NMR spectroscopy in lignocellulose analysis

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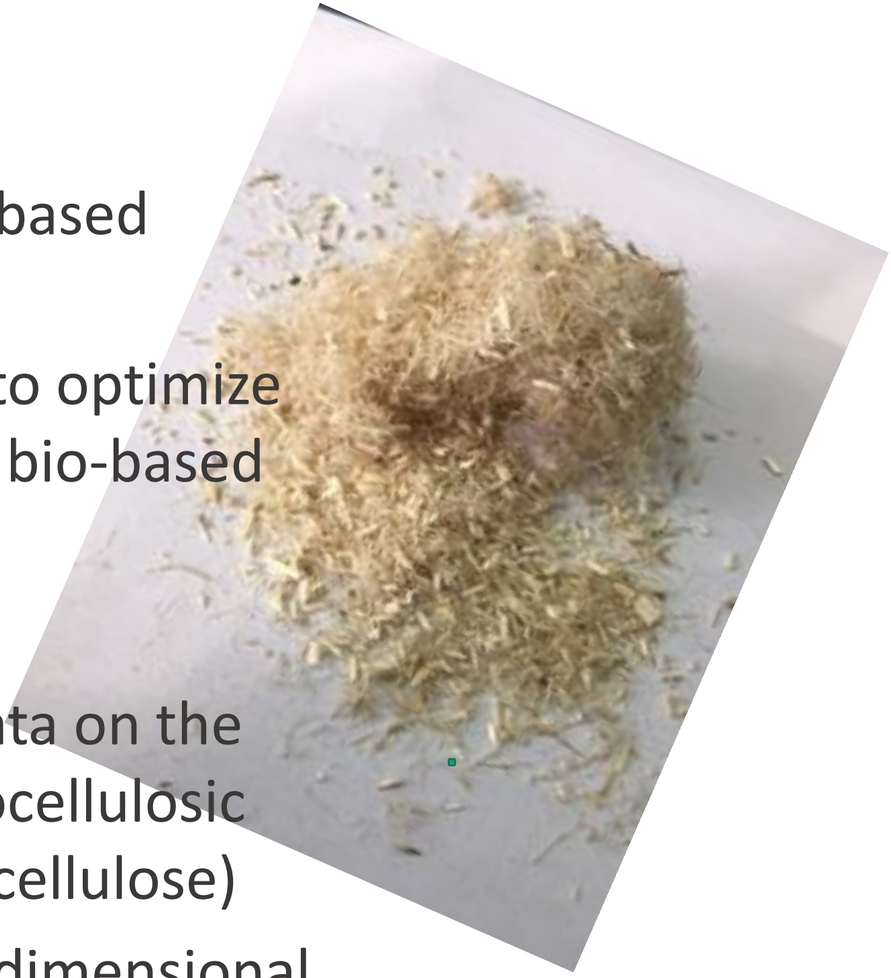
GOAL

- *possibilities of using NMR techniques in the analysis of complex mixtures*
- *lignocellulosic biomass*
- *biofuels*
- *main drawbacks and future perspectives*

lignocellulosic biomass - NMR spectroscopy



- promising feedstock for the production of various bio-based products
- necessary to know its structural composition in order to optimize pretreatment process and further (bio)conversion into bio-based products
- NMR lignocellulose chemistry for more than 30 years
- fast and reliable method that can provide advanced data on the molecular architecture and composition of whole lignocellulosic biomass and its structural units (lignin, cellulose, hemicellulose)
- analysis of the solution and solid state, one- and two- dimensional NMR methods
- spectra can provide qualitative and quantitative information

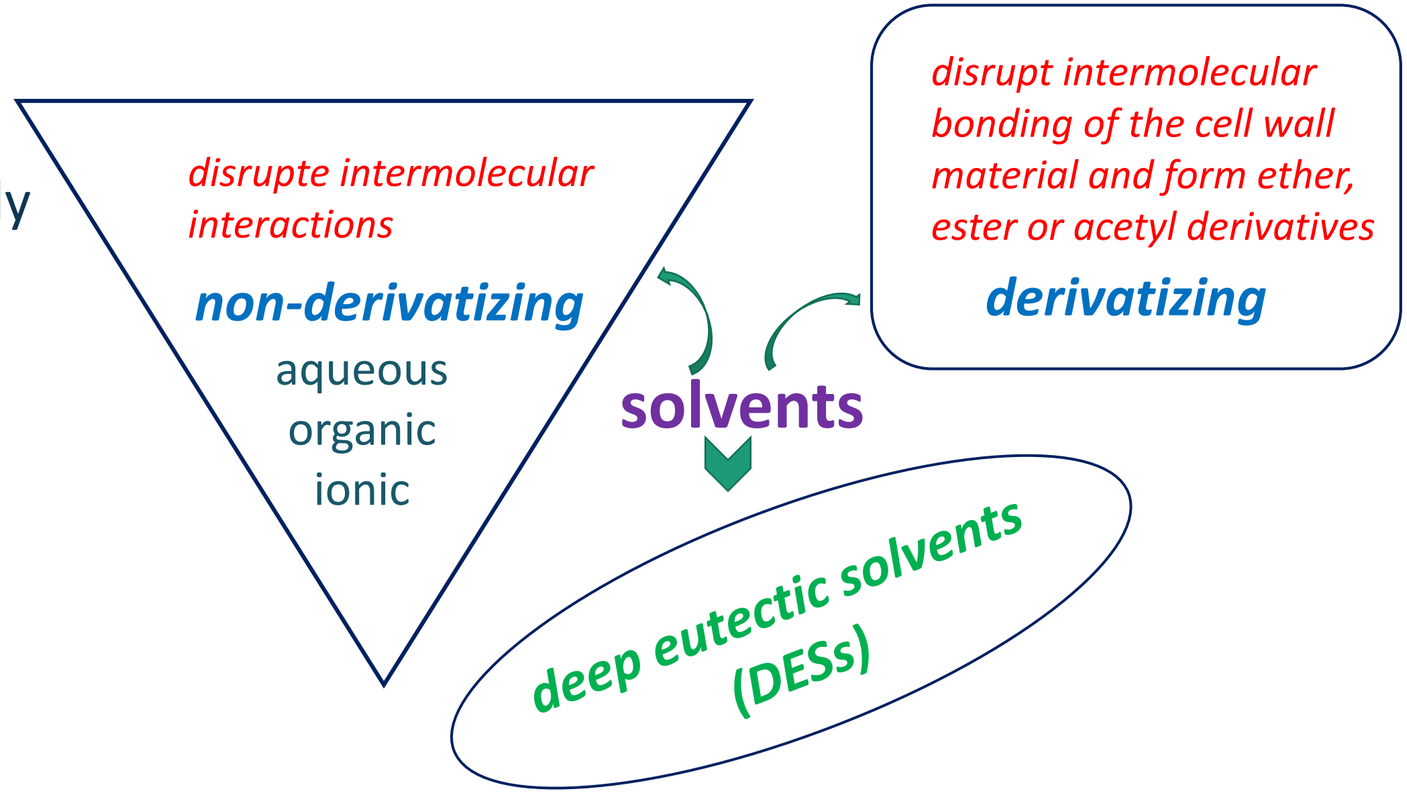
Advantages of NMR spectroscopy in analysis of complex organic samples:

- identification and structural analysis of compounds and compound mixtures without physical separation
- simple sample preparation, no external standards
- fast analysis, plenty of information in one spectum:
  - ✓hydrocarbon distribution,
  - ✓H/C ratio,
  - ✓content of aromatics, olefins, naphtenes, paraffins, oxygenates
  - ✓aromaticity

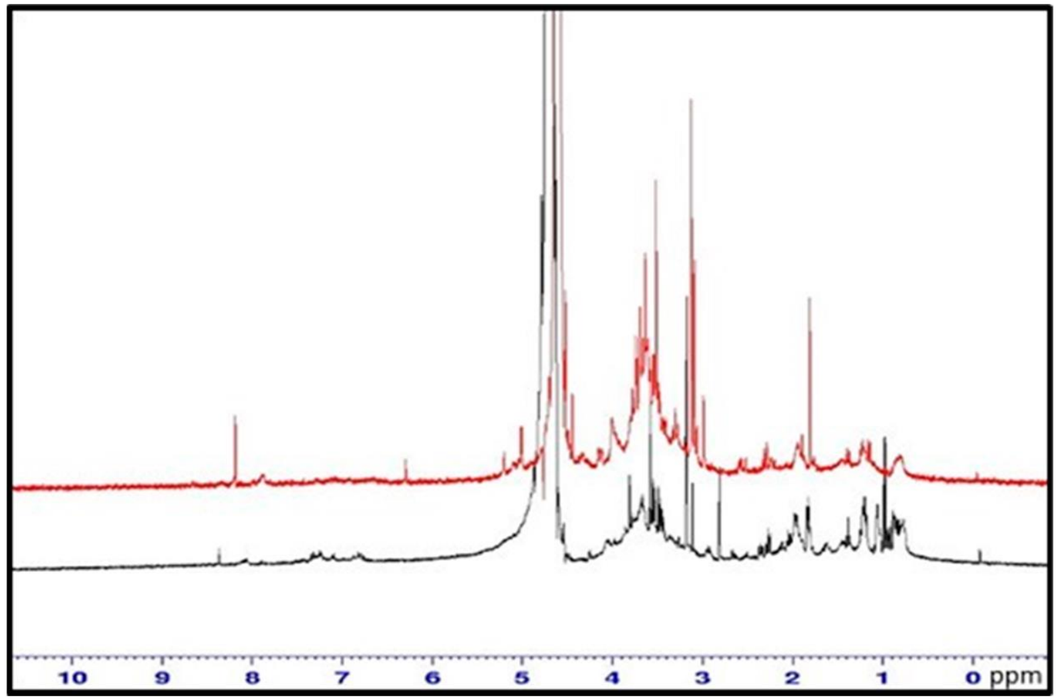
Sample preparation

- one of the most important steps to achieve reliable results with NMR spectroscopy
- the preparation of samples starts with the removal of waxes, fats, salts, non-volatile hydrocarbons, etc. by extraction with various solvents
- solution state NMR requires a sufficiently dissolved or swollen biomass sample
- solid state NMR does not require solubilization of the lignocellulose biomass
- NMR rotors are filled with dried and ground lignocellulosic biomass samples or previously separated lignocellulosic biopolymers by pretreatment processes

- due to the complex chemical composition of the biomass, pretreatment is a crucial step in converting the lignocellulosic biomass into a less recalcitrant form



Brewer's Spent Grain



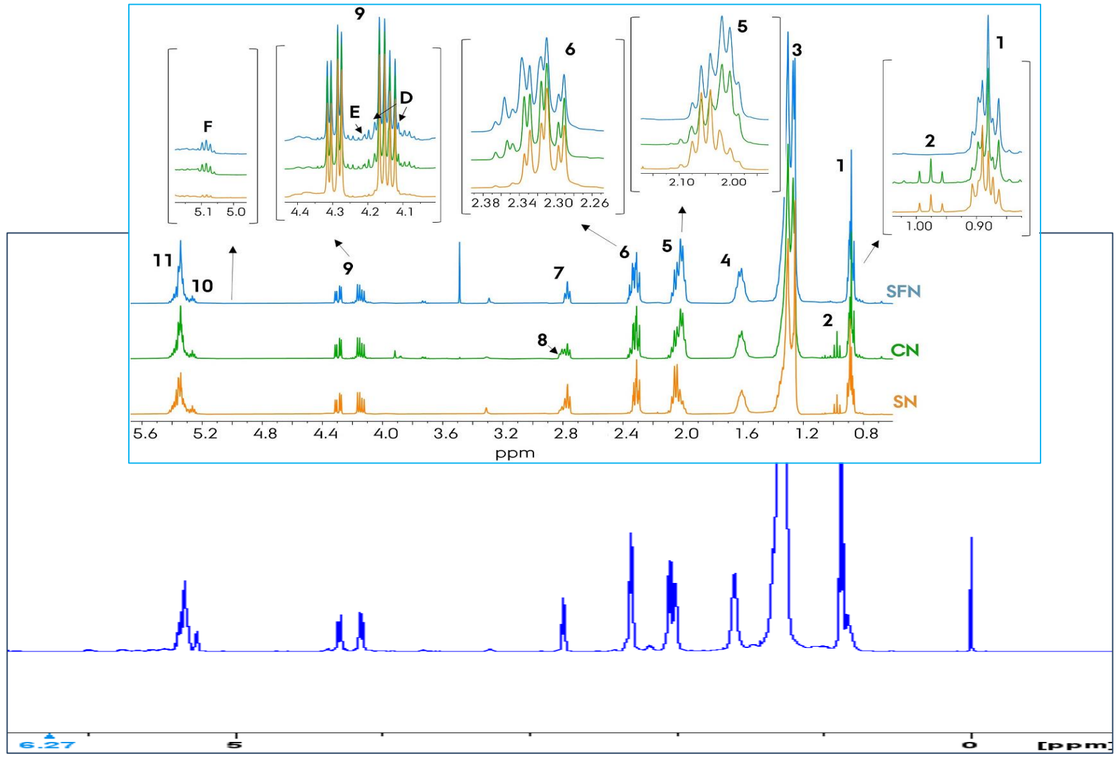
Typical ^{1}H NMR spectra of BSG before and after treatment with *T.v.;* at 25°C in CDCl3

δ / ppm	H-atom	BSG %	BSG + *T.v.* %
0,50-3,00	alifatski	47,94	26,56
3,00-4,40	ugljikohidrati, metoksi	45,10	65,92
6,70-7,65	aromatski	3,50	0,45
7,94-8,50	aromatski, amino kiseline, mravlja kiselina	0,36	1,18

- composition of Brewer's Spent Grain before and after Treatment with *Trametes versicolor*
- despite the fact that BSG components were not separated and the complexity of proton NMR spectra, some significant differences in composition among samples can be noticed

Lipid extracts

- molar percentage of linolenic, linoleic, unsaturated, monounsaturated, saturated + modified acyl groups
- molar percentage of triglycerides (NTG), 1,2-diglycerides (N1,2-DG), 1,3 diglycerides (N1,3-DG), 1-monoglycerides (N1-MG), fatty acids (NFA), and glycerol (NG), total number of moles of the different glyceride structures detected (NT).



Tipični spektar ^{1}H NMR lipida ekstrahiranih iz lignocelulozne mase

Table 1 (Vidal et al, 2023)
^{1}H NMR signals of protons of acyl groups (AG) and fatty acids (FA) from the lipid extracts, together with their chemical shift, and type of protons that generate the signal.

Signal	Chemical shift (ppm)	Type of protons	Compounds
1	0.88	-(C<u>H</u>$_3$)	Saturated, monounsaturated ω-9 and ω-7 in AG and FA
	0.89	-(C<u>H</u>$_3$)	Unsaturated ω-6 in AG and FA
2	0.97	-(C<u>H</u>$_3$)	Unsaturated ω-3 in AG and FA
3	1.19–1.42	-(C<u>H</u>$_2$)$_n$-	AG and FA
4	1.61–1.74	-OCO–CH$_2$–C<u>H</u>$_2$-	AG in TG and FA
5	1.92–2.15	-C<u>H</u>$_2$-CH=CH-	AG and FA
6	2.26–2.38	-OCO-C<u>H</u>$_2$-	AG in TG and FA
7	2.77	= HC-C<u>H</u>$_2$-CH =	Di-unsaturated ω-6 AG and FA
8	2.77-2.90	= HC-C<u>H</u>$_2$-CH =	Polyunsaturated ω-6 and ω-3 acyl groups and FA
9	4.04–4.38	ROC<u>H</u>$_2$-CH(OR')-C<u>H</u>$_2$OR"	Glycerol group in TG
10	5.27	ROCH$_2$-C<u>H</u>(OR')-CH$_2$OR"	Glycerol group in TG
11	5.28–5.46	-C<u>H</u>=C<u>H</u>-	AG and FA
A	3.65	ROCH$_2$-CHOH-C<u>H</u>$_2$OH	Glycerol group in 1-MG
B	3.73	ROCH$_2$-CH(OR')-C<u>H</u>$_2$OH	Glycerol group in 1,2-DG
C	3.94	ROCH$_2$-C<u>H</u>OH-CH$_2$OH	Glycerol group in 1-MG
D	4.05-4.21	ROC<u>H</u>$_2$-C<u>H</u>OH-C<u>H</u>$_2$OR'	Glycerol group in 1,3-DG
E	4.18	ROC<u>H</u>$_2$-CHOH-CH$_2$OH	Glycerol group in 1-MG
F	5.08	ROCH$_2$-C<u>H</u>(OR')-CH$_2$OH	Glycerol group in 1,2-DG

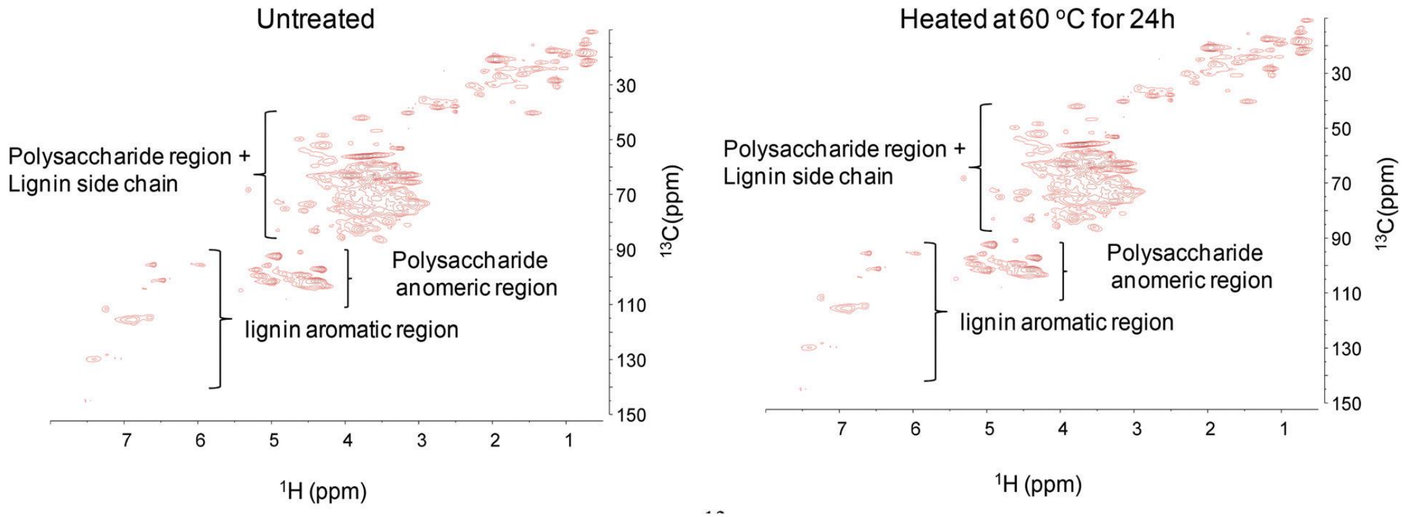
AG: acyl groups; FA: free fatty acids; TG: triglycerides; 1,2- and 1,3-DG, 1,2- and 1,3-diglycerides; 1-MG, 1-monoglycerides. The assignment of the signals is based on other studies (Vidal et al., 2012; Nieva-Echevarría et al., 2017).

2D NMR

- due to severe signal overlapping, a complete assignment of 1H and 13C NMR spectra of lignocellulosic samples is almost impossible
- nowadays, the application of 2D NMR techniques provides more detailed signal assignment of natural polymers
- solution-state 2D HSQC NMR technique provide information on polysaccharide components profile, lignin composition and interunit linkage distribution, acetylated and native call walls

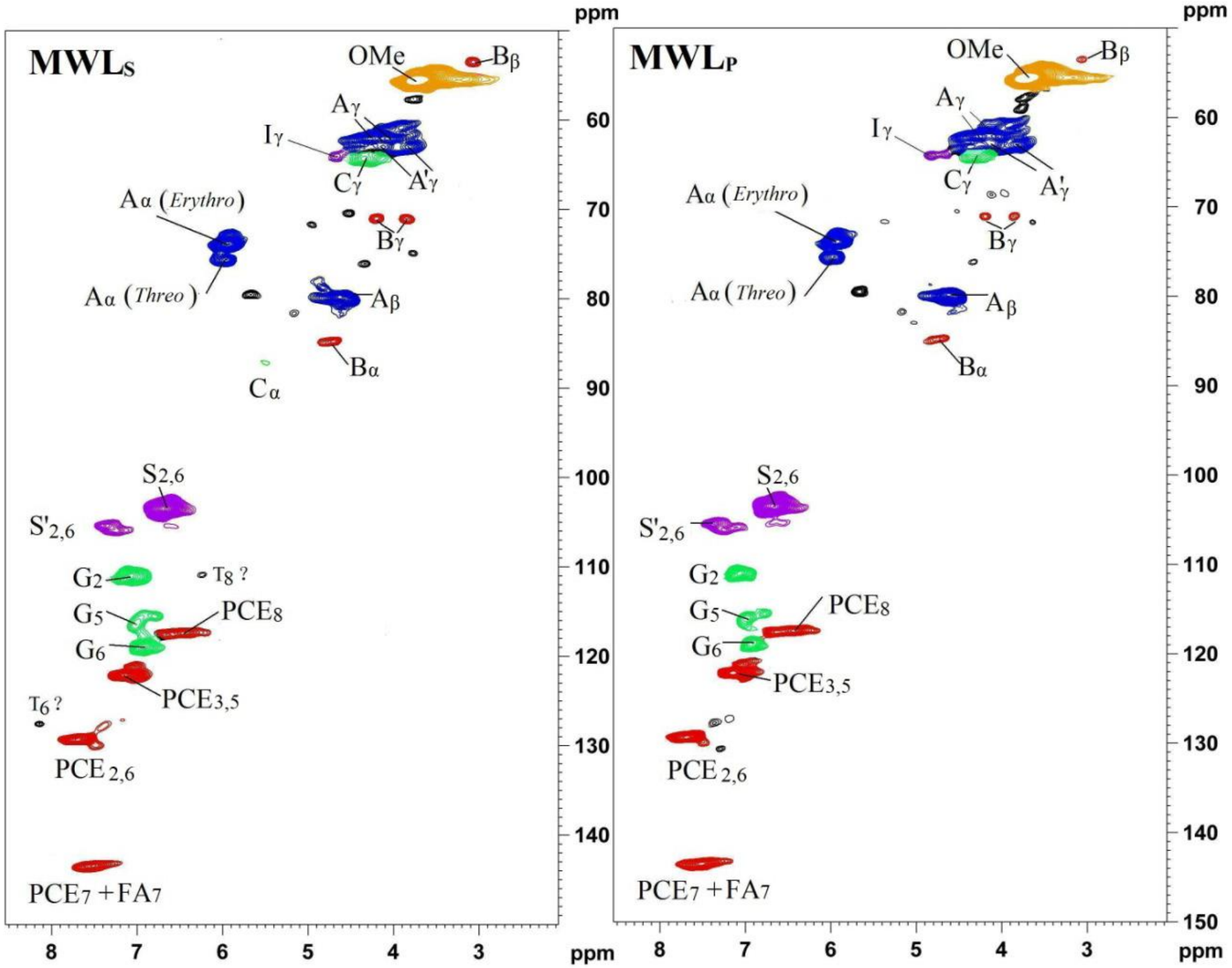
δ_C/δ_H (ppm)	Assignment
55.7/3.8	CH_3 in methoxyl group
60.6/3.6	C_6 polysaccharide + A_γ
71.0/4.8	C_α/H_α in β-O-4 linkage (A)
74.0/4.5	C_2/H_2 in 2-OAc-β-D-Xylp
75.0/4.8	C_3/H_3 in 3-OAc-β-D-Xylp
81.8/3.5	C_4 polysaccharides
86.0/4.4	C_β/H_β in β-O-4 linkage (A)
87.5/5.5	C_α/H_α in phenylcoumaran substructure (B)
83.4/4.95	C_α/H_α in dibenzodioxocin substructure (D)
102.4/4.4	Internal 1–4 linked β-D-glucopyranoside (β-D-Glucop^I)
102.5/4.5	Internal 1–4 linked β-D-xylopyranoside (β-D-Xylp^I)
105.3/6.6	$C_{2,6}/H_{2,6}$ in etherified syringyl units (S)
111.4/7.0	C_2/H_2 in guaiacyl units (G)
115/6.7	C_5/H_5 in guaiacyl units (G)
119.5/6.9	C_6/H_6 in guaiacyl units (G)
128.0/7.2	$C_{2,6}/H_{2,6}$ in *p*-hydroxyphenyl units (H)
145.0/7.5	*p*-Coumaric (*p*-CA) and ferulic (FA) acids

The typical chemical shift assignments of 1H–13C HSQC NMR spectra of biomass in PyCl-d6 : DMSO-d6 system (*Kim & Ralph, 2010*)



1H–13C 2D HSQC NMR spectra corn stover in PyCl-d6 - DMSO-d6 solvent system (*Foston et al, 2016*).

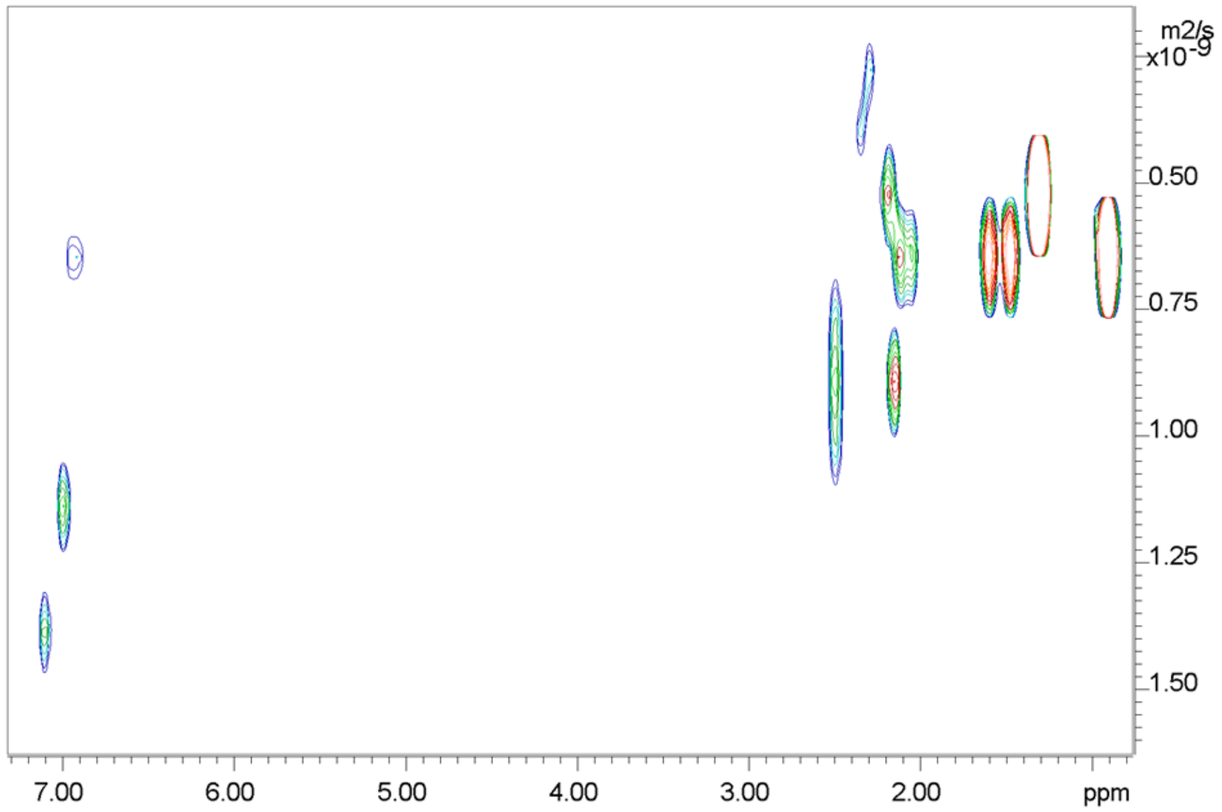
2D NMR



Main structures present in bamboo lignin (Wen et al,2013):
(**A**) β-O-4 alkyl-aryl ethers; (**A**') β-O-4 alkyl-aryl ethers with acylated γ-OH with *p*-coumaric acid; (**A**'') Cα-oxidized β-O-4' structures; (**B**) resinols; (**B**') tetrahydrofuran; (**C**) phenylcoumarans; (**D**) spirodienones; (**E**) α,β-diaryl ethers; (**T**) a incorporation of tricin into the lignin polymer through a G-type β-O-4 linkage; (**I**) *p*-hydroxycinnamyl alcohol end-groups; (**I**') *p*-hydroxycinnamyl alcohol end-groups acylated at the γ-OH; (**J**) cinnamyl aldehyde end-groups; (PCA) *p*-coumarates; (FA) ferulates; (**H**) *p*-hydroxyphenyl units; (**G**) guaiacyl units; (**S**) syringyl units; (**S**') oxidized syringyl units bearing a carbonyl at Cα

DOSY, *diffusion ordered spectroscopy*

- a pseudo-two-dimensional technique in which proton chemical shifts represent one dimension and translational diffusion coefficients the other
- signals of different types separated according to their diffusion coefficients
- analysis of complex and heterogeneous mixtures such as crude oil samples or food and drinks
- it is used in the research of proton-ligand molecular interactions and the characterization of reactive intermediates
- suppression of the effects of eddy and convection currents can be achieved by applying a suitable pulse sequence
- an advanced diffusion NMR method PSYCHE-iDOSY (Pure Shift Yielded by CHirp Excitation DOSY)
- information on the mass, size and composition of the mixture



A typical DOSY spectrum of oil sample

Hydrodynamic radii and molecular masses of mixture constituents

- ✓ Hydrodynamic radii of asphaltenes were calculated from diffusion coefficients by applying Stokes-Einstein equation

Boltzmann constant

Thermodynamic temperature

$$D = \frac{k_B T}{6 \pi \eta R_H}$$

Visosity

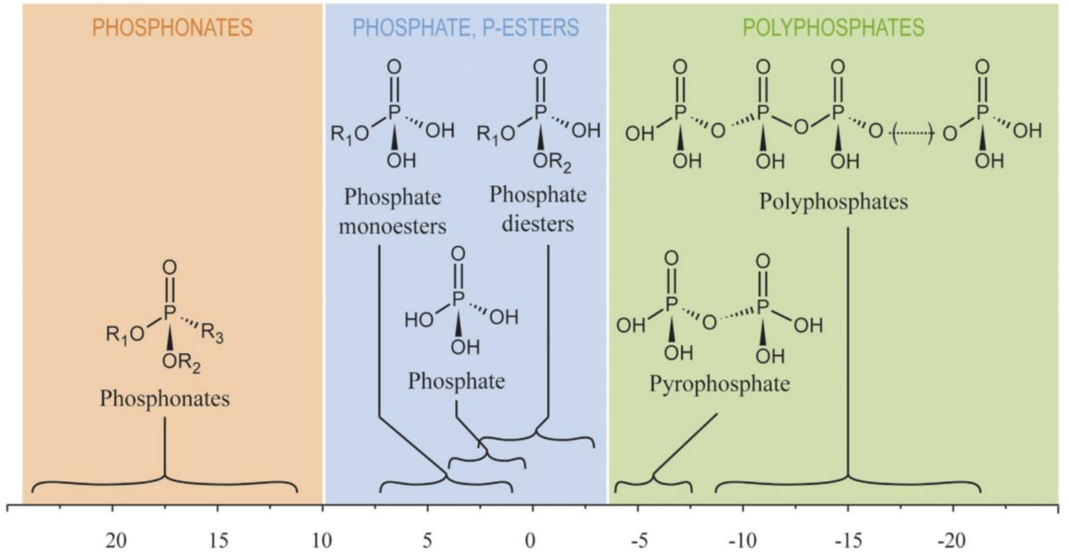
Hydrodynamic radius

- ✓ Molecular masses were estimated from Evans et al model

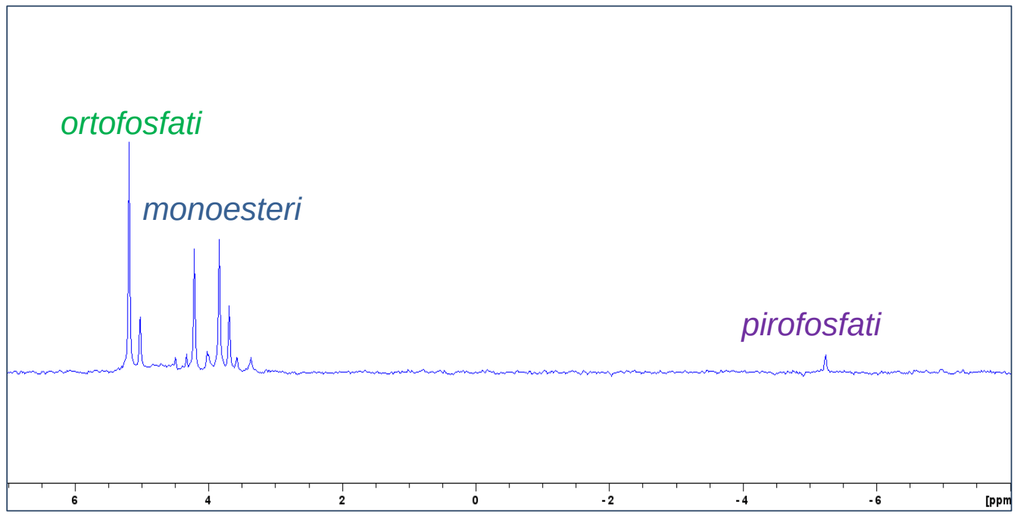
$$D = \frac{k_B T \left(\frac{3\alpha}{2} + \frac{1}{1+\alpha} \right)}{6\pi\eta \sqrt[3]{\frac{3M_W}{4\pi\rho_{eff} N_A}}}, \alpha = \sqrt[3]{\frac{M_{Ws}}{M_W}}$$

Solution ^{31}P NMR spectroscopy of NaOH–EDTA extract of lignocellulose

- phosphorus is one of the most important essential macronutrient for plant
- it is important for appropriate physiological and biochemical conditions in plants
- inorganic phosphorus is present in plants in the form of orthophosphates, pyrophosphates and polyphosphates
- organic phosphorus in plants is usually in the form of phosphate monoesters and diesters, α- and β-glycerophosphates and organic polyphosphates
- the ^{31}P NMR can identify and quantify most of the organic and inorganic forms of phosphorus
- ^{31}P NMR spectroscopy offers major advances in the determination of hydroxyl groups in a single spectrum with high signal resolution compared to conventional wet chemical methods
- the phenolic hydroxyl groups attached to syringyl, guaiacyl and p-hydroxyphenyl units can be distinguished in a short experimental time with small sample amounts
- this method has some limitations, such as the presence of amine groups, which can interfere with the quantification of hydroxyl groups in some cases.



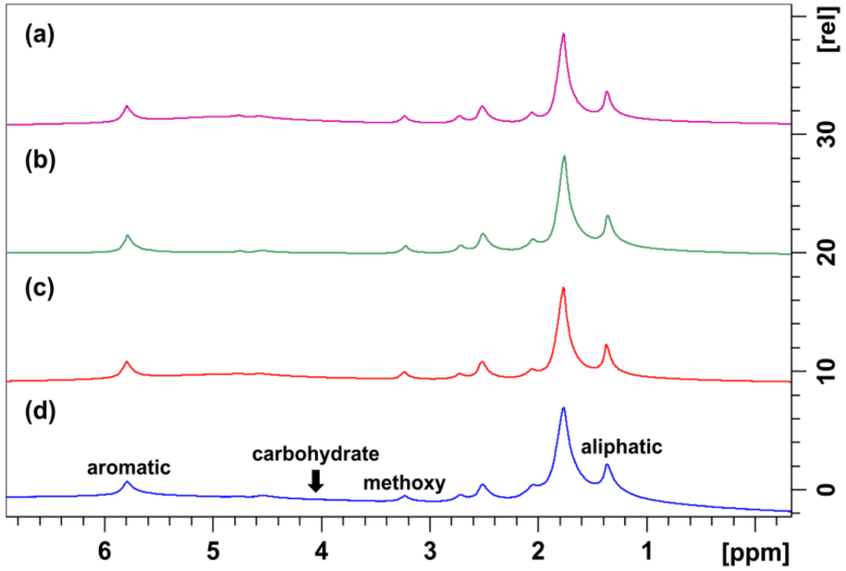
Tipični spektar ^{1}H NMR lipida ekstrahiranih iz lignocelulozne mase



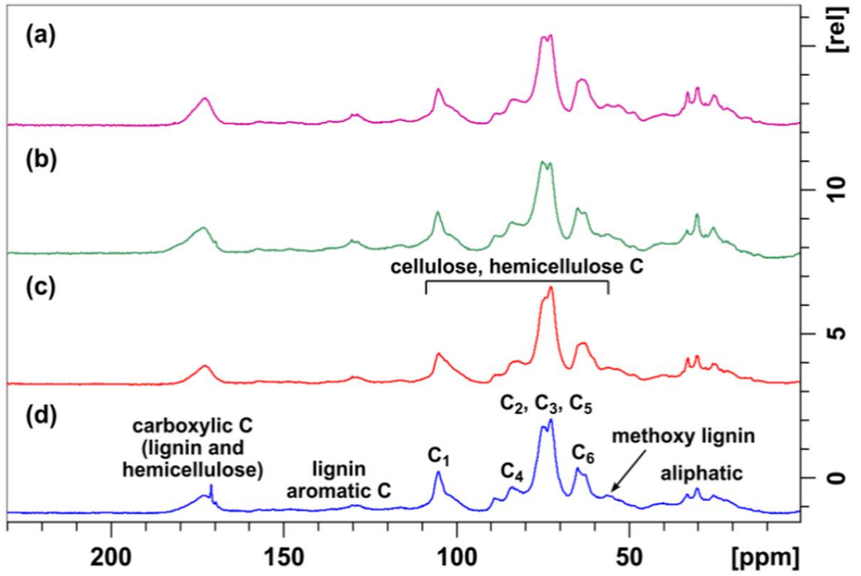
Tipični spektar ^{31}P NMR fosfata ekstrahiranih iz lignocelulozne mase

Solid state nmr

- the main advantage of solid state NMR is that it measures polymers in its native state (without destroying its native structure) compared to liquid state NMR analysis
- various solid state NMR techniques are employed in structural and mechanistic studies of lignocellulose material
- according to the literature, it has been evidenced that solid state NMR has become a promising non-destructive technique that provides a wealth of information on the structure, conformation, packing and polymorphism of lignocellulosic biomass and thus can serve as reliable technique for lignocellulose characterization



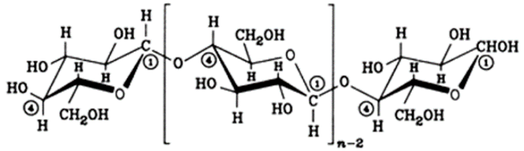
^{1}H CP MAS NMR spectra of brewery spent grain



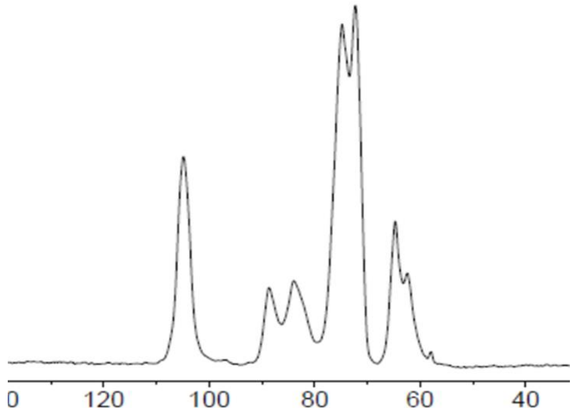
^{13}C CP MAS NMR spectra of brewery spent grain

Alkyl-C (0-45 ppm)/%	NCH/OCH_3 (45-65 ppm)/%	O-Alkyl C (65-90 ppm)/%	O-C-O (90-110 ppm)/%	Aromatic-C (110-145 ppm)/%	Aromatic C-O (145-165 ppm)/%	COO/N-C=O (165-190ppm)/%	Total Al-C (0-110 ppm)/%	Total Ar-C (110-165 ppm)/%

Miscanthus



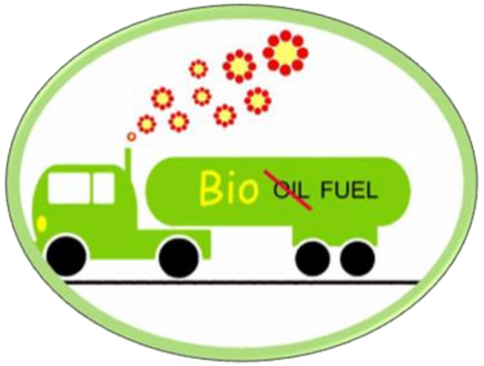
- generally, a cellulosic material has excellent properties such as low cytotoxicity, biocompatibility, good mechanical properties, high chemical stability and low cost, which makes them perfect candidates for many different applications
- one problem with crystalline cellulose materials is their low solubility in most solvents
- by splitting the C4 region of the spectrum into crystalline and amorphous parts it was possible to calculate CI (cellulose crystallinity index)

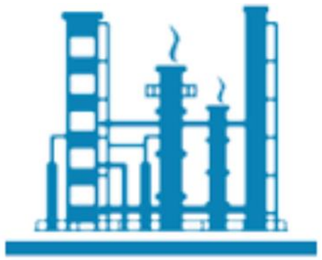


Typical ^{13}C CP MAS NMR spectrum of cellulose isolated from miscanthus

Alternative fuels

- global climate changes
- rising crude oil price
- rapid depletion of fossil fuel reserves
- development of alternative fuels that could partially or fully replace the existing fossil fuels
- el. energy, hydrogen, biofuel, synthetic and paraffin fuel, natural and liquefied petroleum gases
- an alternative fuel has to be technologically acceptable, economically competitive, environmentally friendly and easily accessible





✓Transport sector produces around 20% of the GHG (greenhouse gas) emissions in the EU (gCO_2eq/MJ)

Biodiesel

- biodiesel is now the most commonly used renewable energy source
- by definition a mixture of mono-alkyl esters of long chain fatty acids derived from different types of oils or animal fats
- commercially, it is produced by chemical transesterification of oil with alcohol in the presence of a catalyst
- previous studies showed that the combustion of biodiesel greatly reduced emissions
- based on the type of feedstock biodiesel can be categorised as first-, second- and third- generation

- **Edible oil and fats** — 1st

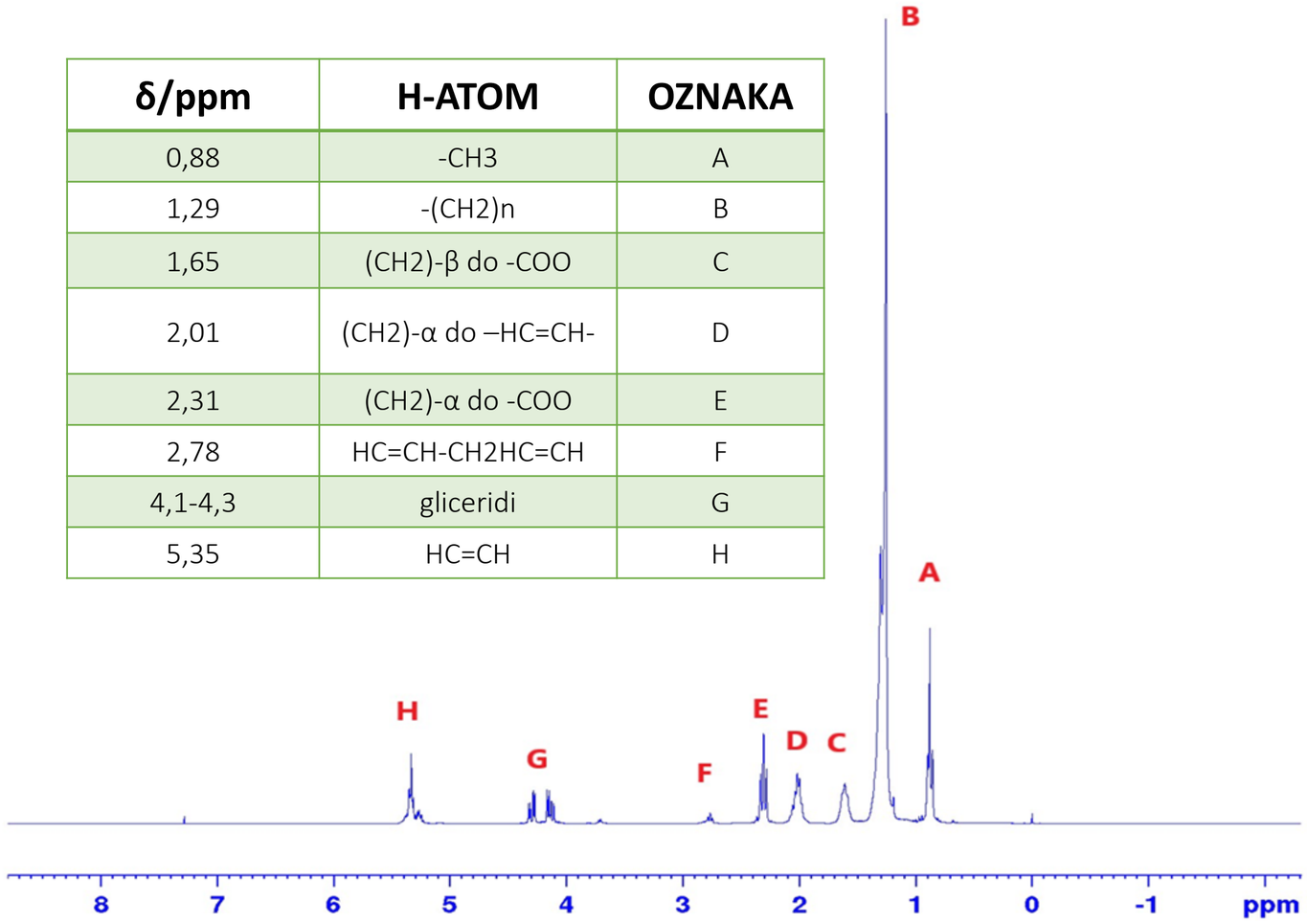
- **Non food crops** — 2nd

- **Algae** — 3rd

Biodiesel

- feedstock analysis
- monitoring the transesterification process
- content of fatty acid methyl esters (FAME)
- distribution of saturated and nonsaturated fatty acids
- triglycerides, monoglycerides and diglycerides
- content of free fatty acids
- iodine number
- study biodiesel degradation

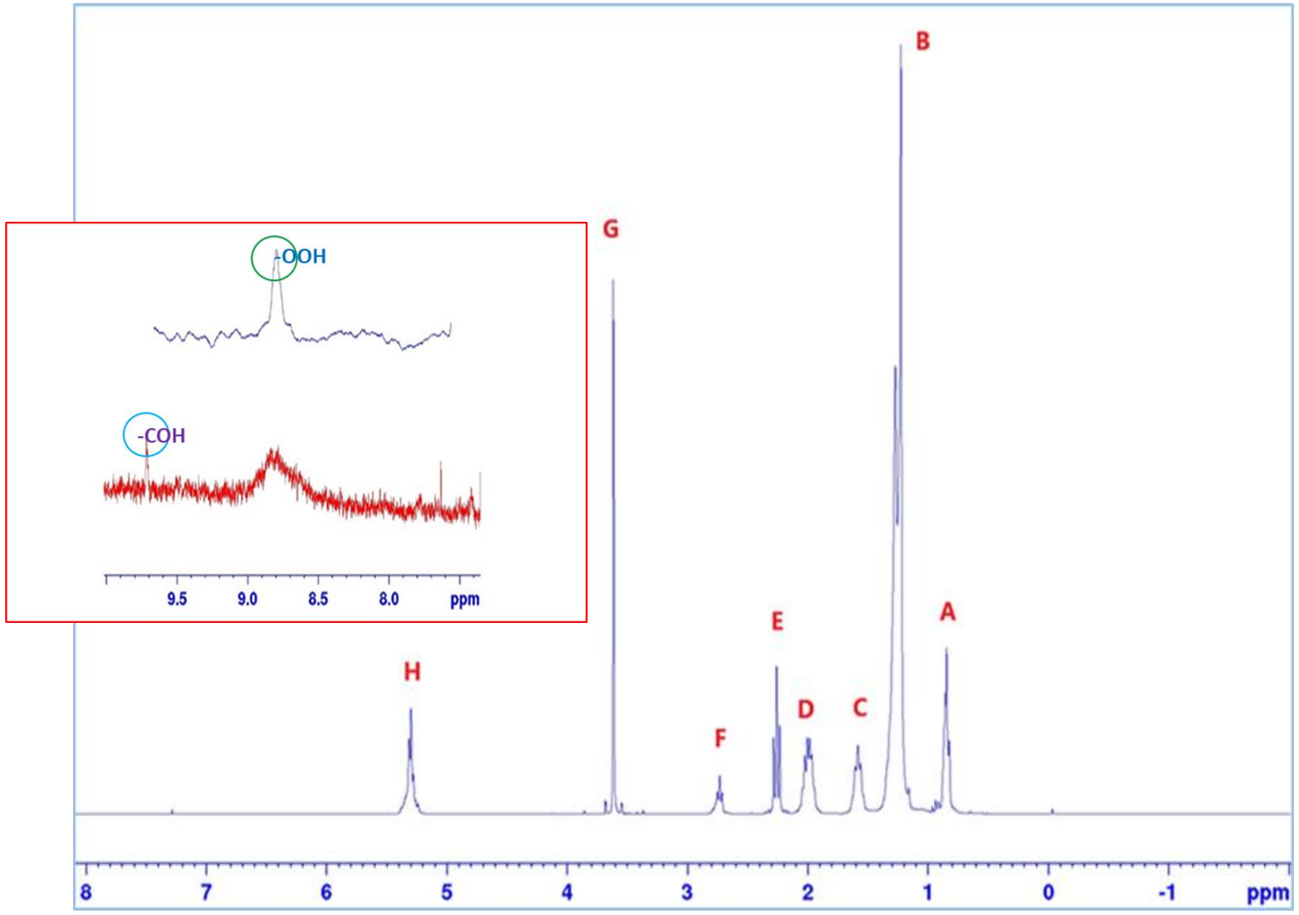
δ/ppm	H-ATOM	OZNAKA
0,88	-CH3	A
1,29	-(CH2)n	B
1,65	(CH2)-β do -COO	C
2,01	(CH2)-α do –HC=CH-	D
2,31	(CH2)-α do -COO	E
2,78	HC=CH-CH2HC=CH	F
4,1-4,3	gliceridi	G
5,35	HC=CH	H



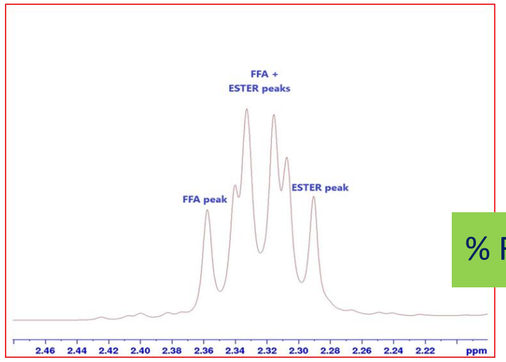
- A. Sander, M.A.Košćak, D.Kosir, N.Milosavljević,J.Parlov Vuković, L.Magić ; *Renewable Energy* **118** (2018) 752-760
- A. Sander, A. Petračić, J. Parlov Vuković, L. Husinec, *Separations* **7** (2020) 2; 22, 18.

Biodiesel oxidation stability

- stability is one of the important criteria concerning fuel properties
- lower than common diesel fuel
- biodiesel obtained from different oil sources has different physico-chemical characteristics and chemical compositions
- during storage in the presence of air, heat, light and pro-oxidants oxidative degradation can occur, which leads to the formation of insoluble sediments that can negatively affect engine operability and reduces the quality of lubrication
- result of the oxidation: acids, aldehydes, ketones, peroxides and alcohols

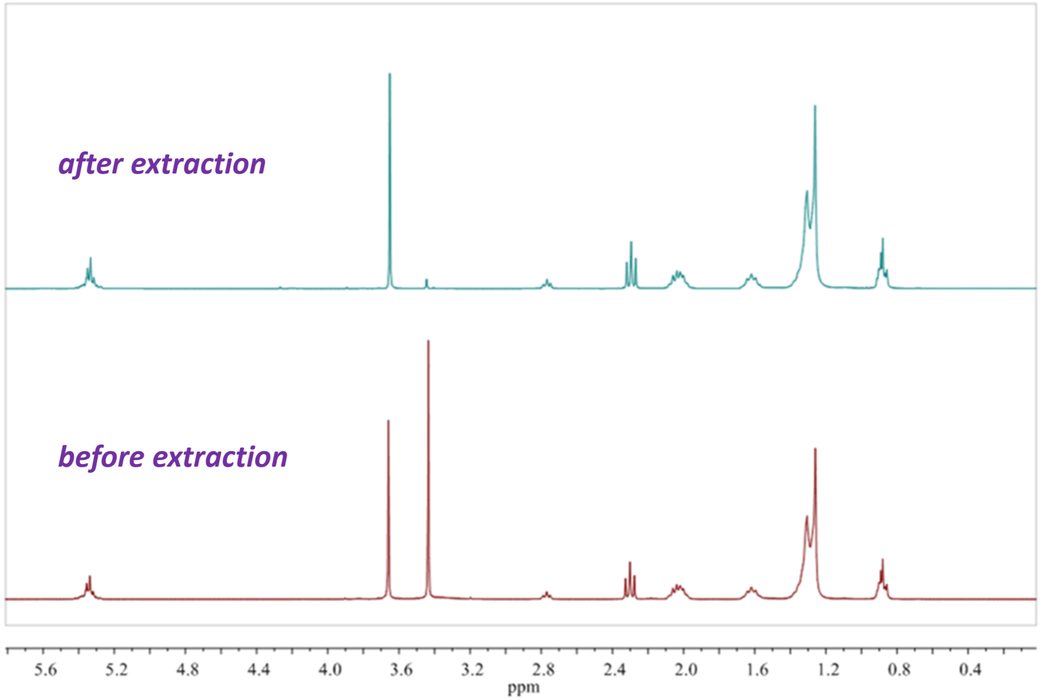


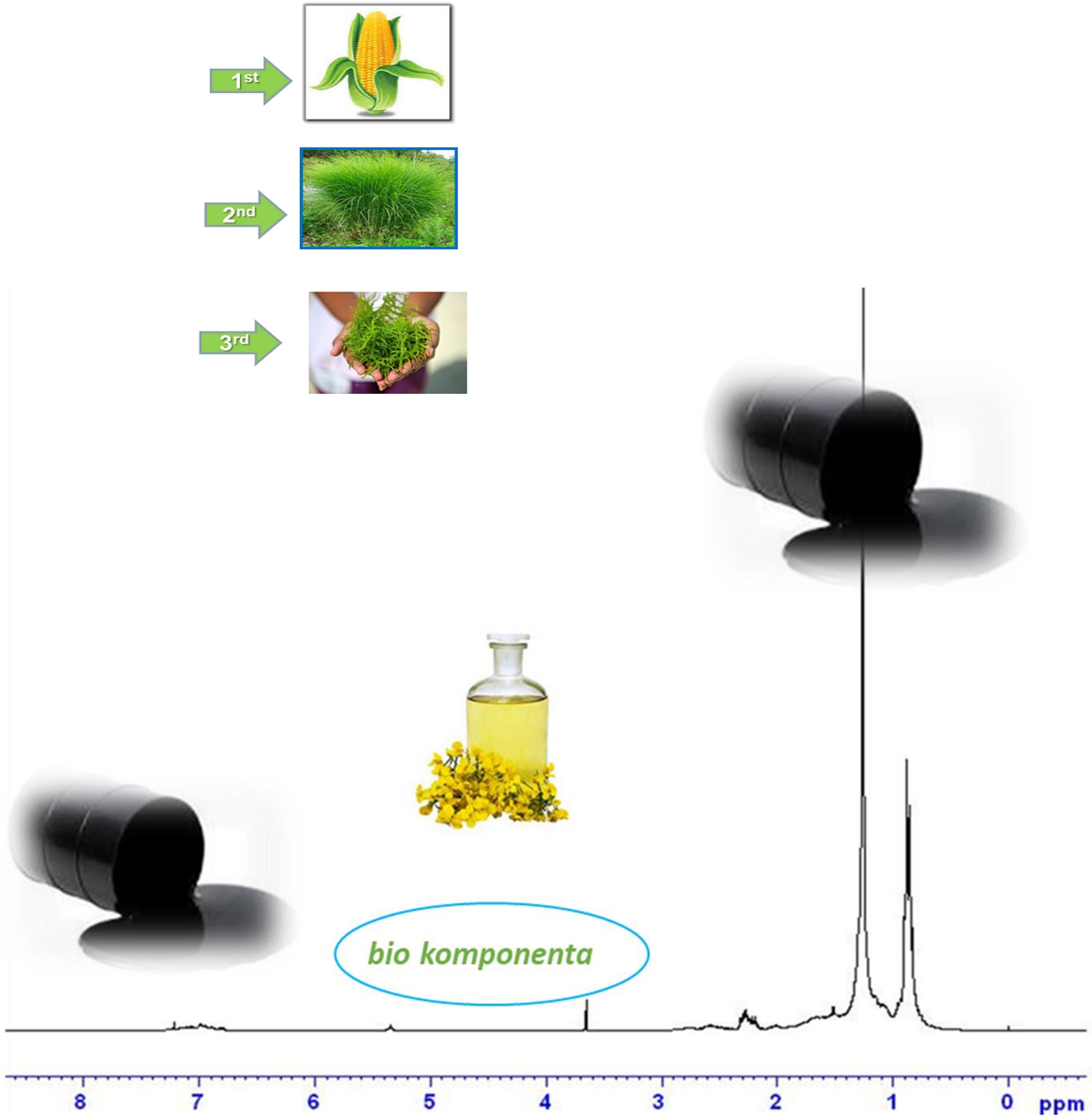
- a higher content of FFA affects the loss during refining, lower quality and stability of the oil
- saponification can occur, which affects the reduction of catalyst activity



$$\% \text{ FFA} = \frac{4\ x\ područje\ signala\ \alpha{-}CH2}{ukupno\ područje\ \alpha{-}CH2\ FFA\ i\ estera}$$

- ^{1}H NMR spectra of biodiesel synthesized from coffee grounds even after purification
- 10-15% w/w of coffee beans is oil
- Coffee waste represents a great potential for biodiesel production





Typical ^{1}H NMR spectrum of diesel with biocomponent

- *many open questions still remain*
- *due to a complex, insoluble and disordered lignocellulose biomass structure, further improvements in lignocellulosic sample preparation and characterization are expected*
- *advancements in modern liquid- and solid-state NMR methods will enable further steps to clarify and elucidate unresolved structural data, polymorphism architecture, water-matrix interactions and packing of lignocellulosic material*
- *since lignocellulosic biomass represents the most promising feedstock for the future biofuels and value-added products production, their detailed structural characterisation will be even more important*

P. Novak
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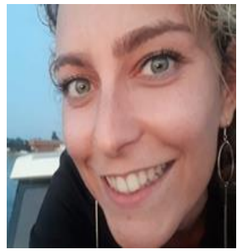
K. Zangger

J. Plavec



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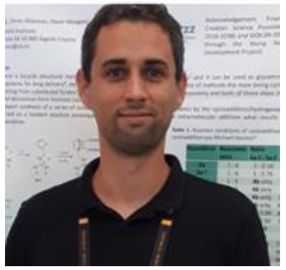

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Thank you!!!