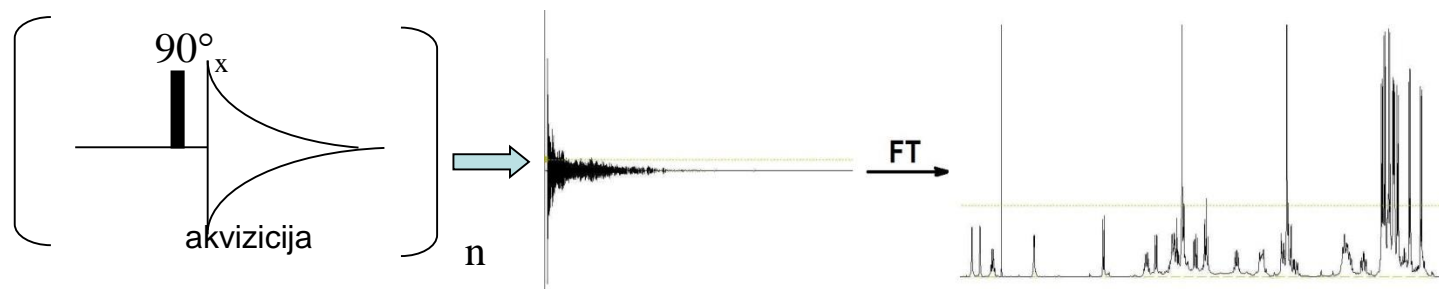
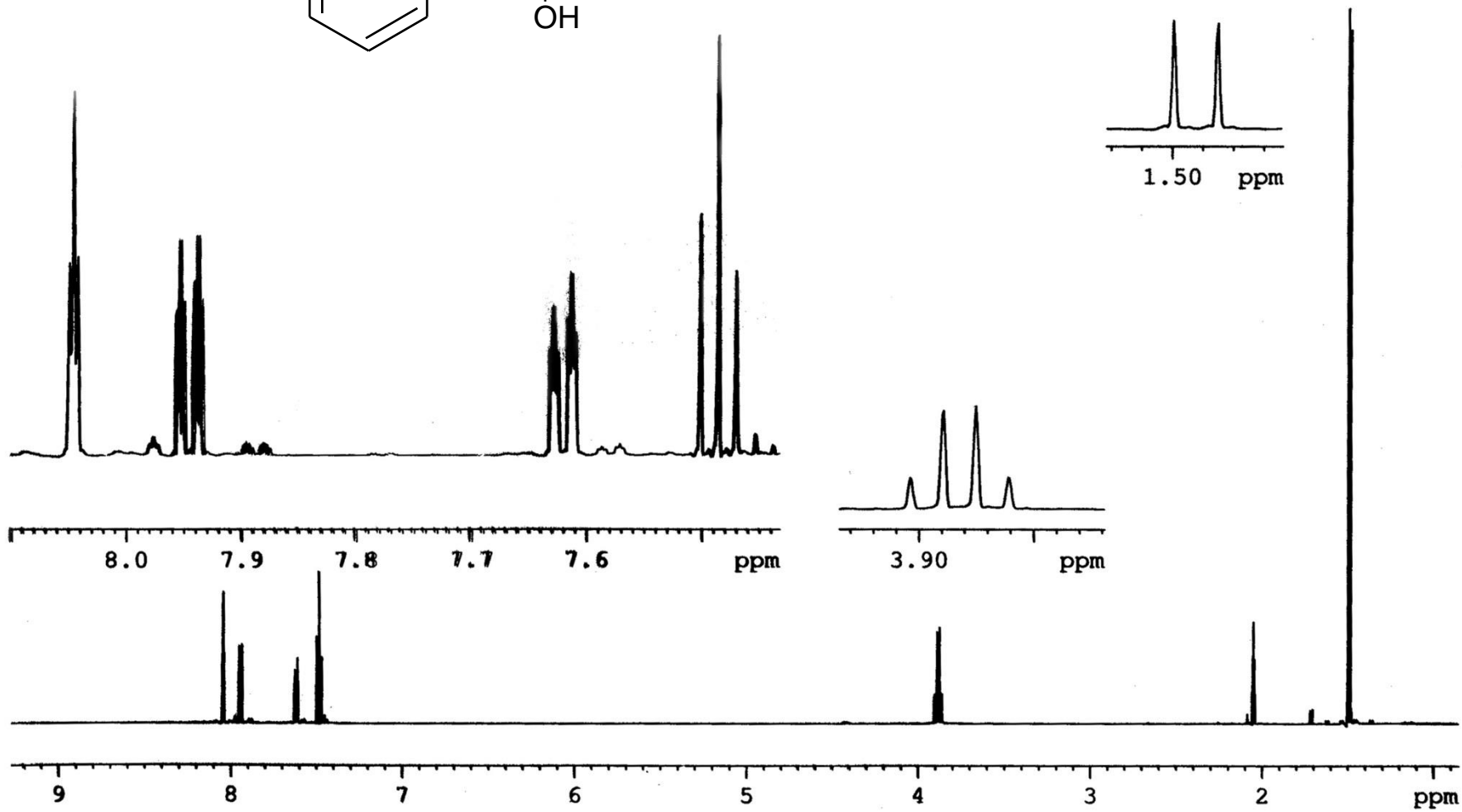
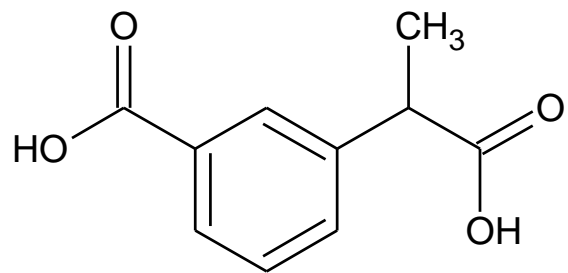


Jednodimenzijske tehnike



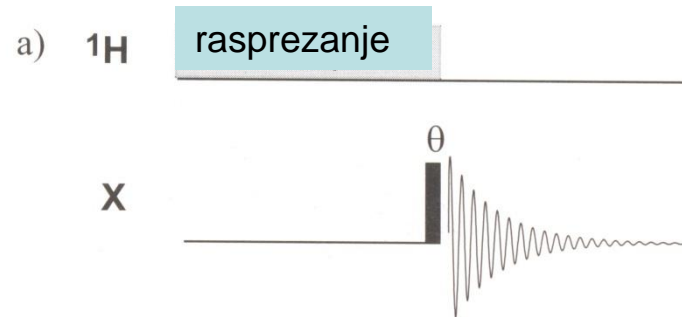
Osnovne višepulsne jednodimenzijske tehnike

Tehnika	primjena
J-modulirana spinska jeka ili APT (Attached proton test)	Editiranje spektara heterojezgri (^{13}C) prema multiplicitetu
INEPT (Insensitive nuclei enhanced by polarisation transfer)	pojačavanje signala jezgri s malim γ (^{13}C) pomoću prijenosa polarizacije sa jezgri s velikim γ (^1H , ^{19}F). Editiranje spektara heterojezgri prema multiplicitetu.
DEPT (Distortionless enhancement by polarisation transfer)	pojačavanje signala jezgri s malim γ (^{13}C) pomoću prijenosa polarizacije sa jezgri s velikim γ . Editiranje spektara heterojezgri prema multiplicitetu. Robusniji od INEPT-a. Nema informacija o kvarternim ^{13}C
DEPT-Q	Isto kao DEPT ali sadrži kvarterne ^{13}C
PENDANT	pojačavanje signala jezgri s malim γ (^{13}C) pomoću prijenosa polarizacije sa jezgri s velikim γ (^1H , ^{19}F). Editiranje spektara heterojezgri prema multiplicitetu. Sadrži informacije o kvarternim ^{13}C

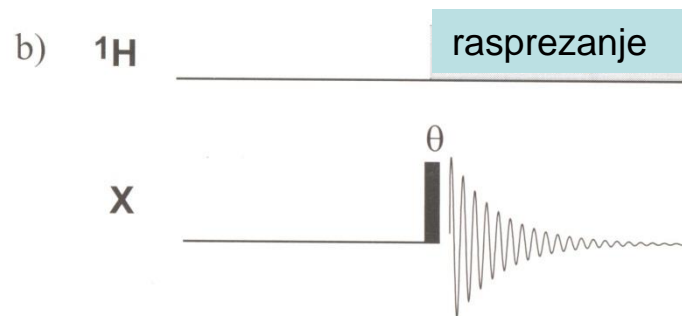


Tehnike dvostruke rezonancije

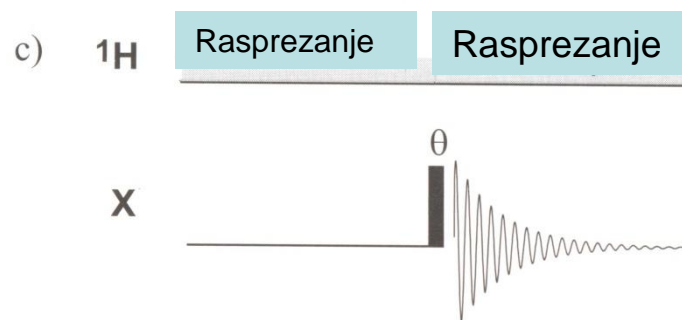
a) Spregnuti spektar s NOE



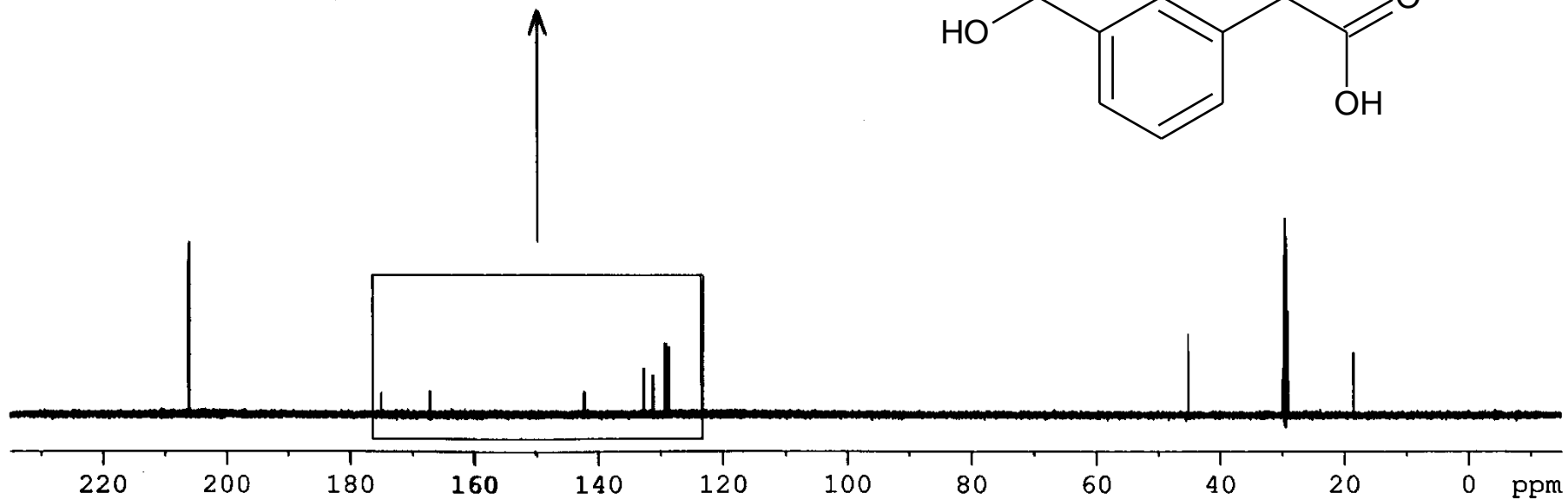
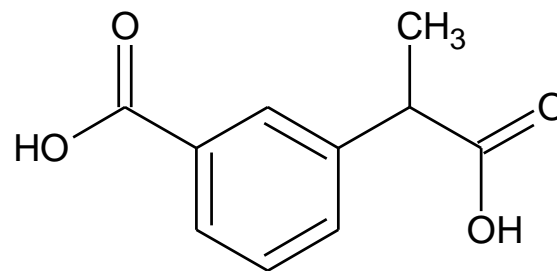
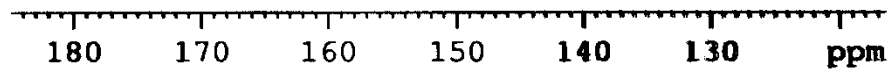
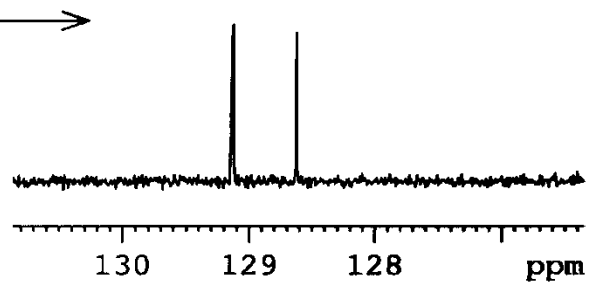
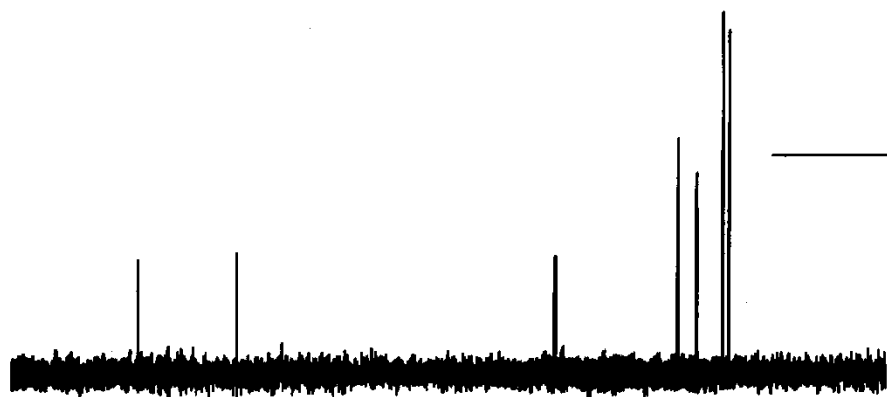
b) Raspregnuti spektar bez NOE

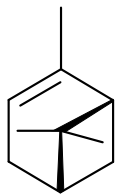


c) Raspregnuti spektar s NOE (rf) polje primjenjeno s dvije različite snage-power gated



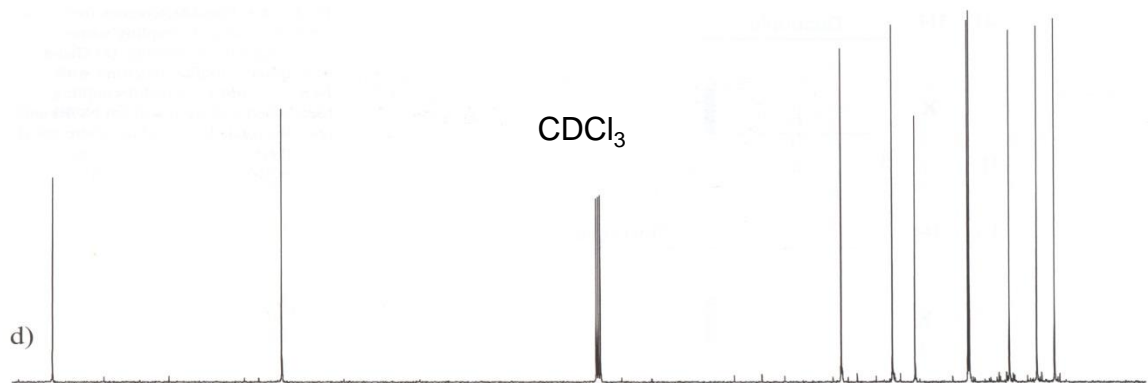
13C proton raspregnuti spektar
otapalo aceton





α -pinen

d) Raspregnuti spektar s
NOE-power gated



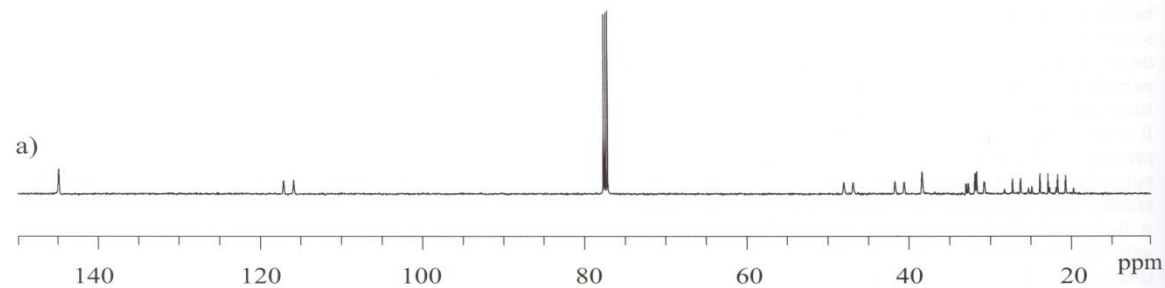
c) Raspregnuti spektar bez
NOE



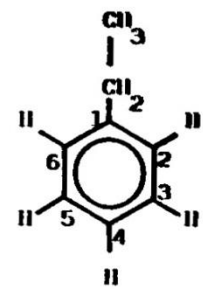
b) Spregnuti spektar s NOE



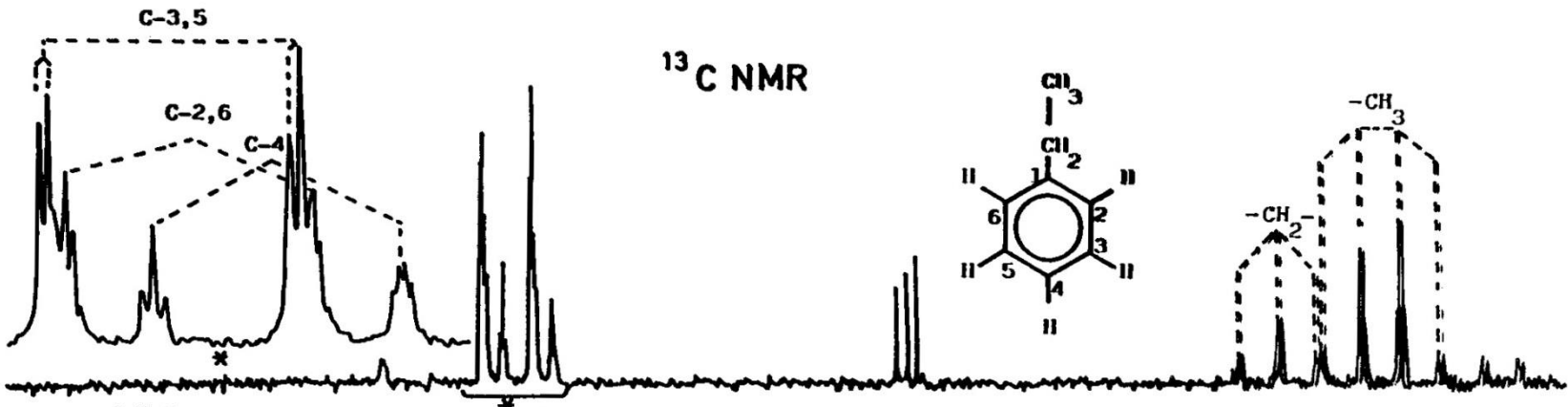
a) Spregnuti spektar bez NOE



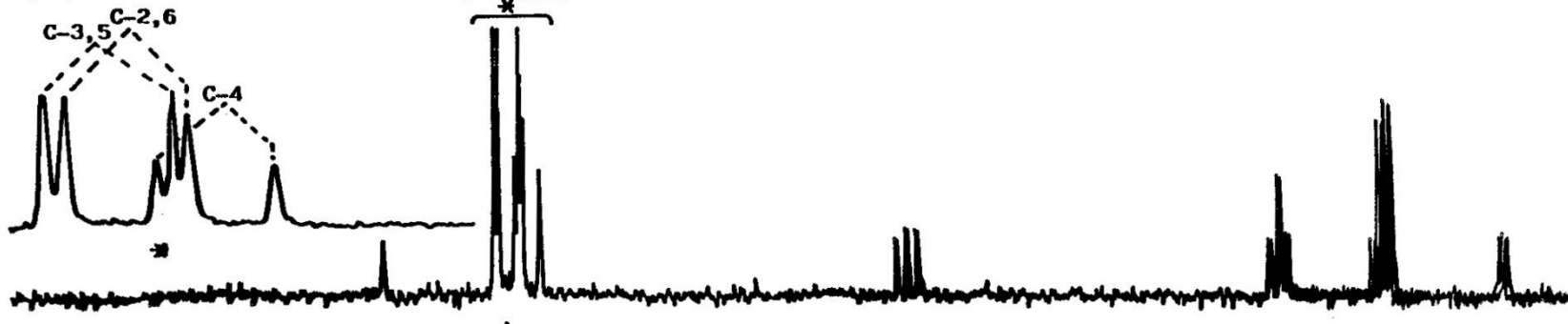
¹³C NMR



NOE



OFR



NNE



COM



C-1
C-3 C-2 C-4
(C-5) (C-6)

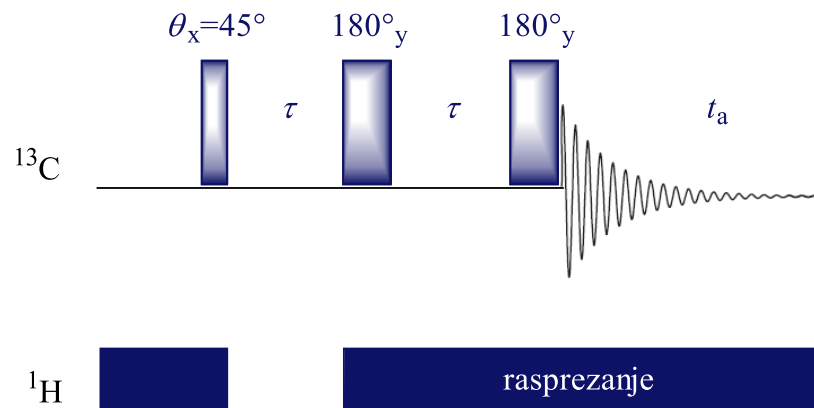
CDCl₃

-CH₂-

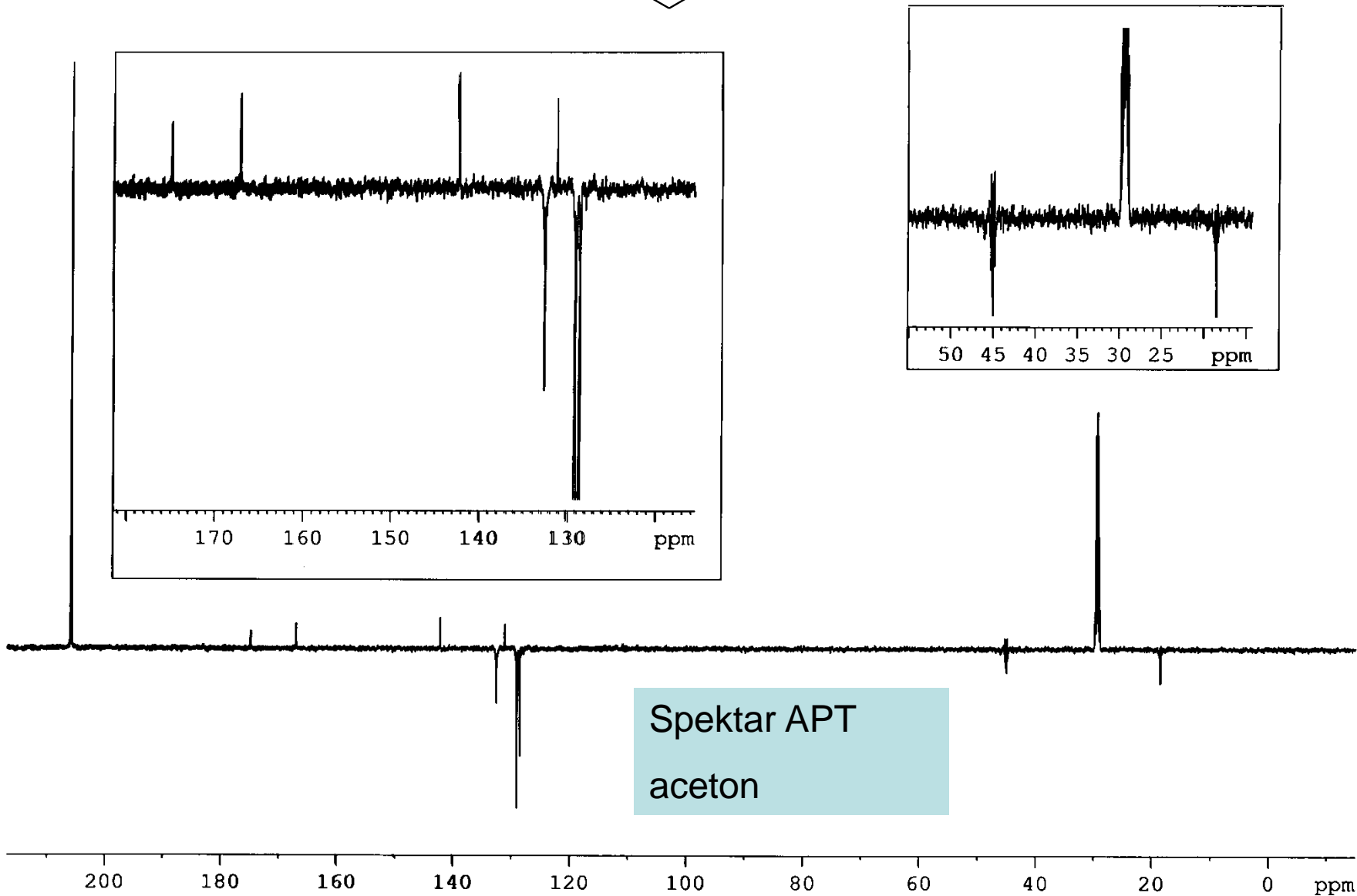
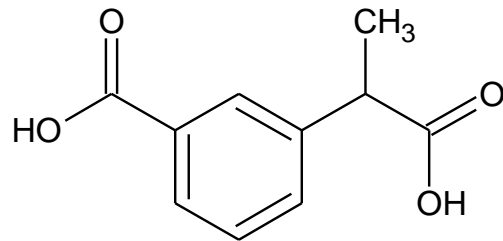
-CH₃

TMS

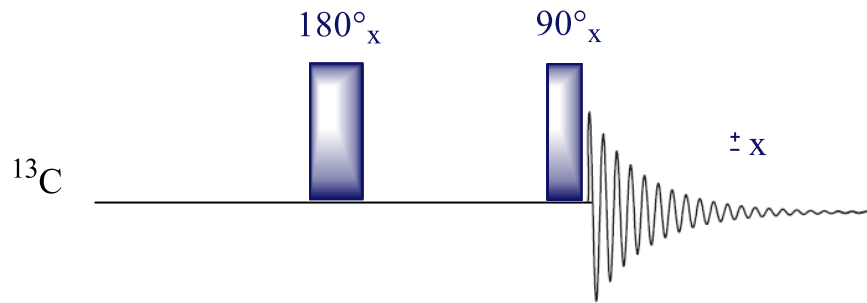
Tehnika APT (attached proton test)



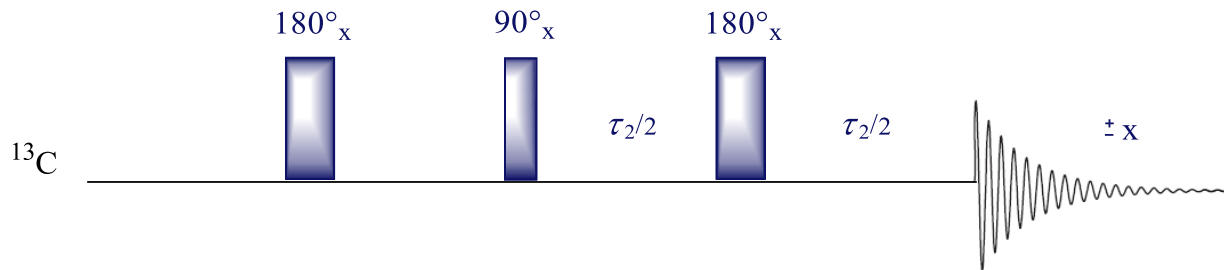
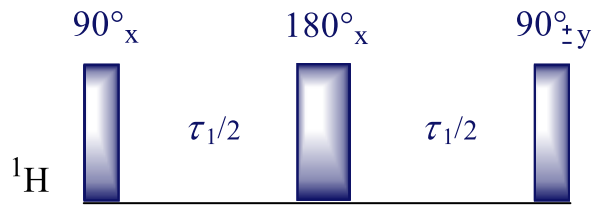
odabirom vremena evolucije τ moguće je razlikovati ugljikove atome prema multiplicitetu te odrediti broj protona direktno vezanih za pojedini ugljik.



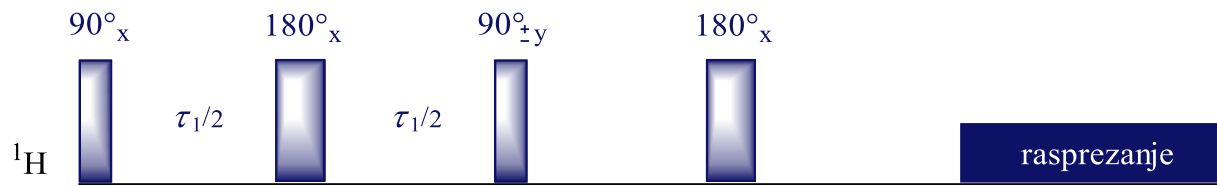
Tehnika INEPT



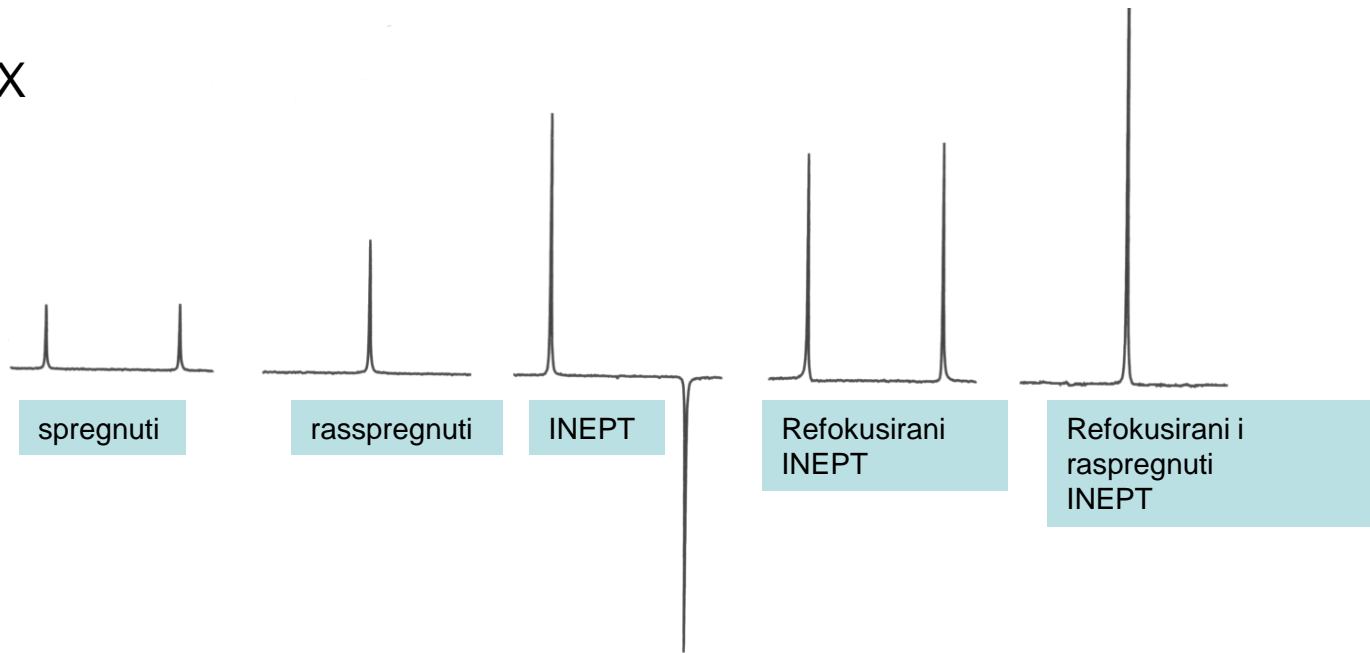
Prijenos polarizacije s ^1H na ^{13}C



Refokusirani INEPT



AX



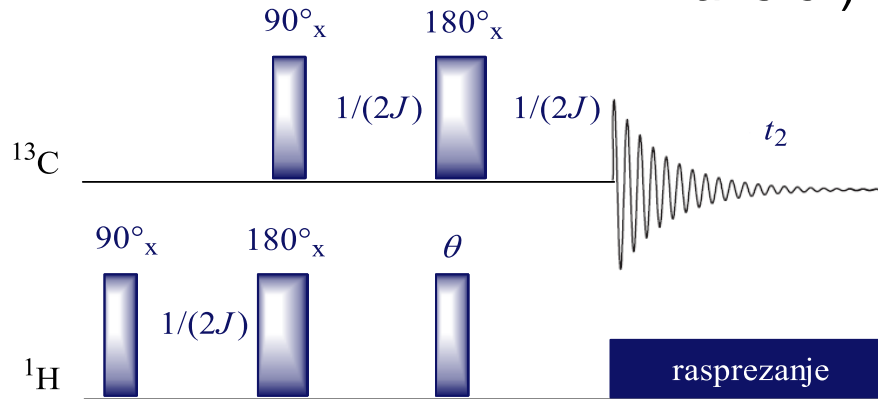
Povećanje osjetljivosti!

$$I_{\text{INEPT}} = I_0(\gamma_H/\gamma_X)$$

$$I_{\text{NOE}} = I_0(1 + \gamma_H/2\gamma_X)$$

X	¹³ C	¹⁵ N	²⁹ Si	³¹ P
I_{INEPT}	3.98	9.87	5.03	2.47
I_{NOE}	2.99	-3.94	-1.52	2.24

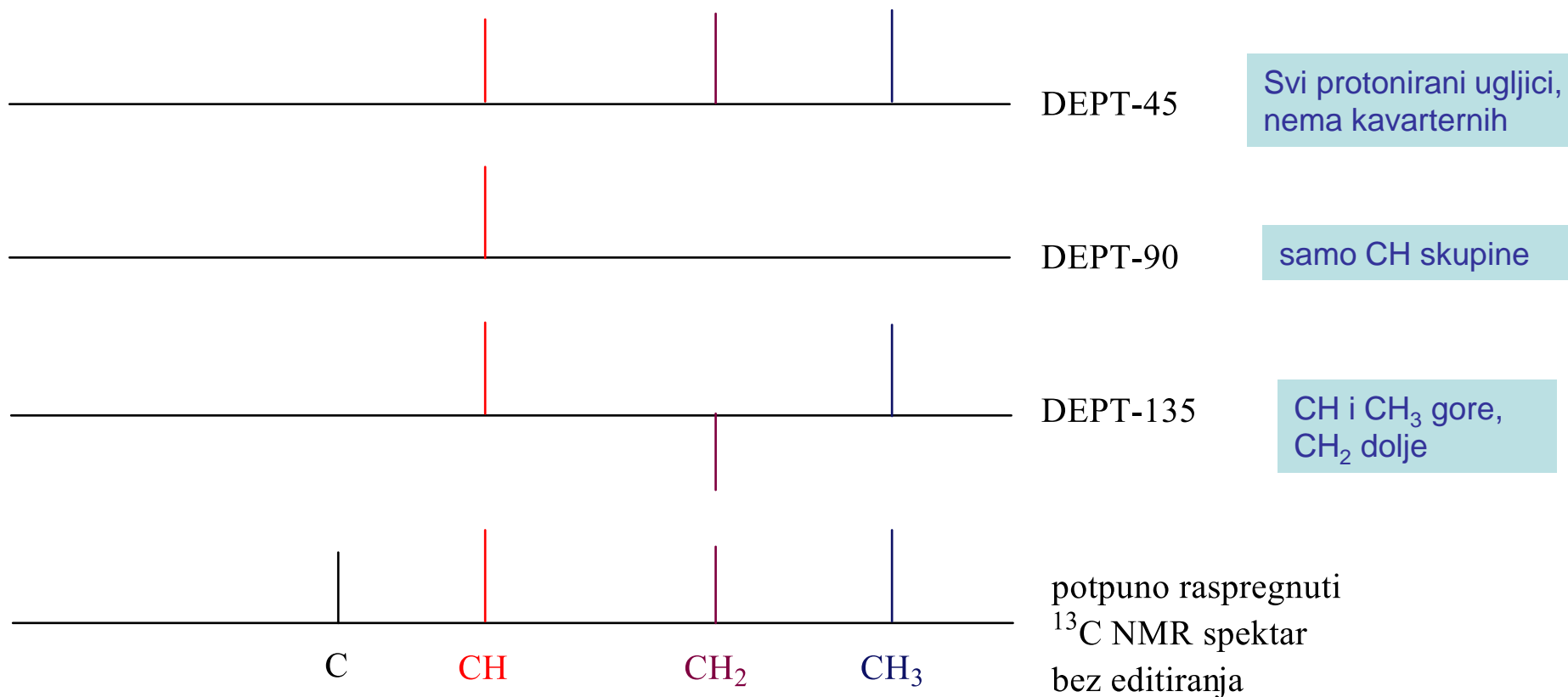
Tehnika DEPT (Distortionless Enhancement by Polarisation Transfer)



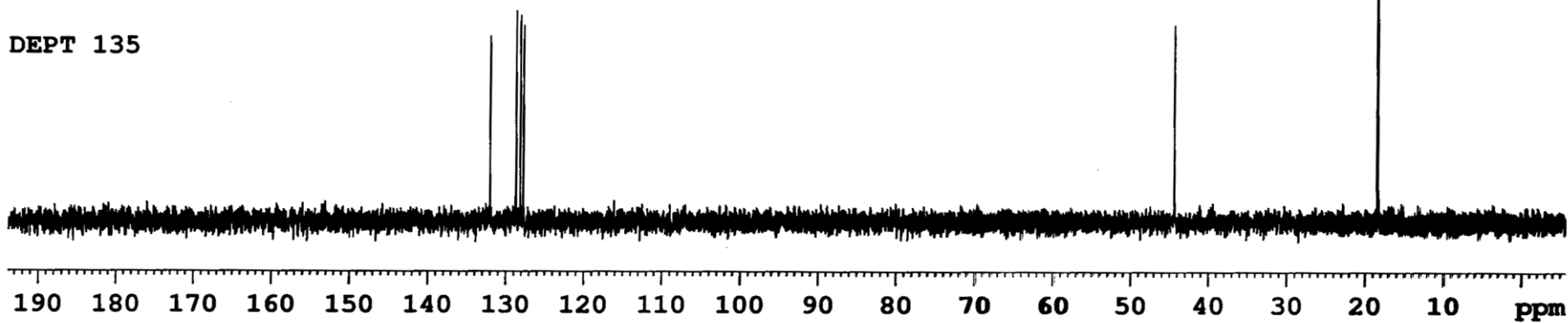
- Faza i intenzitet signala ugljikovih atoma ovisi o kutu θ
- Slično kao i kod INEPT-a, no s tom razlikom što se kod DEPT-a editiranje postiže različitim kutem θ , a ne vrijednostima τ
- Stoga je i učinkovitost editiranja veća za DEPT

	DEPT-45	DEPT-90	DEPT-135
XH	+	+	+
XH ₂	+	0	-
XH ₃	+	0	+

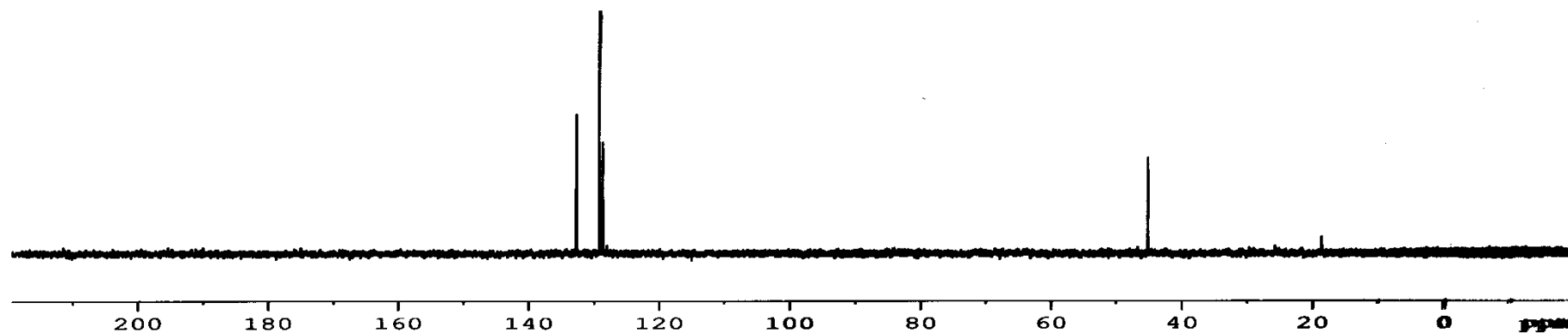
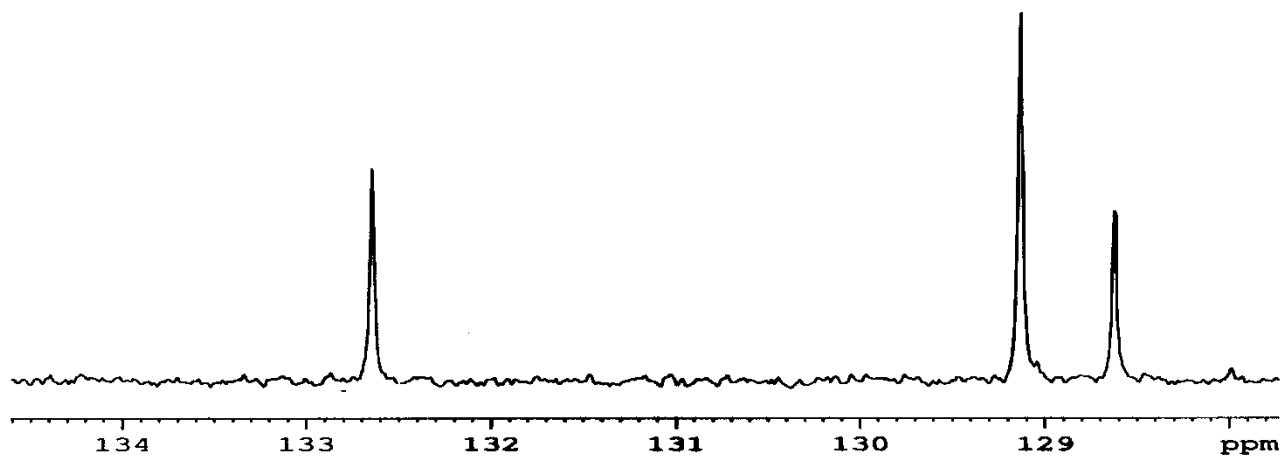
Vrste Tehnike DEPT



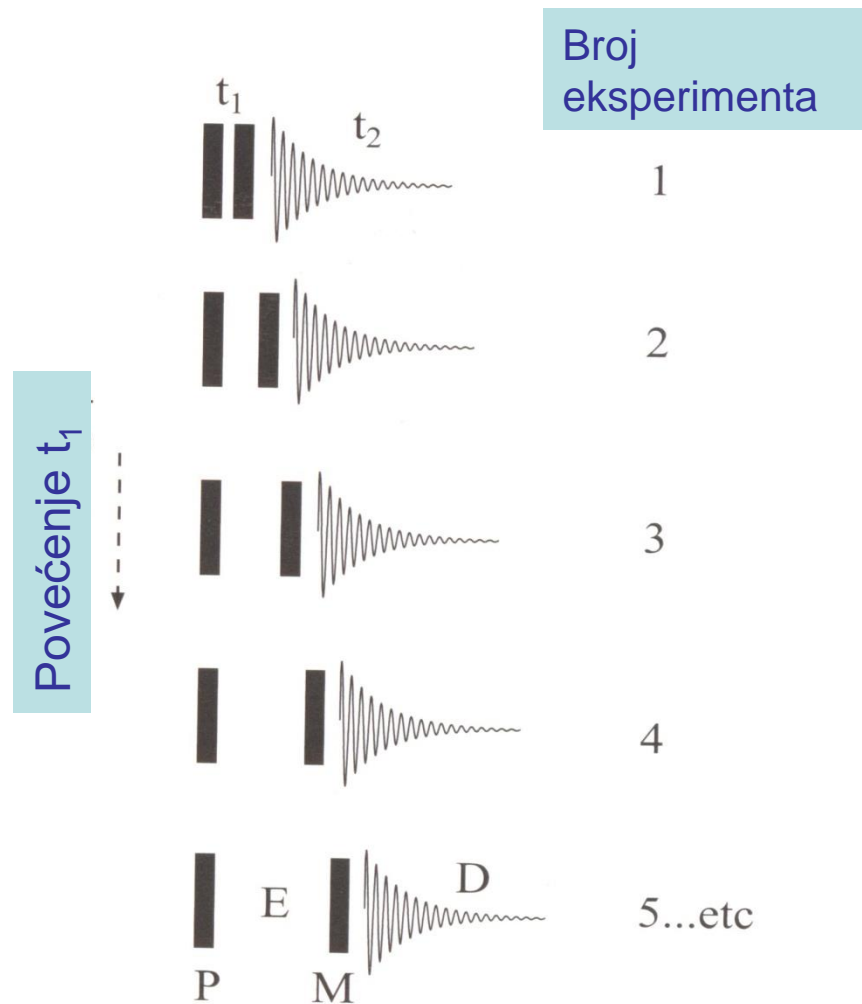
DEPT 135



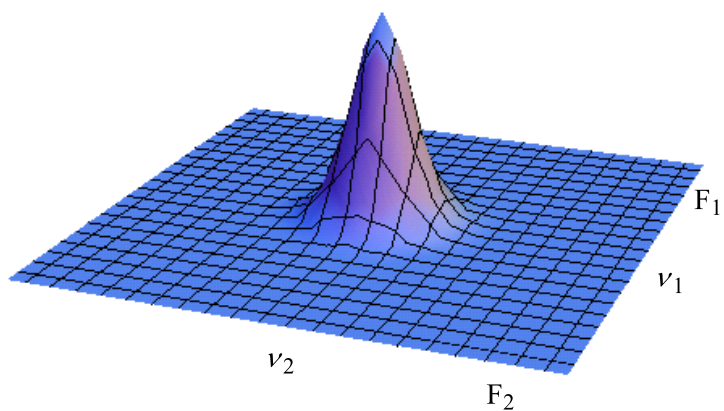
DEPT 90 spekter
otapalo acetone



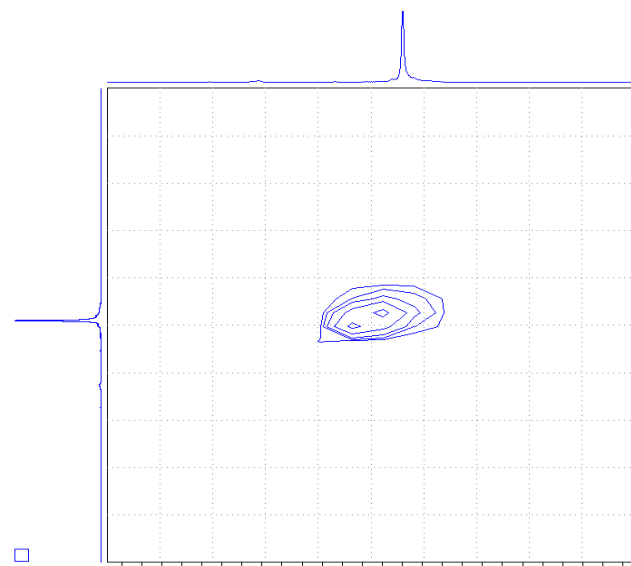
Opća shema za sakupljanje podataka 2D



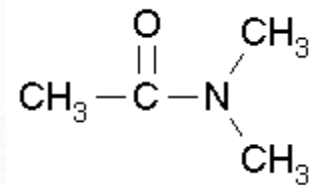
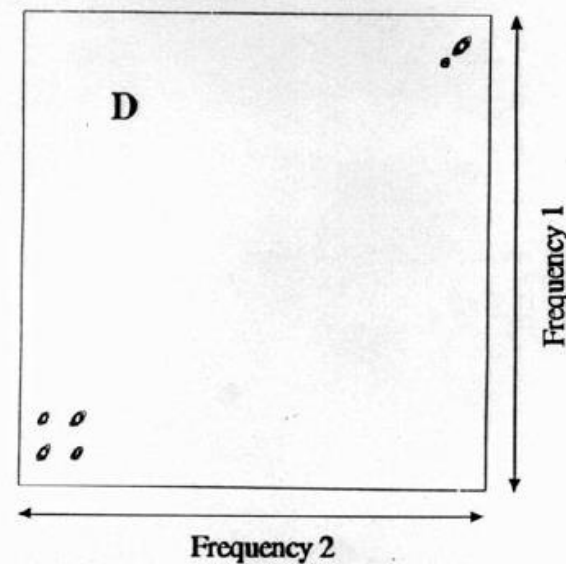
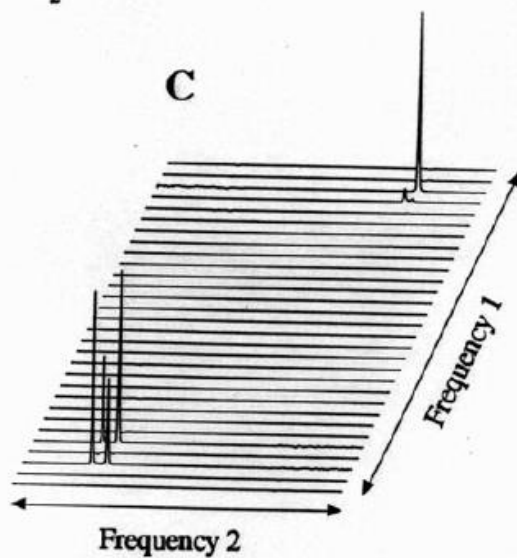
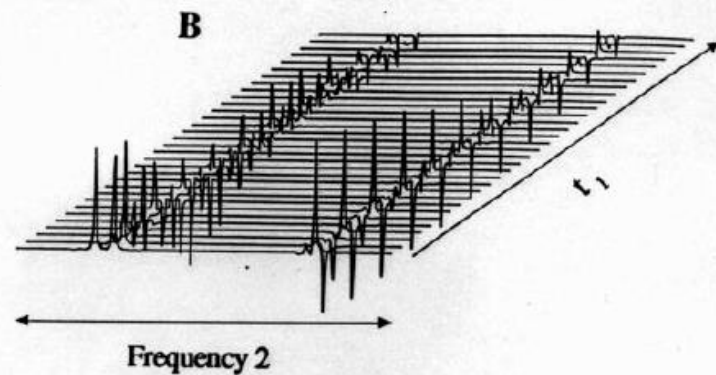
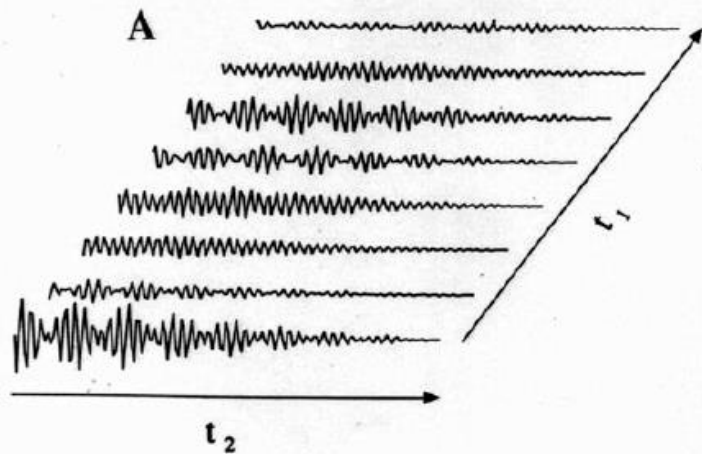
Shematski prikaz signala u dvodimenzijском спекtru NMR

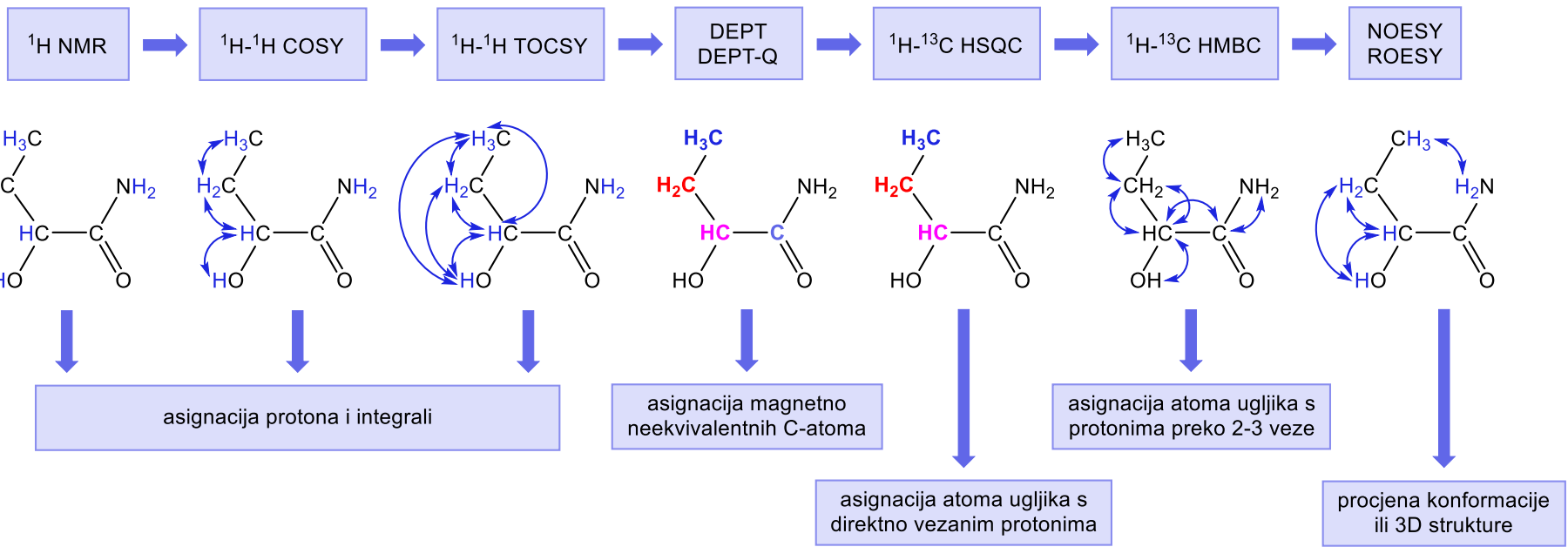


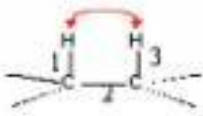
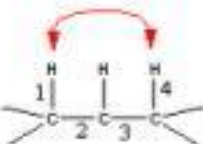
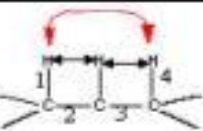
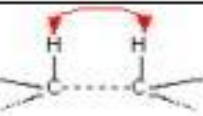
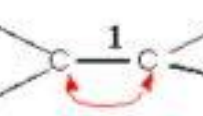
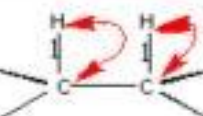
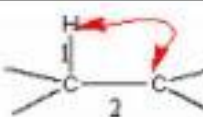
trodimenzijski



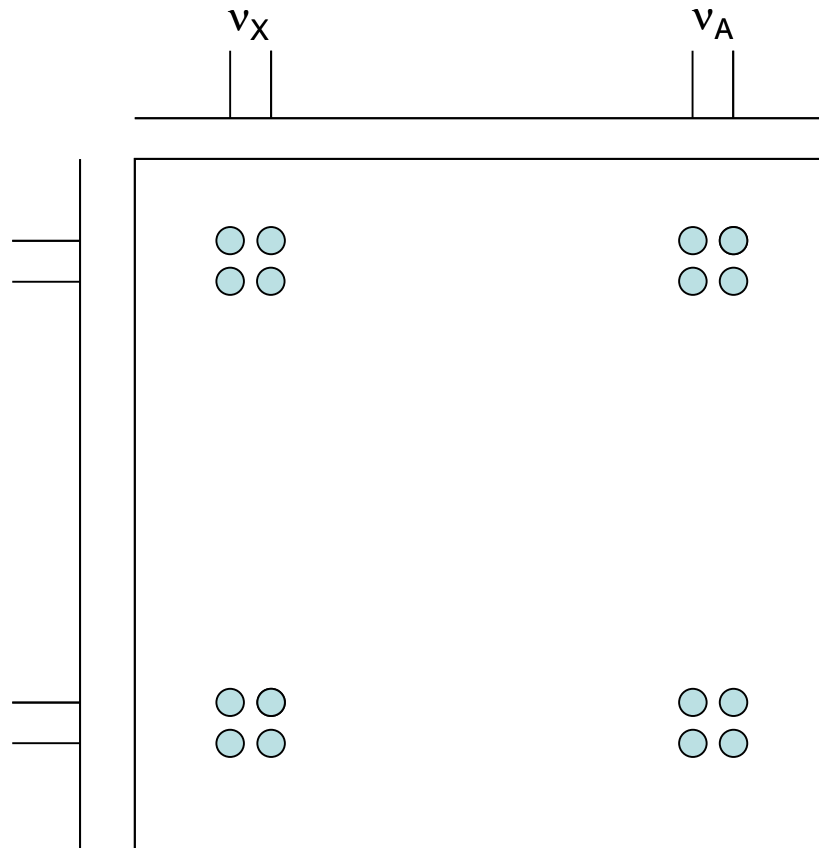
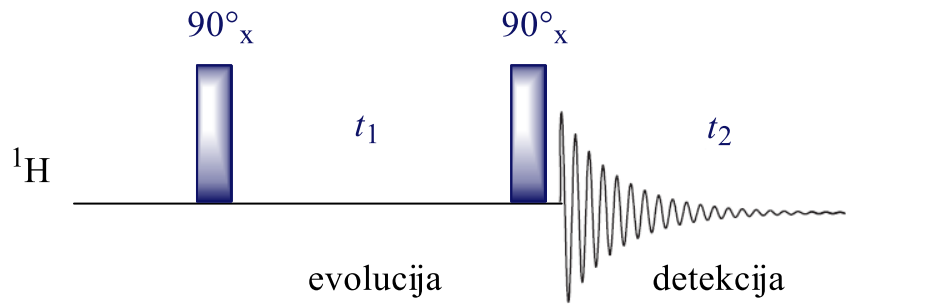
konturni





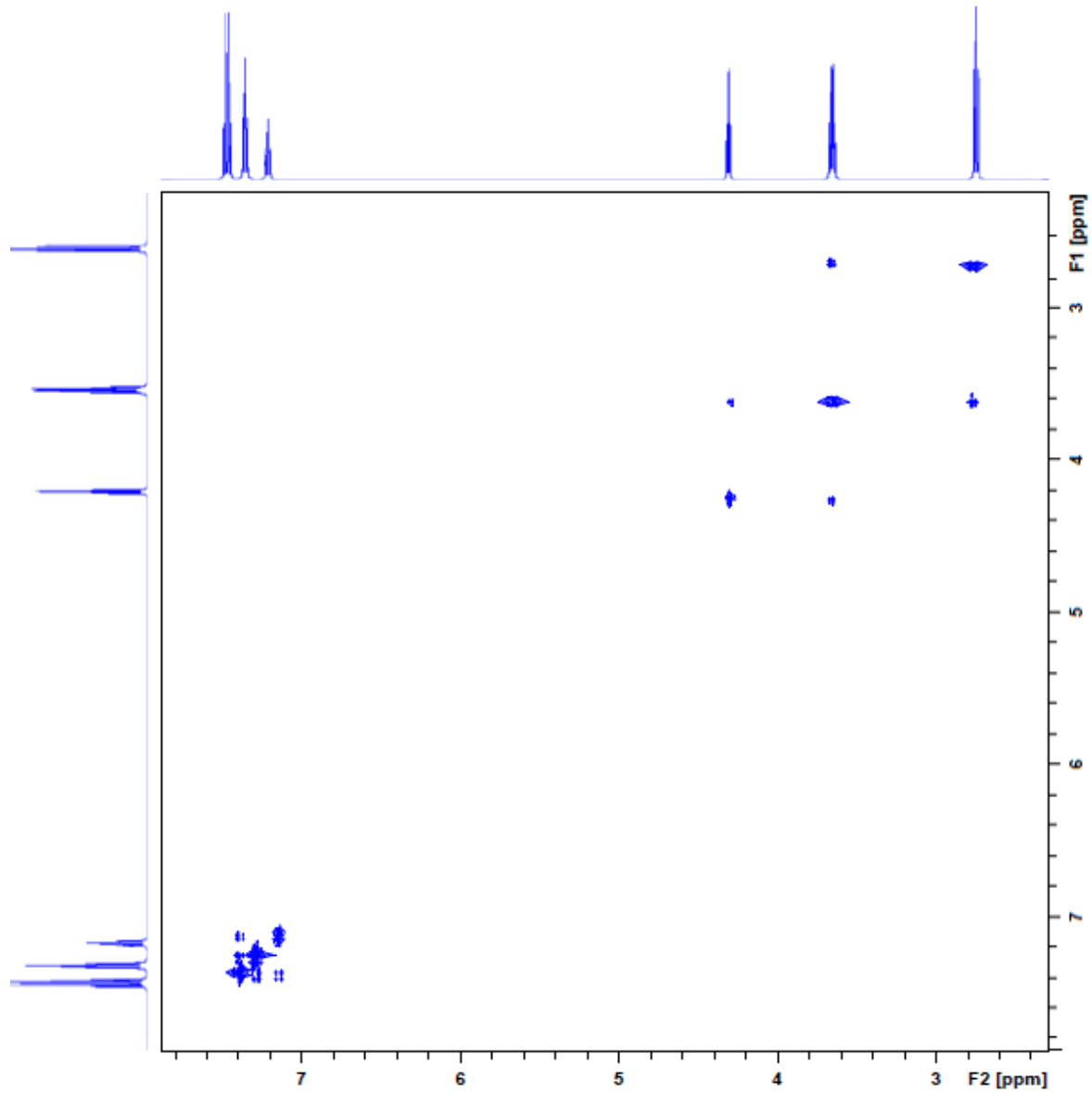
EKSPERIMENT	F2 DIMENZIJA	F1 DIMENZIJA	INFORMACIJE
Homonuklearne korelacije			
COSY TOCSY	$\epsilon_H \cdot J_{HH}$	$\epsilon_H \cdot J_{HH}$	 nJ_{HH} ($n \leq 3$)
COSY LR TOCSY	$\epsilon_H \cdot J_{HH}$	$\epsilon_H \cdot J_{HH}$	 nJ_{HH} ($n > 3$)
Relayed COSY	$\epsilon_H \cdot J_{HH}$	$\epsilon_H \cdot J_{HH}$	 nJ_{HH} ($n > 3$)
NOESY ROESY	$\epsilon_H \cdot J_{HH}$	$\epsilon_H \cdot J_{HH}$	 N.O.E.
2D INADEQUATE	$\delta_C \cdot J_{CC}$	$\delta_C \cdot J_{CC}$	 $1J_{CC}$
Heteronuklearne korelacije			
HETCOR HSQC (inverzni)	ϵ_C	$\epsilon_H \cdot J_{HH}$	 $1J_{CH}$
COLOC HMBC (inverzni)	ϵ_C	$\epsilon_H \cdot J_{HH}$	 nJ_{CH} ($n \geq 1$)

Homonuklearna korelacija COSY (COrrelation SpectroscopY)



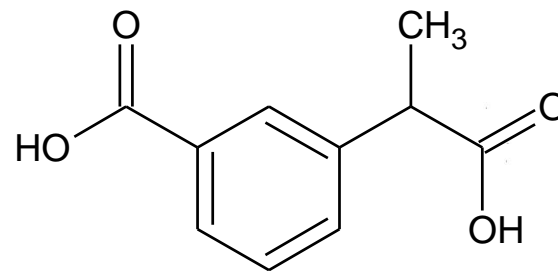
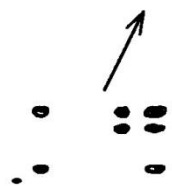
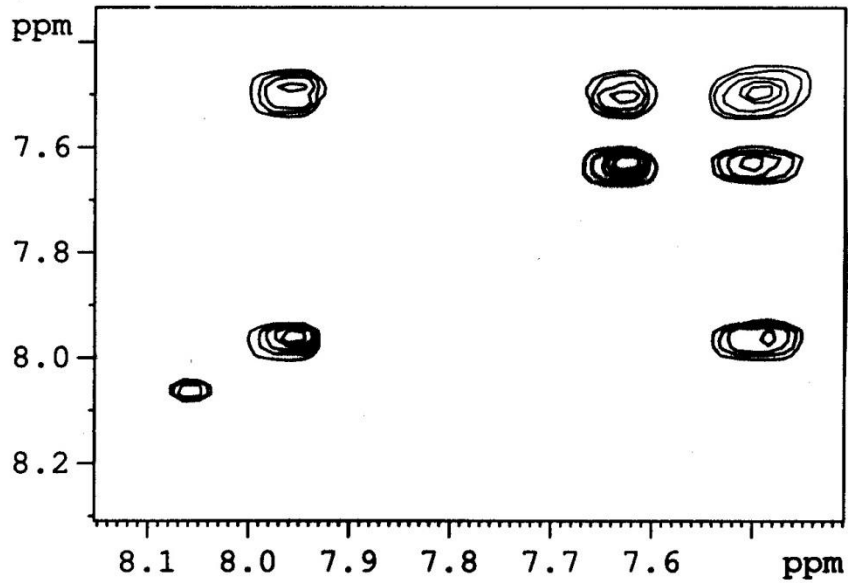
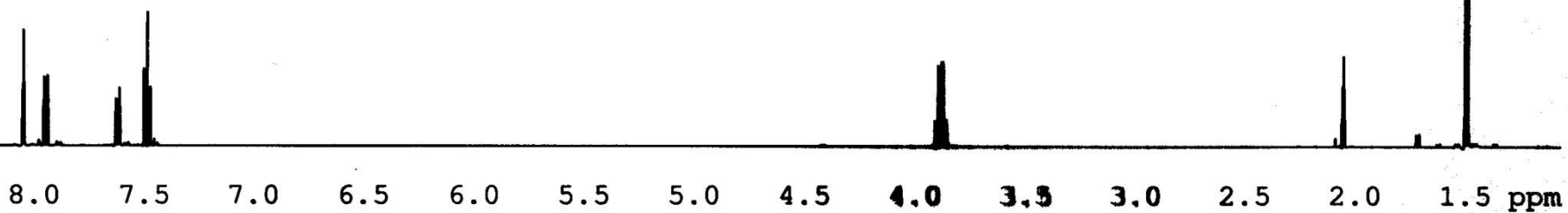
Spinski sustav AX

$$I_A = I_X = 1/2$$

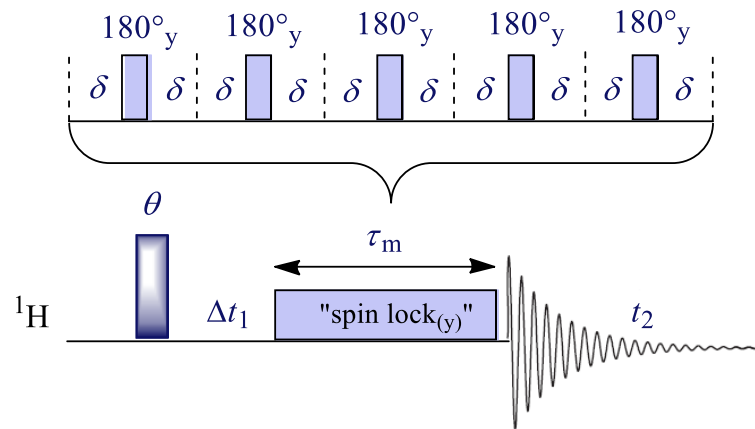


Simulirani spektar COSY 2-feniletanola u dimetilsulfoksidu-d₆. Na F1 odnosno F2 osi nalaze se odgovarajući jednodimenzijski spektri NMR

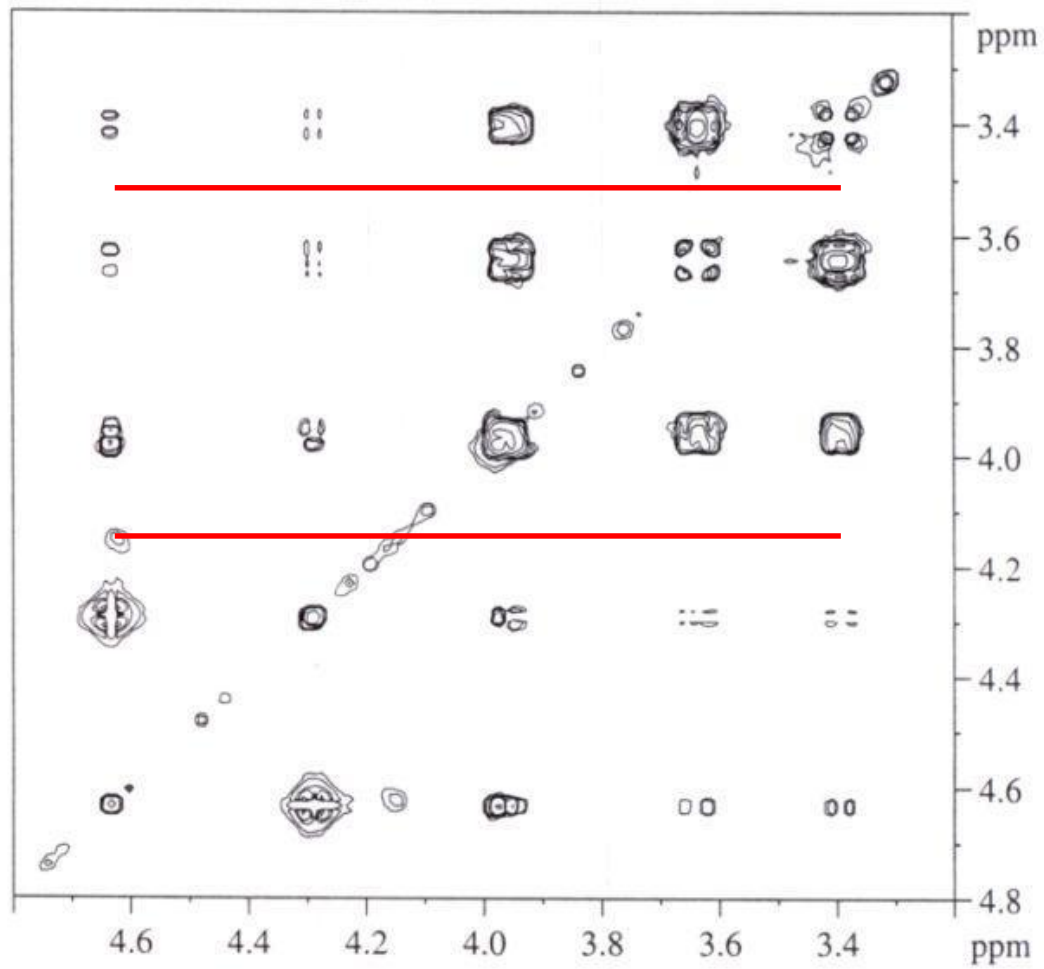
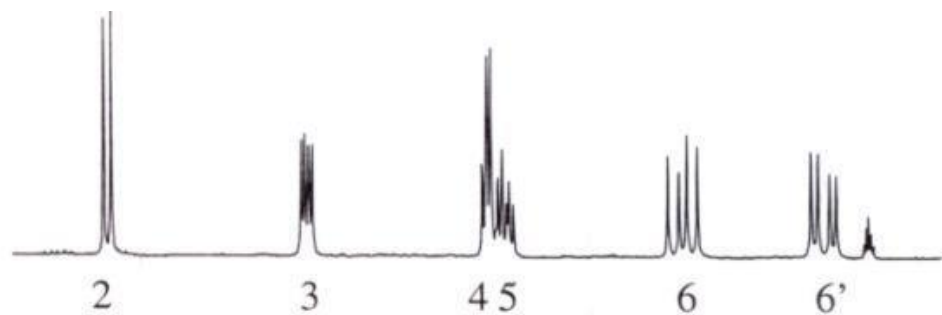
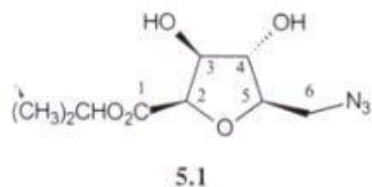
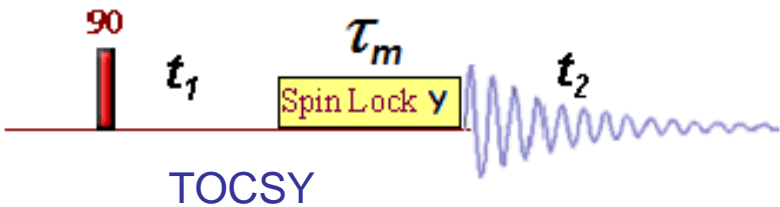
COSY spektar s gradijentima
otapalo aceton



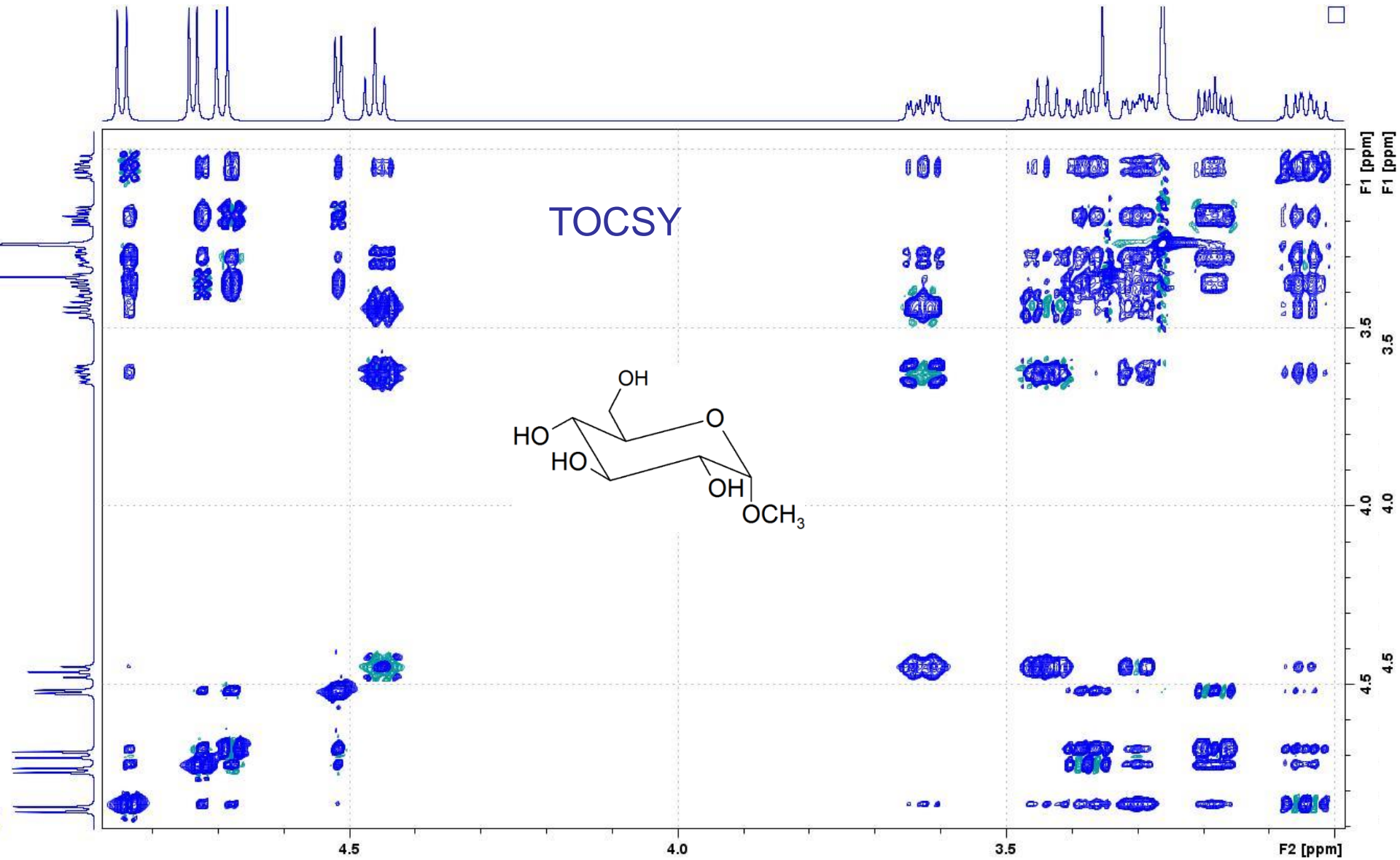
Tehnika TOCSY (TOtal COrrelation SpectroscopY)



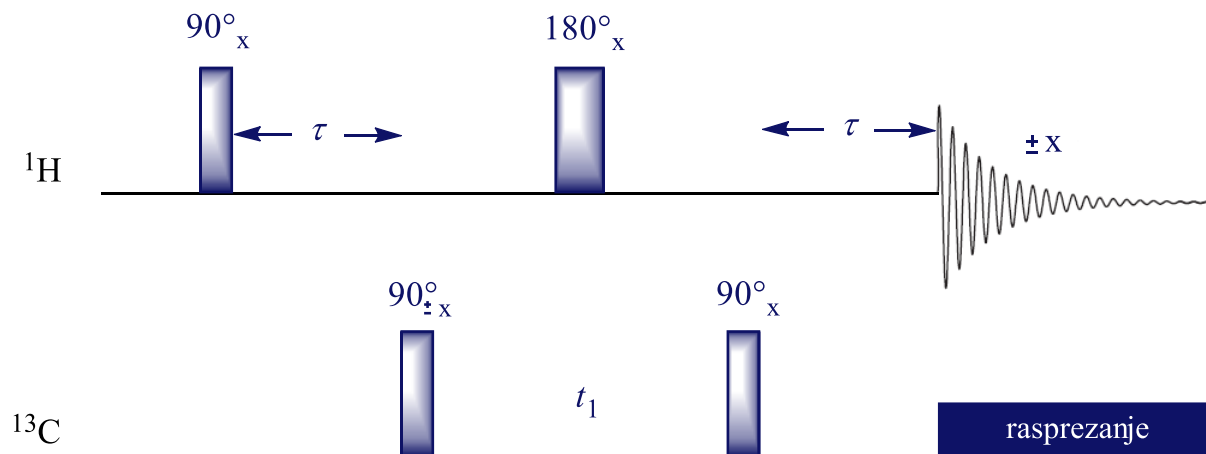
- Postoje korelacije među spinovima unutar spinskog sustava, bez obzira da li su međusobno spregnuti
- Ako postoje neprekinuti lanac spinova A-B-C-D, TOCSY slijed će prenositi magnetizaciju od spina A na spinove B, C, D
- Homonuklearna križna polarizacija (HOHAHA)
- t_m - vrijeme mješanja ili spin-lock ili izotropno mješanje, potrebno za prijenos magnetizacije unutar spinskog sustava, tj svi spinovi dijele koherenciju
- Vrijeme t_m je u ms što omogućava višestupanjski prijenos koherencije
- za vrijeme t_m primjenjuje se rf polje ili pulsevi uzduž osi y koji zaključavaju spinove uzduž te osi i kreiraju homonuklearnu spinsku jeku
- Homonuklearnom spinskom jekom sprege nastavljaju evoluirati, što znači da su na kraju vremena t_m kemijski pomaci odstranjeni dok sprege ostaju aktivne
- Zahtjev da jezgre osjećaju ista lokalna polja za vrijeme mješanja da bi se omogućio prijenos zove se i Hartman-Hahn uvjet, odnosno vrijedi $\gamma_A B_{1A} = \gamma_X B_{1X}$

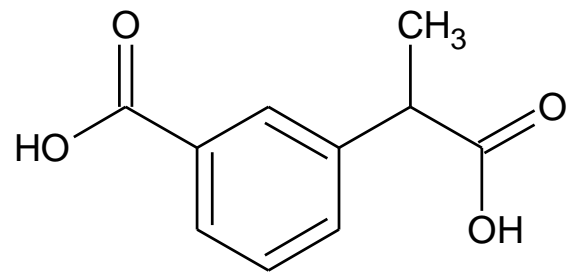


COSY

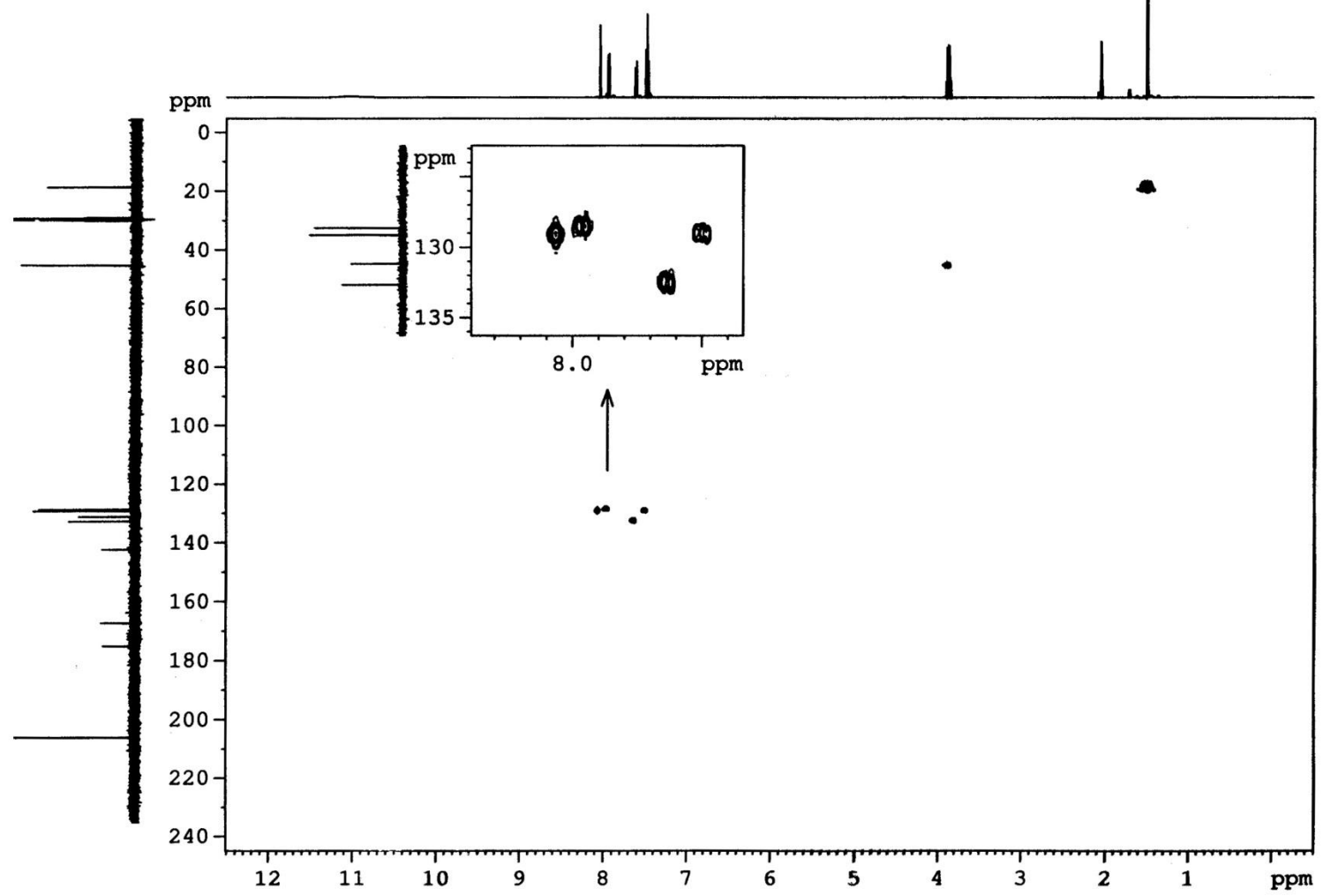


Tehnika HMQC (Heteronuclear Multiple Quantum Coherence)

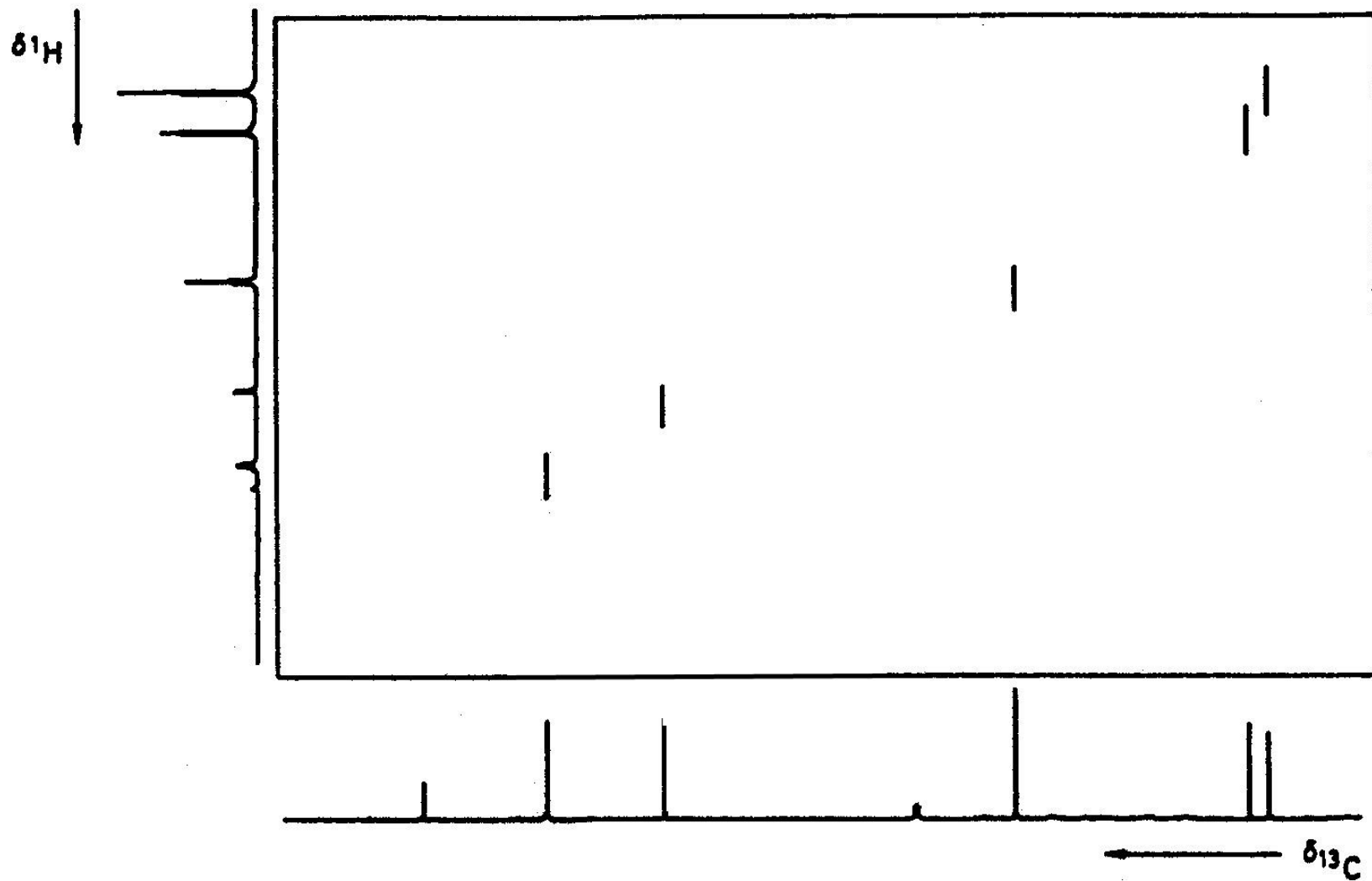




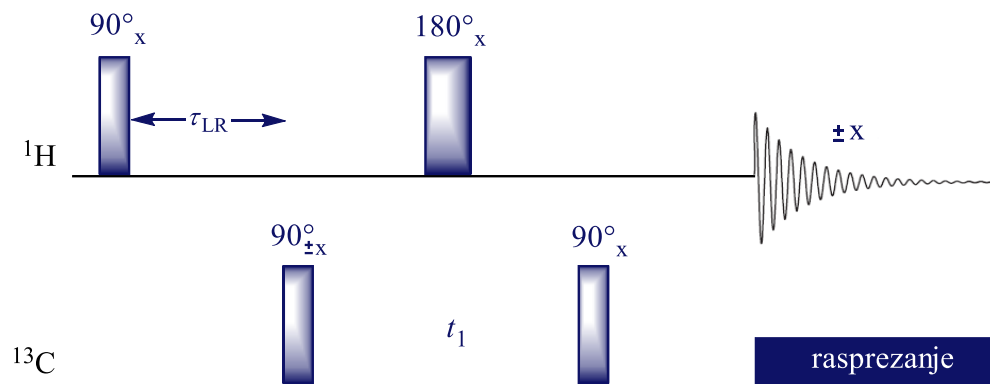
HMQC spektr otapalo aceton



HETCOR

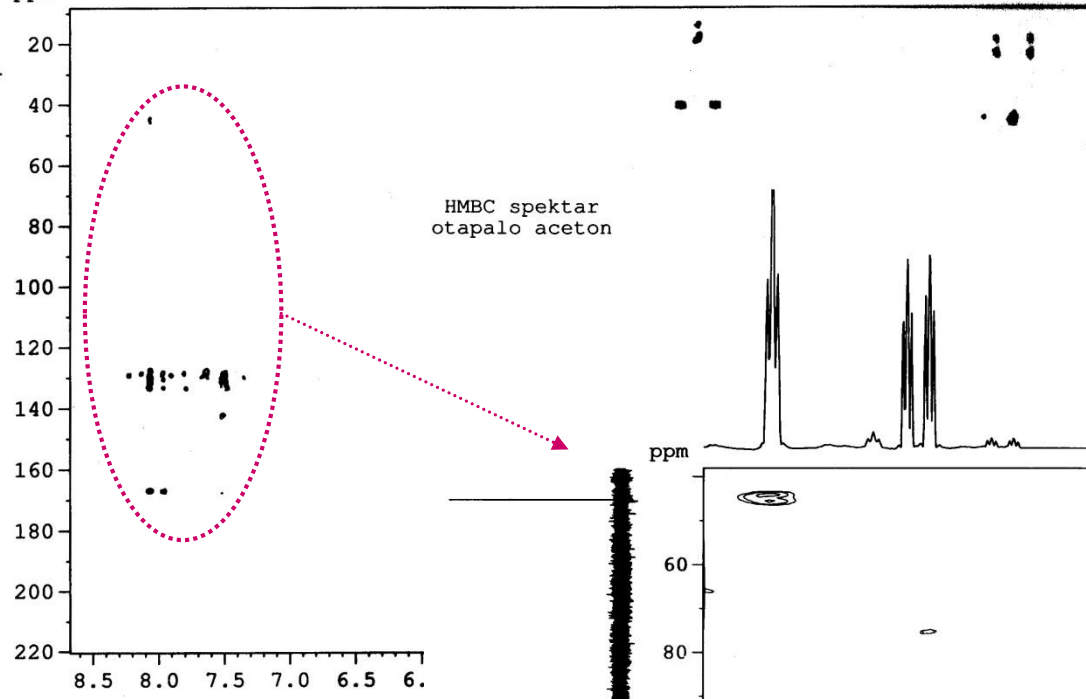
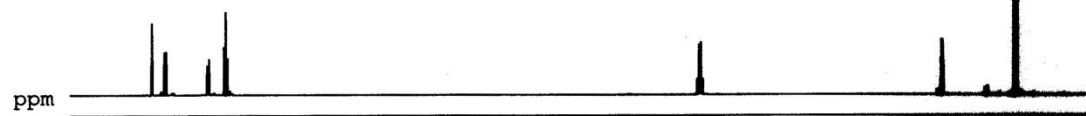
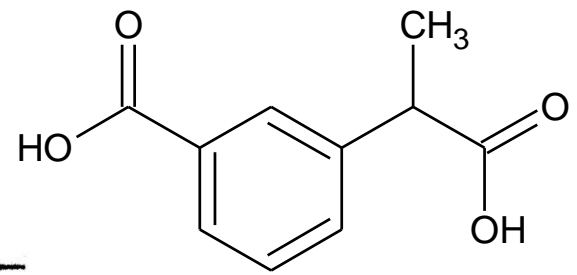


Tehnika HMBC (Heteronuclear Multiple Bond Coherence)

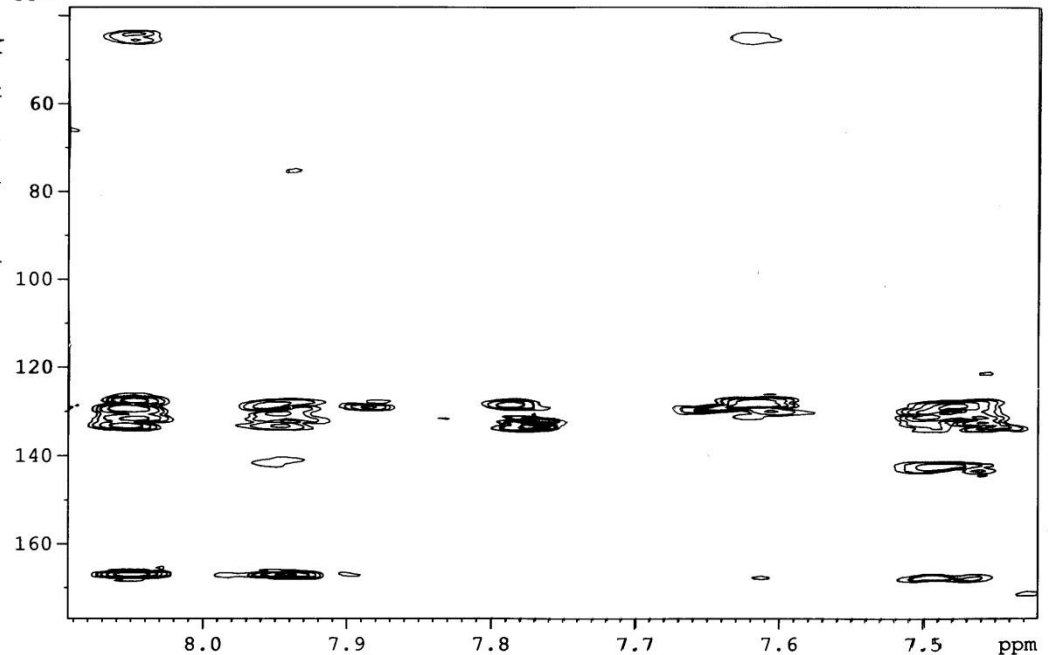
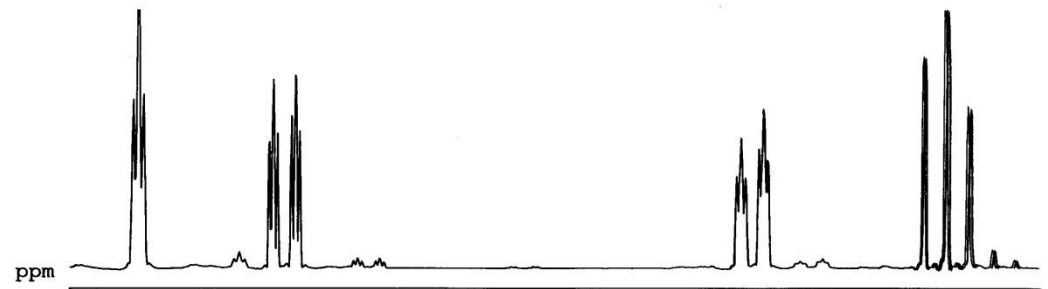


Vrijeme odgode τ_{LR} optimirano je za spregu kroz više veza, koja je puno manja od one kroz jednu vezu.

HMBC spektar
otapalo aceton

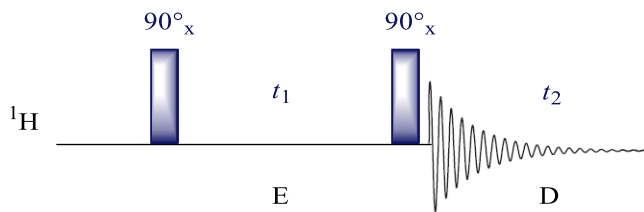


HMBC spektar
otapalo aceton



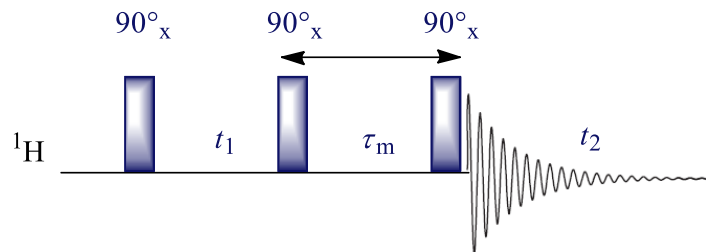
Interakcije kroz prostor- križna relaksacija (NOESY, ROESY)

COSY

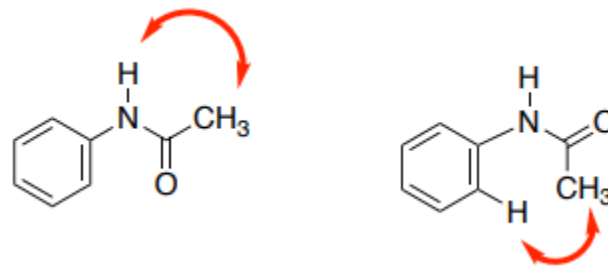


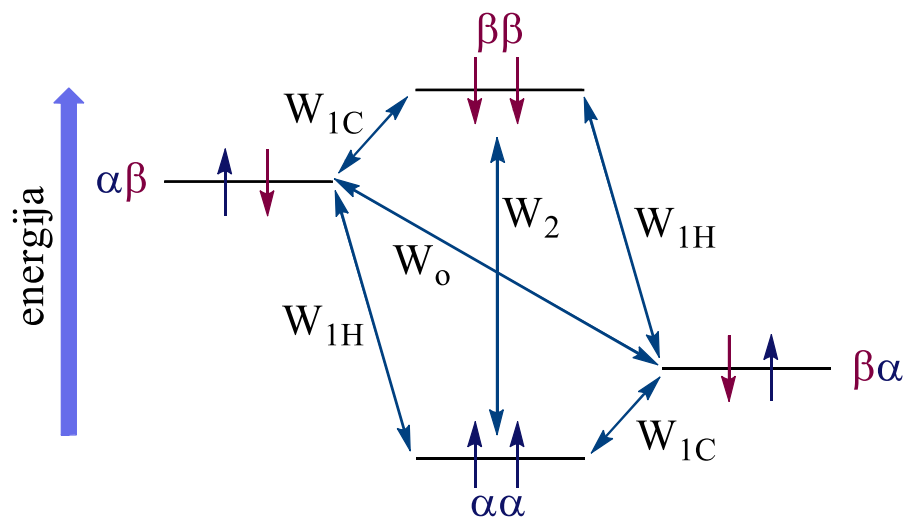
1. Skalarnе interakcije

NOESY



1. Skalarnе interakcije, dipolne interakcije, kemijska izmjena
2. Vrijeme miješanja τ_m
3. Prijenos magnetizacije kroz prostor





W_0 = prijelaz od nula kvanta

W_1 = prijelaz od jednog kvanta

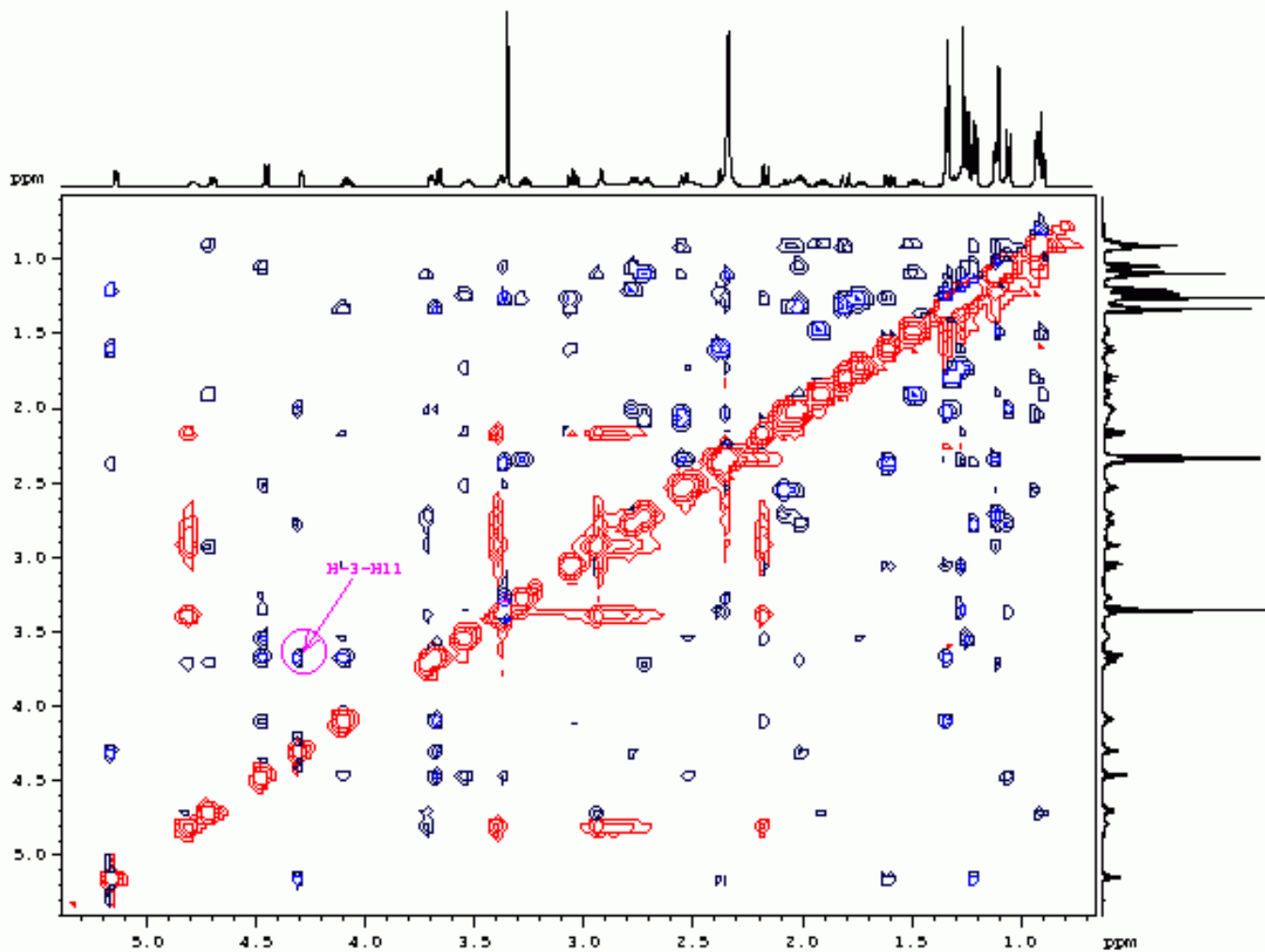
W_2 = prijelaz od dva kvanta

W_0 i W_2 imaju istodobnu promjenu oba spina

kvantno su zabranjeni jer $\Delta M \neq 1$

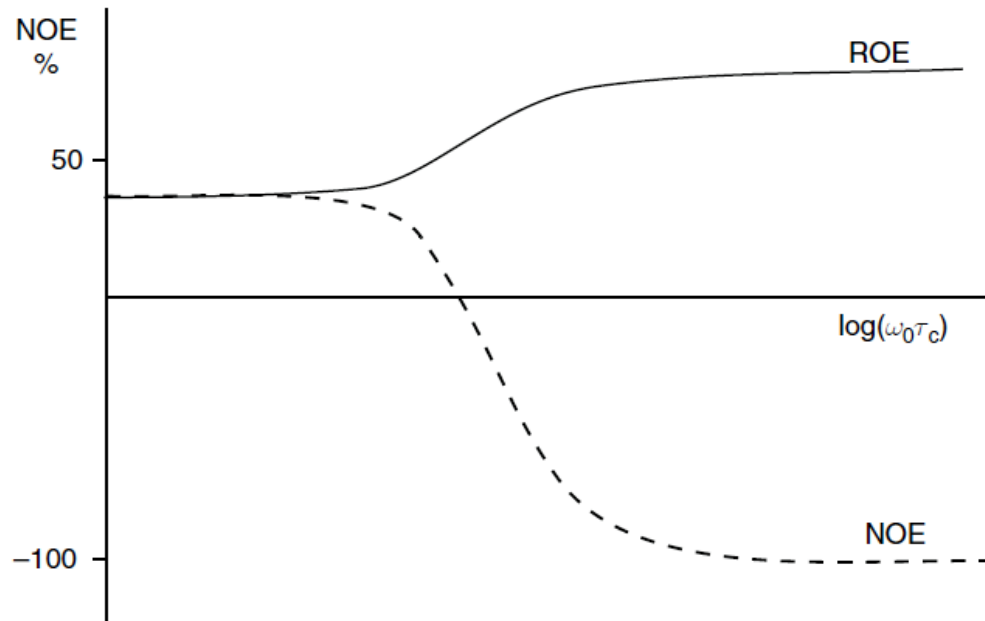
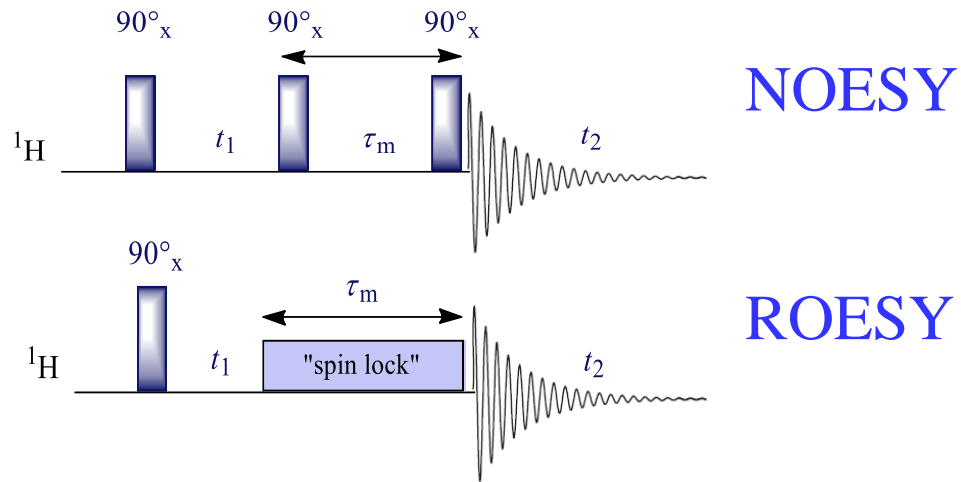
W_0 i W_2 predstavljaju puteve križne relaksacije

Križna relaksacija mehanizmom W_2 rezultira povećanjem intenziteta signala i pozitivnim učinkom NOE, dok relaksacija mehanizmom W_0 rezultira smanjenjem intenziteta signala i negativnim učinkom NOE



NOESY spektar azitromicina

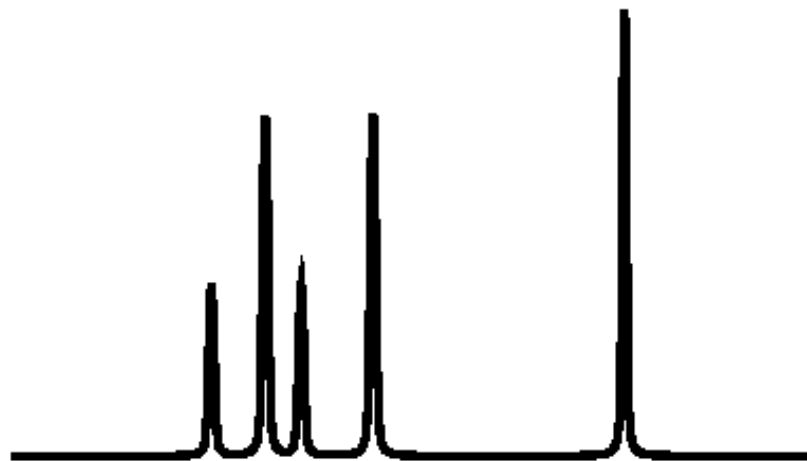
NOESY vs. ROESY



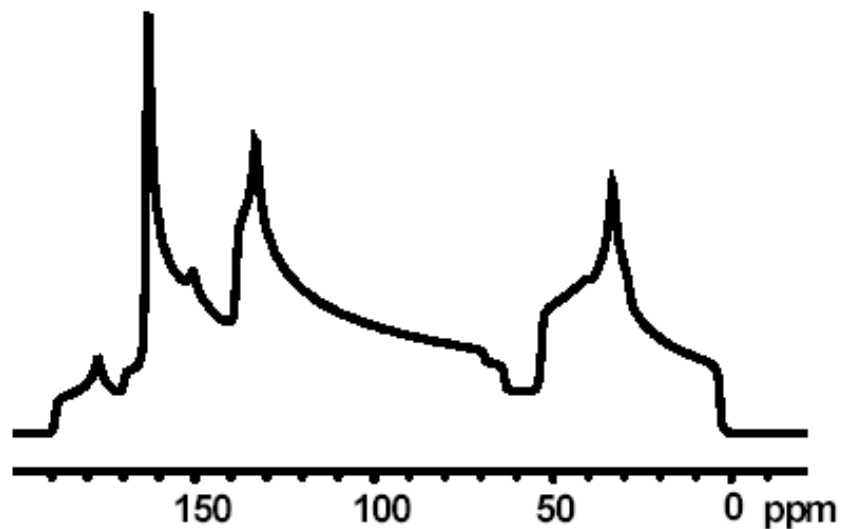
Protokol za određivanje ili potvrdu strukture molekula

postupak	tehnika	informacija
Spektar ^1H	1D	kemijski pomaci, konstante sprega, integrali
Korelacija ^1H - ^1H	COSY	povezanost protona preko sprega (asignacija)
spektar s editiranjem ^{13}C	DEPT, APT, PENDANT	broj atoma C i multipletnost (C, CH, CH ₂ , CH ₃) često nepotrebno ako se koriste inverzne 2D tehnike s editiranjem
spektri hetero-jezgri (^{31}P , ^{19}F ..)	1D	kemijski pomaci i homo-/hetero-nuklearne konstante sprega
korelacija kroz jednu kemijsku vezu ^1H - ^{13}C	HMQC, HSQC	asignacija atoma C i H, multipletnost iz HSQC spektra s editiranjem
korelacija kroz više kemijskih veza ^1H - ^{13}C	HMBC	povezanost atoma C i H kroz više veza, povezanost preko hetero-atoma, identifikacija strukturnih fragmenata
korelacija kroz prostor preko efekta NOE	1D ili 2D NOESY, ROESY	stereokemija: konfiguracija i konformacija

Spektroskopija NMR u čvrstom stanju

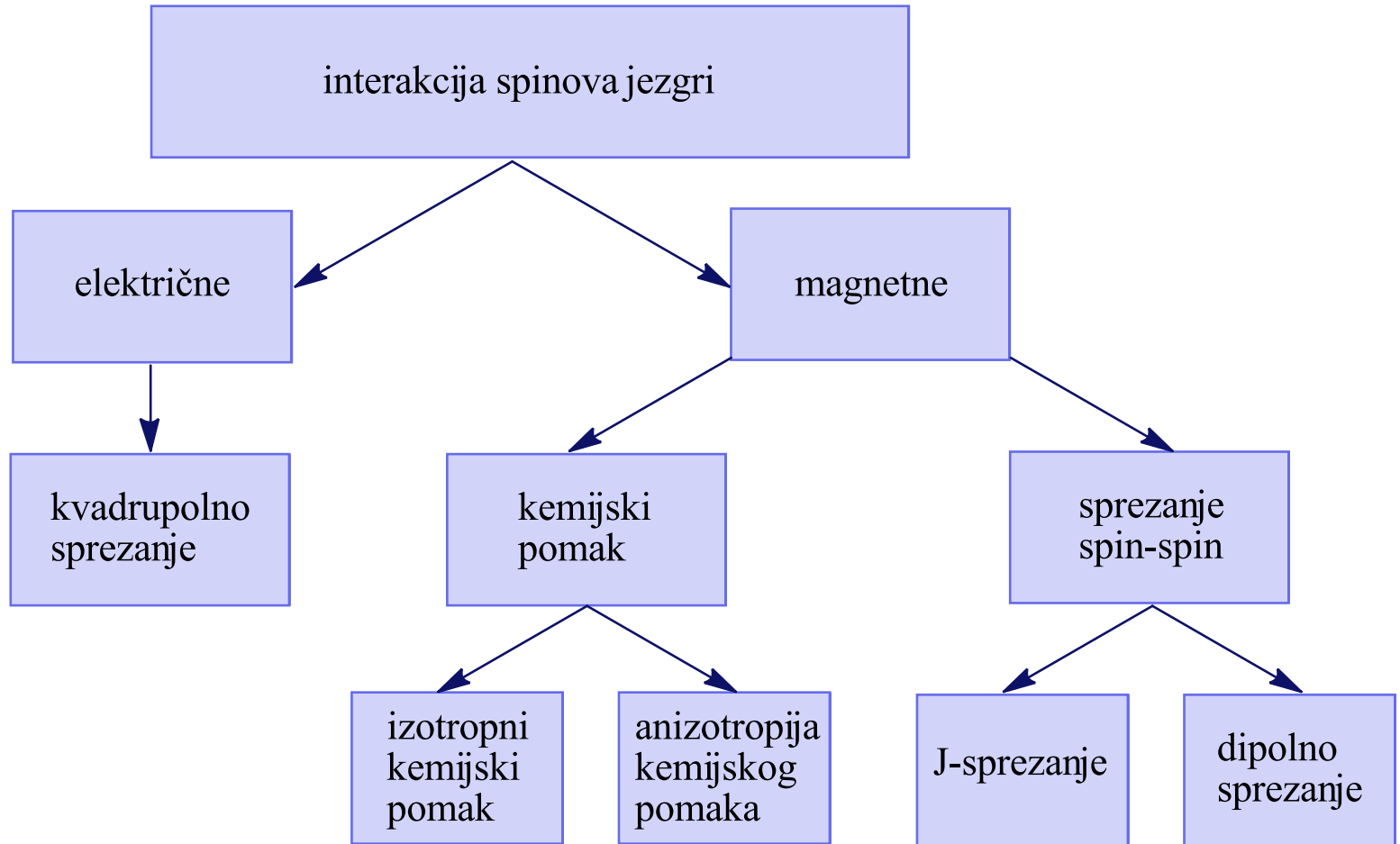


➤ Spektar ^{13}C NMR u otopini



➤ Spektar ^{13}C NMR u čvrstom stanju

Interakcije spinova



$$H = H_{CS} + H_{CSA} + H_J + H_D + H_Q$$

interakcije spinova **anizotropnog** su karaktera
(orijentacijski ovisne)



TENZORI 2. REDA

Spektri NMR u čvrstom stanju imaju vrlo široke linije zbog:

- Anizotropije kemijskih pomaka
- dipolnih interakcija
- kvadrupolnih interakcija

- U otpini su te interakcije uprosječene na nulu zbog brzog gibanja molekula

- Uske linije

Spektri visoke rezolucije u čvrstom stanju:

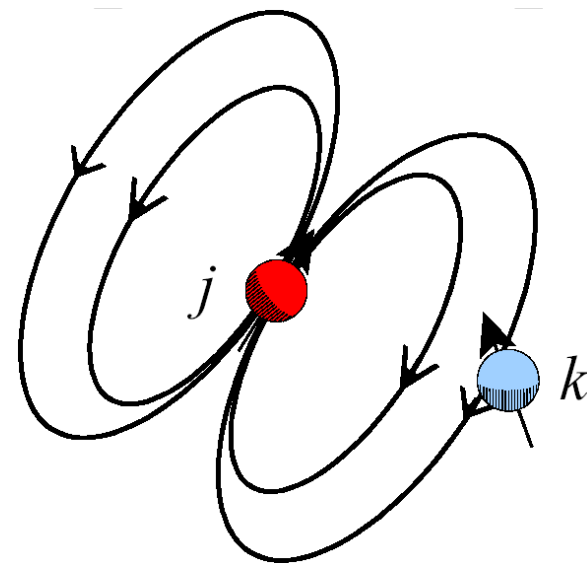
- Magic Angle Spinning (MAS)
- dipolno raspredanje (DD)
- križna polarizacija (cross polarisation, CP)

Interakcija NMR	Fizikalna osnova	Informacija	Red veličine	Izotropna vrijednost
<i>anizotropija kemijskog pomaka</i>	interakcija spina jezgre i lokalnog magnetnog polja posredstvom elektrona	koordinacijski broj vezni kutevi i udaljenosti	10 kHz	σ_{iso}
<i>indirektna (skalarna) spreaga</i>	interakcija spinova jezgri posredstvom veznih elektrona	kemijska veza	1 – 100 Hz	J
<i>dipolna spreaga</i>	prostorna interakcija spinova jezgri	prostorna udaljenost	10 – 100 kHz	0
<i>kvadrupolna spreaga ($I > 1/2$)</i>	interakcija električnog kvadrupolnog momenta jezgre s gradijentima električnog polja u molekuli	geometrija	1 – 10 MHz	0

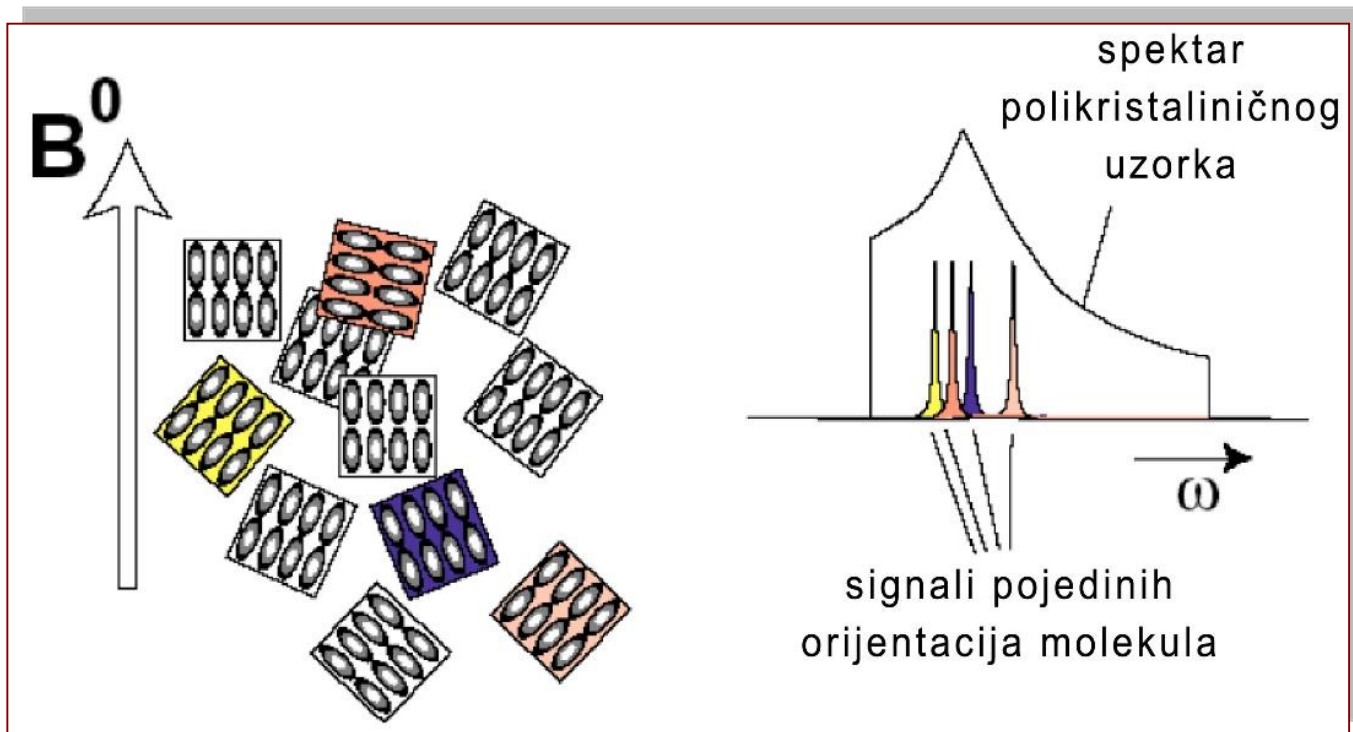
➤ Dipolne interakcije

$^1\text{H}, ^1\text{H} \approx 40 \text{ kHz} - 120 \text{ kHz}$

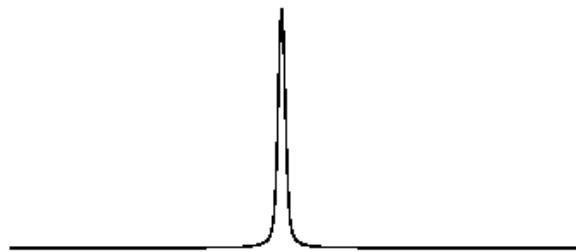
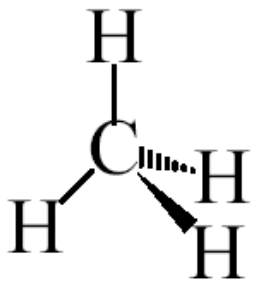
$^{13}\text{C}, ^1\text{H} \approx 3 \text{ kHz} - 30 \text{ kHz}$



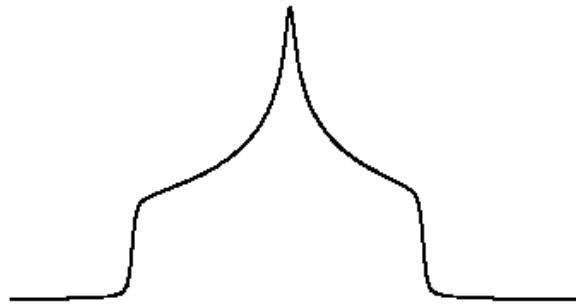
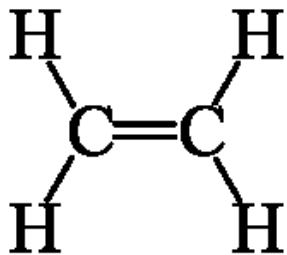
Utjecaj anizotropije kemijskog pomaka (CSA) na spektre NMR u čvrstom stanju



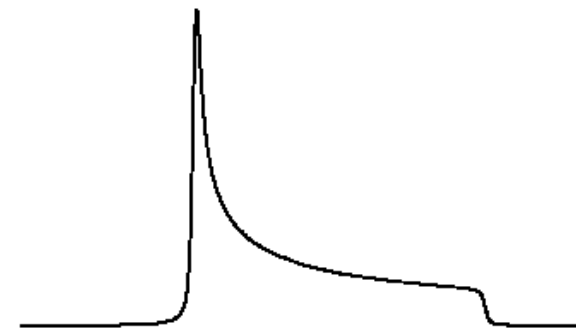
➤ Anizotropija kemijskog pomaka



➤ kubična simetrija



➤ aksijalna simetrija

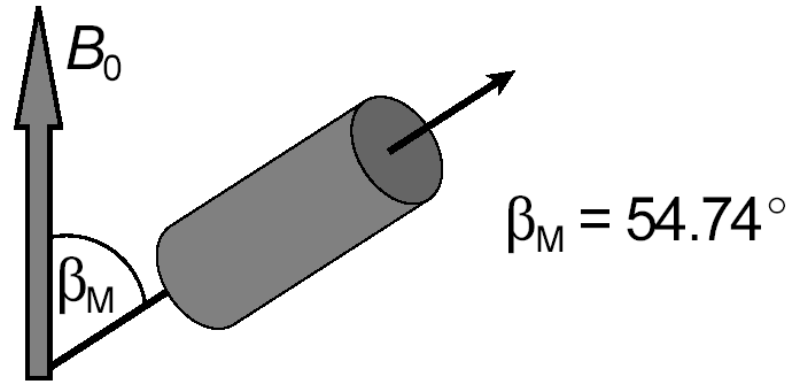


➤ neaksijalna simetrija

Čarobni trik...

$$(3\cos^2\theta - 1) = 0 \quad \text{kada je } \theta = 54.74^\circ$$

- beskonačno dugačak cilindar



- E. R. Andrew i I. J. Lowe

Magic Angle Spinning

MAS
CP-MAS

- dipolne interakcije

$^1\text{H}, ^1\text{H} \approx 40 \text{ kHz} - 120 \text{ kHz}$

$^{13}\text{C}, ^1\text{H} \approx 3 \text{ kHz} - 30 \text{ kHz}$

Križna polarizacija uz vrtnju pri magičnom kutu (CP-MAS)

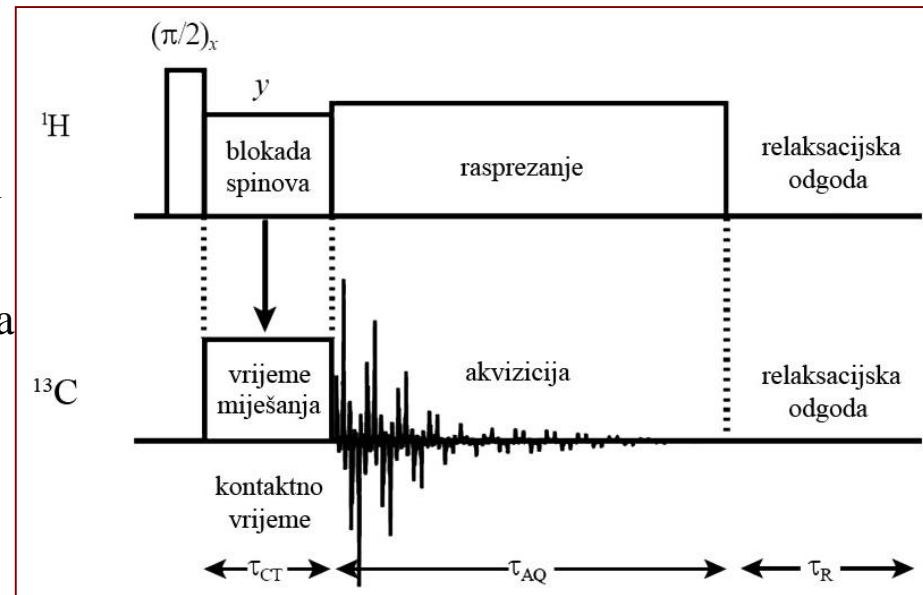
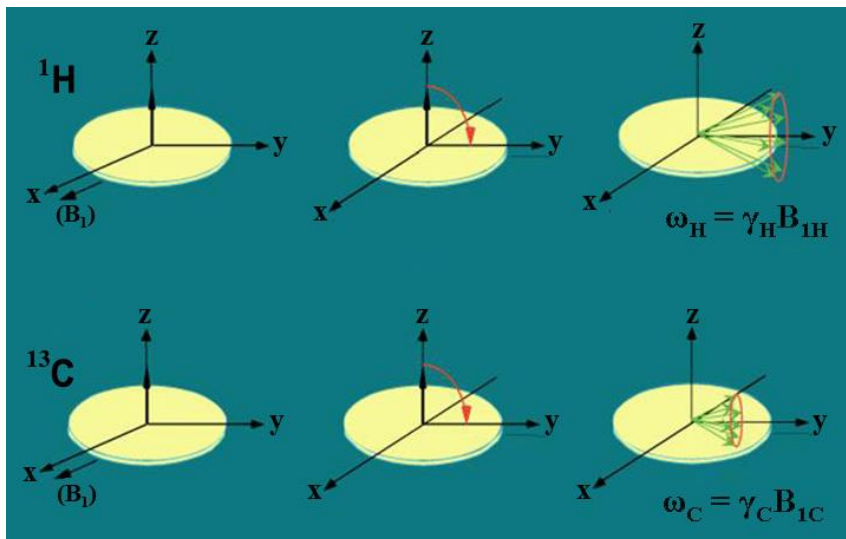
□ **CP-MAS: križna polarizacija (CP) + MAS + rasprezanje**

□ **CP:** povećanje osjetljivosti (intenziteta signala) jezgri s malom konstantom γ (^{13}C , ^{15}N) prijenosom magnetizacije jezgri s velikom konstantom γ (^1H)

- maksimalno povećanje intenziteta: $\gamma_{\text{H}}/\gamma_{\text{C}}$
- jezgre su dipolno spregnute

□ **rasprezanje** uklanja **heteronuklearnu dipolnu i skalarnu spregu**

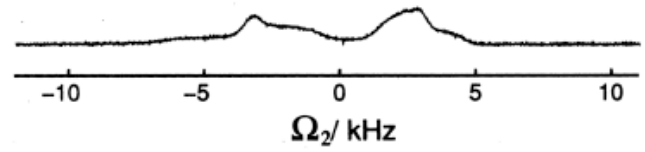
□ **homonuklerna dipolna sprega nije značajna** za prirodno slabo zastupljene jezgre



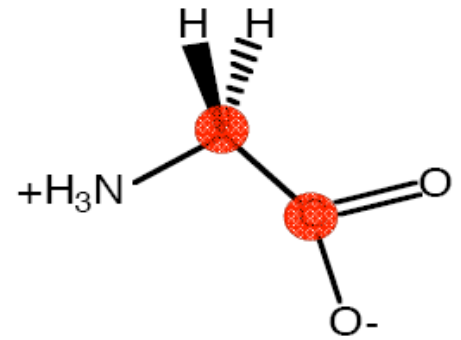
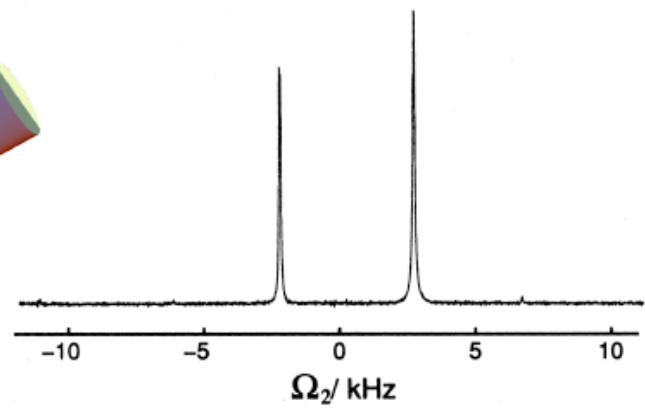
- ^1H 90° puls generira xy -magnetizaciju ($B_{1\text{H}}$)
- Spin-lock puls zadržava magnetizaciju u xy -ravnini
brzina precesije $\gamma_{\text{H}}B_{1\text{H}}$
- ^{13}C puls generira xy -magnetizaciju
brzina precesije $\gamma_{\text{C}}B_{1\text{C}}$
- **Prijenos polarizacije** : kontaktno vrijeme (ms)
Hartmann Hahn uvjet $\gamma_{\text{H}}B_{1\text{H}} = \gamma_{\text{C}}B_{1\text{C}}$

Spektri ^{13}C NMR glicina

Bez vrtnje



S vrtnjom od 12 kHz



Učinak MAS-a na spektar ^{13}C

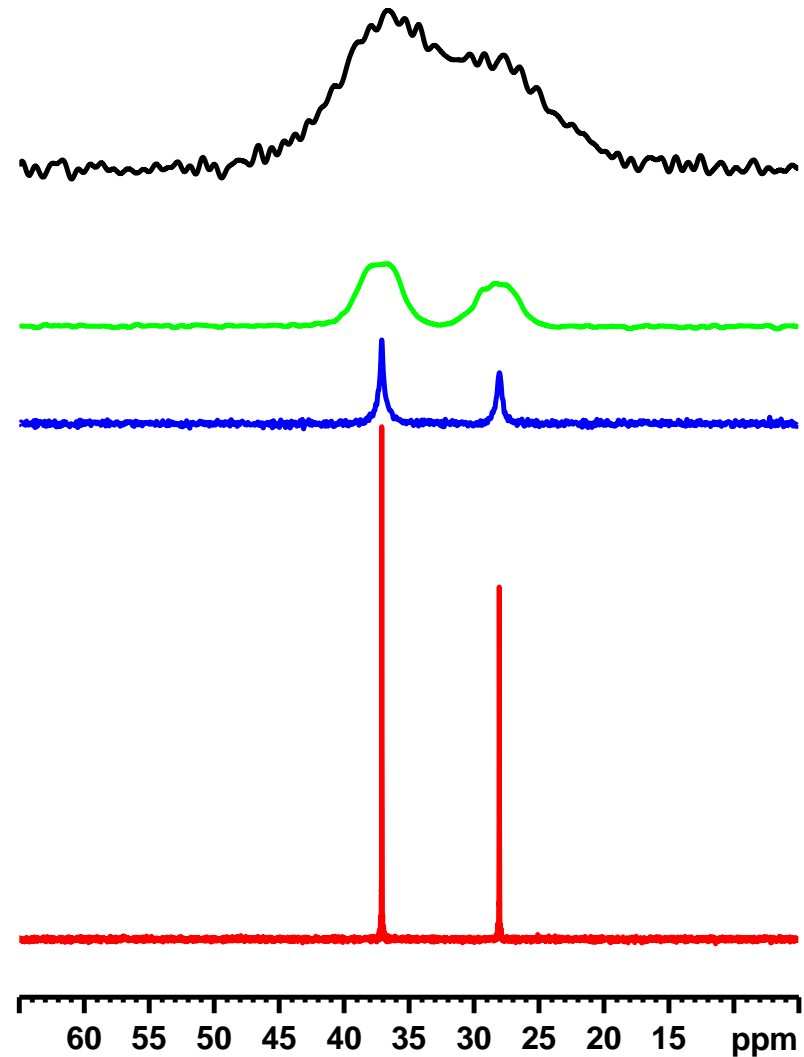
Adamantan (^{13}C) pri različitim uvjetima

statično + bez ^1H raspredanja
(CSA.+ dipolno sprezanje)
→ **1000 Hz** širina linije

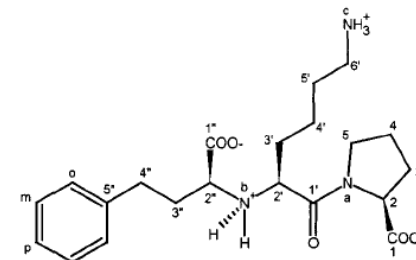
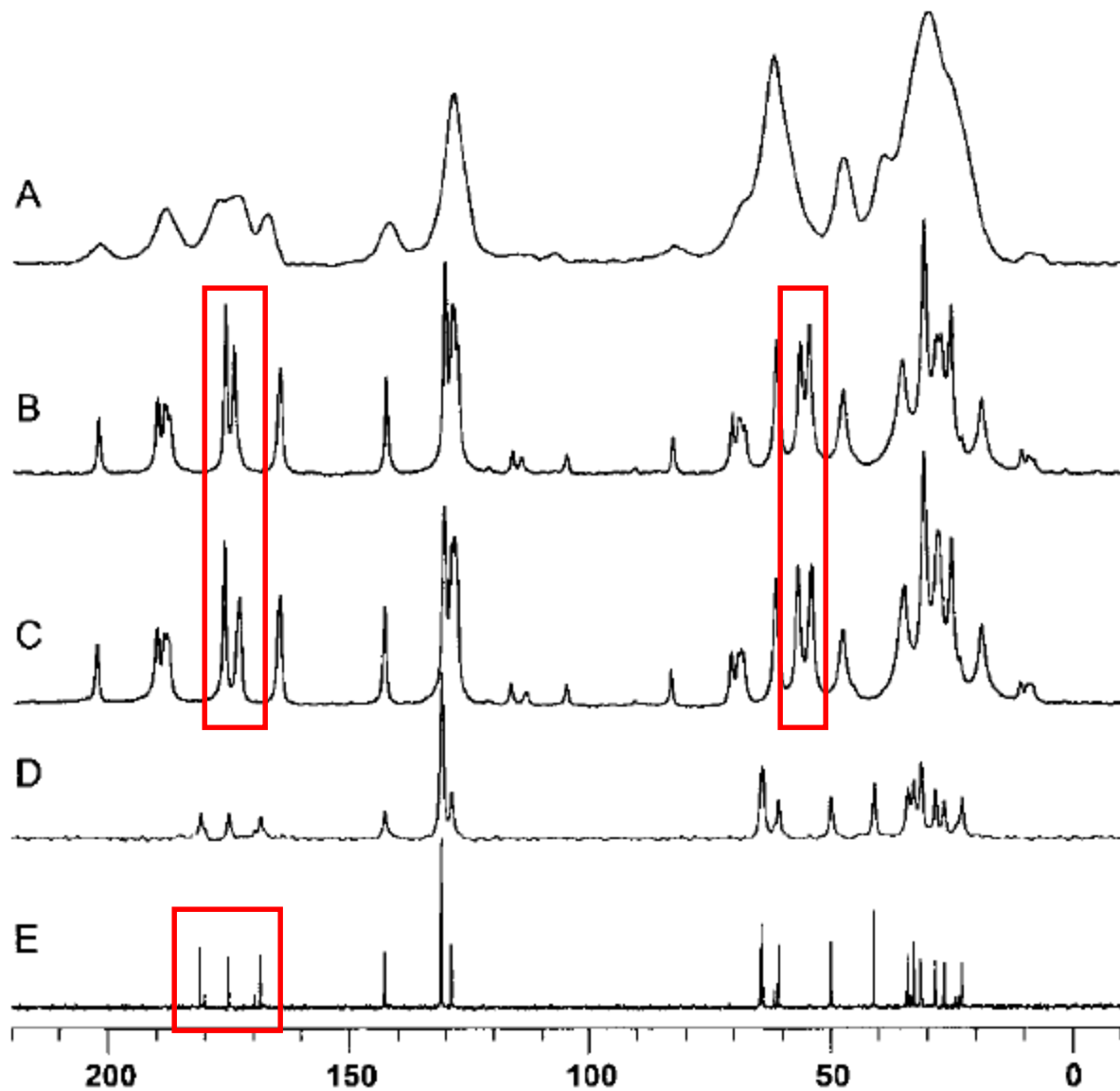
statično + ^1H raspredanje
(ACS, nema dipolnog sprezanja)
→ **500 Hz** širina linije

MAS + bez ^1H raspredanja
(nema ACS + hetero dipolno sprezanje)
→ **50 Hz** širina linije

MAS + ^1H raspredanje
(nema ACS, nema dipolnog sprezanja)
→ **5 Hz** širina linije



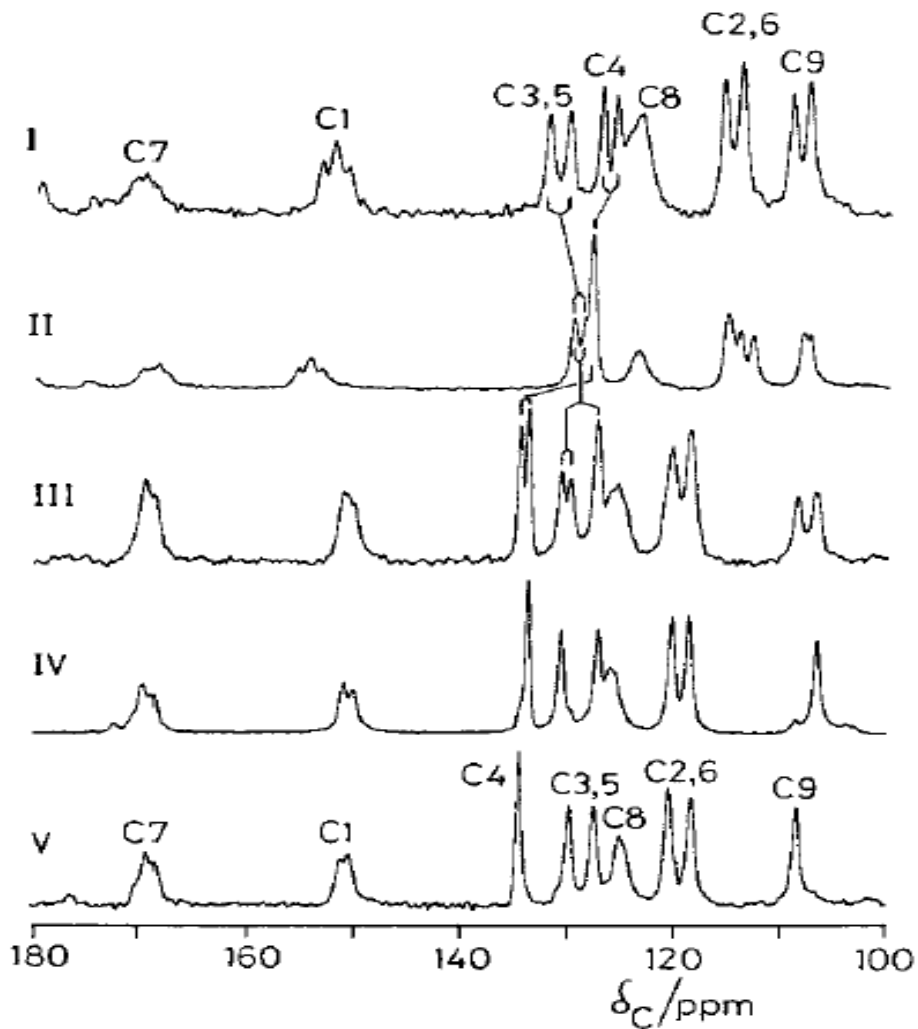
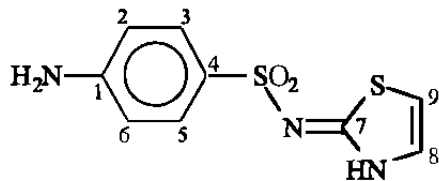
Analiza svojstava krutine – solvati (lizinopril)



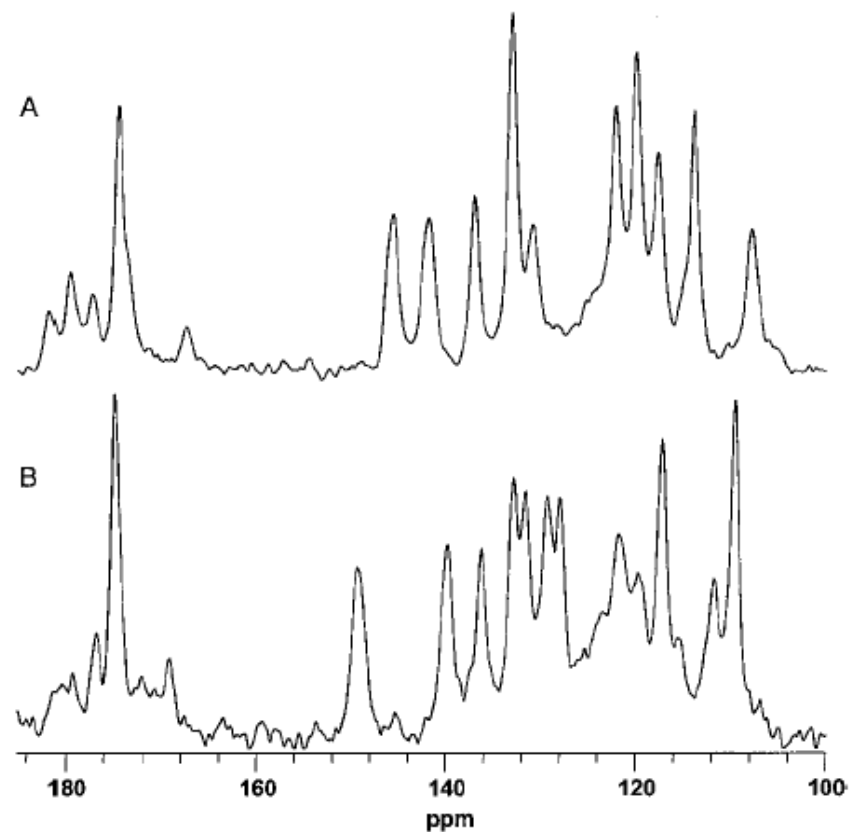
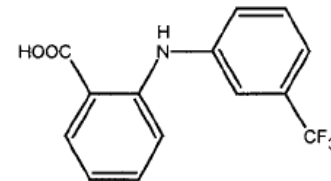
- A. Amorfni oblik
- B. Kristal anhidrat
- C. Kristal dihidrat
- D. D₂O otopina
- E. D₂O otopina

Analiza svojstava krutine – polimorfi

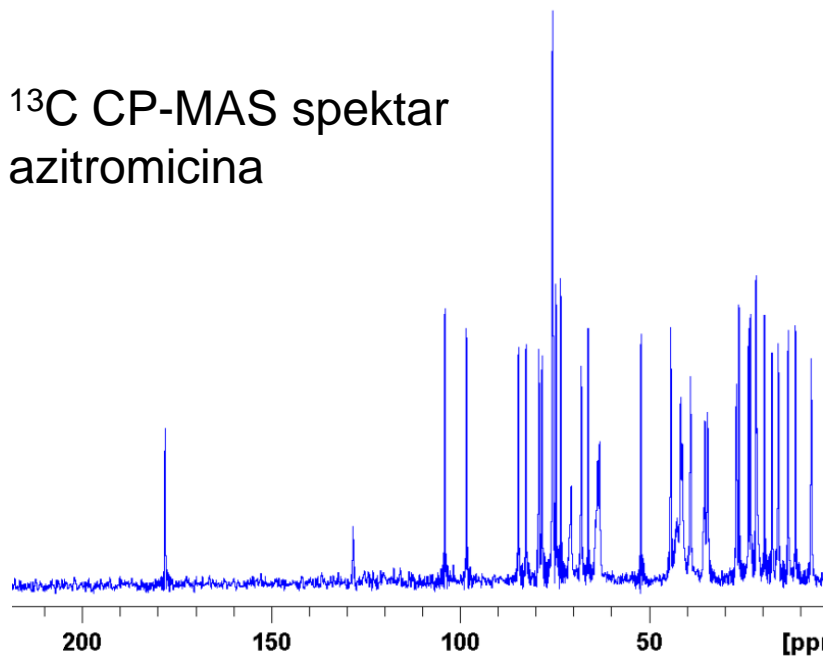
sulfatiazol



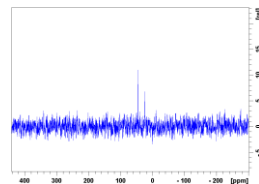
flufenamska
kiselina



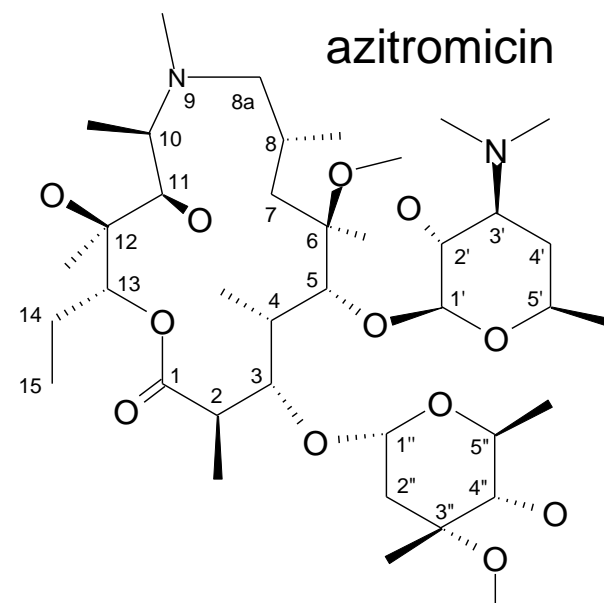
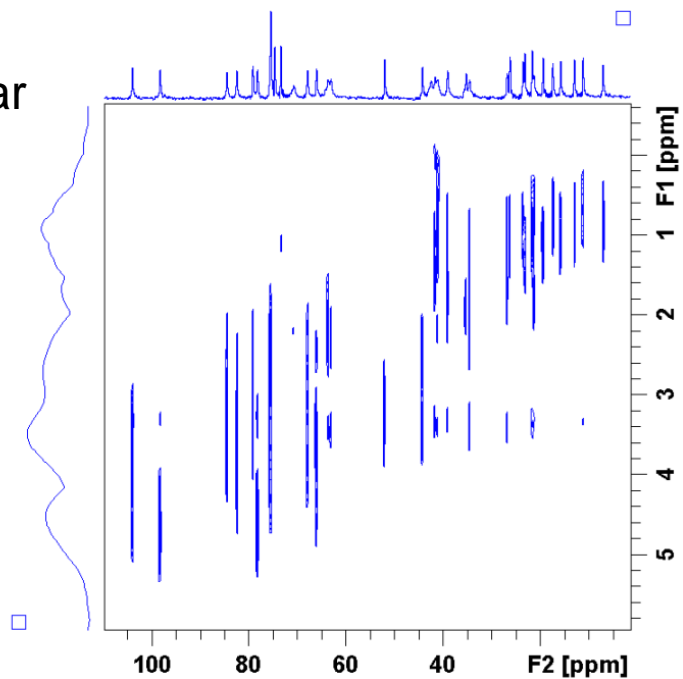
^{13}C CP-MAS spektr
azitromicina



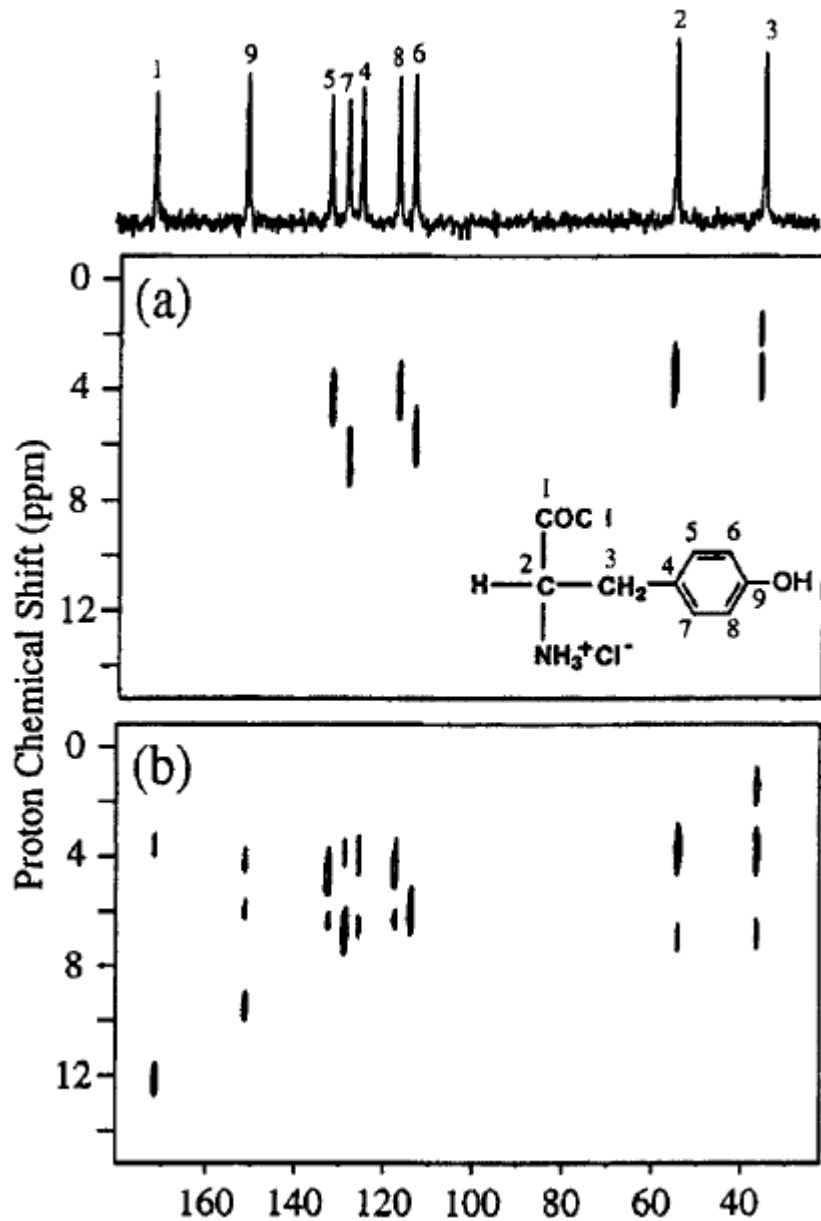
^{15}N CP-MAS spektr
azitromicina



HETCOR spektr
azitromicina



Strukturna analiza – L-tirozin hidroklorid



a) MAS-J-HMQC

b) dipolarni HETCOR

Sprega tekućinske kromatografije i NMR-a

LC-NMR

HPLC

pumpa

detektor

Otopalo
(deuterirano)

kolona

NMR

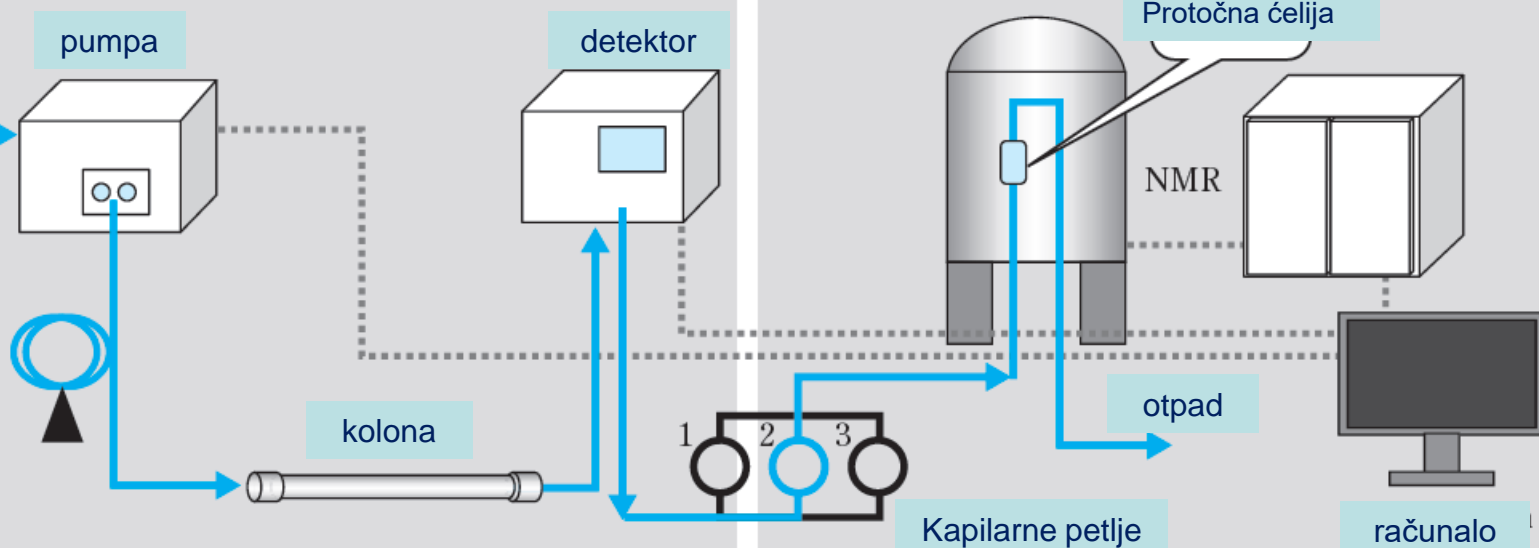
Protočna ćelija

NMR

otpad

Kapilarne petlje

računalo



Načini izvedbe LC-NMR-a

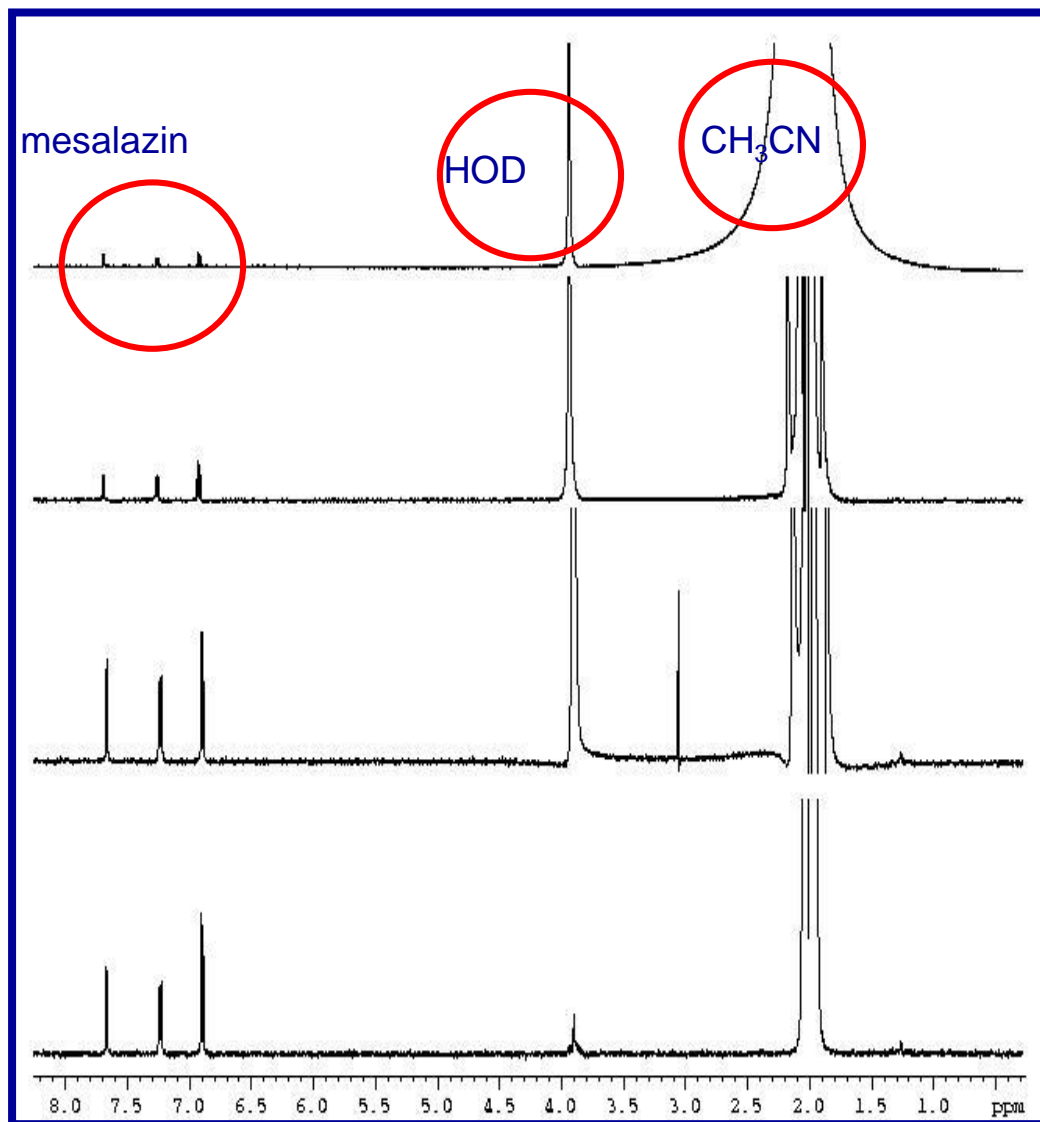
- Kontinuirani protok (on-flow)
 - Izvedivo za ^1H i ^{19}F (osjetljivost)
 - Za uzorke nepoznatih kromatografskih svojstava
 - Labilni spojevi (razgradnja, izomerizacija)
- Zaustavljeni protok u vremenskim intervalima (time-slice)
 - Loše odvojive komponente u smjesi
 - Slaba ili nikakva kromofora UV
 - Slabo definirana retencijska vremena
- Zaustavljeni protok (stopped-flow)
 - Poznata retencijska vremena komponenata u smjesi
 - Poznate kromofore UV
- Skladištenje u kapilarnim petljama (loop transfer)
 - LC pikovi se skladište u kapilarne petlje
- LC-SPE-NMR
 - Pikovi LC se skladište u SPE (solid phase extraction) kolonicama (cartridge)

Tehnike supresije signala otapala

- Prezasićenje (NOESY tip, “shaped” pulsevi)
 - Jedno-, dvo-, i višestruka supresija signala
- WET (water suppression enhanced trough T_1 effects)
- WATERGATE (water suppression by gradient-tailored excitation)
- Excitation sculpting (dvostruka pfg spinska-jeka)

Protonski spektri mesalazina u 71% CH₃CN i 29% D₂O

a) običan spektar



b) prezasićenje CH₃CN

c) dvostruko prezasićenje CH₃CN i HDO

d) Dvostruko prezasićenje CH₃CN i HDO uz ¹³C rasprezanje

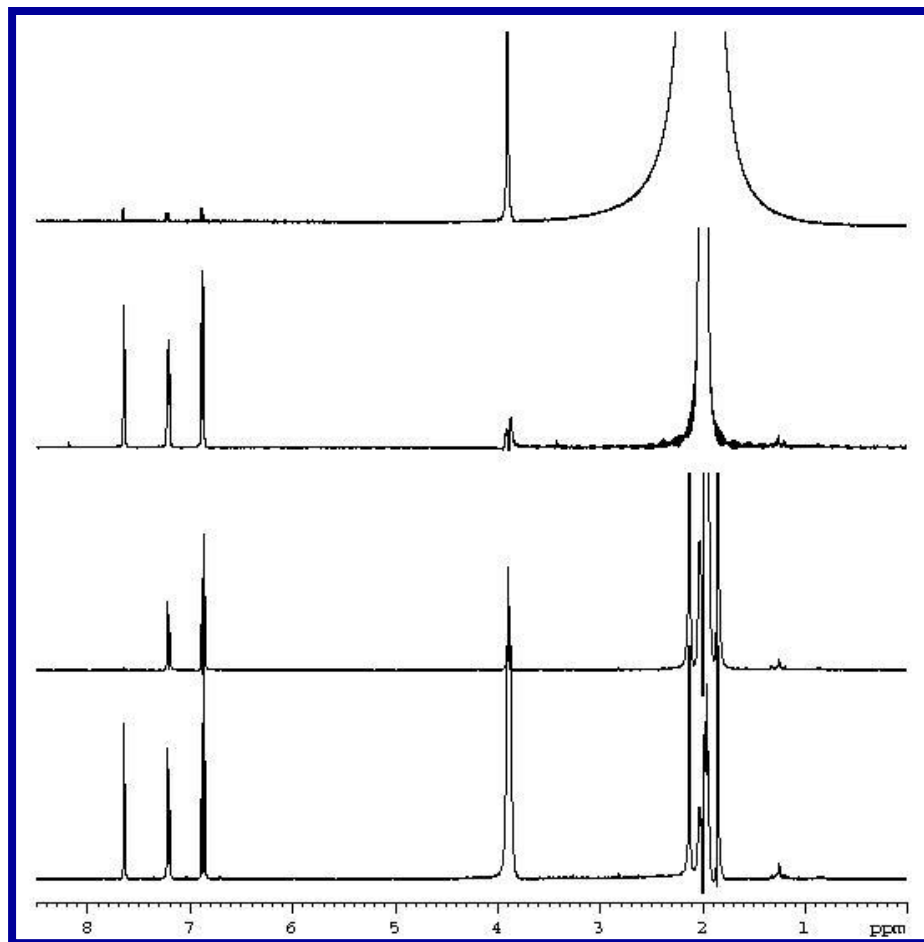
Protonski spektri mesalazina u 71% CH₃CN i 29% D₂O

a) Običan spektar

b) WET višestruka supresija otapala

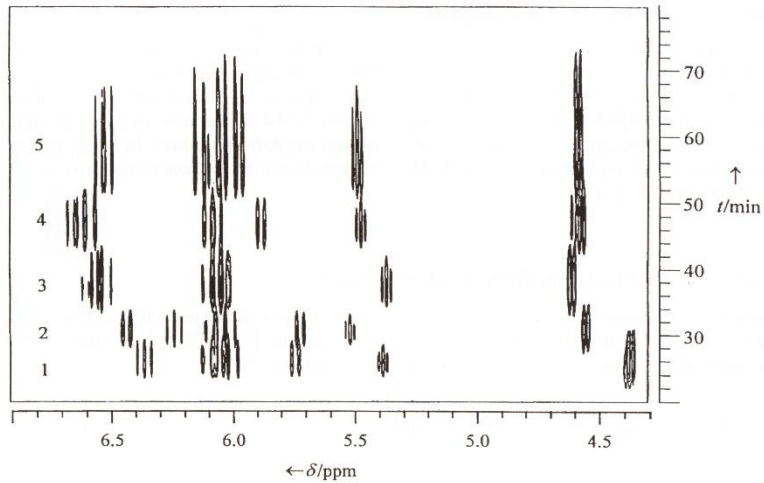
c) 3-9-19 WATERGATE (W3)

d) 3-9-19 WATERGATE (W3)

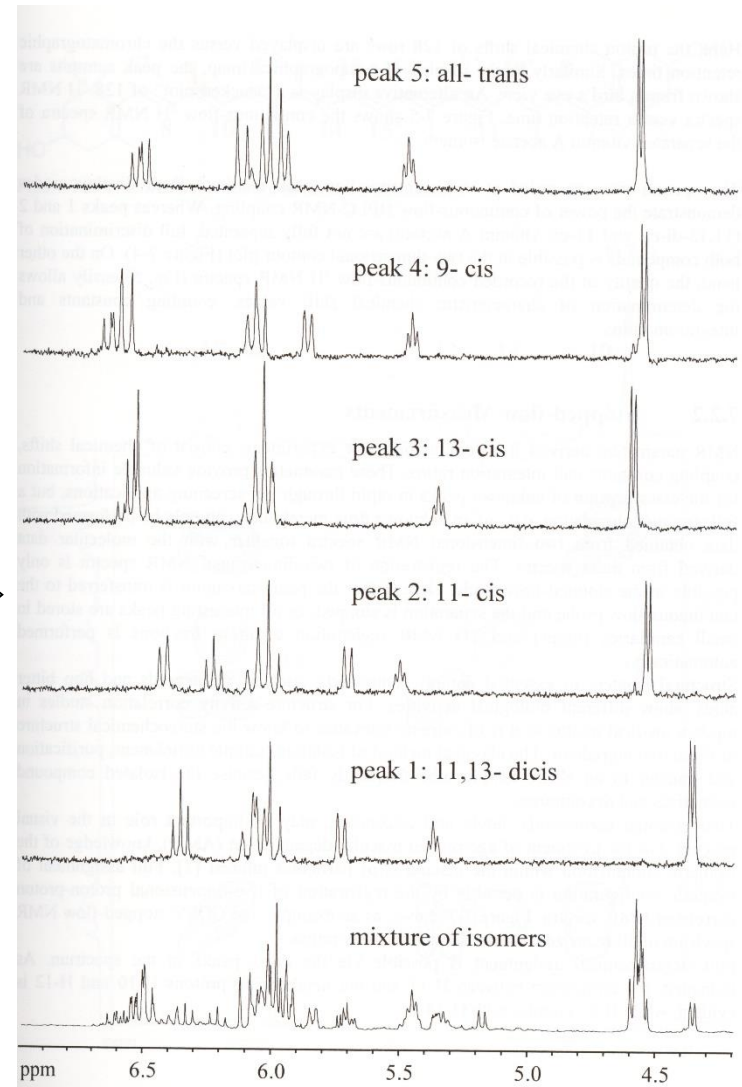


Tipični spektri LC-NMR kontinuiranoga protoka

spektri kontinuiranog protoka ^1H LC-NMR



Odvajanje izomera vitamina A



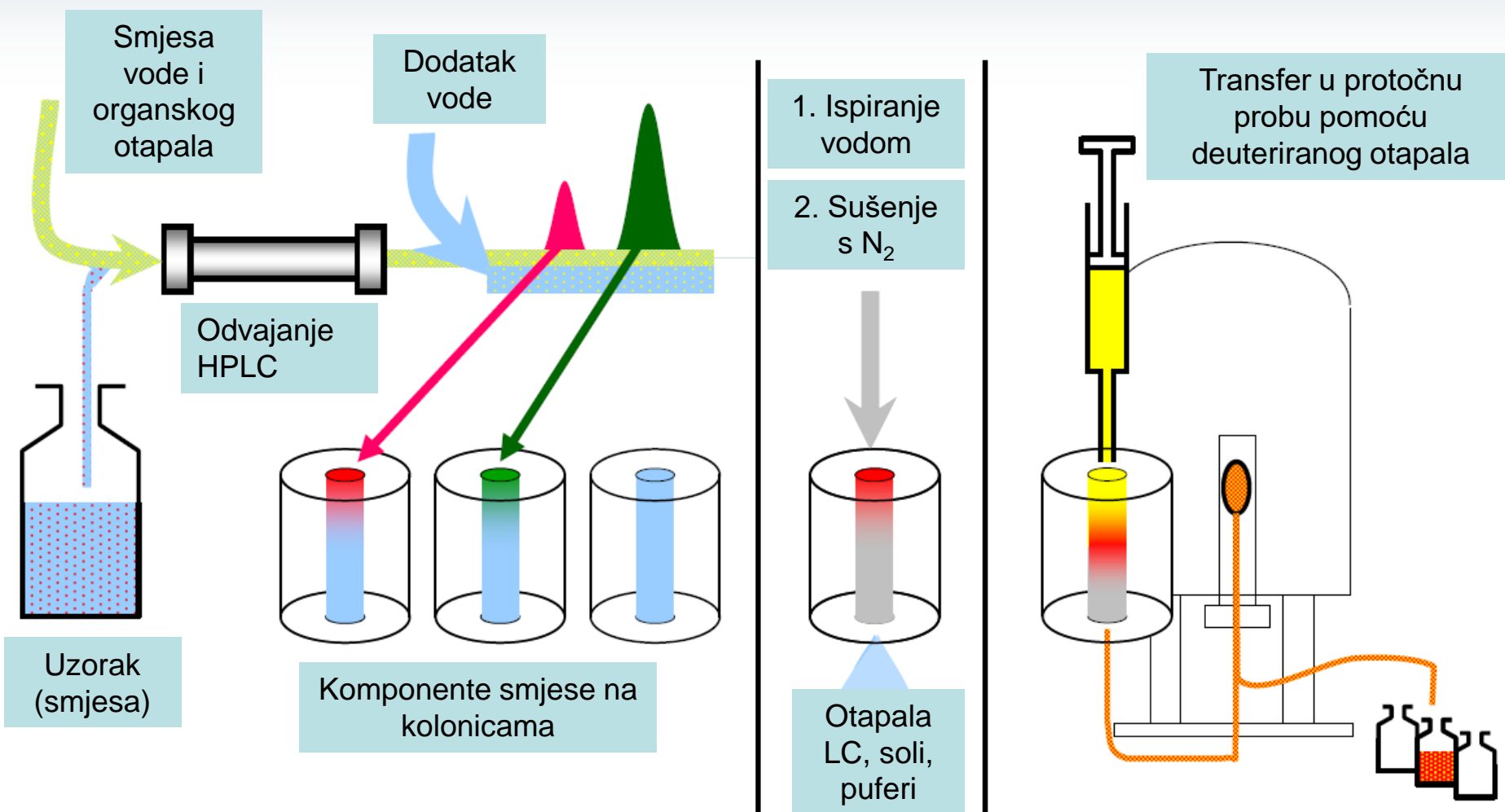
Ograničenja LC-NMR-a

- Količina izorka za NMR limitirana je kromatografijom
- Samo dio signala LC nalazi se unutar protočne probe NMR
- Količina od samo jednog injektiranja je dostupna za NMR
- Nedeuterirana otapala daju jake pozadinske signale
- Deuterirana otapala (obično D_2O) preskupa su za kromatografiju i onemogućuju opažanje izmjenjivih protona

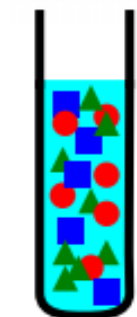
Rješenje: sprega ekstrakcije na čvrstoj fazi (SPE)

- Nakon kromatografskog odvajanja za skladištenje pikova u kolonicama SPE (cartridge)
- Kolonice se suše u struji dušika za uklanjanje otapala
- Nedeuterirana otapala se mogu koristiti za odvajanje komponenti smjese u LC-u
- Oblik pika LC nije bitan za NMR
- Deuterirana otapala se mogu koristiti za ispiranje uzoraka s kolonica i uvođenje u protočnu probu NMR
- Nije potrebna supresija signala otapala
- Mogu se opažati izmjenjivi ptoni
- Manji volumen eluiranja, povećanje S/N
- Mogućnost višestrukog skladištenja signala LC za povećanje koncentracije za NMR

SPE- ukoncentriravanje za NMR



Sustav LC-SPE / krio NMR



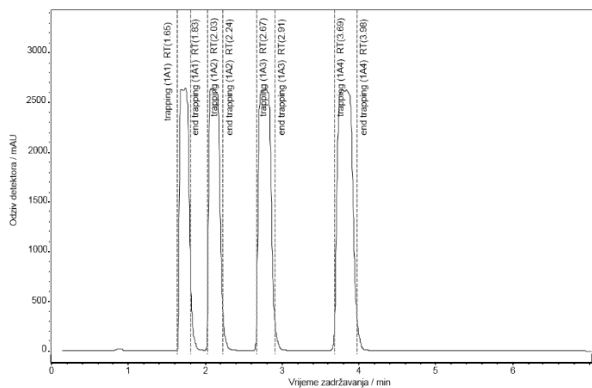
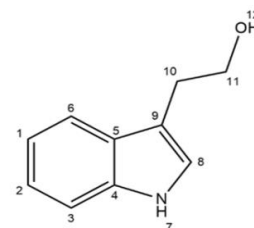
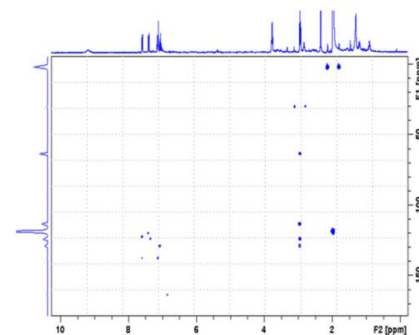
Uzorak



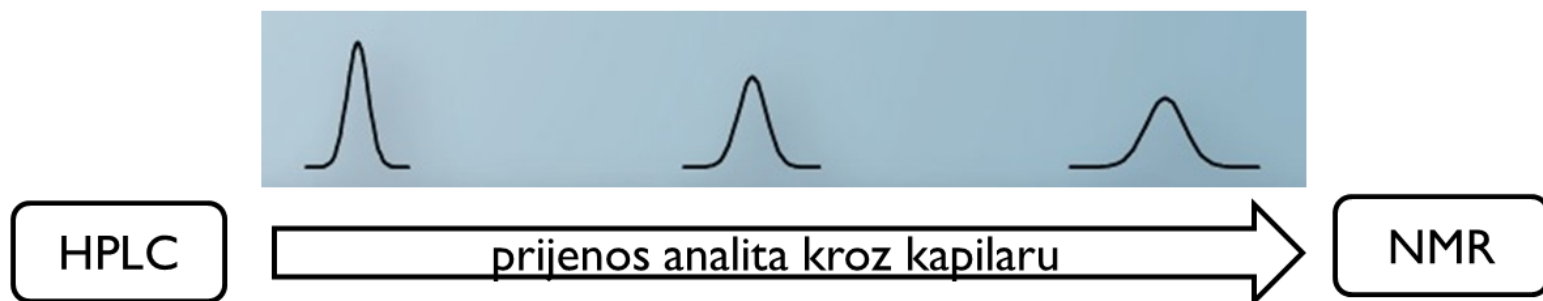
Odjeljivanje

Izdvajanje (ekstrakcija)

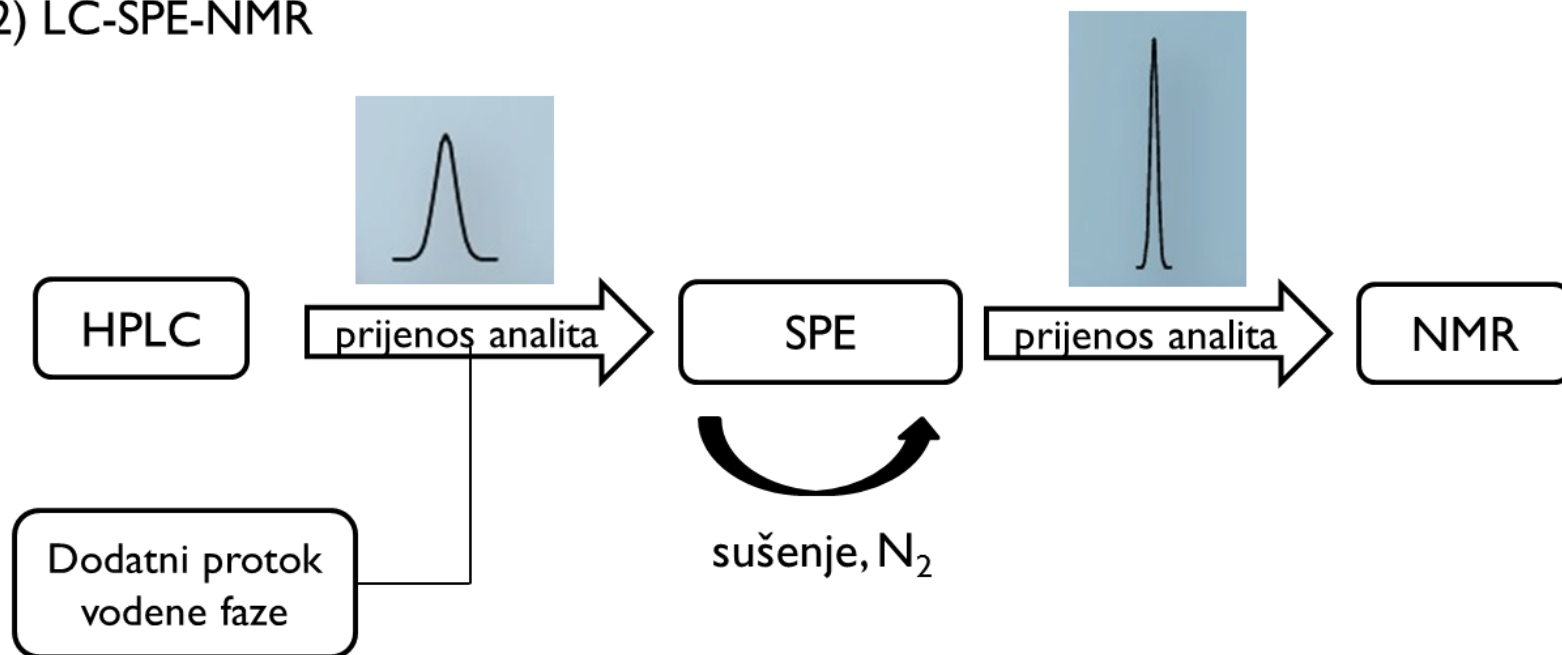
Detekcija



1) LC-NMR



2) LC-SPE-NMR



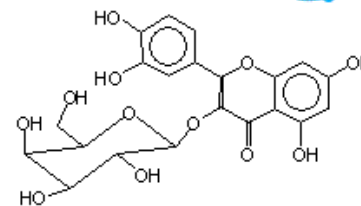
Oblik kromatografskog signala u 1) tehnici LC-NMR i 2) tehnici LC-SPE-NMR

LC-SPE-NMR

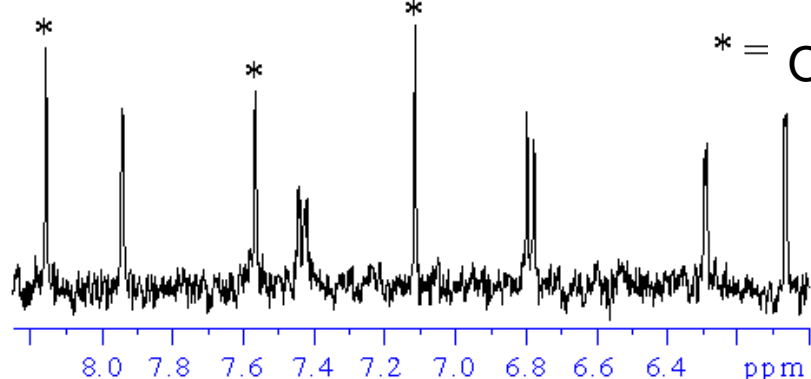
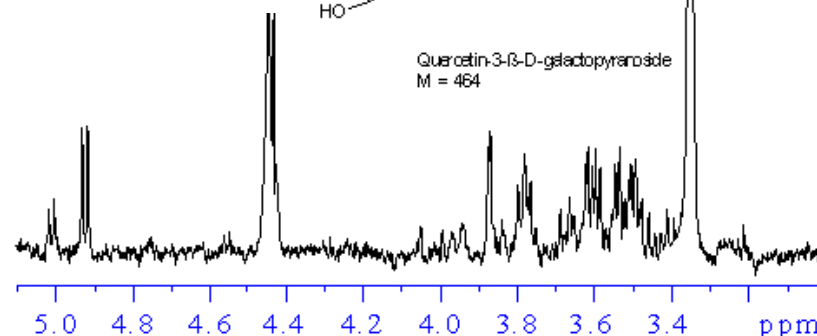
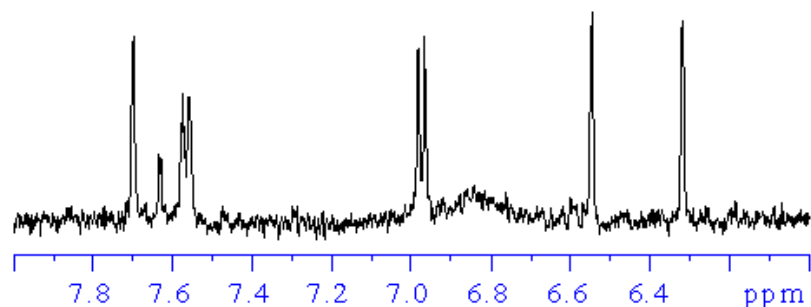


LC-NMR vs. LC-SPE-NMR

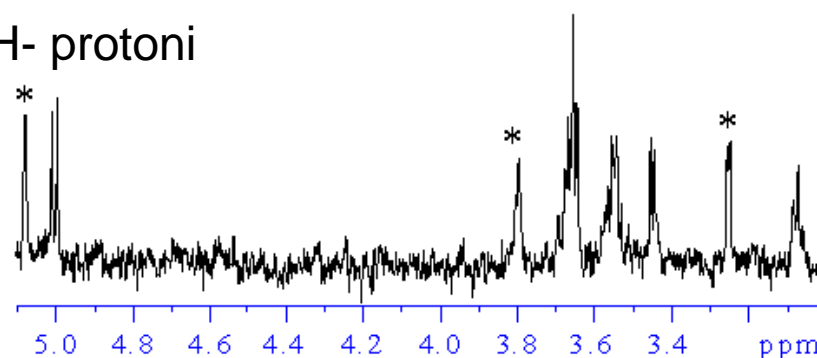
- LC-NMR sa 100 μ L injektiranja, NS = 128
- LC-SPE-NMR sa 20 μ L injektiranja, NS = 128



Quercetin-3- β -D-galactopyranoside
M = 464



* = OH- protoni



Primjena tehnike LC-SPE-NMR

analiza biljnih ekstrakata

analiza maslinovog ulja

analiza tropskog voća

analiza metabolita *in vitro*

analiza organskog sastava tla i sedimenata

analiza onečišćenja i razgradnih produkata u aktivnim farmaceutskim pripravcima

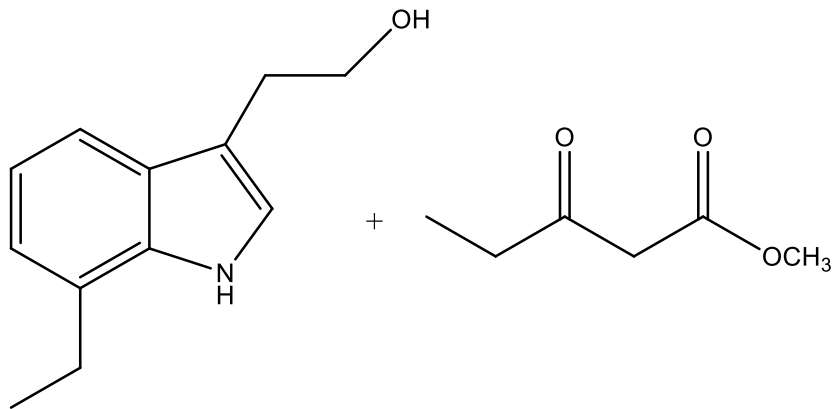
LC²-SPE-NMR za analizu složenih smjesa i onečišćenja s udjelom oko 0,1 %

LC-NMR u farmaceutskim istraživanjima

- **Određivanje strukture bioaktivnih molekula**
- **Brza analiza smjesa**
 - Nečistoće u lijekovima (>0.1%)
 - Degradacijski produkti
- **Analiza prirodnih spojeva**
 - Komponente ekstrakata
- **Kombinatorijska kemija**
- **Praćenje kemijskih reakcija**
- **Metabolizam lijekova**
 - NMR-matabonomika

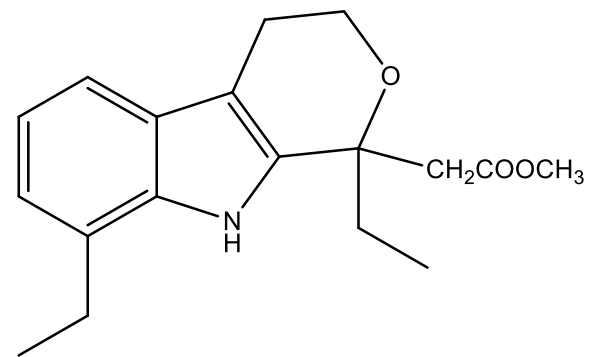
Primjer: LC-SPE / krio-NMR analiza 7- etiltryptofola

- Prekursor u sintezi etodolaka
- Lječenje reumatoidnog artritisa

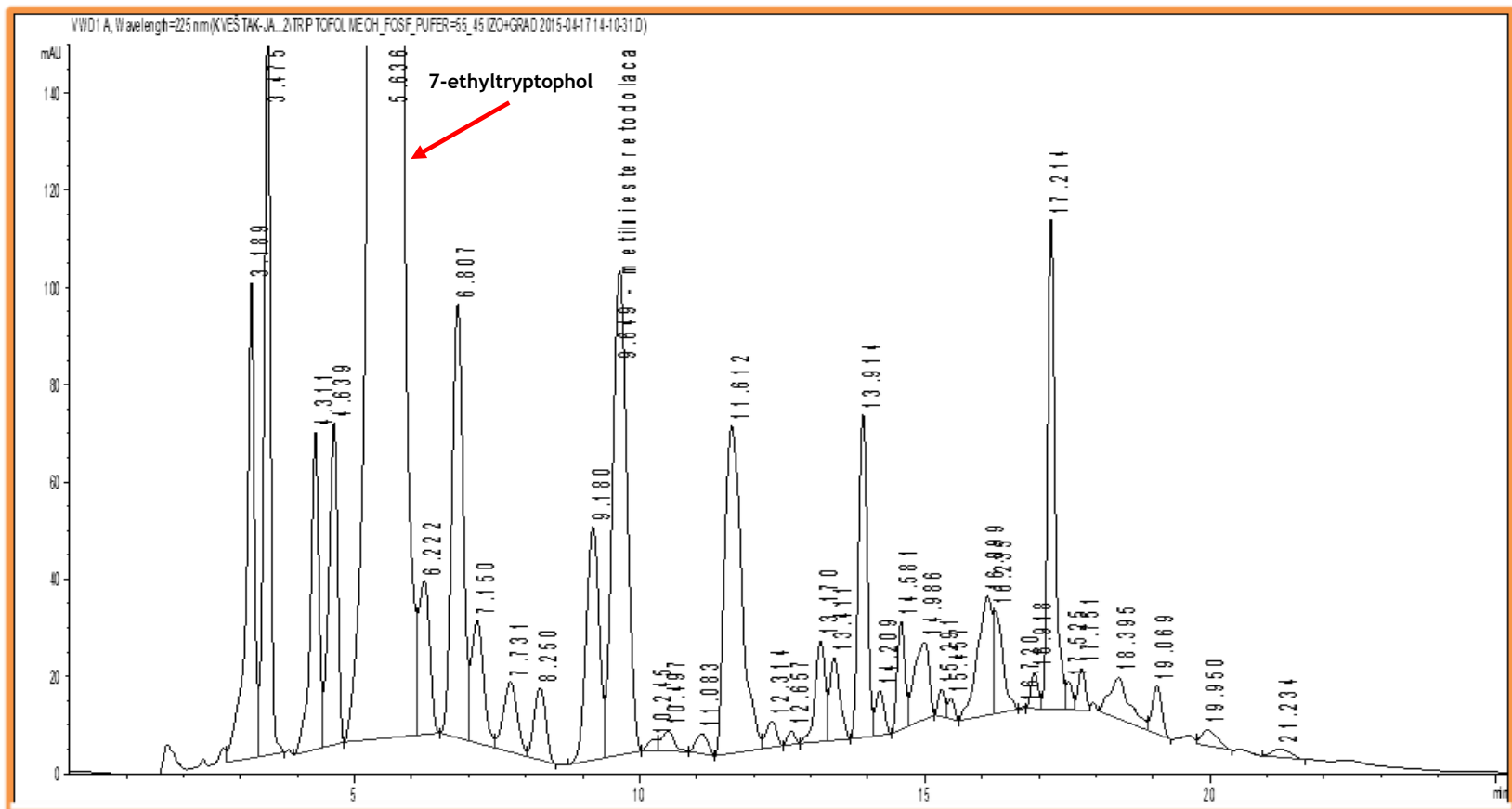


7-ethyltryptophol

Methyl 3-oxopentanoate



Methyl ester of Etodolac



LC-kromatogram uzorka 7-etiltriptofola

Optimal uvjeti

kolona: Waters XBridge Phenyl (150 mm × 4.6 mm; 3.5 μm)

mobilna faza: metanol-fosfatni pufer (pH 7), gradientna metoda

protok: 1 ml/min

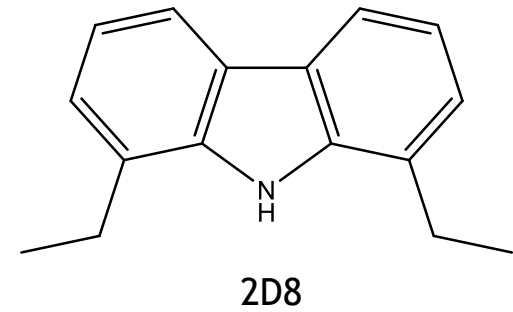
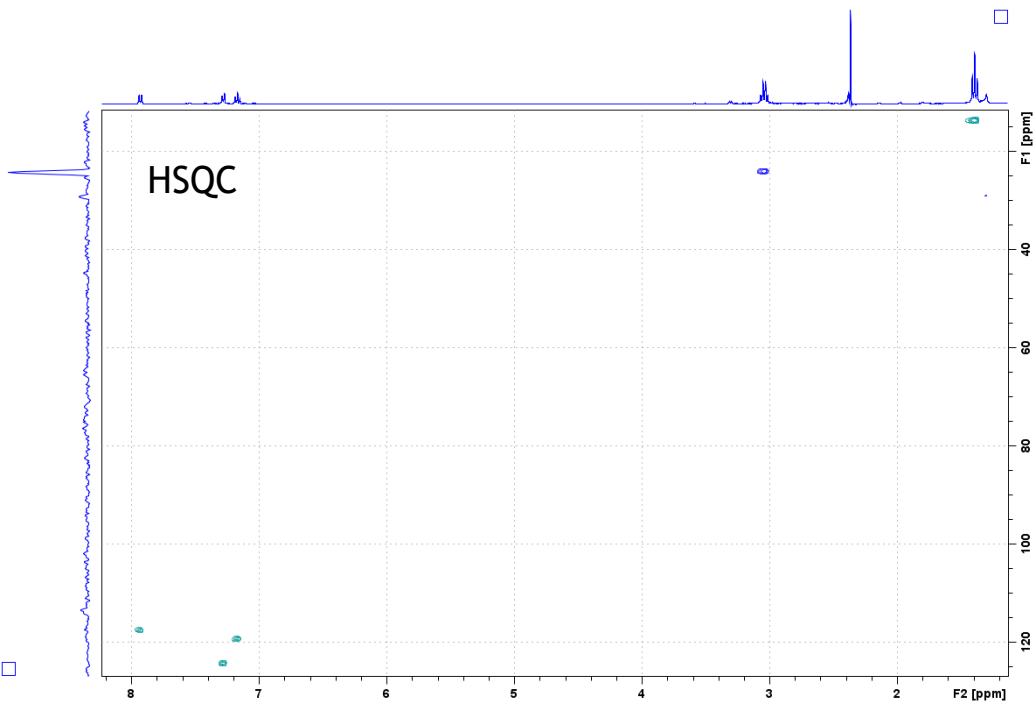
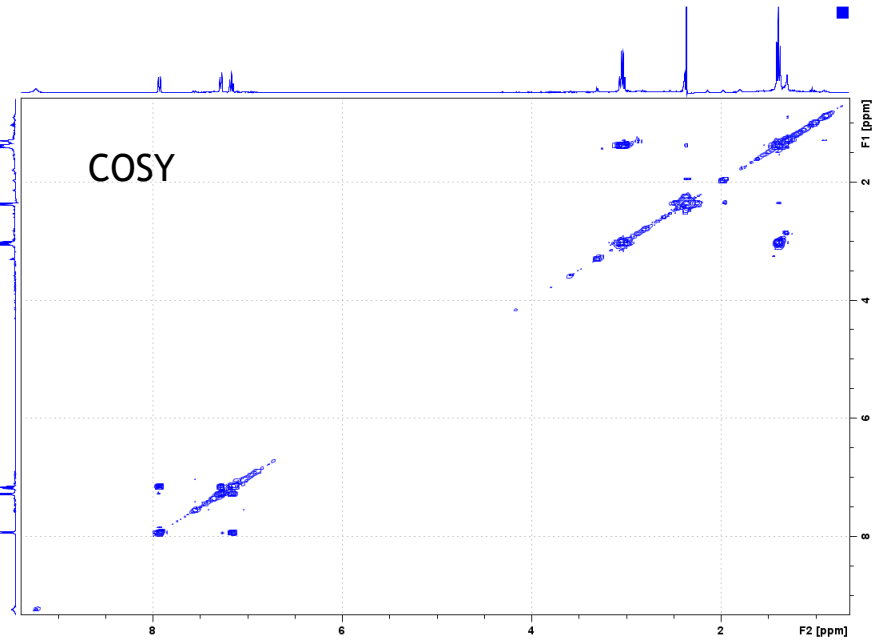
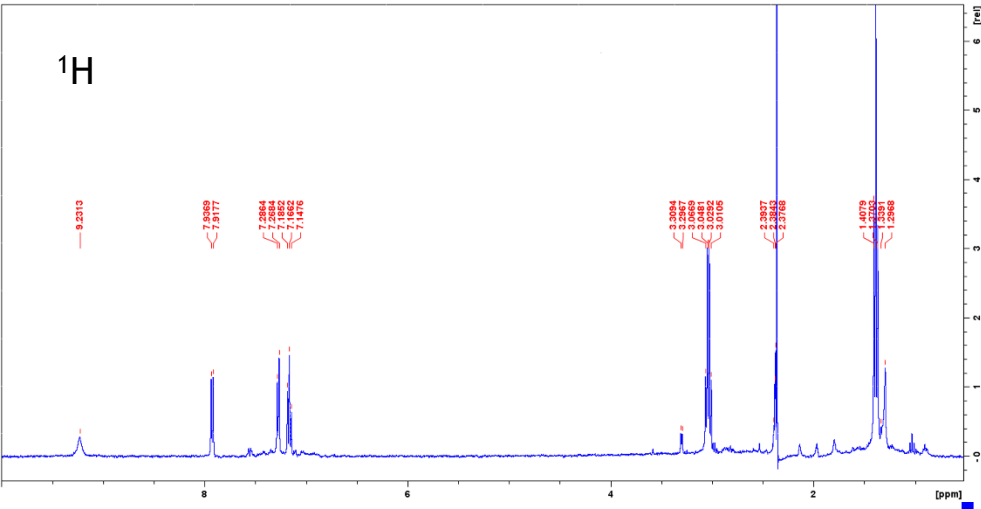
temperatura: 25 °C

injektirani volumen: 20 μl

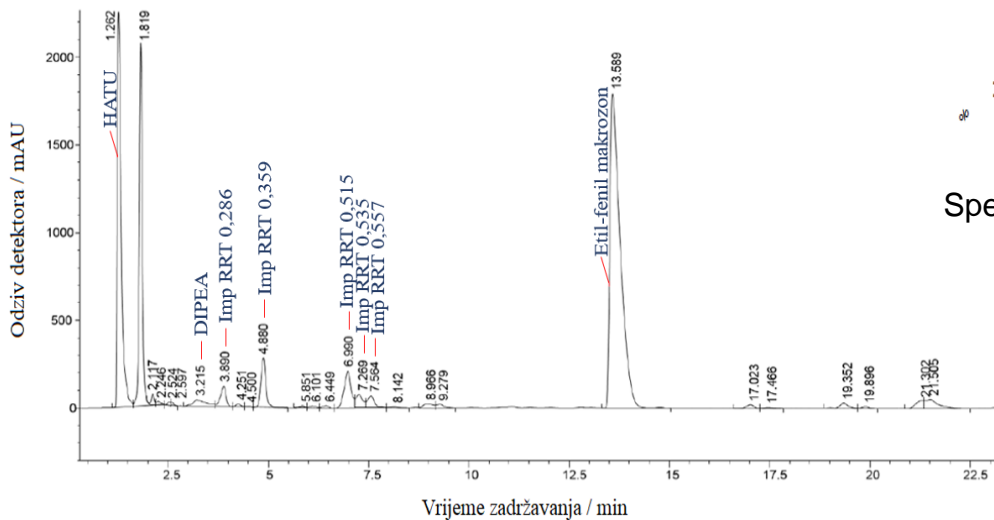
UV detekcija: 225 nm, 273 nm

početna masa: 10 mg

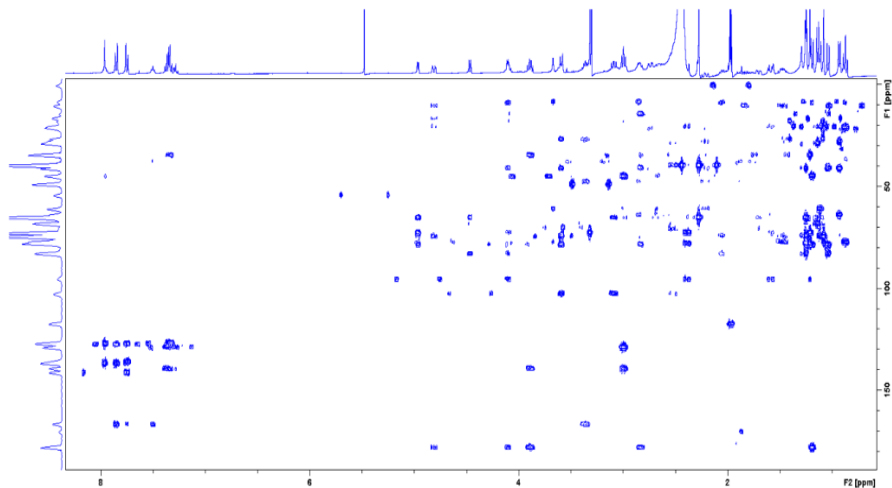
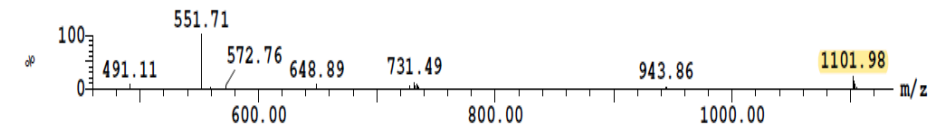
onečišćenje 2D8



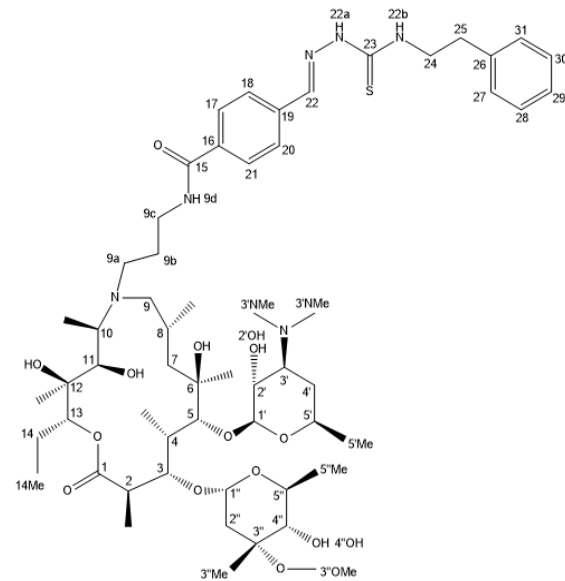
Izolacija i identifikacija nepoznatih onečišćenja u reakcijskoj smjesi pripreve makrozona



Kromatogram reakcijske smjese 9a-etil-fenil makrozona



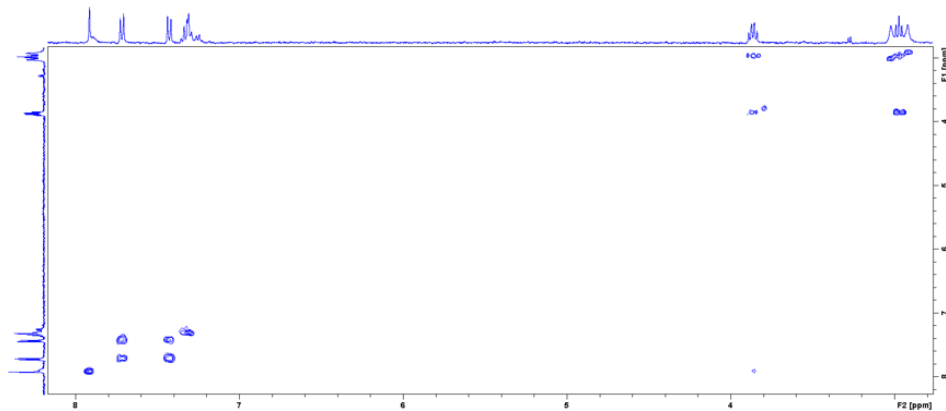
Spektar HMBC snimljen u CD_3CN pri 25 °C korištenjem sustava LC-SPE/krio NMR



Struktura 9a-etil-fenil makrozona

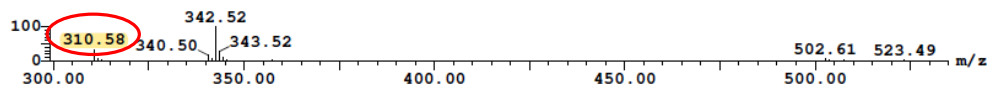
Imp RRT 0,286

Spektar LC-SPE COSY

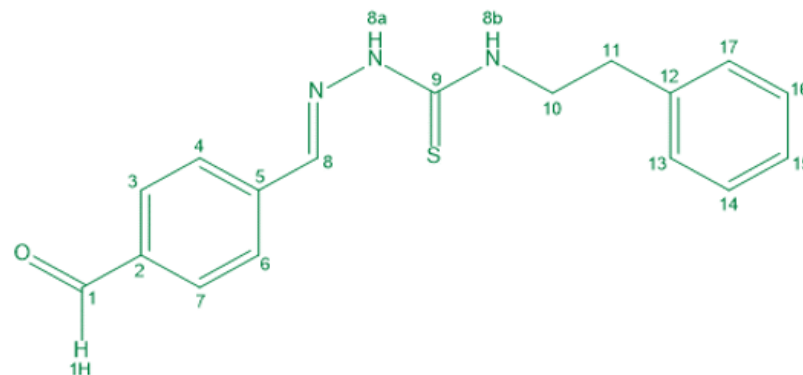


Slika 14. Spektar COSY onečišćenja Imp RRT 0,286 snimljen u acetonitrilu-*d*3 pri 25 °C.

Spektar MS



Slika 15. Spektar ESI-MS onečišćenja Imp RRT 0,286.



Slika 16. Predložena struktura onečišćenja Imp RRT 0,286