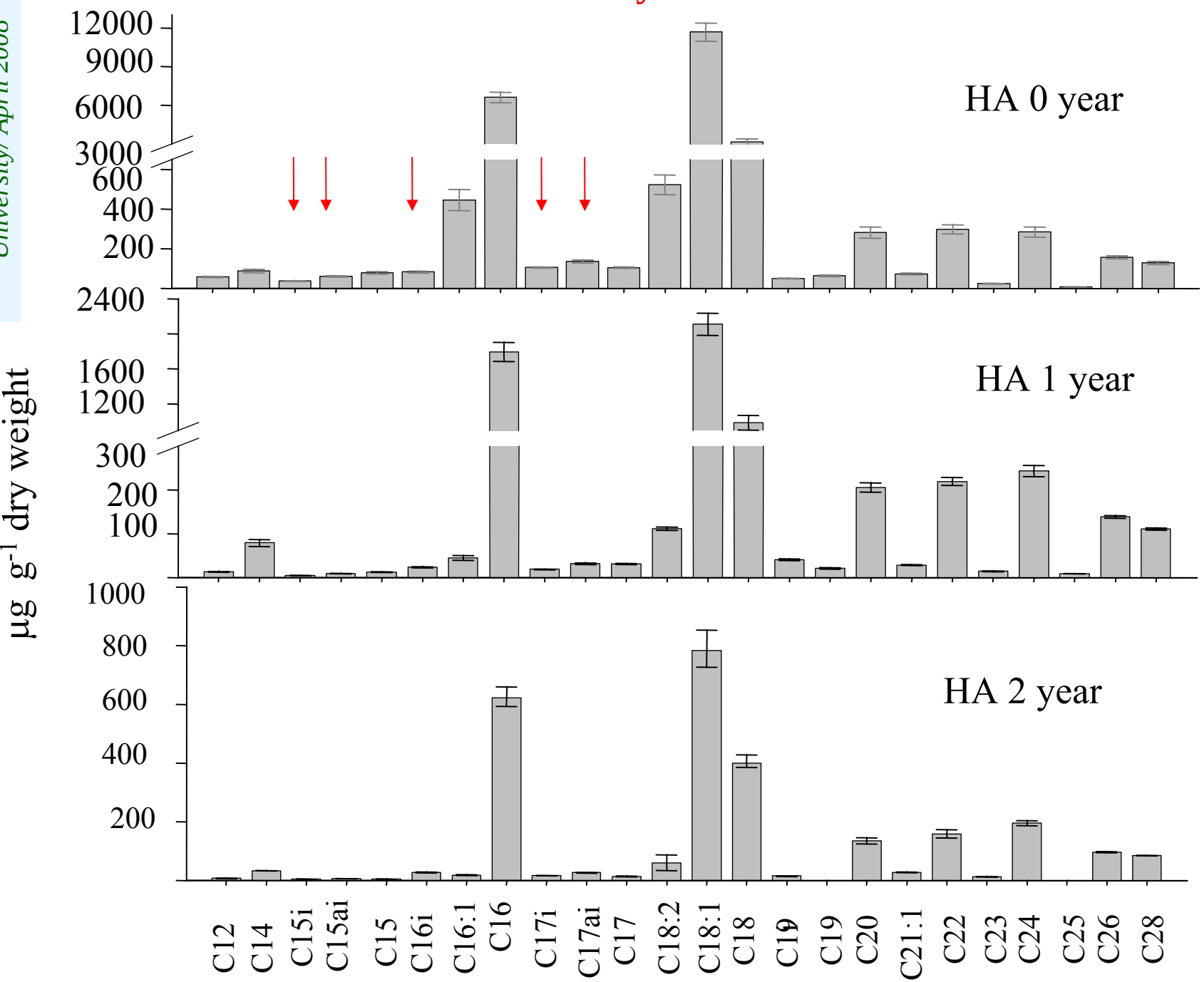


plant Biomarkers identified in unbound components of soil humic acids

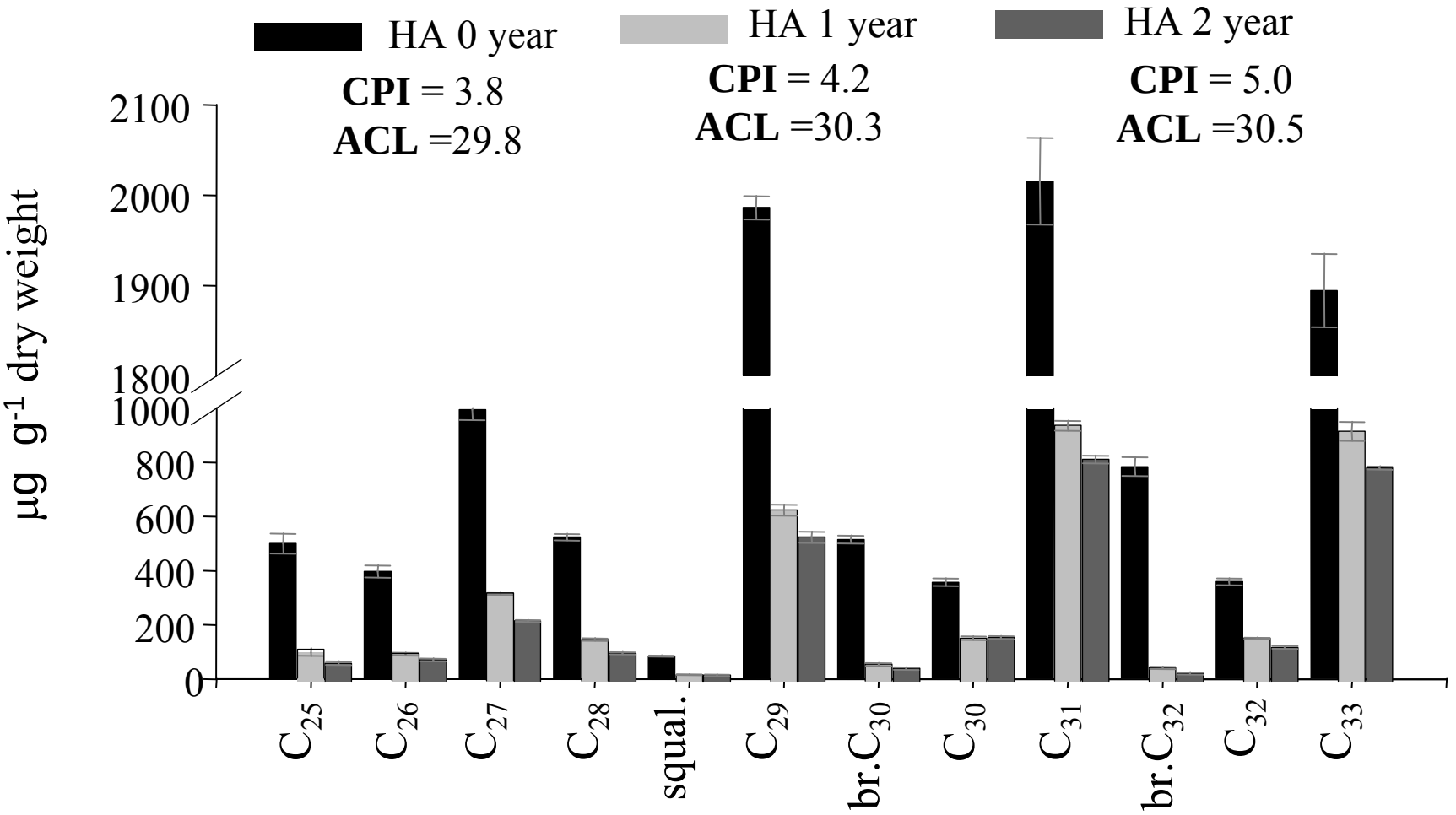
tricyclic Diterpenes- tetra- e penta-ciclyc Triterpeni

Dehydroabietane	270, 255, 185, 173, 159, 69, 43
Dehydroabietic acid m.e.	314, 299, 255, 239
Abietic acid m.e.	316, 301, 273, 256, 241, 121, 105
Pimaric acid m.e.	316, 301, 256, 241, 173, 157, 145, 121
Isopimaric acid m.e.	316, 301, 287, 257, 241, 187, 148, 121
CAS number 55515-21-4 as m.e.	348, 330, 288, 273, 235181, 161, 121
Ferruginol TMS	356, 343, 301, 273, 261, 247, 73
Dehydroabietol TMS	358, 253, 239, 185, 173, 73
CAS number 13346-06-0 as di-m.e.	364, 304, 289, 273, 221, 181, 161, 121
Stigmasta-3,5-dien-7-one*	410, 269, 218, 187, 174, 161, 91, 55
Stigmasta-4-en-3-one**	412, 398, 370, 289, 271, 229, 124, 55
Friedoolean-14-en-3-one**	424, 409, 273, 203, 191, 189, 81, 69
C(14a)-Homo-27-nor-14 β -gammaceran-3-one	426, 411, 340, 274, 232, 205, 123, 81
24-Methylcholest-5-en-3β-ol TMS	472, 457, 382, 343, 261, 255, 213
Urs-12-en-28-oic acid, 3-methoxy, m.e.*	484, 452, 424, 262, 221, 203, 189
Stigmasta-5-22-dien-3β-ol TMS	469, 396, 379, 255, 213, 159 129, 89
Stigmast-5-en-3β-ol TMS	471, 396, 381, 357, 275, 255, 129
Friedoolean-14-en-3β-ol TMS	498, 483, 408, 393, 359, 284, 269, 189
Triterpenyl acid, m.e.	514, 483, 467, 437, 262, 221,203, 189

Variation of fatty acids content of humic acids



Variation of alkanes content of humic acids



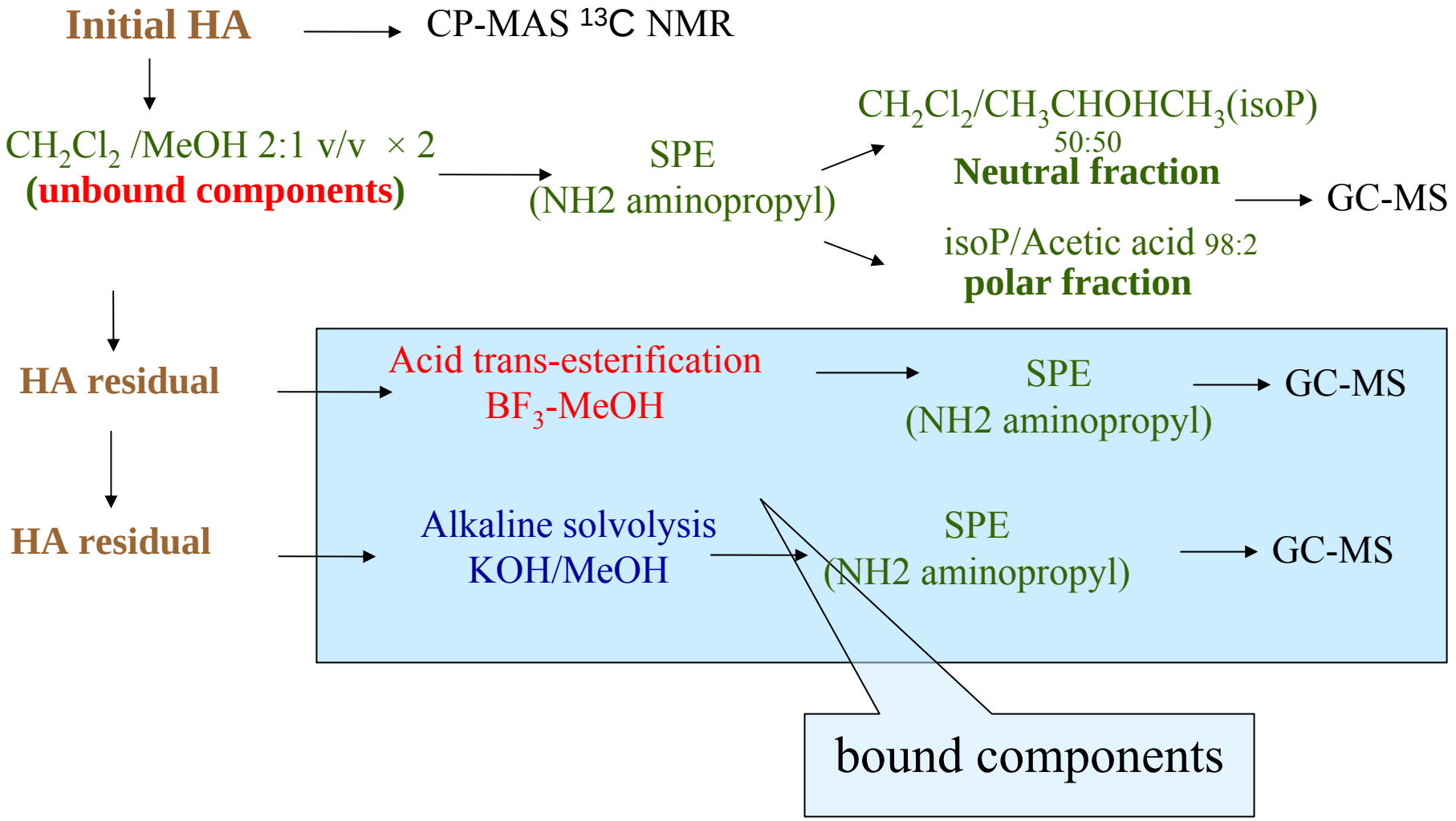
CPI CarbonPreferenceIndex = (Σ odd C25÷C31+ Σ odd C27÷C33)/2Σeven C26÷C32;

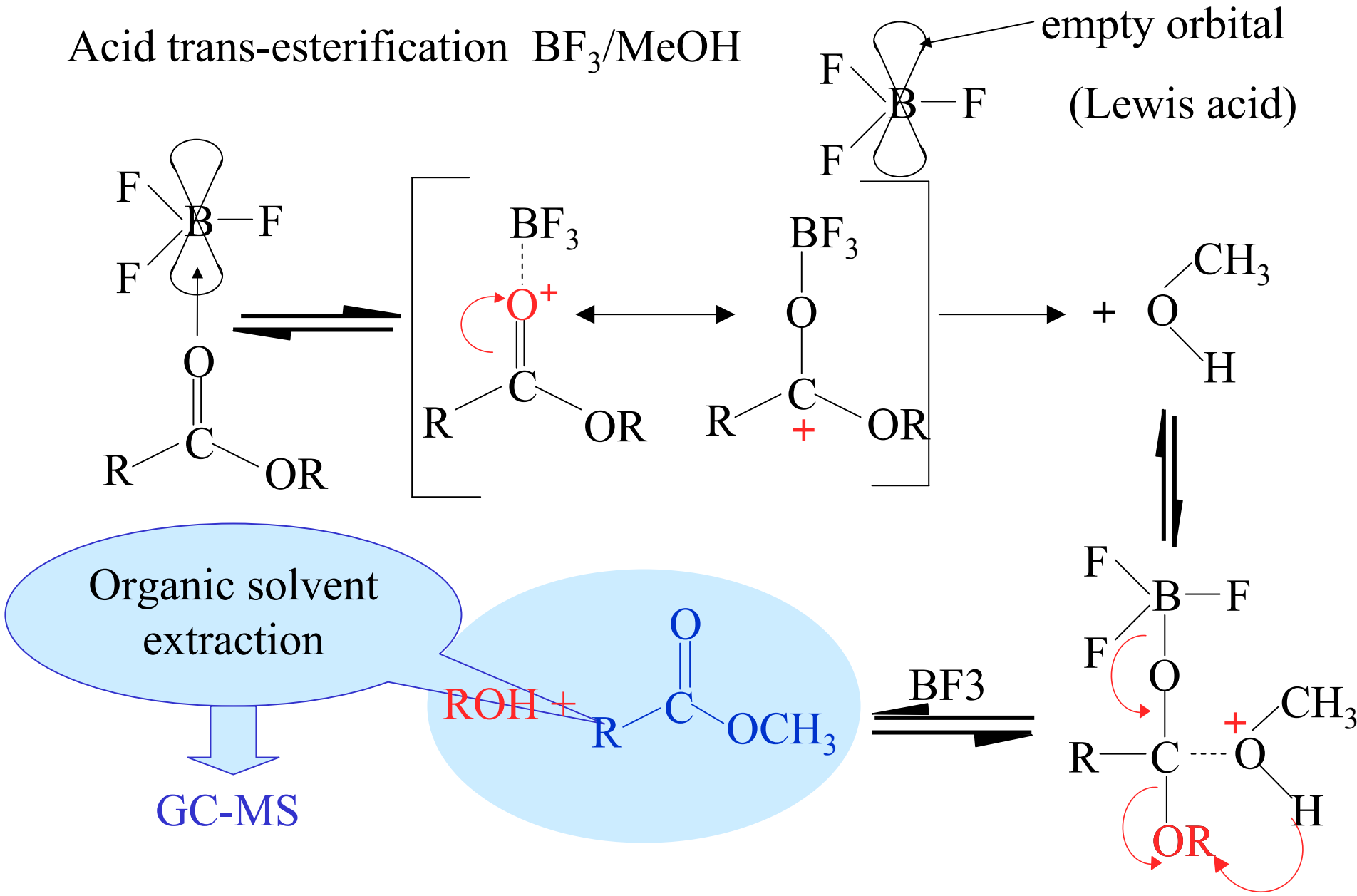
ACL Average Chain Lenght = Σ([Ci] x i)/ Σ[Ci] [Ci]=concentration of *n*-alkane containing i carbon atoms

Yield (μg g^{-1} dry weight) and composition of main classes of unbound components extracted from soil humic acids

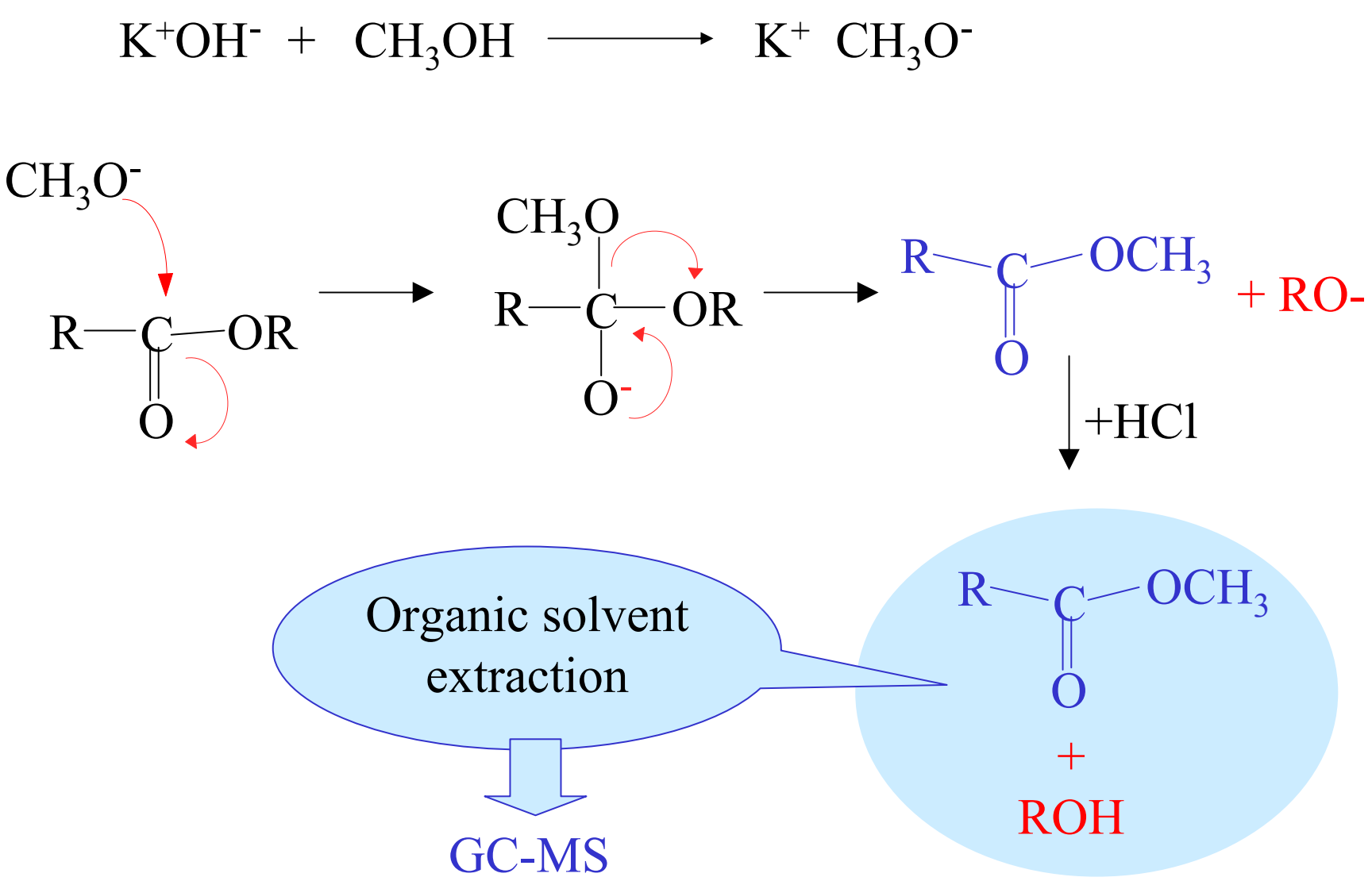
	HA 0 year	HA 1 year	HA 2 year
fatty acids	24225; $C_{12} \div C_{28}$ (a)	6288; $C_{12} \div C_{28}$ (b)	4770; $C_{12} \div C_{28}$ (c)
unsaturated (%)	51.9	36.5	32.2
> C20 (%)	5.2	15.4	25.8
branched fatty acids	472; $C_{15} \div C_{19}$ (a)	510; $C_{15} \div C_{19}$ (a)	490; $C_{15} \div C_{19}$ (a)
Alkanes	7540; $C_{25} \div C_{33}$ (a) **CPI** = 3.8; **ACL** =29.8	3500; $C_{25} \div C_{33}$ (b) **CPI** = 4.2; **ACL** =30.3	2944; $C_{25} \div C_{33}$ (b) **CPI** = 5.0; **ACL** =30.5
Alcohols	2370; $C_{22} \div C_{28}$ (a)	1570; $C_{22} \div C_{28}$ (b)	1360; $C_{22} \div C_{28}$ (b)
Sterols	1810 (a)	1720 (a)	1740 (a)
Resin acids	1530 (a)	1490 (a)	1480 (a)

sequential extraction



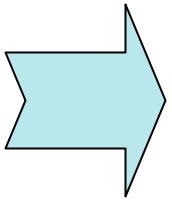

Acid trans-esterification BF3/MeOH
empty orbital
(Lewis acid)
F
B
F
F
O
C
R
OR
BF3
O+
+
CH3
H
Organic solvent extraction
GC-MS
ROH +
OCH3
BF3

Alkaline solvolysis KOH/MeOH

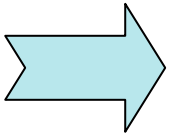


Yields (μg g^{-1}dry weight) and composition of main classes of bound components extracted from soil HAs

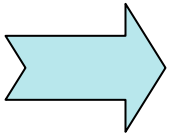
	HA 0 year	
	BF_3/ MeOH	KOH/ MeOH
ω-hydroxy acids	6100 $C_{12} - C_{26}$	4070 $C_{14} - C_{26}$
mid-chain hydroxyacids	7300 C_{16} C_{18}	2430 $C_{16} - C_{18}$
Alkane-Dioic acids	3070 $C_8 - C_{24}$	5650 $C_{14} - C_{24}$
aromatic	620	2000
α/β hydroxyacids	1060 $C_{10} - C_{26}$	490 $C_{14} - C_{26}$
fatty acids	3770 $C_{12} - C_{28}$	1620 $C_{14} - C_{34}$
alcohols	610 $C_{12} - C_{24}$	510 $C_{12} - C_{24}$



Plant biopolyesters cutin ad suberin

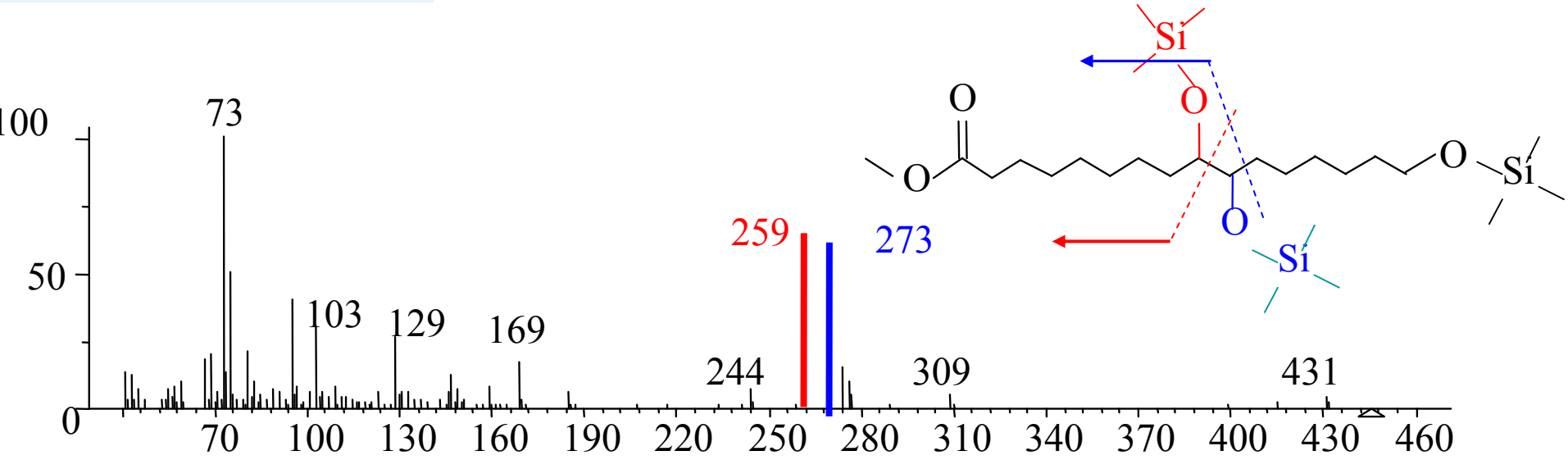


biopoliesters /lignin

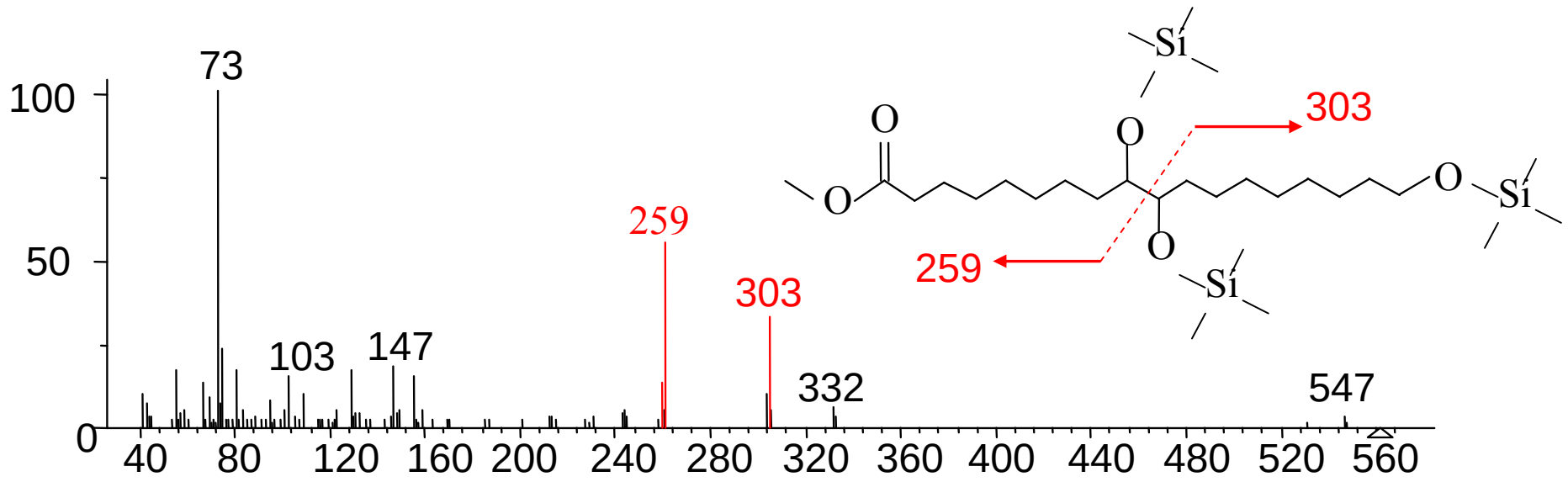


microbial markers

mid-chain hydroxy acids (plant cutin components)

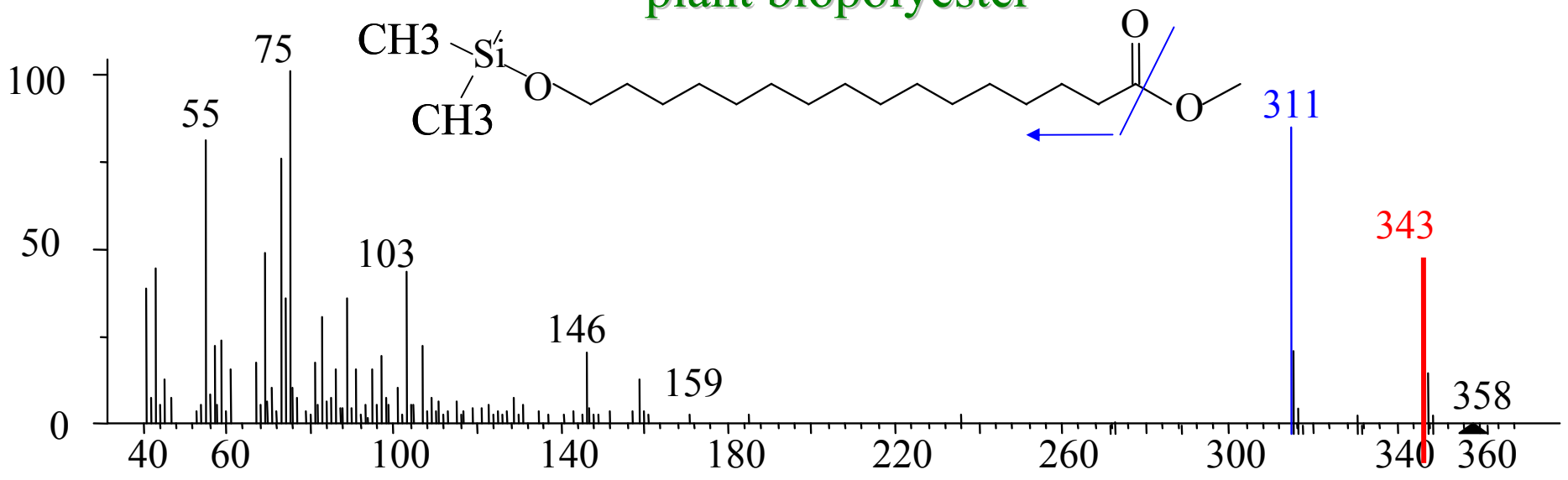


Hexadecanoic acid, 9(10) 16-bis[trimethysilylether]-, methyl ester



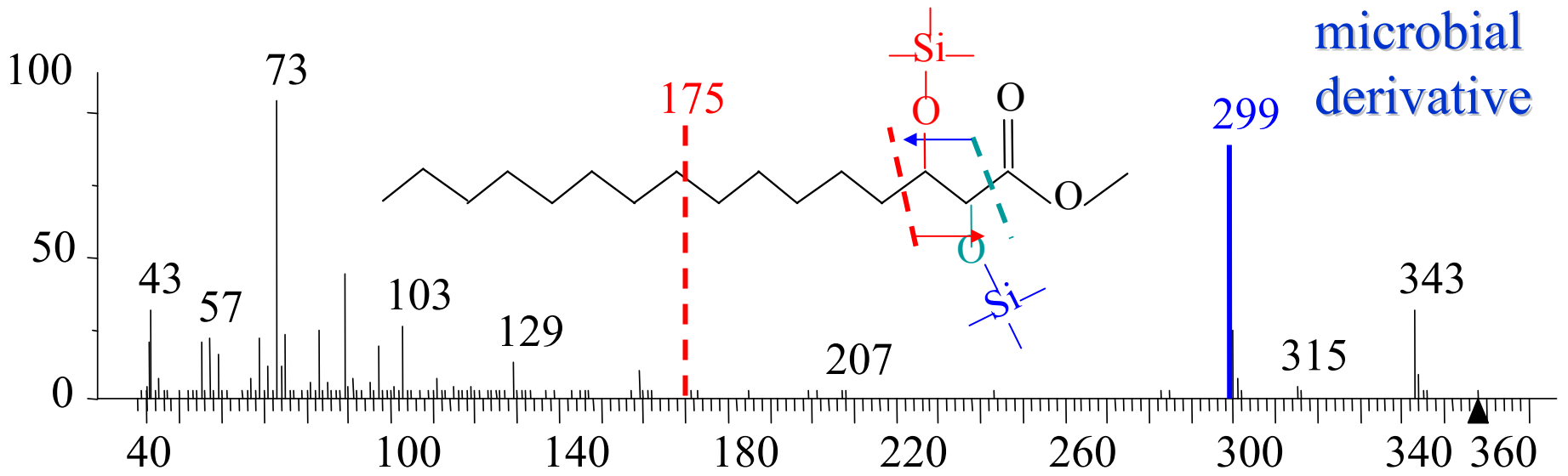
Octadecanoic Acid, 9,10,18-tris[trimethylsilyleter]-, methyl ester

plant biopolyester



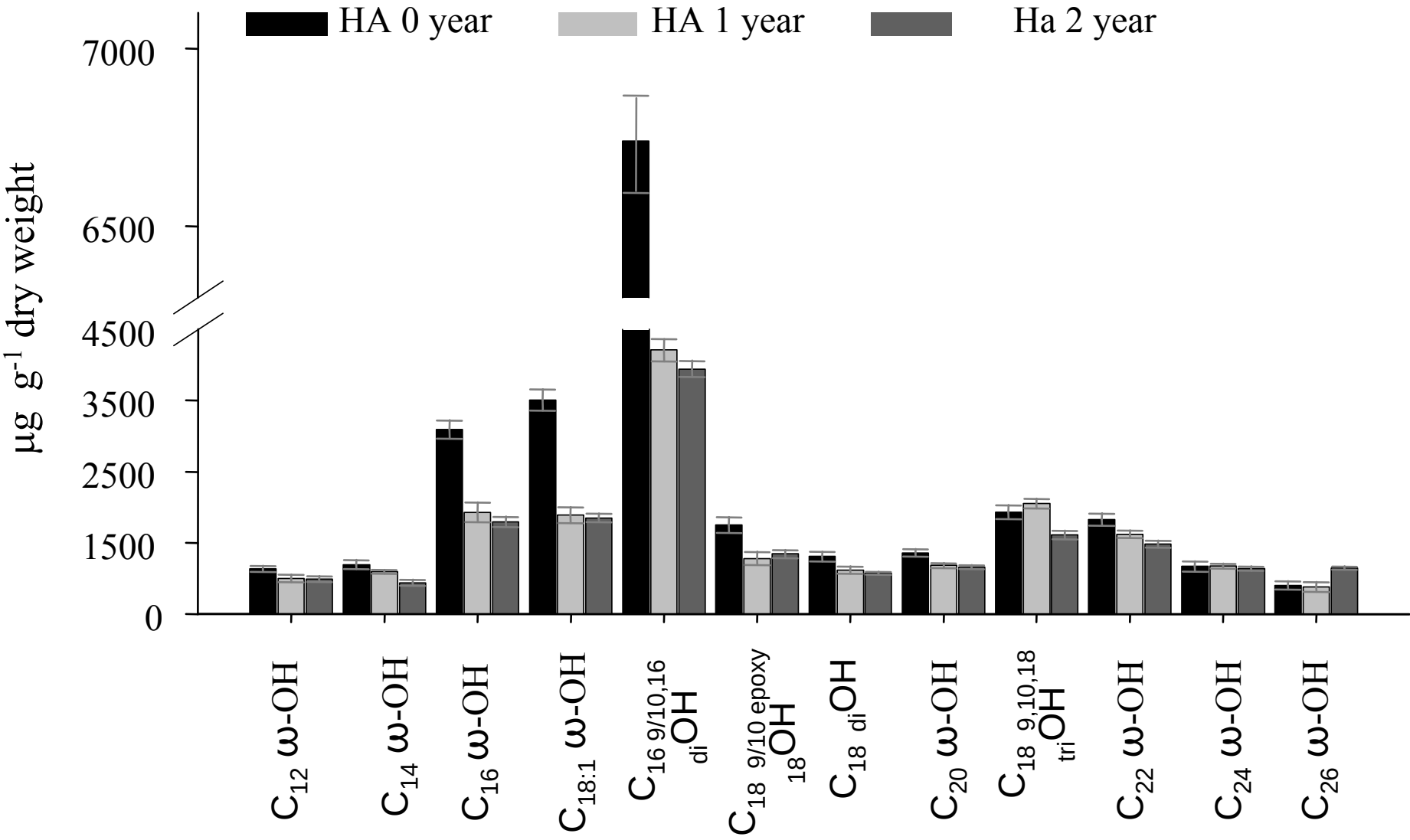
Hexadecanoic Acid, 16-(trimethylsililether)-, methyl ester

microbial derivative

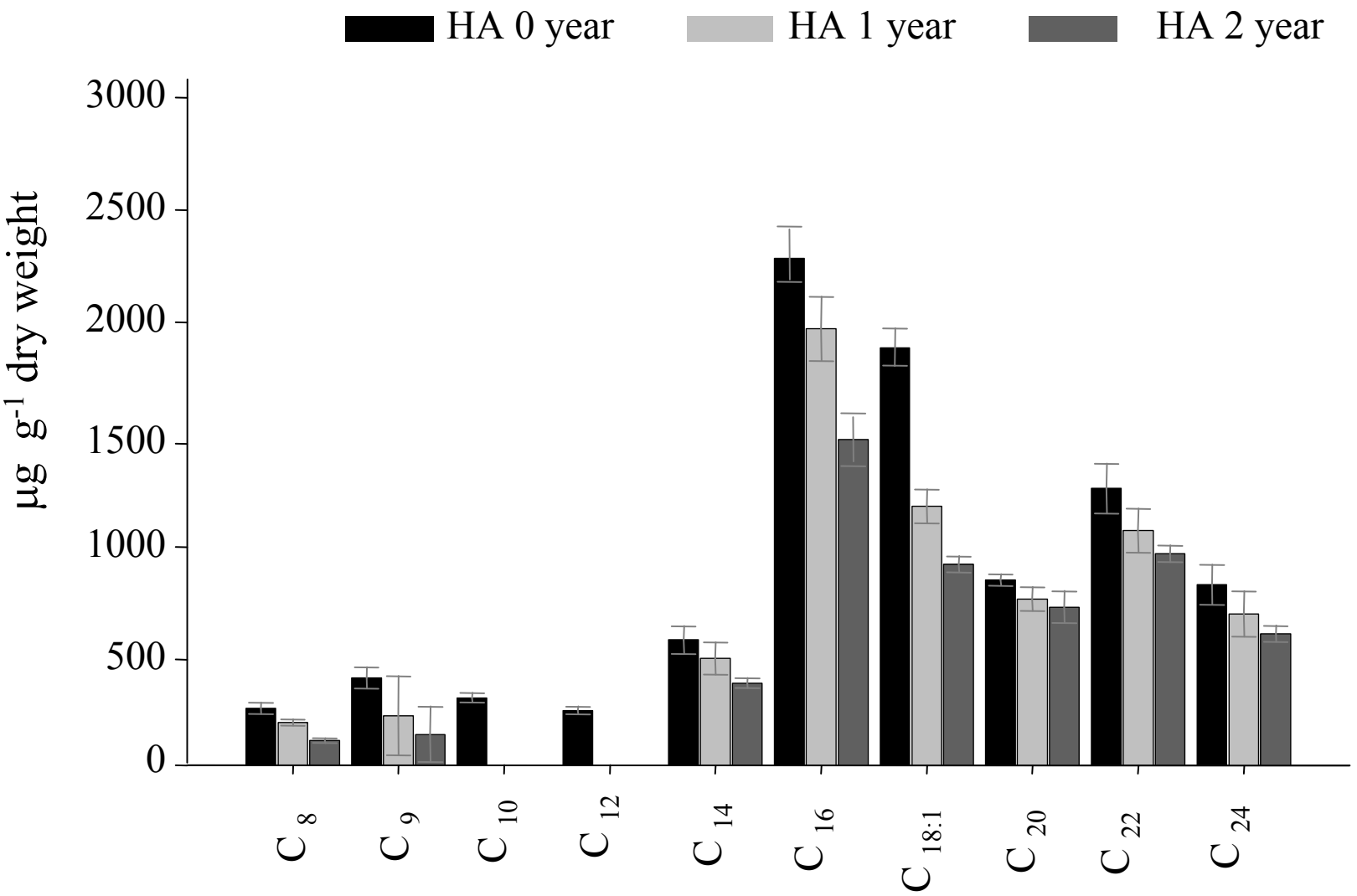


Hexadecanoic acid, 2(3)-[(trimethylsilyl)oxy]-, methyl ester

Variation of ω-hydroxyacids mid-chain- hydroxyacids in soil humic acids



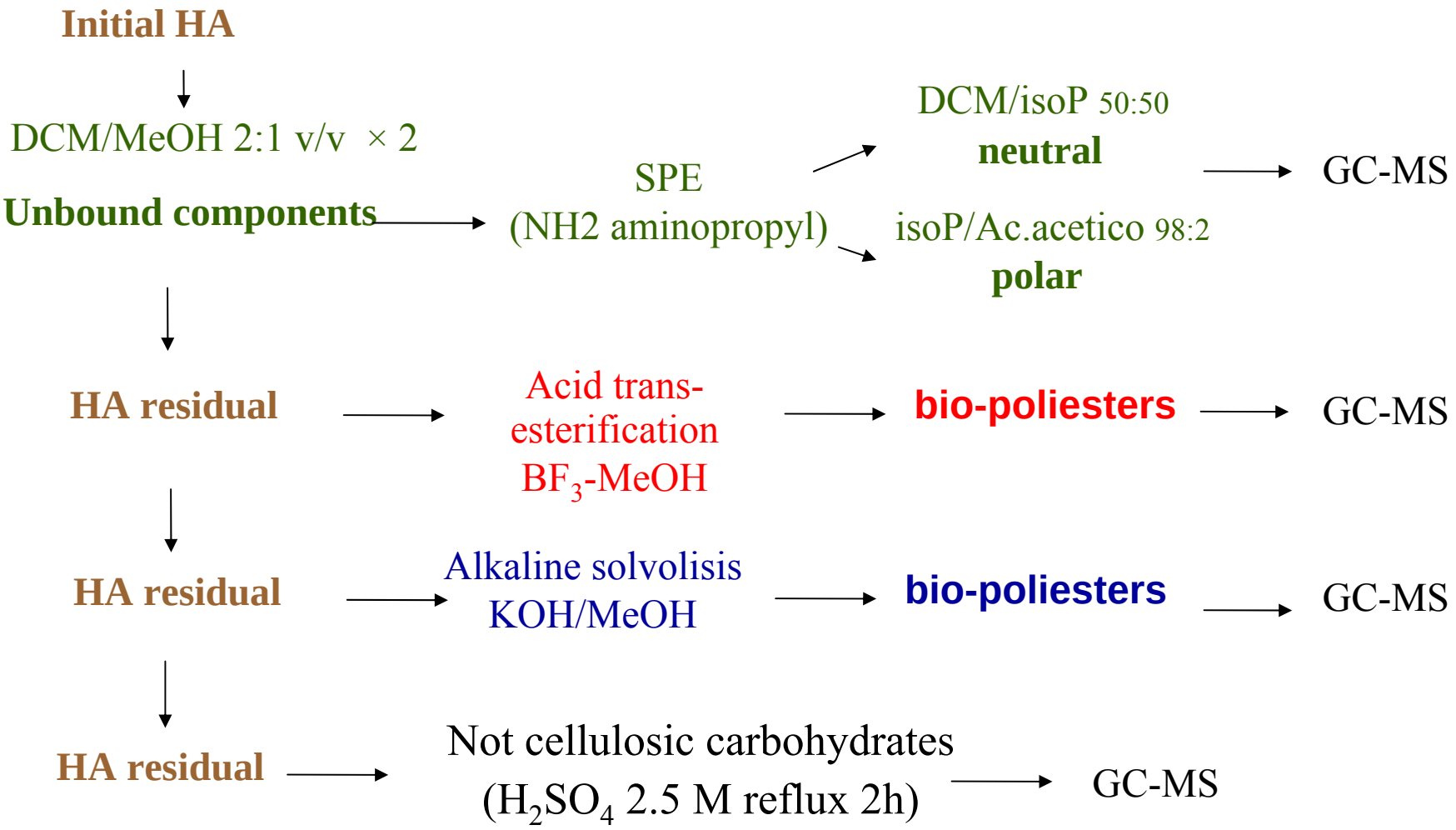
Variation of alkane dioic acids in soil humic acids

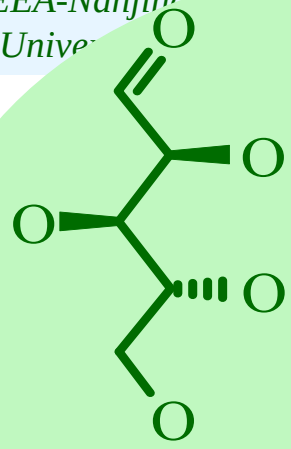


Yields (μg g^{-1}d. w.) and composition of main classes of bound components extracted from soil HAs

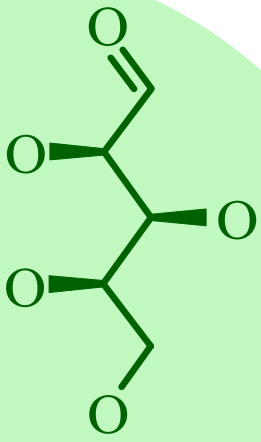
	HA 0 year		HA 1 year		HA 2 year	
	BF_3/ MeOH	KOH/ MeOH	BF_3/ MeOH	KOH/ MeOH	BF_3/ MeOH	KOH/ MeOH
ω-hydroxy acids	6200 (a) $C_{12} - C_{26}$	4100(a) $C_{14} - C_{26}$	4600 (b) $C_{12} - C_{26}$	2160(b) $C_{14} - C_{26}$	4520 (b) $C_{12} - C_{26}$	2570 (b) $C_{14} - C_{26}$
mid-chain hydroxyacids	7300 (a) C_{16} C_{18}	2600 (a) C_{16} C_{18}	4500 (b) C_{16} C_{18}	2200 (a) C_{16} C_{18}	4300 (b) $C_{12} - C_{26}$	1670 (b) $C_{12} - C_{26}$
alkanedioic acids	3070 (a) $C_8 - C_{24}$	4650(a) $C_{14} - C_{24}$	2250 (b) $C_8 - C_{24}$	3400(b) $C_{14} - C_{26}$	2200 (b) $C_8 - C_{24}$	3150 (b) $C_{14} - C_{26}$
aromatics	620 (a)	2000 (a)	510 (a)	1890 (a)	530 (a)	1900 (a)
α/β hydroxyacids	1060 (a) $C_{10} - C_{26}$	490 $C_{14} - C_{26}$	730 (b) $C_{12} - C_{26}$	**n.d.**	610 (b) $C_{12} - C_{26}$	**n.d.**
fatty acids	3770 (a) $C_{12} - C_{28}$	1620 (a) $C_{14} - C_{34}$	2980 (a) $C_{12} - C_{26}$	1600 (a) $C_{14} - C_{34}$	1920 (b) $C_{12} - C_{28}$	1300 (b) $C_{14} - C_{34}$
alcohols	610 (a) $C_{12} - C_{24}$	510 (a) $C_{12} - C_{24}$	400 (b) $C_{16} - C_{24}$	**n.d.**	420 (b) $C_{16} - C_{24}$	**n.d.**

Sequential extraction



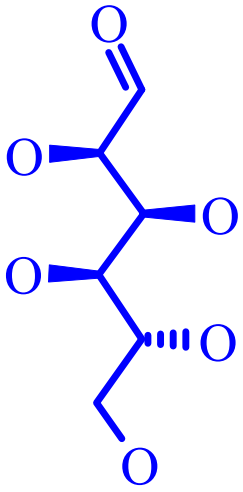


arabinose

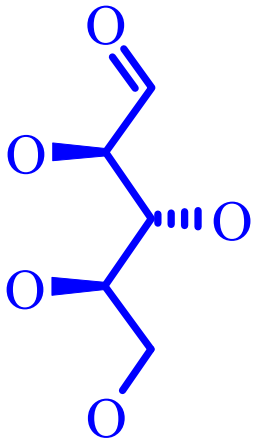


xilose

plant origin

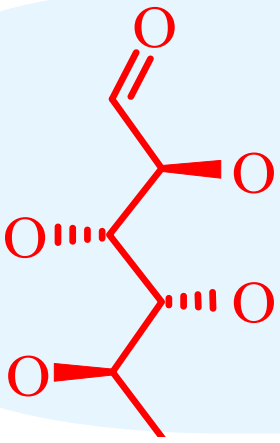


glucose

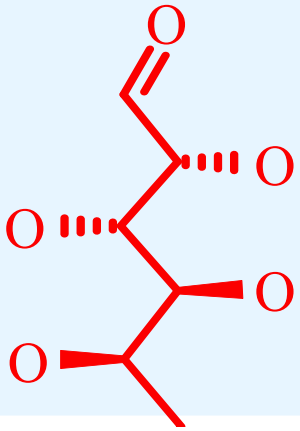


ribose

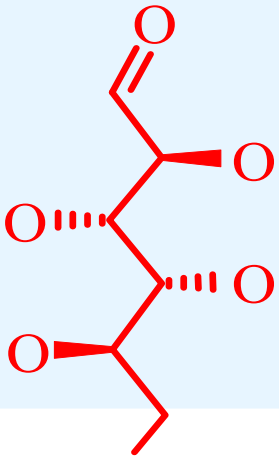
microbial origin



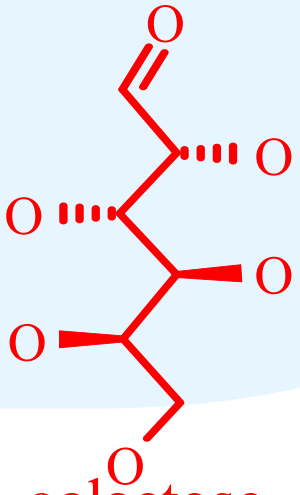
rhamnose



fucose

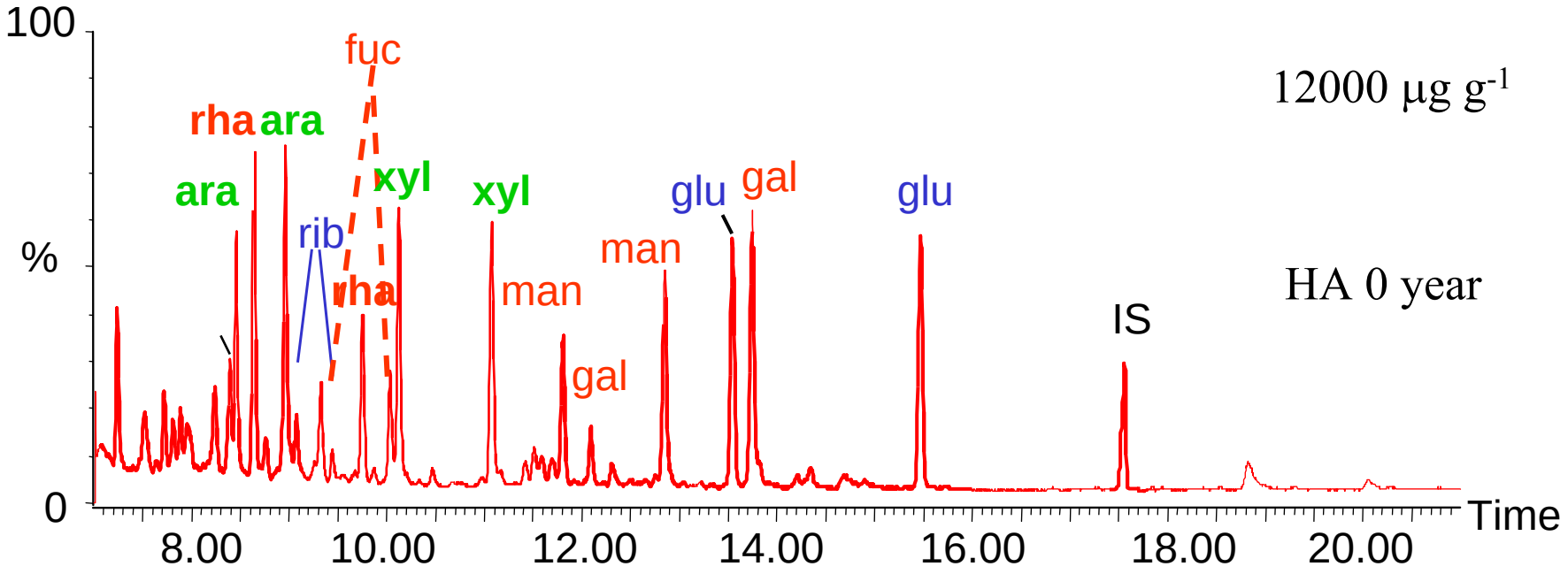


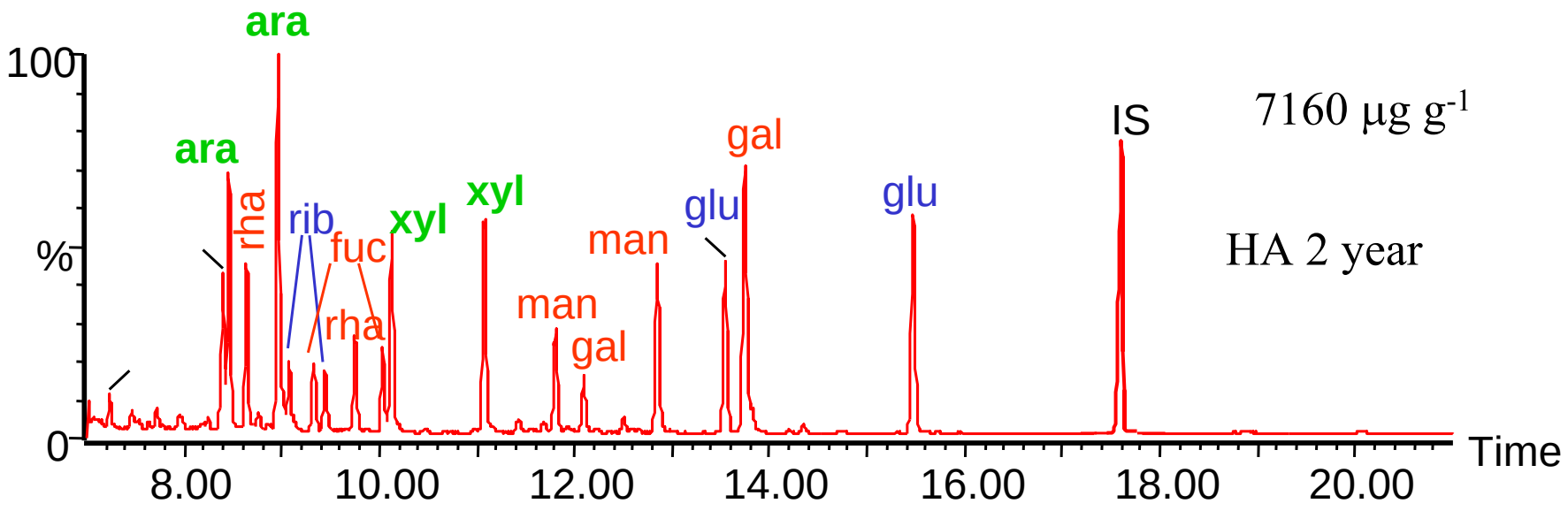
mannose



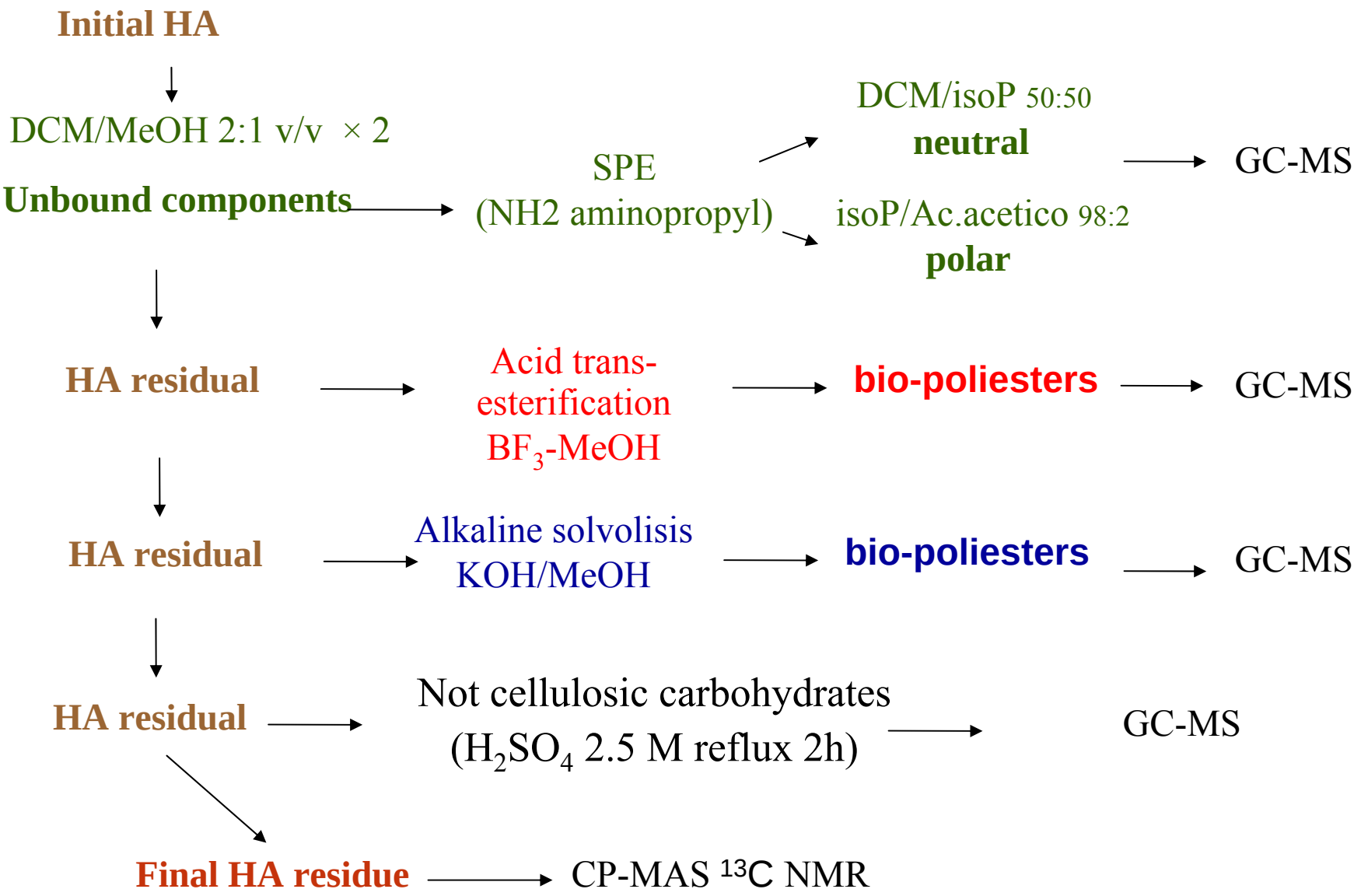
galactose

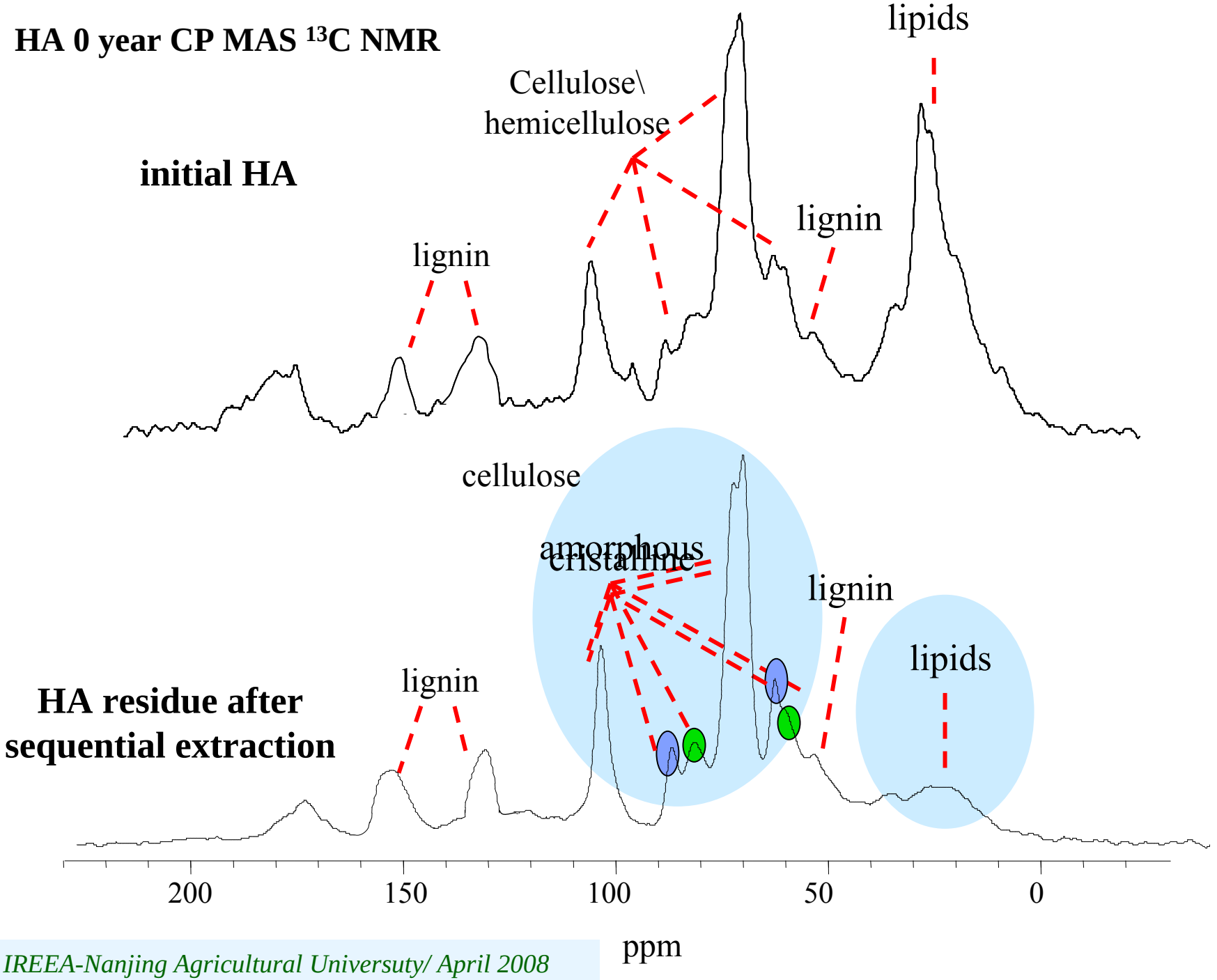
Non cellulosic carbohydrates

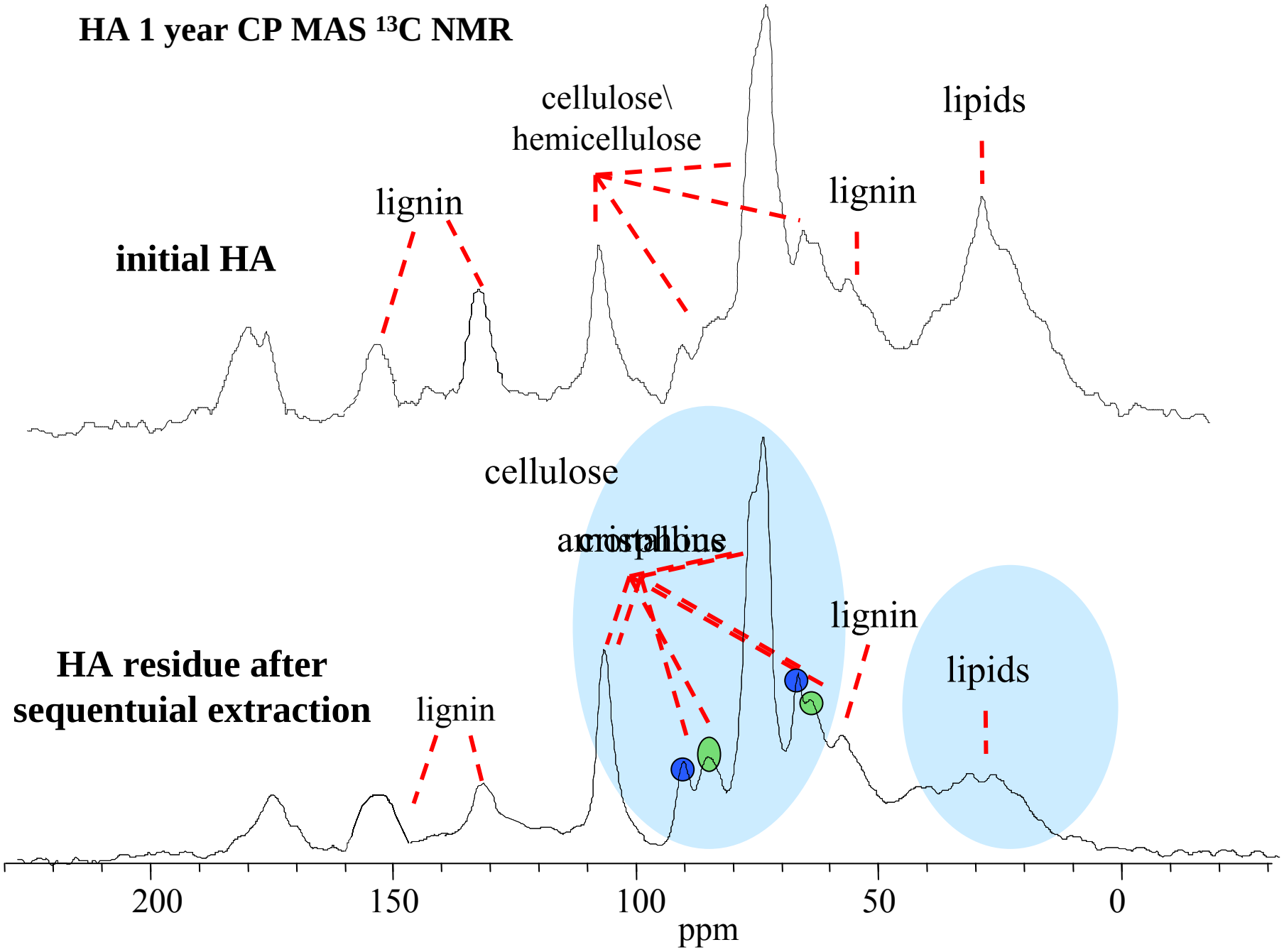




Sequential extraction




HA 0 year CP MAS 13C NMR
initial HA
lipids
Cellulose\
hemicellulose
lignin
lignin
cellulose
amorphous
cristalline
lignin
lipids
HA residue after
sequential extraction
lignin
200
150
100
50
0
ppm


HA 1 year CP MAS 13C NMR
cellulose\
hemicellulose
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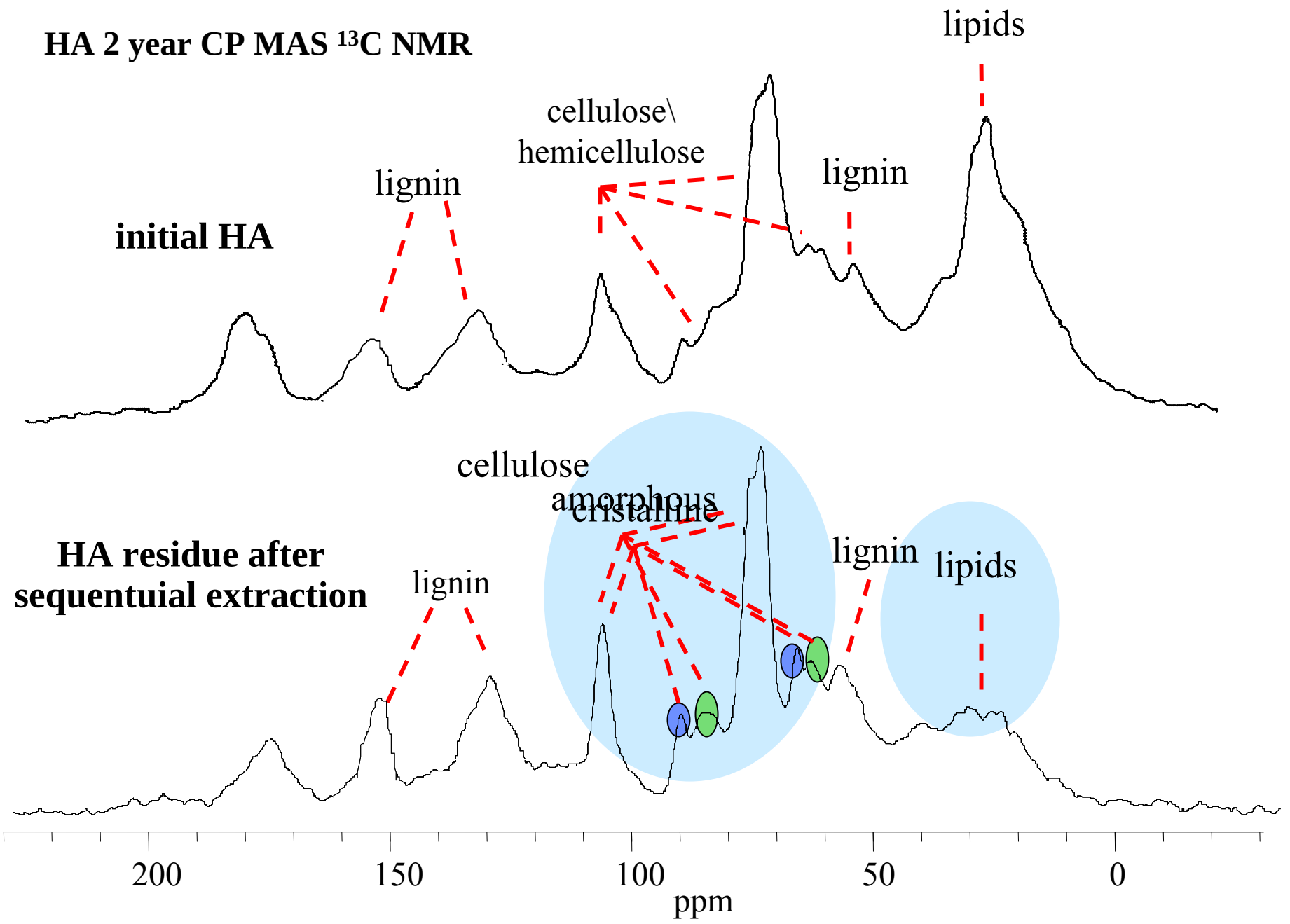
lignin
lignin
initial HA
cellulose
lignin
lignin
lipids
HA residue after
sequentuial extraction
200
150
100
50
0
ppm


HA 2 year CP MAS 13C NMR
lipids
cellulose\
hemicellulose
lignin
lignin
initial HA
cellulose
amorphous
cristalline
HA residue after
sequentuial extraction
lignin
lignin
lipids
200
150
100
50
0
ppm

- The humic acid extracted after OM addition was characterized by a large content of alkyl lipids compounds, carbohydrates and lignin components sequential fractionation released a large amount of unbound and bound molecules; plant biopolymers like lignin, cellulose and aliphatic polyesters were recognized as the main sources of humic acids
- after 1 year the main variations in HA composition were represented by a large decrease of bio-available wax components such as fatty acids and linear hydrocarbon followed in the second year by a decrease of carbohydrates deriving mainly from hemicellulose
- Lower decrease and large persistence were found for biopolyester and lignin components – these findings confirm previous results on the formation of Humic substances through the selective accumulation of these recalcitrant organic molecules

Advantage and drawbacks of Sequential extraction

- Powerful and selective qualitative and quantitative characterization of some organic components (lipids, biopolyesters, carbohydrates) overall s.d. < 7-9%

- the sequential extraction allow to distinguish among bound and unbound components and different form of carbohydrates

- high effectiveness towards ester bonds and alkyl components and ether bonds of carbohydrates
- lower effectiveness towards aromatic components (lignin): need of additional step for lignin detection

- slow and multi-step experimental protocol

- sample purification and derivatization before MS analysis
- 1 sample (3 replicates 2 weeks)

Thanks for
your attention