1. To Registered Participants

Bari (Italy), September 29th, 2025

Subject: Magnethon 2025 - Guidelines

Dear Participant,

Welcome to *Magnethon* – an exciting and innovative challenge designed to bring together PhD students, researchers, and passionate scientists in a unique collaborative competition. This initiative offers a rare opportunity to be directly engaged with NMR spectroscopy, guided by international experts and immersed in a dynamic academic environment.

Throughout of four expert-led sessions, you'll explore the entire analytical workflow, from sample handling to data interpretation, while working in teams, sharing ideas, and testing your skills. Participants will have the opportunity to engage actively in discussions by asking questions or making comments, with a maximum duration of 5 minutes per talk. While formal talks will not be permitted, discussions are encouraged and may be supported by PowerPoint slides or other visual aids to enhance communication effectiveness. To ensure a smooth flow of the session, participants are encouraged to reserve their talk slots in advance.

Whether you're deeply experienced or just beginner in your NMR journey, Magnethon promises an intellectually stimulating and hands-on experience. To help you navigate the challenge effectively, we have prepared the following guidelines. Please read them carefully, as submitting your results implies full understanding and acceptance of the procedures and conditions outlined. The **deadline for result submission is October 15th, 2025**.

Should you have any questions or require support at any stage, we are here to help.

Thank you for being part of Magnethon 2025 – and good luck!

Best regards,

The Magnethon Team

Contents

1. Scope

This document specifies the procedures for achieving the two analytical objectives of the Magnethon 2025: to determine the molar concentration of (2-hydroxypropyl)-β-cyclodextrin (HPBCD) and to identify its degree of substitution (DS) by means of ^{1}H NMR spectroscopy.

HPBCD has been chosen because it is widely used as an excipient in pharmaceutical formulations and extensively investigated as a potential active pharmaceutical ingredient. It has a closed circular structure formed of seven glucopyranose (GP) rings linked by α-1,4-glycosidic bond (Figure 1). HPBCD is synthesized by the reaction of β-cyclodextrin (βCD) with propylene oxide under aqueous basic conditions. Thus, HPBCD is the product of a random substitution of the secondary (OH-2 and OH-3) and the primary (OH-6) alcohol moieties. For this reason, the determination of its degree of substitution and its precise concentration are tasks of primary importance for the proper use of βCD in pharmaceutical formulations.

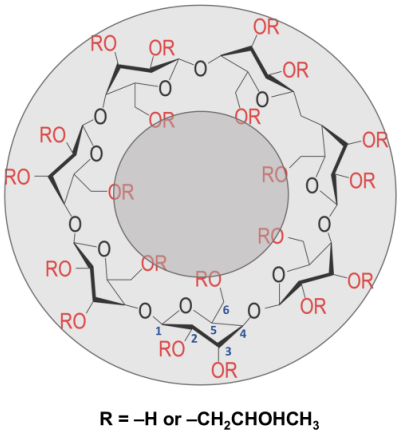

Figure 1. Schematic structure of 2-hydroxypropyl)-β-cyclodextrin

All materials and instructions are described in the following pages.

2. General Information

Differently from what initially stated on the website www.magnethon.com, the preparation of the tubes was managed by the provider and no sample preparation was requested to the participants.

The delivered tubes must be used exclusively for the Magnethon interlaboratory study. No other use is permitted without the written authorization of the Polytechnic University of Bari. After use, disposal of NMR tubes must be carried out following local legislation. Polytechnic University of Bari is not responsible for incorrect procedures for the disposal of NMR tubes. No costs can be charged to Polytechnic University of Bari for the disposal of NMR tubes.

3. Packing list

Each participating team will receive a 3D printed tube-holder containing 5 sealed NMR tubes labelled as A, B, C, D and T.

Tube A contains a D_2O solution of (2-Hydroxypropyl)-β-cyclodextrin (HPBCD) at an unknown concentration.

Tube B contains a D_2O solution of (2-Hydroxypropyl)-β-cyclodextrin (at unknown concentration) alongside three reference compounds, i.e. dimethyl sulfone ($DMSO_2$, alternatively named as Methylsulfonylmethane, MSM), fumaric acid (FA), and 3-(trimethylsilyl)propionic-2,2,3,3-d_4 acid, sodium salt (TSP-d_4) at known concentration values.

Tube C contains a D_2O solution of the three reference standards ($DMSO_2$, FA, and TSP-d_4) at known concentration values.

Tube D contains a D_2O solution of the reference standards ($DMSO_2$, FA, mannitol and TSP-d_4), all at known concentration.

Tube T contains methanol-d_4 and must be used as a spectroscopic thermometer.

All materials used are ACS grade or CRM grade. Deuterated methanol (CD_3OD, CAS 811-98-3, 99.80 %D), deuterium oxide (D_2O, CAS 7789-20-0, 99.9 %D), (2-hydroxypropyl)-β-cyclodextrin (HPBCD, CAS 128-446-35-5, Pharmaceutical Secondary Standard; Certified Reference Material), 3-(trimethylsilyl)propionic-2,2,3,3-d_4 acid sodium salt (TSP-d_4, CAS 24493-21-8), dimethyl sulfone ($DMSO_2$, CAS Number 67-71-0), fumaric acid (CAS 110-17-8), mannitol (CAS 69-65-8), and NMR tubes (Wilmad® WG-1000-7) were generously provided by Merck KGaA (Darmstadt, Germany).

NMR tubes must be handled by qualified technical staff. All tubes contain 700 µL of solution. Specific composition of Tubes A, B, C and D, with the known values of concentration, is reported in Table 1.

X_A, X_B, Y_A and Y_B indicate the unknown values to be determined by the participants.

Table 1. List of substances contained in the Tubes A, B, C and D and their respective concentrations.

Tube	Substances	MW (g/mol)	Degree of Substitution (DS)	Concentration (mmol/g)
A	HPBCD	nd[a]	X_A	Y_A
B	HPBCD	nd[a]	X_B	Y_B
	$DMSO_2$	94.1		$5.635 \cdot 10^{-3}$
	FA	116.1		$4.580 \cdot 10^{-3}$
	TSP-d_4	172.3		$3.525 \cdot 10^{-3}$
C	$DMSO_2$	94.1		$9.466 \cdot 10^{-3}$
	FA	116.1		$6.830 \cdot 10^{-3}$
	TSP-d_4	172.3		$4.041 \cdot 10^{-3}$
D	Mannitol	182.2		$80.95 \cdot 10^{-3}$
	$DMSO_2$	94.1		$9.416 \cdot 10^{-3}$
	FA	116.1		$6.606 \cdot 10^{-3}$
	TSP-d_4	172.3		$4.036 \cdot 10^{-3}$

[a]nd: not declared

4. NMR measurements

After the temperature calibration, all spectra can be recorded according to the acquisition procedure deemed most appropriate by each participating group, following the next steps.

4.1 Temperature calibration using the tube T

Use tube T as a nuclear magnetic resonance thermometer to assess the correct calibration of the Temperature Control Unit (TCU).

Please, follow the procedure described below, as reported in the literature (M. Findeisen, T. Brand, S. Berger, *Magnetic Resonance in Chemistry*, 2007, *45*, 175-178, https://doi.org/10.1002/mrc.1941).

On the temperature control unit of your spectrometer, set the sample temperature to 298 K.

Record a routine ^{1}H spectrum by 90° single excitation pulse sequence ("s2pul" for Agilent/Varian; "zg" for Bruker; "proton" for Jeol). Pick the two signals of methanol-d_4 at ca. 4.8 and 3.3 ppm and calculate the difference of their chemical shifts ($\Delta\delta$, considering 4 decimal digits). Use the $\Delta\delta$ value for calculating the sample temperature T [K] according to the following equation:

$$T = -16.7467 * (\Delta\delta)^2 - 52.5130 * \Delta\delta + 419.1381$$

Adjust the temperature control unit in a way to obtain a value suitable to reach T = 298.0 ± 0.1 K. Once optimized, use the temperature control unit setup for all further measurements described in the following paragraphs.

4.2 Experiment setup

According to European Pharmacopeia 11.0, Monographs H, Hydroxypropylbetadex, 01/2017:1804, 3041-3043, "*use the appropriate instrument settings (frequency, gain, digital resolution, sample rotation, shims, probe tuning, resolution/data point, receiver gain, etc.) to obtain a suitable spectrum for quantitative analysis (good FID and no distortion of the spectrum after Fourier transform and phase correction). The*

relaxation delay must be adapted to the pulse angle to have sufficient relaxation of the protons of interest between 2 pulses (for example: 10 s for a 90° pulse)".

Set a spectral window (SW) of 12 ppm, approximately centred at ca 4.7 ppm, the residual hydrogen signal of deuterated water.

Use the same acquisition parameters for all the tubes (A, B, C and D).

Register five spectra for each NMR tube to meet repeatability conditions, i.e. same NMR tube, same spectrometer, same user, consecutive repetitions. Different tubes can be recorded in different sessions. For each tube, follow the instructions below:

- load the tube in the magnet;
- ***wait for the temperature stabilization at 298.0 ± 0.1 K for at least 5 min;***
- perform 5 consecutive runs of the same tube to obtain 5 replicates;
- do not take out the NMR tube from the magnet between replicates.

4.3 Naming of the individual spectra

Please, name each spectrum according to the scheme: "LabID_TubeID_RepetitionID".

LabID has been assigned and communicated (along with username and password) to each participating team by the organizing committee.

TubeID is the label of the NMR tube indicated on its upper part.

RepetitionID is the repetition number you will assign to the spectrum preceded by the letter R.

For instance, if a team equipped with the spectrometer named "Lab33" receives the tubes labelled "A47, B47, C47 and D47", the five repetitions executed on the tube "A47" must be named as follows:

- Lab33_A47_R01 (repetition 1),
- Lab33_A47_R02 (repetition 2),
- Lab33_A47_R03 (repetition 3),

- Lab33_A47_R04 (repetition 4),
- Lab33_A47_R05 (repetition 5),

and the same procedure must be followed for all the other tubes “B47, C47 and D47”.

WARNING!

ONLY FOR SPECTROMETERS EQUIPPED WITH OLDER MODEL AUTOSAMPLERS.

Due to sealing, the NMR tubes have slightly variable lengths, and the nipper could fail while catching the tube in proximity to the magnet. To avoid any inconvenience, please pay attention to the autosampler configuration.

4.4 Processing parameters (Tubes A - D)

Participants can choose their preferred procedure for processing NMR spectra.

5. Analytical targets

5.1 Determination of the degree of substitution

The degree of substitution (DS) of (2-Hydroxypropyl)-β-cyclodextrin must be determined in both Tubes A and B, producing two values indicated as X_A and X_B in Table 1, respectively. The DS calculation is based on the comparison between the signal of the methyl groups (around 1 ppm) and the two signals of the anomeric hydrogens (around 5 ppm) indicated as Me and Gl, respectively (Figures 2 and 3).

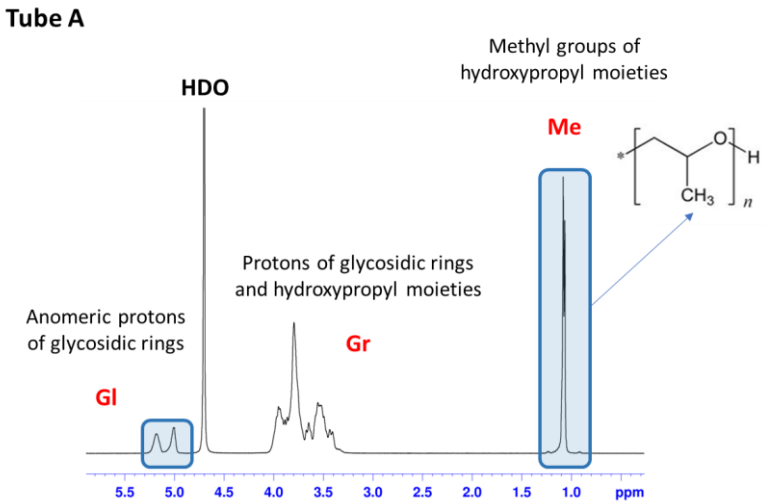

Figure 2. Typical ^{1}H NMR spectrum of Tube A (400 MHz, D_2O, 298 K)

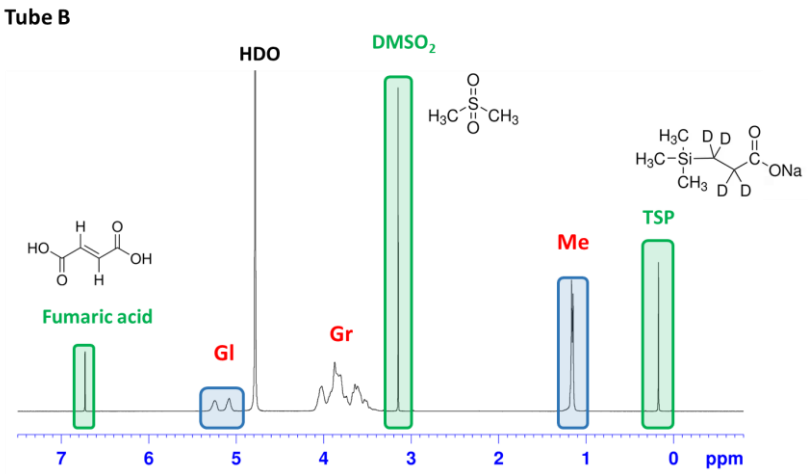

Figure 3. Typical ^{1}H NMR spectrum of Tube B (400 MHz, D_2O, 298 K)

According to *European Pharmacopeia 11.0, Monographs H, Hydroxypropylbetadex, 01/2017:1804, 3041-3043* and *E. Kalydi, M. Malanga, T. T. Nielsen, R. Wimmer, S. Béni, Carbohydrate Polymers, 338 (2024)* 122167 (https://doi.org/10.1016/j.carbpol.2024.122167), DS must be calculated as follows:

1) measure the area under the signals assigned to the methyl groups in the hydroxypropyl moieties around 1.2 ppm (Me), and the area under the signals assigned to the seven anomeric hydrogens between 5 ppm and 5.4 ppm (Gl);

2) calculate the degree of substitution (DS) using the equation Eq. 1:

Eq.1
$$DS = \frac{\frac{Me}{3}}{\frac{Gl}{7}}$$

Each participant can freely define the limits of the integration regions based on their assessment.

5.2 Calculation of HPBCD concentration

The concentration of HPBCD must be determined for both Tube A and Tube B, producing two values indicated as Y_A and Y_B in Table 1, respectively. HPBCD concentration must be expressed as mmol/g units.

Each participant can freely define the limits of the integration regions based on their assessment.

5.2.1 Calculation of Y_A based on the external standard method

The determination of HPBCD concentration Y_A by using the external standard approach must be performed on Tube A. Participants are free to choose one or more reference tubes among Tube B (Figure 3), Tube C (Figure 4) and Tube D (Figure 5) containing different quantities (Table 1) of the three standards, i.e. FA, $DMSO_2$, and TSP. Mannitol was included in Tube D to simulate the matrix conditions as in Tube B.

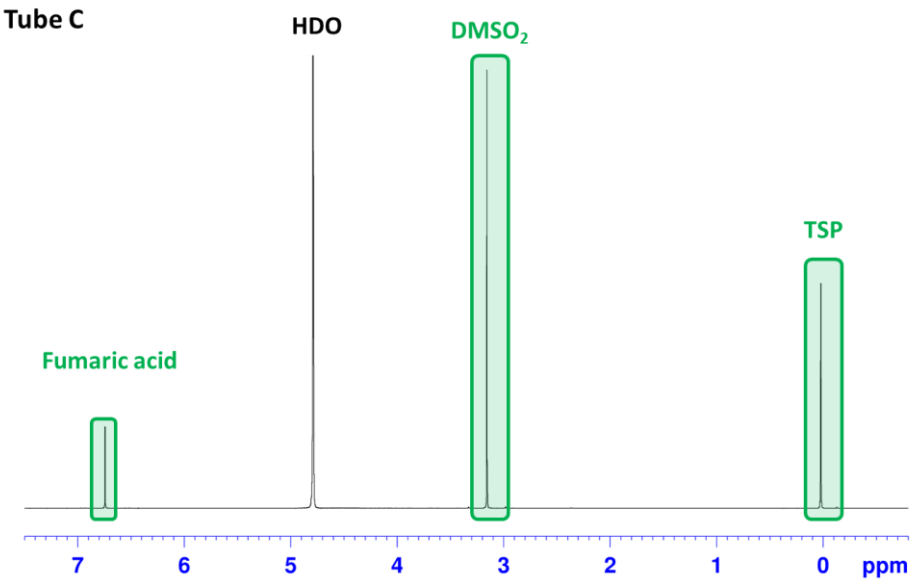

Figure 4. Typical ^1H NMR spectrum of Tube C (400 MHz, D_2O, 298 K)

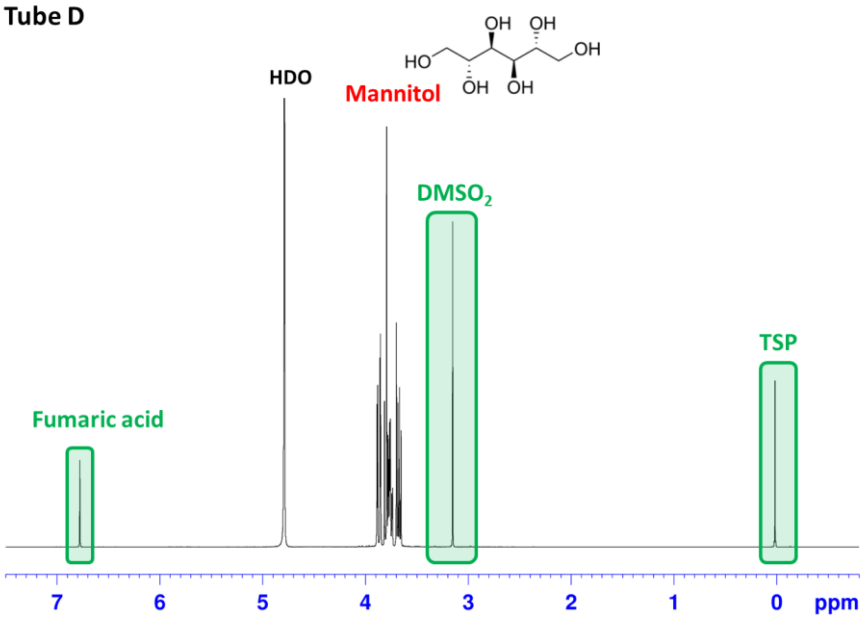

Figure 5. Typical ^{1}H NMR spectrum of Tube D (400 MHz, D_2O, 298 K)

5.2.2 Calculation of Y_B based on the internal standard method

In this task, participants must use only Tube B, which contains HPBCD, $DMSO_2$, FA, and TSP (Figure 3). The use of one of the following equations might be considered:

Eq. 3.1 $$Y_B = m_{HPBCD} = m_{STD} \frac{nH_{STD}}{nH_{GI}} \frac{A_{GI}}{A_{STD}}$$

Eq. 3.2 $$Y_B = m_{HPBCD} = m_{STD} \frac{nH_{STD}}{nH_{Me}} \frac{A_{Me}}{DS \cdot A_{STD}}$$

m_{HPBCD} = HPBCD concentration expressed as molality (mmol/g);

nH_{STD} = number of hydrogen atoms generating the signal selected as reference (FA: 2; $DMSO_2$: 6; TSP: 9);

nH_{GI} = 7, that is the number of anomeric hydrogen atoms (hydrogen bound to C1 carbons) of the 7 glucopyranosic rings;

A_{GI} = area under the signals of the anomeric hydrogen atoms of the 7 glucopyranosic rings;

nH_{Me} = 3, that is the number of methyl hydrogen atoms belonging to the hydroxypropyl moieties;

A_{Me} = area under the signals of the methyl hydrogen atoms belonging to the hydroxypropyl moieties;

DS = degree of substitution indicated as X_B and calculated following the procedure described in section 5.1;

A_{STD} = area under the signal selected as internal reference;

m_{STD} = concentration of the selected internal standard expressed as molality (mmol/g) as reported in Table 1.

6. Results and spectra submission

Participants must log in to the website https://nmr.istracer.it/index.php by entering their username and password and clicking on “Data input” (left side menu). Participants will find two separate links titled “Magnethon Conc” and “Magnethon DS” to upload the concentration and degree of substitution values, respectively. The results must be inserted by using at least 4 decimal places in the blanks (see the example in Figure 6).

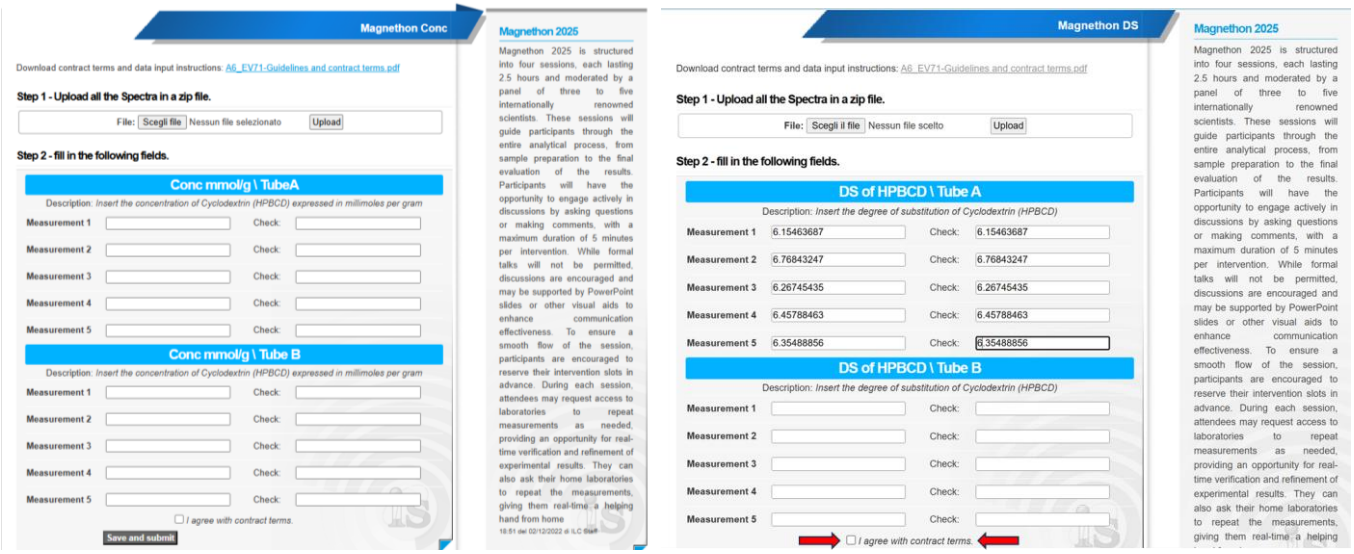

Figure 6. Page for “Data Input”

Please, consider that “Measurement” stands for “Repetition”. In the “Check” field insert again the value of the corresponding measurement.

Consider the opportunity to proceed by partial data input.

Once a block of data (for instance, the values in “DS of HPBCD \ Tube A”) has been uploaded, the participants must agree to the contract terms (as indicated by the red arrows at the bottom of Figure 6),

and then click on “Save and submit”. In this way, the data will be definitively uploaded and saved in the platform. Please consider saving frequently, as participants may lose their data due to the timeout limits of the webpage, and they are asked to restart filling in the data.

Participants must upload in the webpage “Magnethon Conc” a zip folder containing all the recorded NMR spectra as raw data (FID), named as described in Paragraph 4.3. The total number of spectra must be 21 [5 spectra x 4 tubes (A, B, C, and D) + 1 spectrum for tube T].

Should you have any questions or require support at any stage, send an e-mail to magnethon@gmail.com.

7 And the winner is …

The winner will be the team obtaining the highest score calculated according to the following equation:

$$Magnethon\ Winner = \max_{0 \le z \le 3} \left[40 \cdot \left\{ \frac{3 - \left| Z_{X_A} \right|}{3} \right\} + 30 \cdot \left\{ \frac{3 - \left| Z_{X_B} \right|}{3} \right\} + 15 \cdot \left\{ \frac{3 - \left| Z_{Y_A} \right|}{3} \right\} + 15 \cdot \left\{ \frac{3 - \left| Z_{Y_B} \right|}{3} \right\} \right]$$

where $Z_{X_A}, Z_{X_B}, Z_{Y_A}, Z_{Y_{AB}}$ are the z-scores calculated for each team based on the uploaded values of X_A, X_B, Y_A and Y_B, respectively. The z-score will be referred to the consensus value and will be automatically calculated when the submission session will be closed. In case of z values higher than 3, z will be set to 3.