Joint EDQM-USP Webinar on "*Orthogonal Analytical Methods for the Characterisation of Pharmacopoeial Reference Standards*"

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Webinar, 9 October 2025, from 15:00 to 16:00 (CEST, Paris, France)

Overview of the programme

- ***Welcome***
- ***Case Study – Orthogonal Analytical Methods for Chemical Identity Confirmation and Value Assignment***
- ***qNMR as a 'Selective' Analytical Balance – Case Study: Freeze-dried Oxytocin***
- ***Questions & Answers***

Definition – Orthogonal

ὀρθός(orthos) - straight, right, proper

γωνία (gonia) - angle, corner

= from a different angle

Orthogonal Analytical Methods exploit

different chemical or physical measurement principles

for analysis, thus **quality and reliability** of the measurement are **increased**

ISO 17034:2016 - Characterization

Chapter 7.12.3 The RMP shall select a characterization strategy appropriate for the intended use of the RM

Characterization can include, but is not limited to, the following approaches:

a) using a single reference measurement procedure in a single laboratory

b) characterization of a non-operationally defined measurand using two or more methods of demonstrable accuracy in one or more competent laboratories

c) characterization of an operationally-defined measurand using a network of competent laboratories

d) value transfer from an RM to a closely matched candidate RM performed using a single measurement procedure performed by one laboratory

e) characterization based on mass or volume of ingredients used in the preparation of the RM

ISO 33405:2024 - Purity

Chapter 9.6

The purity of substances can either be determined directly (by measuring the amount of the substance in question) or indirectly by subtracting the mass or mole fractions of all impurities from 100%.

9.6.2 Direct Determination of Purity

- Suitable methods include coulometry, titrimetry, and calorimetry (freezing point depression) as well as qNMR
- Methods requiring calibration (e.g., HPLC, GC) can in principle be used for purity assignment

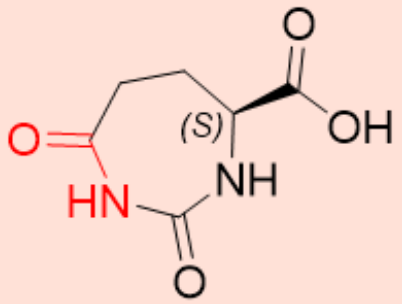
9.6.3 Indirect Determination of Purity

- Purity can be determined by difference, using a set of orthogonal analytical techniques capable of detecting and quantifying all the major classes of impurities in the material
- The purity of the main component is computed by difference

Case #1 – Structure Identification

An Impurity RS

Initially Proposed struture
Structure-1



Chemical Formula: $C_6H_8N_2O_4$
Exact Mass: 172.05
Molecular Weight: 172.14
Elemental Analysis: C, 41.86; H, 4.68; N, 16.27; O, 37.18

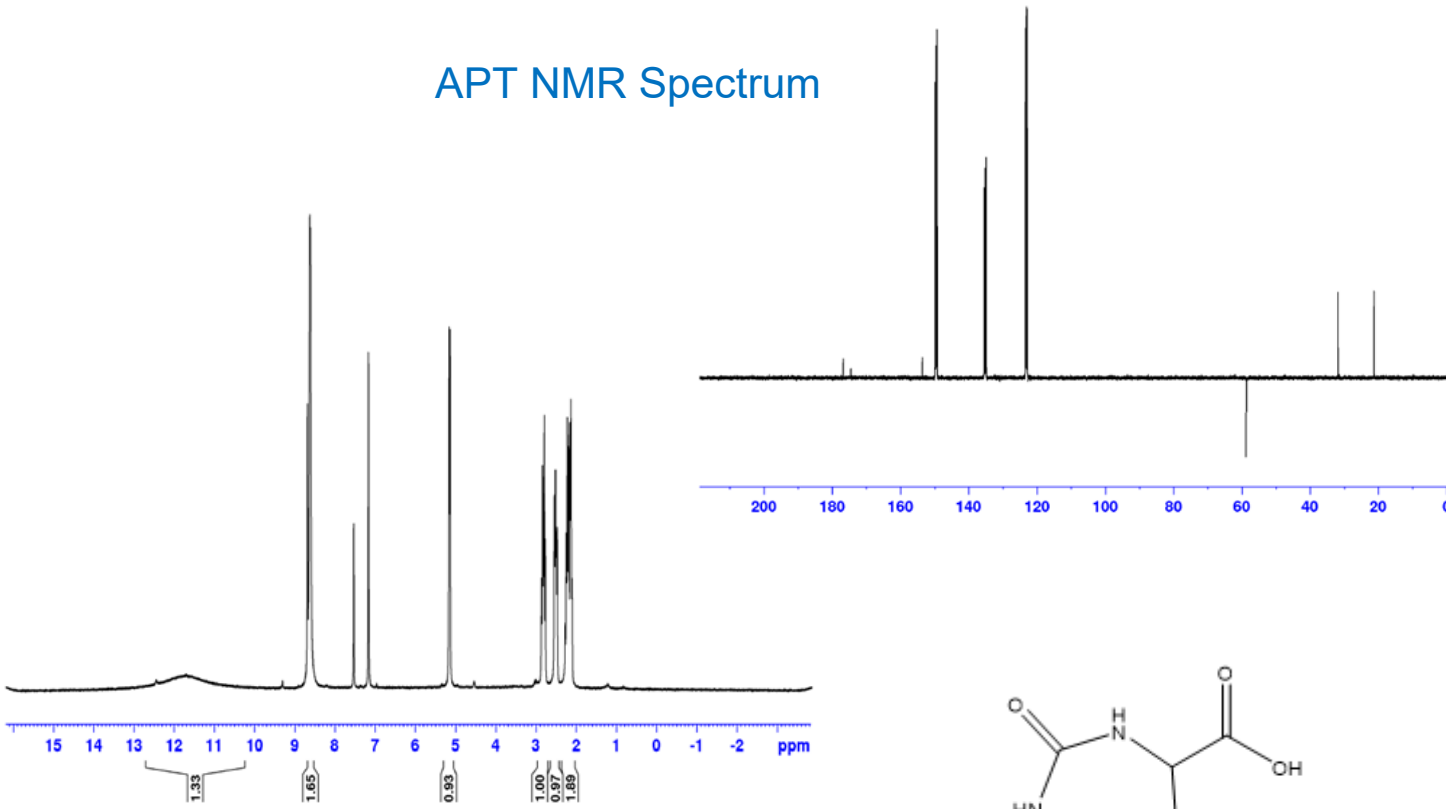
Identification Tests:

IR, Mass Spec.,NMR, CHN Analysis, and RRT comparison with the value listed in the HPLC method in the monograph

Case #1 – Structure Identification

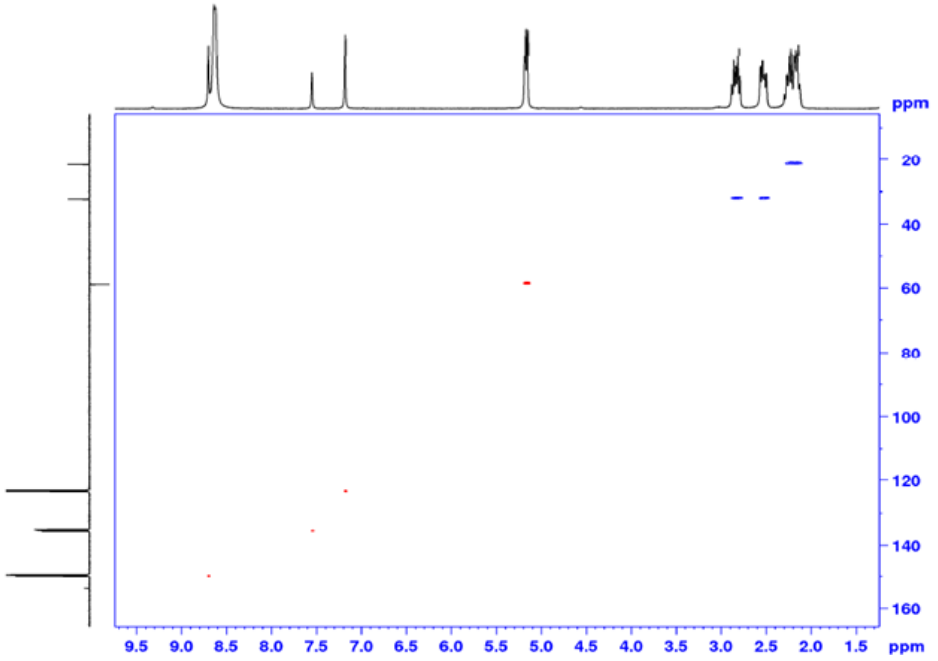
NMR Solvent: Pyridine-d_5

APT NMR Spectrum



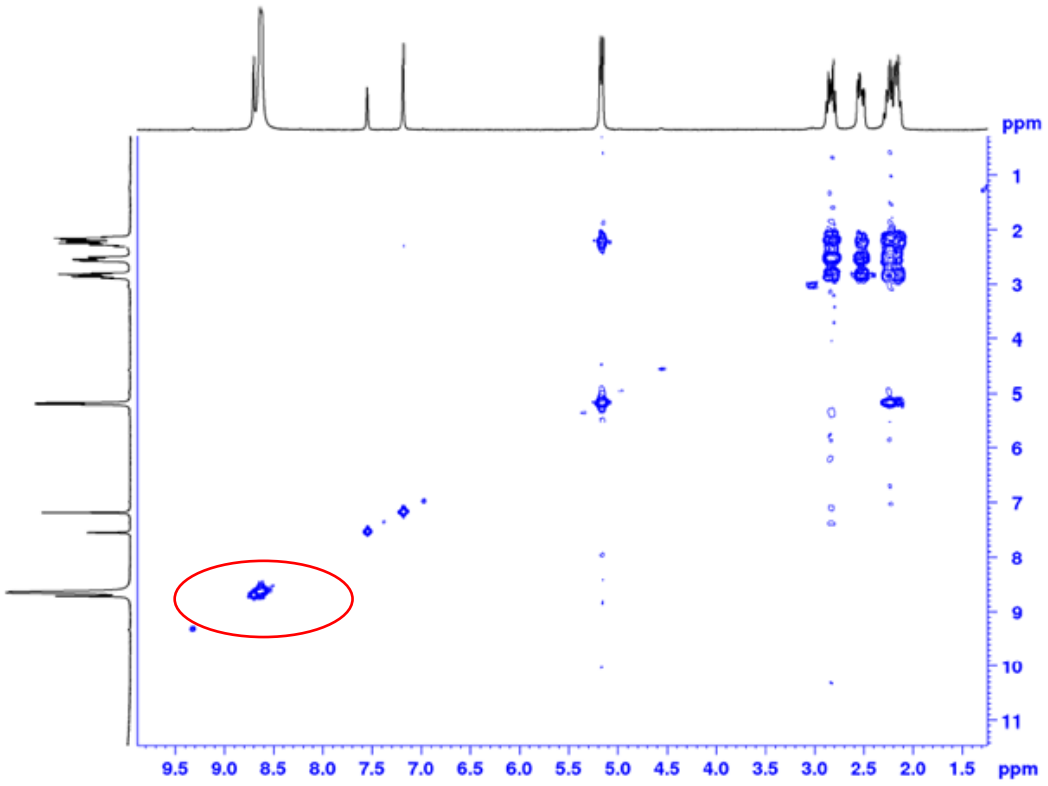
Proton NMR Spectrum

$^{1}H/^{13}C$ HSQC NMR Spectrum

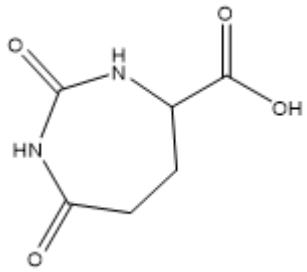


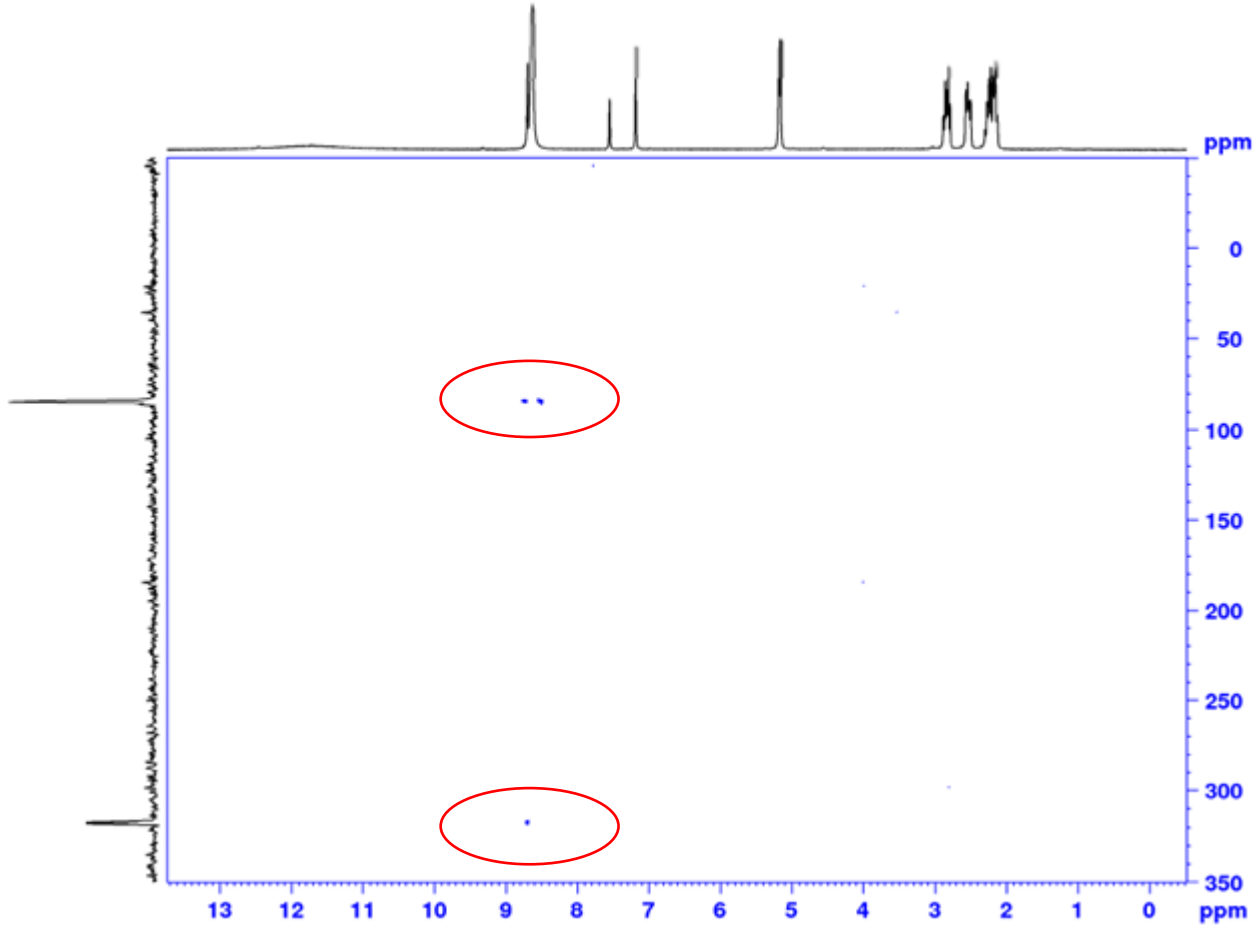
Case #1 – Structure Iden

NMR Solvent: Pyridine-d_5



$^1H/^1H$ Cosy NMR Spectrum



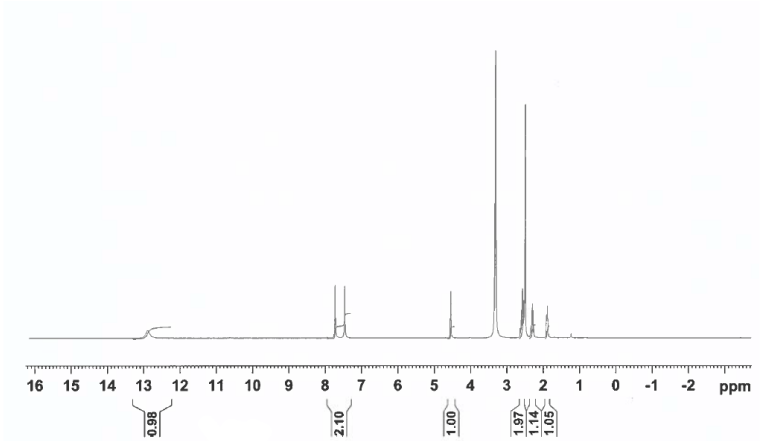


$^1H/^{15}N$ HMBC NMR Spectrum

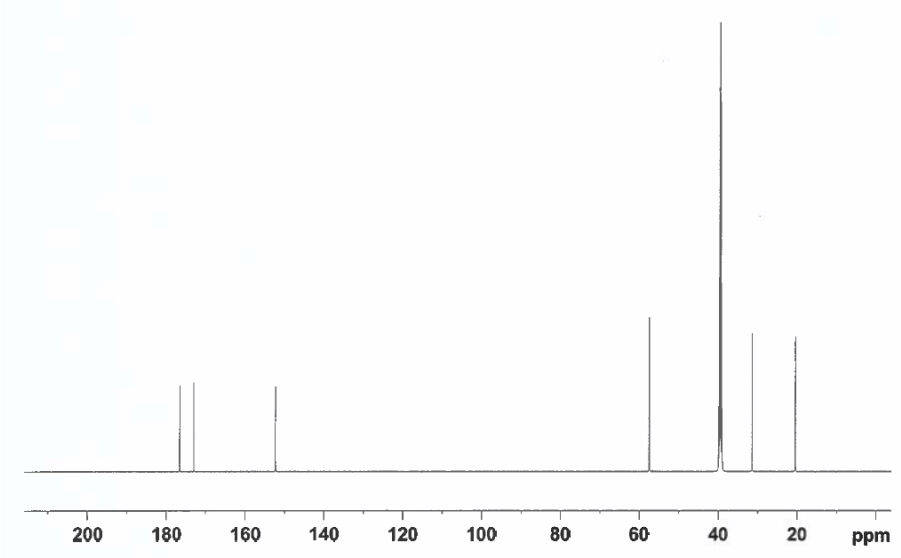
Case #1 – Structure Identification

NMR Solvent: DMSO-d_6

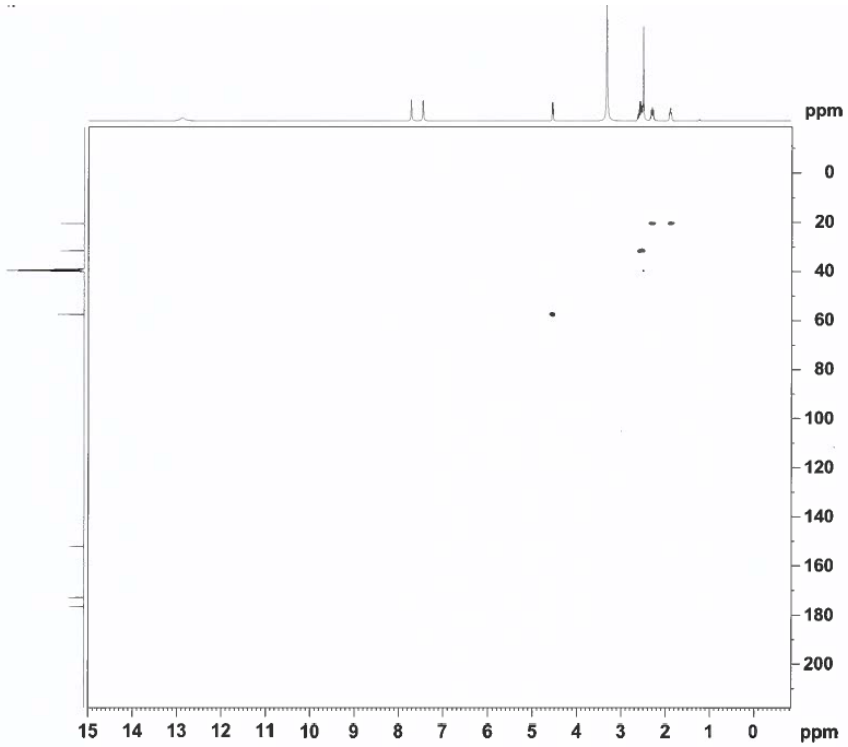
Proton NMR Spectrum



^{13}C NMR Spectrum

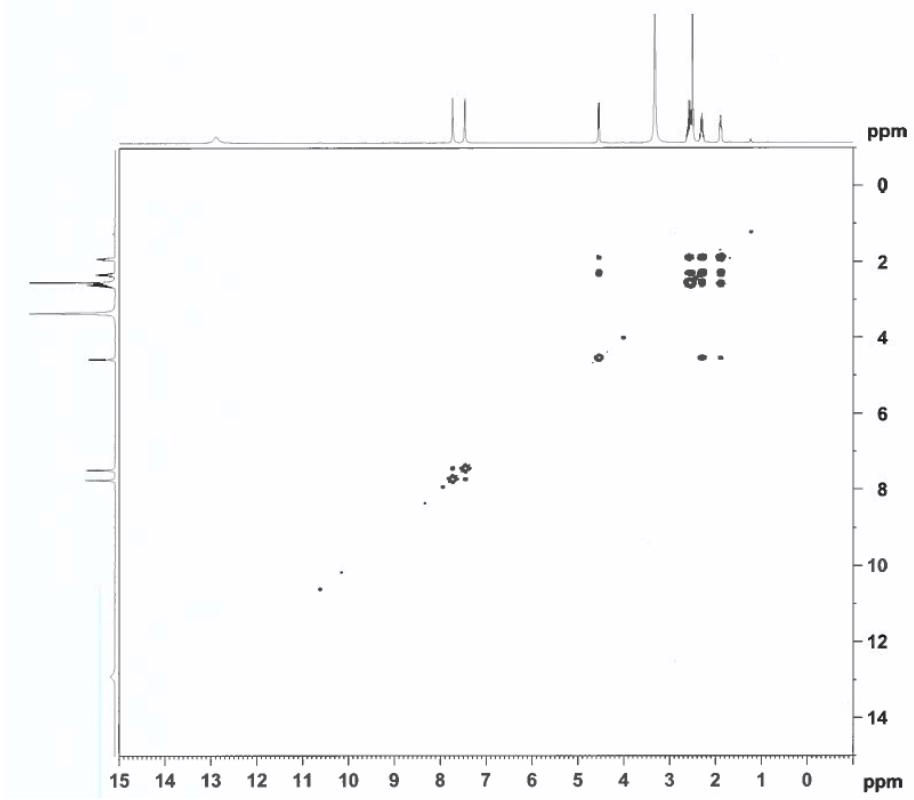


^{1}H/^{13}C HSQC NMR Spectrum

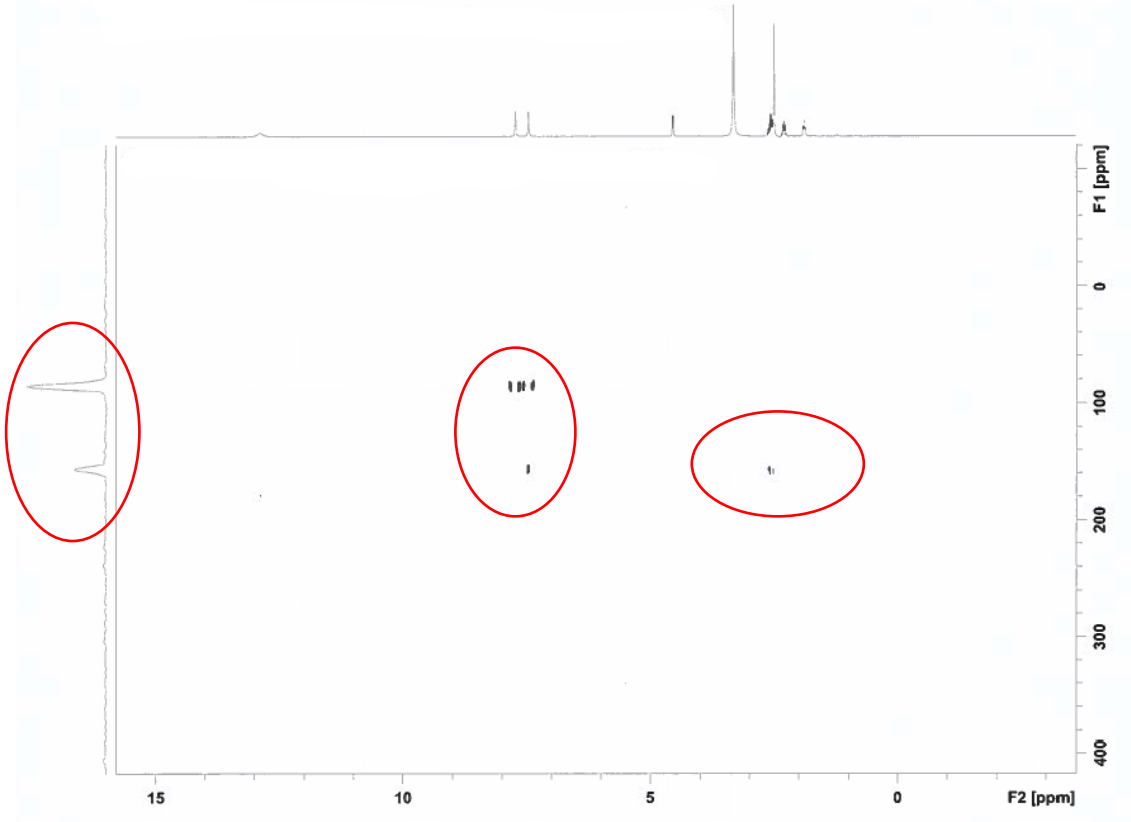


Case #1 – Structure Identification

NMR Solvent: DMSO-d_6



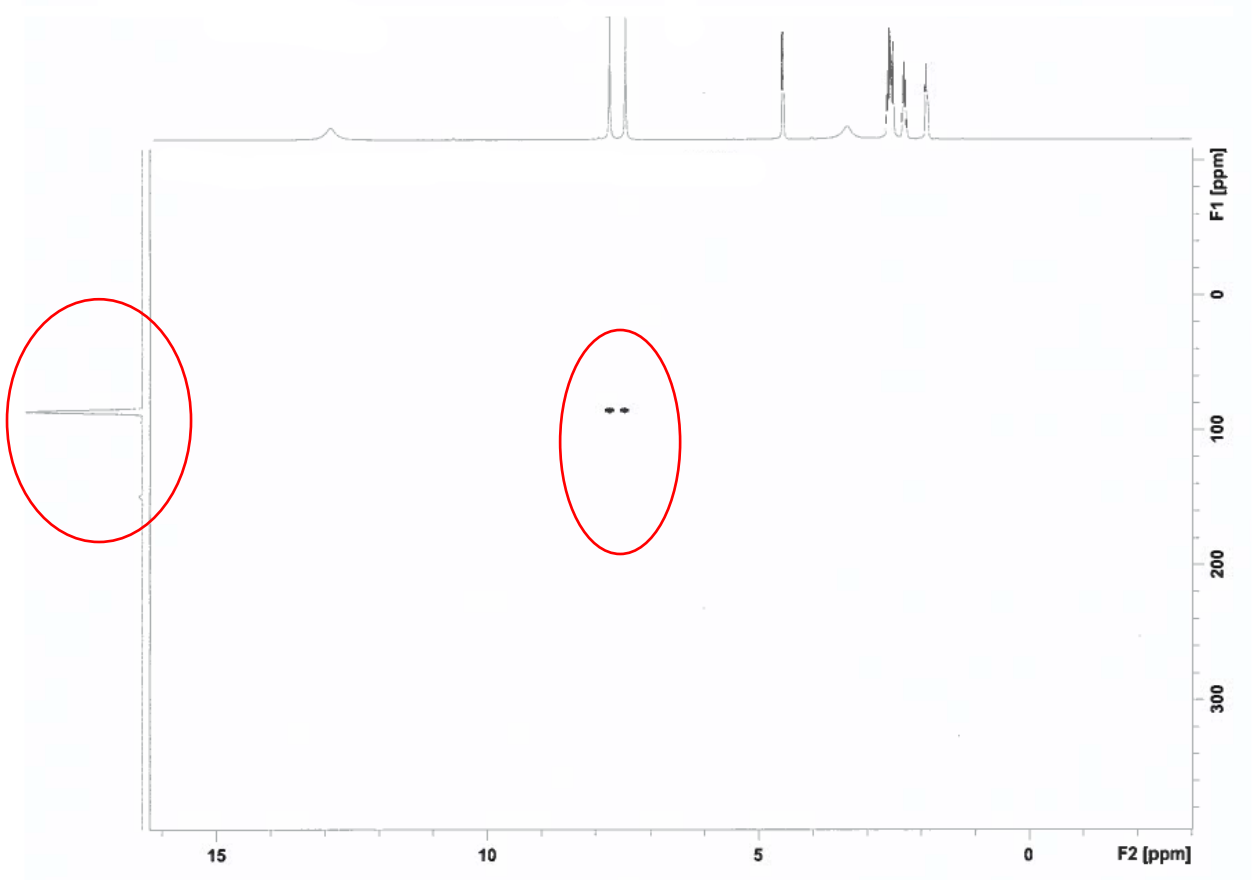
$^{1}H/^{1}H$ COSY correlation observed among the amide protons



$^{1}H/^{15}N$ HMBC - The nitrogen signal at about 158 ppm indicative of the presence of tertiary amide

Case #1 – Structure Identification

NMR Solvent: DMSO-d_6



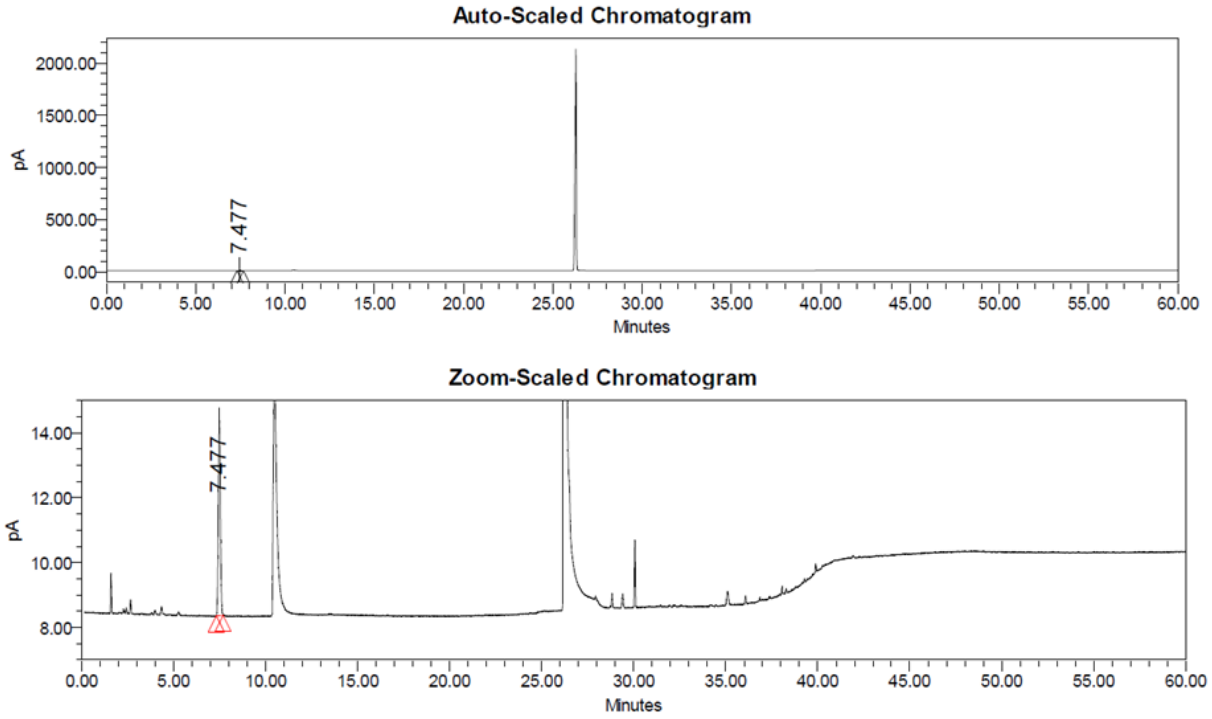
$^{1}H/^{15}N$ HSQC indicating primary amine protons correlation to the same nitrogen environment

Re-Proposed Structure
Structure-2

Case #2 – Assigned Value Accuracy

Assigned Value Determined by the Mass Balance Approach:

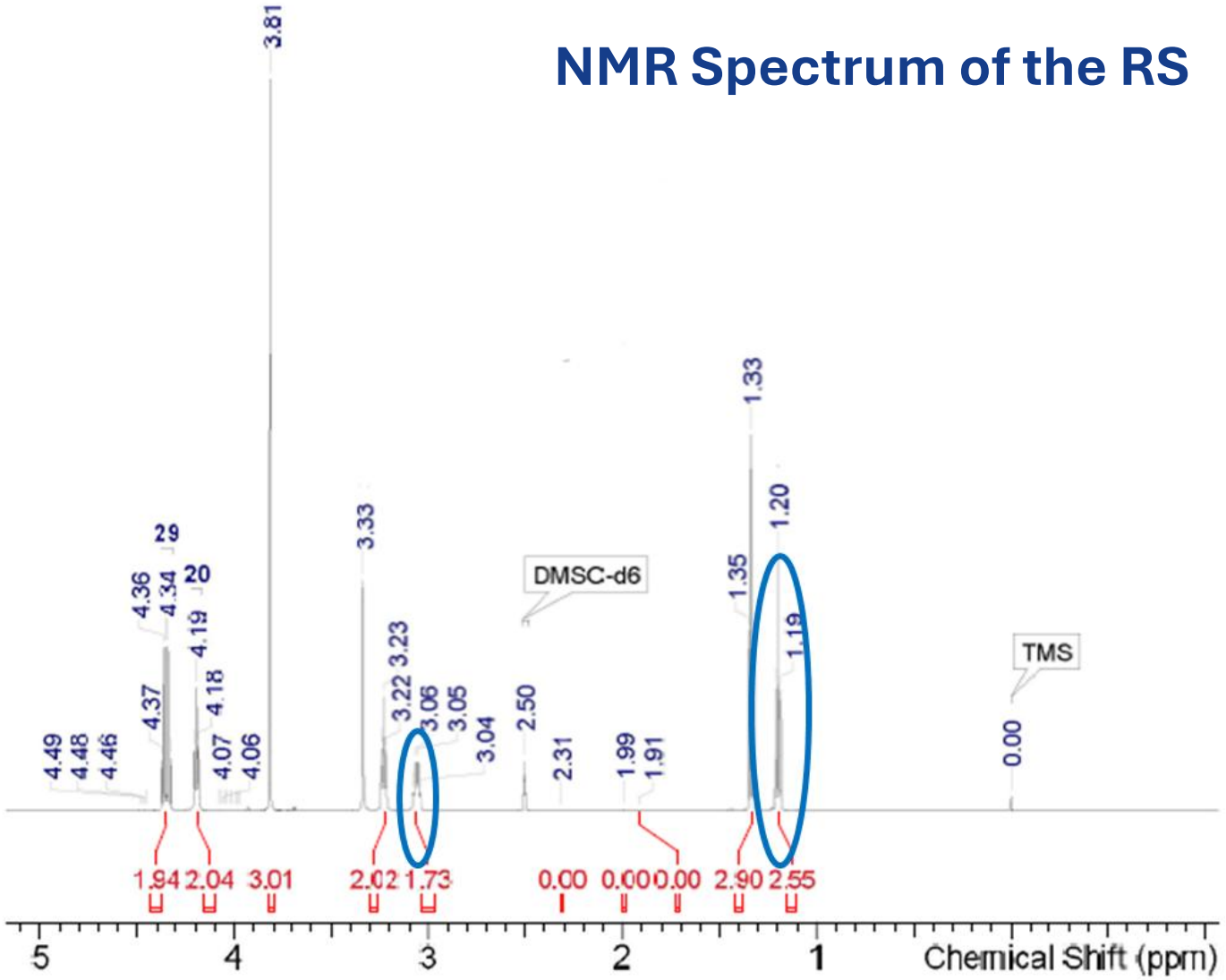
Assigned Value of the Candidate Lot				
Test	**Reported As**	**Lab [A]**	**Lab [I]**	**Average**
Organic impurities, Total (I1)	%TDA	0.11	0.11	0.11
Organic impurities, Total (I2)	% w/w	0.00	0.00	0.00
Water Content	% w/w	0.09	0.15	0.12
Residual solvents, Total <467> Residual Solvents, Current USP-NF	% w/w	--	0.02	0.02
Residue on Ignition	% w/w	--	0.01	0.01



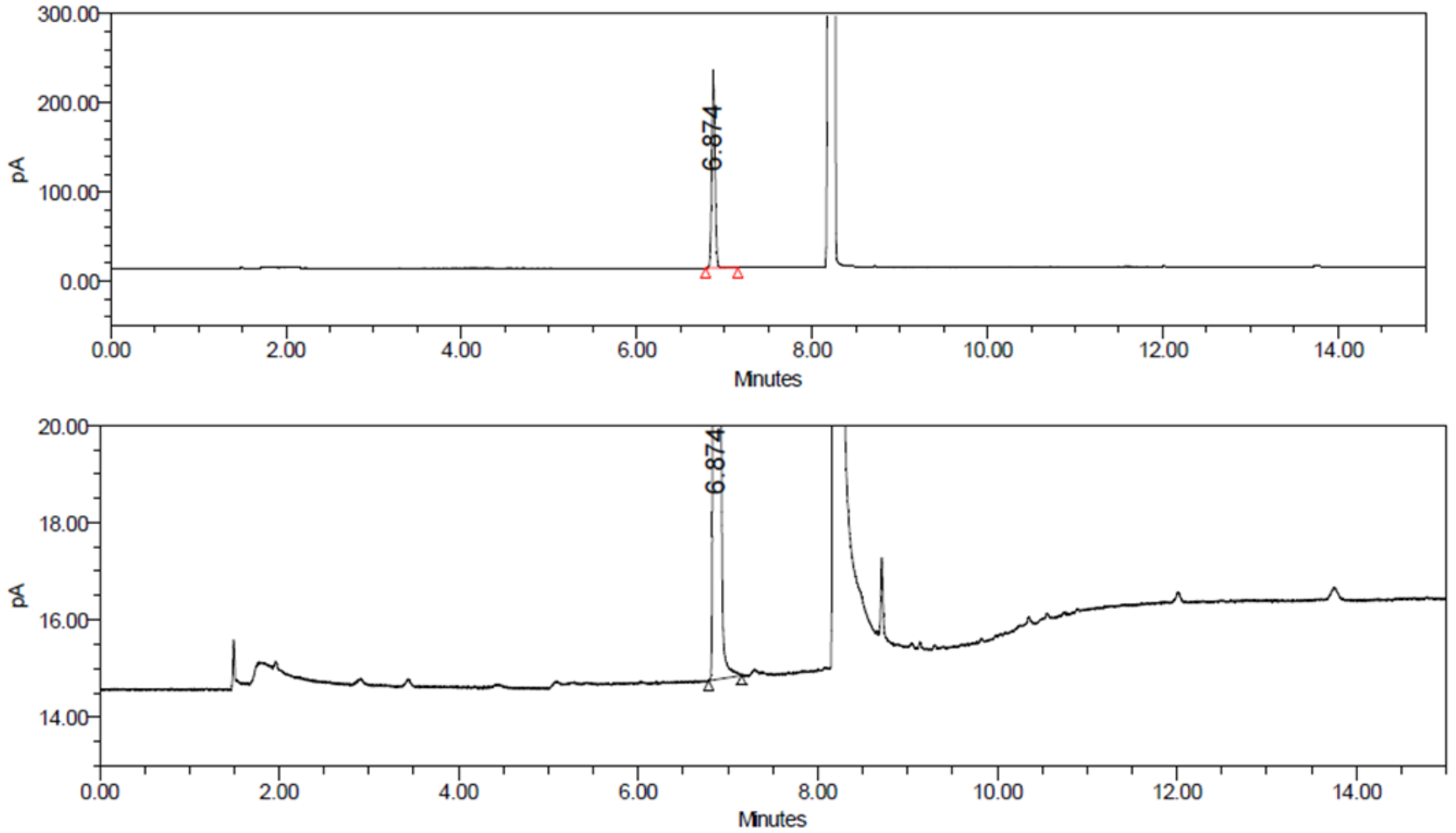
0.02%w/w ethyl acetate at RT ~7.5 minutes

Case #2 – Assigned Value Accuracy

NMR Spectrum of the RS



Case #2 – Assigned Value Accuracy



7.0%w/w triethylamine at RT ~6.9 minutes

Method Reference: Adapalene, Impurities, Limit of Triethylamine, Current USP-NF

Case #2 – Assigned Value Accuracy

Assigned Value of the Candidate Lot				
Test	**Reported As**	**Lab [A]**	**Lab [I]**	**Average**
Organic impurities, Total (I1)	%TDA	0.11	0.11	0.11
Organic impurities, Total (I2)	% w/w	0.00	0.00	0.00
Water Content	% w/w	0.09	0.15	0.12
Residual solvents, Total <467> Residual Solvents, Current USP-NF	% w/w	--	0.02	0.02
Residue on Ignition	% w/w	--	0.01	0.01

Assigned Value of the Candidate Lot				
Test	**Reported As**	**Lab [A]**	**Lab [I]**	**Average**
Organic impurities, Total (I1)	%TDA	0.11	0.11	0.11
Organic impurities, Total (I2)	% w/w	0.00	0.00	0.00
Water determination	% w/w	0.09	0.15	0.12
Residual solvents, Total <467> Residual Solvent, Current USP-NF	% w/w	--	0.02	0.02
Residual solvents, Triethylamine Adapalene, Impurities Limit of Triethylamine, Current USP-NF	% w/w	6.99	--	6.99
Residue on Ignition	% w/w	--	0.01	0.01

Assigned Value:

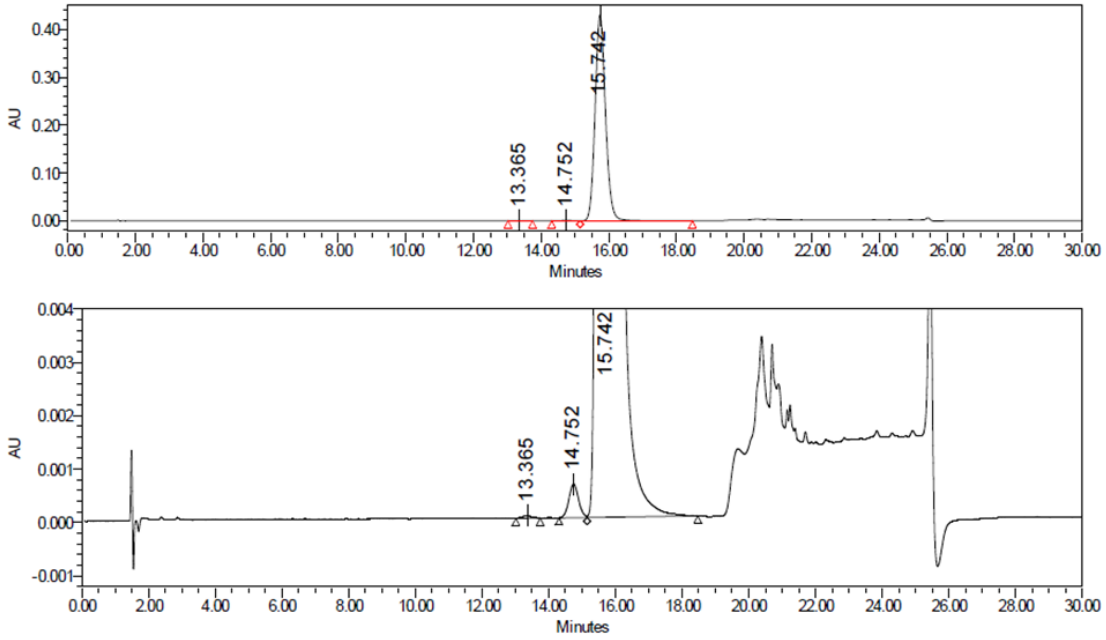
1.00 => 0.93 mg/mg, as is basis

Case #3 – Assigned Value Accuracy

RS : Potassium Salt (New RS)

- **Assigned Value: 0.995 mg/mg, as is basis** (by the mass balance aaproach)

Test	Reported As (units)	Collaborator Test Result			
		[A]	[I]	[Z]	Average
Water Content	%w/w	0.23	0.43	--	0.33
Residual Solvents	%w/w	--	Not detected	--	--
Chromatographic impurities, (T) *Evaluation wavelength: 267nm*	%TDA	0.15	0.14	0.15	0.15



- **Potassium Content:** Align with theoretical value
- **Value by Titration:** 0.998 mg/mg, as is basis
- **Value by qNMR:** 0.995 mg/mg, as is basis

Case #3 – Assigned Value Accuracy

RS : Potassium Salt (New RS)

Assigned Value of the Candidate Lot					
Test	**Reported As**	**Lab [A1]**	**Lab [A2]**	**Lab [D]**	**Average**
Assay against USP Free Acid RS	% w/w	98.37	97.61	98.0	97.99
mg/mg = average of Assay ÷ 100 = 0.9799 0.980 mg per mg of material on the as is basis.					

qNMR as a 'Selective' Analytical Balance – Case Study: Freeze-dried Oxytocin

Matthias Weber

Study director and NMR responsible EDQM Laboratory

Content

- ❑ Introduction
- ❑ Oxytocin CRS
- ❑ **qNMR for lyophilized RS**
- ❑ Combine qNMR and LC
- ❑ Conclusion/Summary

Ph. Eur. Monograph 07/2023:0780 for Oxytocin

OXYTOCIN

Oxytocinum

H – Cys – Tyr – Ile – Gln – Asn – Cys – Pro – Leu – Gly – NH_2

$C_{43}H_{66}N_{12}O_{12}S_2$ M_r 1007
[50-56-6]

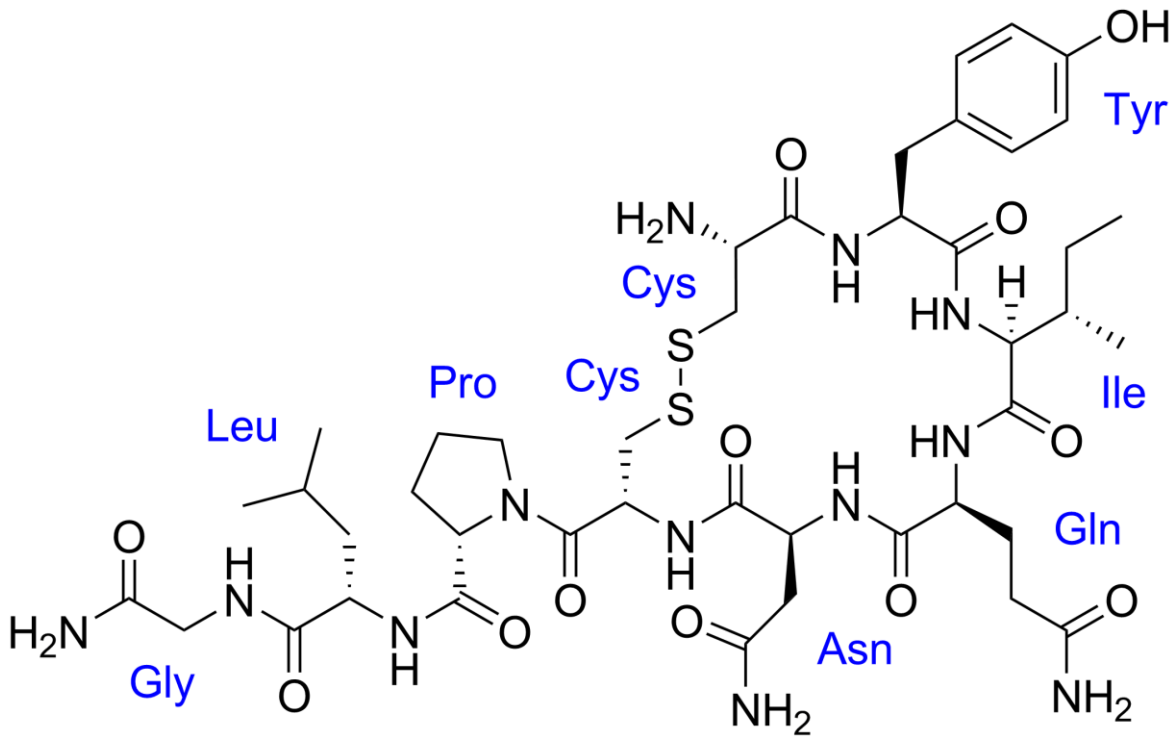
DEFINITION

$S^{3.1}$,$S^{3.6}$-Cyclo(L-cysteinyl-L-tyrosyl-L-isoleucyl-L-glutaminyl-L-asparaginyl-L-cysteinyl-L-prolyl-L-leucylglycinamide).

Synthetic cyclic nonapeptide having the structure of the hormone produced by the posterior lobe of the pituitary gland that stimulates contraction of the uterus and milk ejection in receptive mammals. It is available in the freeze-dried form as an acetate.

Content: 93.0 per cent to 102.0 per cent (anhydrous and acetic acid-free substance).

By convention, for the purpose of labelling oxytocin preparations, 1 mg of oxytocin peptide ($C_{43}H_{66}N_{12}O_{12}S_2$) is equivalent to 600 IU of biological activity.

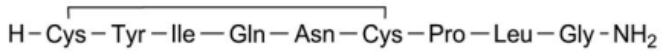


Ph. Eur. Monograph 07/2023:0780 for Oxytocin

07/2023:0780

OXYTOCIN

Oxytocinum



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Related substances. Liquid chromatography (*2.2.29*): use the normalisation procedure.

Test solution. Prepare a 0.25 mg/mL solution of the substance to be examined in a 15.6 g/L solution of *sodium dihydrogen phosphate R*.

Reference solution (a). Dissolve the contents of a vial of *oxytocin for peak identification CRS* (containing impurities B, D, E and I) in 1 mL of a 15.6 g/L solution of *sodium dihydrogen phosphate R*.

Reference solution (b). Dissolve the contents of a vial of *oxytocin impurity F CRS* in 1 mL of a 15.6 g/L solution of *sodium dihydrogen phosphate R*.

Reference solution (c). Dissolve the contents of a vial of *oxytocin CRS* in a 15.6 g/L solution of *sodium dihydrogen phosphate R* to obtain a concentration of 0.25 mg/mL.

Column:

– *size*: l = 0.125 m, Ø = 4.6 mm;

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modifications.

Injection: 25 µL of the test solution and reference solution (c).

Calculate the percentage content of oxytocin ($C_{43}H_{66}N_{12}O_{12}S_2$) taking into account the assigned content of $C_{43}H_{66}N_{12}O_{12}S_2$ in *oxytocin CRS*.

Oxytocin CRS 7(*)

(*) Currently Oxytocin CRS 8 is the valid batch.

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INFORMATION LEAFLET Ph. Eur. Reference Standard

Oxytocin CRS batch 7

1. Identification

Catalogue code: O0700000

2. Scientific Information

2.1 Intended use

Reference Standard for laboratory tests as prescribed in the European Pharmacopoeia only.
Established for use with the monograph(s): 0779, 0780.

2.2 Analytical information related to intended use, when applicable

The "as is" content is : **0.96 mg of oxytocin ($C_{43}H_{66}N_{12}O_{12}S_2$) per vial**

Notes: Oxytocin CRS 7 contains about 40 mg of the excipient sucrose per vial. The unit quantity indicated on the vial represents the approximate total amount of material (active substance and excipients) which has been filled in each vial. This quantity is not to be considered accurate from an analytical point of view.

2.3 Uncertainty of the assigned value, when applicable

The uncertainty of the assigned value is not stated since it is considered to be negligible in relation to the defined limits of the method-specific assays for which the reference standard is used. Please also refer to Ph. Eur. chapter 5.12.

2.4 Validity

Ph. Eur. RS are periodically tested to ensure their continuous fitness for purpose. For each valid Ph. Eur. RS, a Batch Validity Statement at the time of use can be downloaded and printed from the EDQM website (Reference Standards Database).

2.5 Instructions for use

The container should not be opened until required for use. Allow the closed container to equilibrate at ambient temperature before use. This substance has been freeze-dried and is known to be hygroscopic so the container shall not be opened to withdraw its content. Tap the container gently to collect the material at the bottom. Reconstitute the content by injecting the prescribed volume of the prescribed solvent. Mix gently to ensure complete dissolution. When necessary transfer the solution to an appropriate volumetric flask and dilute to the required concentration. Ph. Eur. reference standards are for immediate use. Once the container has been broached, its entire content shall be used immediately. Any further storage and/or re-use are not warranted.

Establishment of Oxytocin CRS 7

The content of Oxytocin CRS 7 stated in the leaflet was assigned

- by an inter-laboratory study
- by LC assay
- against a highly characterised in-house primary standard of pure oxytocin.

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qNMR as orthogonal method

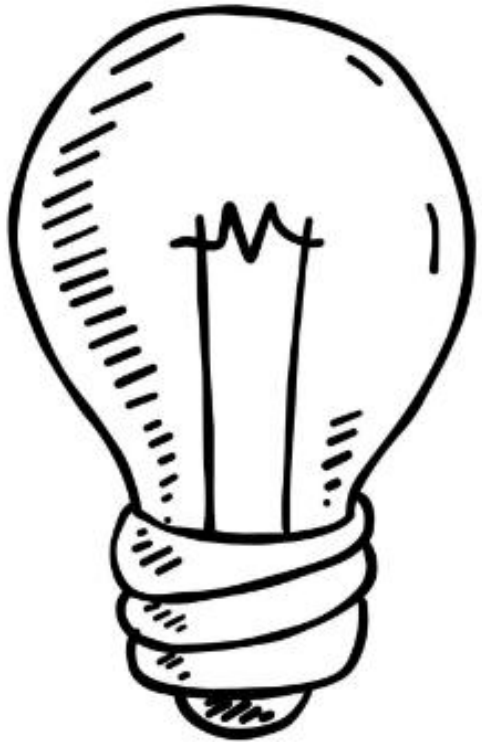
How can quantitative NMR be used to verify the assigned content of the lyophilized reference standard oxytocin CRS as an independent and primary method ?

(qNMR = quantitative NMR in solution using an internal standard of known purity.)

qNMR for lyophilized oxytocin

How can quantitative NMR be used to verify the assigned content of the lyophilized reference standard oxytocin CRS as an independent and primary method ?

(1) Content in mg per vial
(2) Quantitative dissolution of the vial content
(3) Solvent
(4) Internal standard
(5) Signal(s) for NMR quantification
(6) Selectivity -> related substances
(7) Sensitivity -> S/N ratio
(8) Stability in solution
(9) Robustness and repeatability
(10) Estimated uncertainty



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qNMR for lyophylized oxytocin

(1) Content in mg per vial

$$\omega_{sample} = \frac{I_{sample}}{I_{cal}} \frac{m_{cal}}{m_{sample}} \frac{N_{cal}}{N_{sample}} \frac{M_{sample}}{M_{cal}} w_{cal}$$

$$m_{sample} = \frac{I_{sample}}{I_{cal}} m_{cal} \frac{N_{cal}}{N_{sample}} \frac{M_{sample}}{M_{cal}} \frac{w_{cal}}{100\ \%}$$

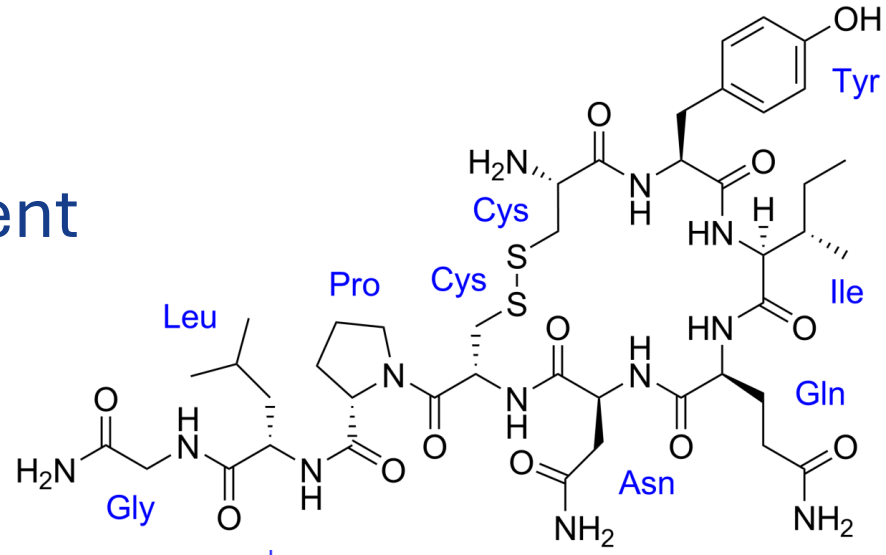
✓ Use of qNMR as an analytical balance!

qNMR for lyophilized oxytocin

(2) Quantitative dissolution of the vial content

(3) Solvent D_2O

(4) Internal standard



qNMR for lyophilized oxytocin

(2) Quantitative dissolution of the vial content
(3) Solvent
(4) Internal standard

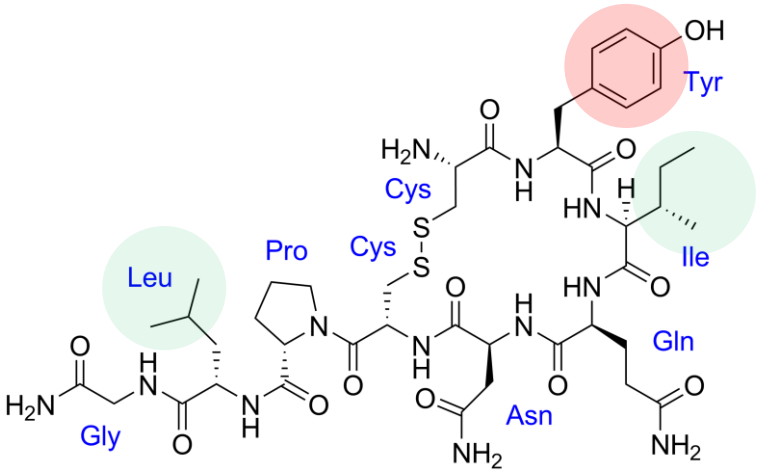
Experimental procedure for the lyophylized CRS vials:

Prepare an internal standard solution of exactly known concentration (0.120 mg/mL) in D_2O by precise weighing and a dilution series.
Add an accurate volume (700 µL) of the internal standard solution directly in the oxytocin CRS vial.
Add some DCl solution (5 µL) and ensure that the entire residue is well dissolved.
Transfer the solution to the NMR tube.

qNMR for lyophilized oxytocin

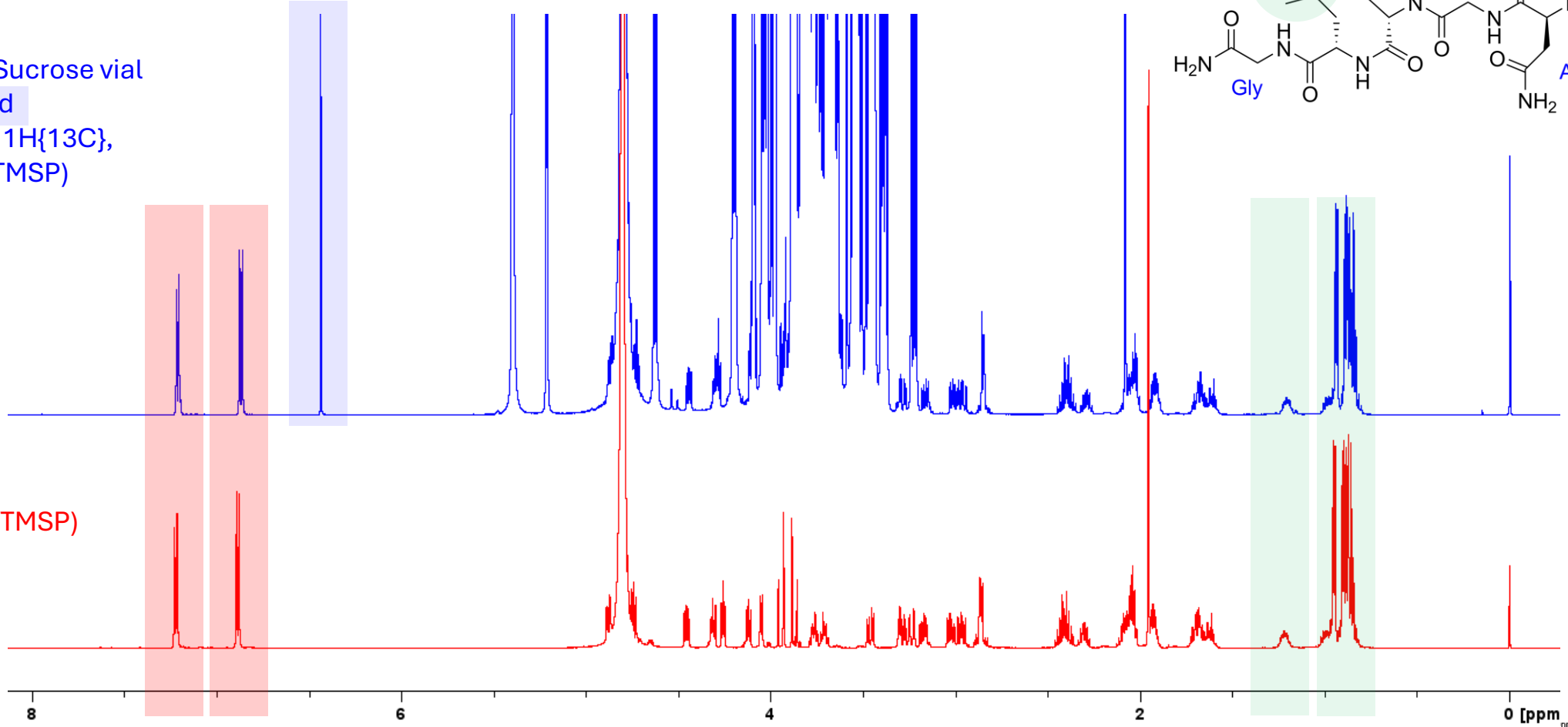
(5) Signal(s) for NMR quantification

(6) Selectivity -> Related substances



Oxytocin/Sucrose vial
Maleic acid
(600 MHz, 1H{13C},
D_2O/HCl/TMSP)

Oxytocin
(600 MHz,
D_2O/pH 5/TMSP)



(^{13}C decoupling GARP4, 600 MHz)

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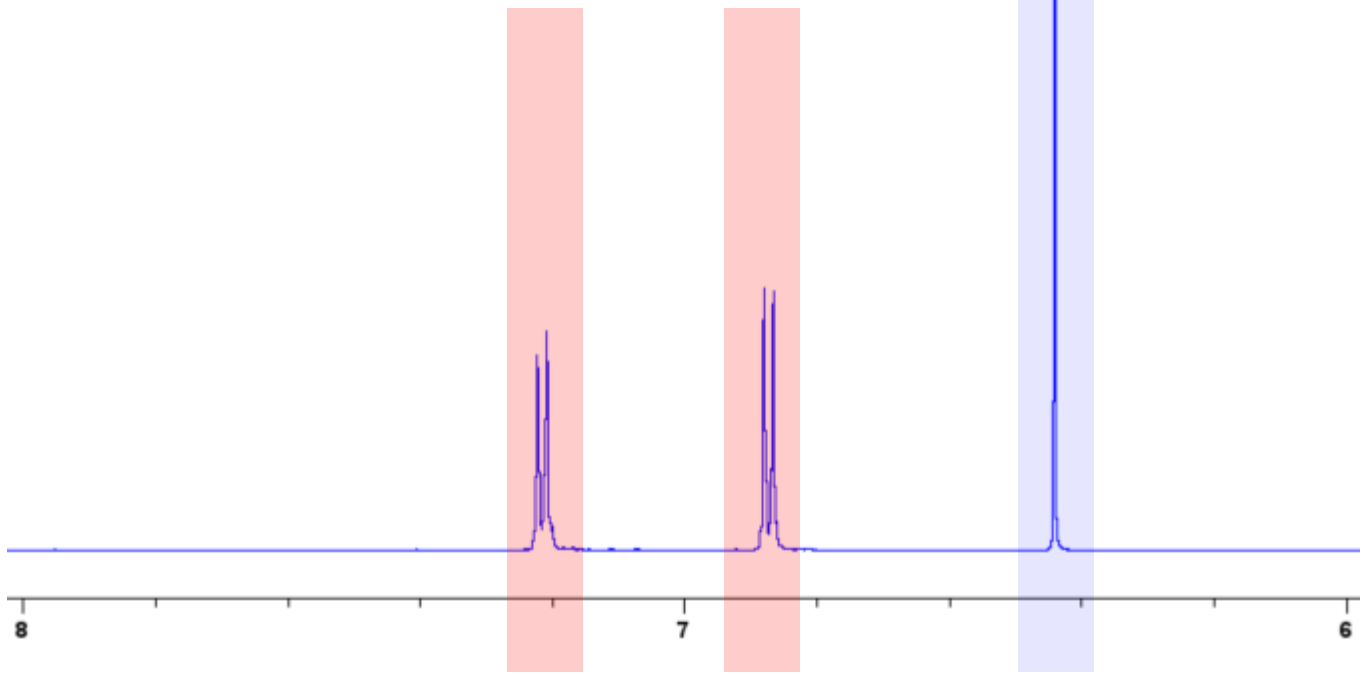
qNMR for lyophilized oxytocin

(5) Signal(s) for NMR quantification

(6) Selectivity -> related substances

Oxytocin/Sucrose vial
Maleic acid
(600 MHz, 1H{13C},
D_2O/HCl/TMSP)



Impurities

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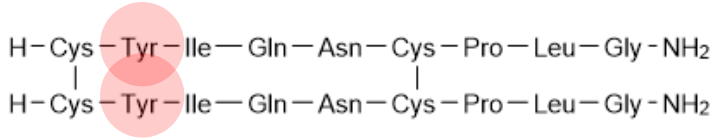
$C_{86}H_{132}N_{24}O_{24}S_4$
Exact Mass: 2012.8729
Mol. Wt.: 2014.3860



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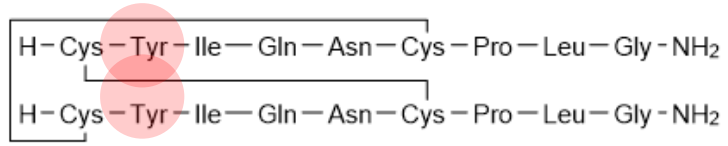

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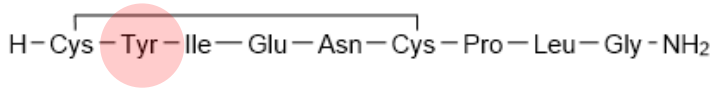
Exact Mass: 1007.4205
Mol. Wt.: 1008.1770



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Exact Mass: 1006.4365
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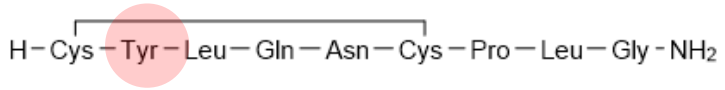

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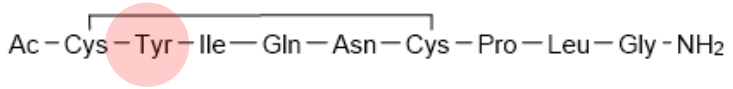
Exact Mass: 1048.4470
Mol. Wt.: 1049.2300



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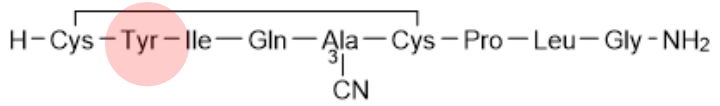

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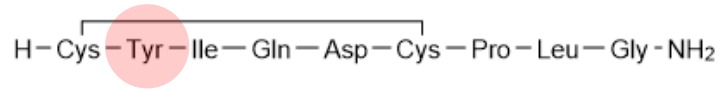
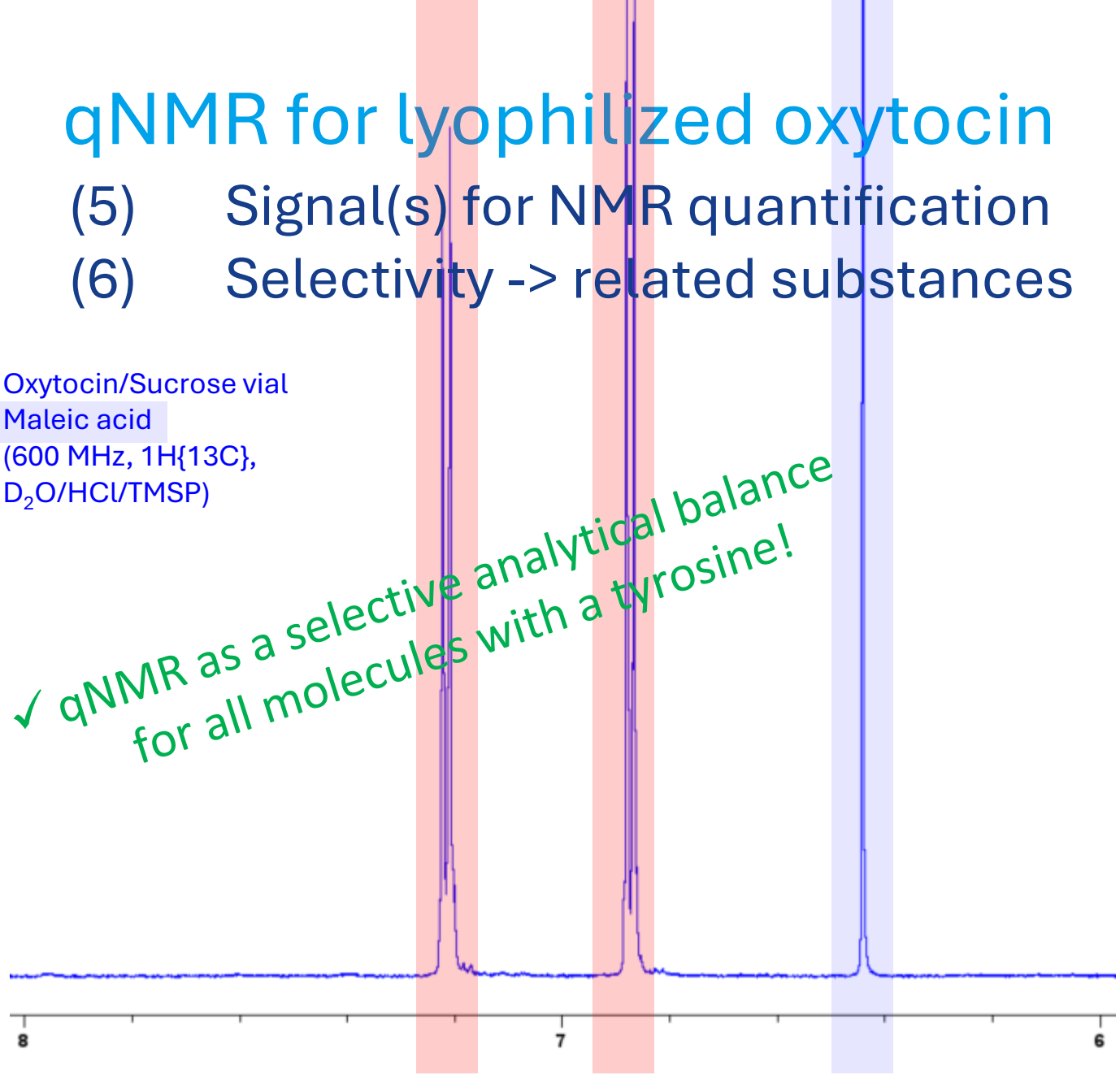


qNMR for lyophilized oxytocin

(5) Signal(s) for NMR quantification

(6) Selectivity -> related substances

Oxytocin/Sucrose vial
Maleic acid
(600 MHz, 1H{13C}, D_2O/HCl/TMSP)



✓ qNMR as a selective analytical balance for all molecules with a tyrosine!

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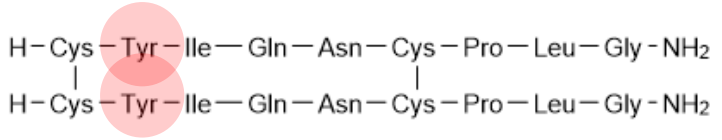

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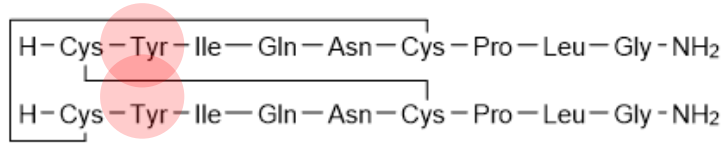

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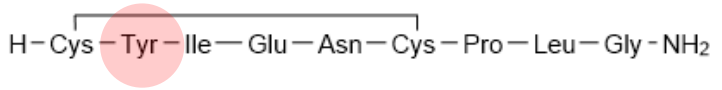

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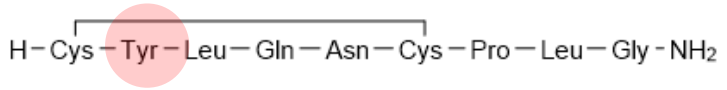

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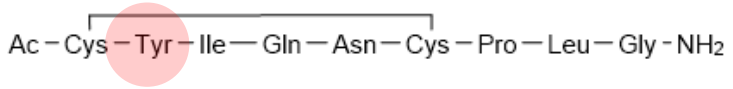

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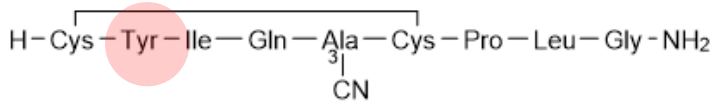

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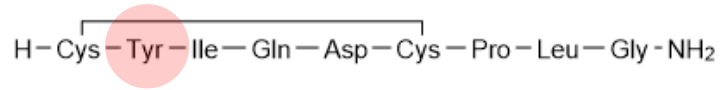
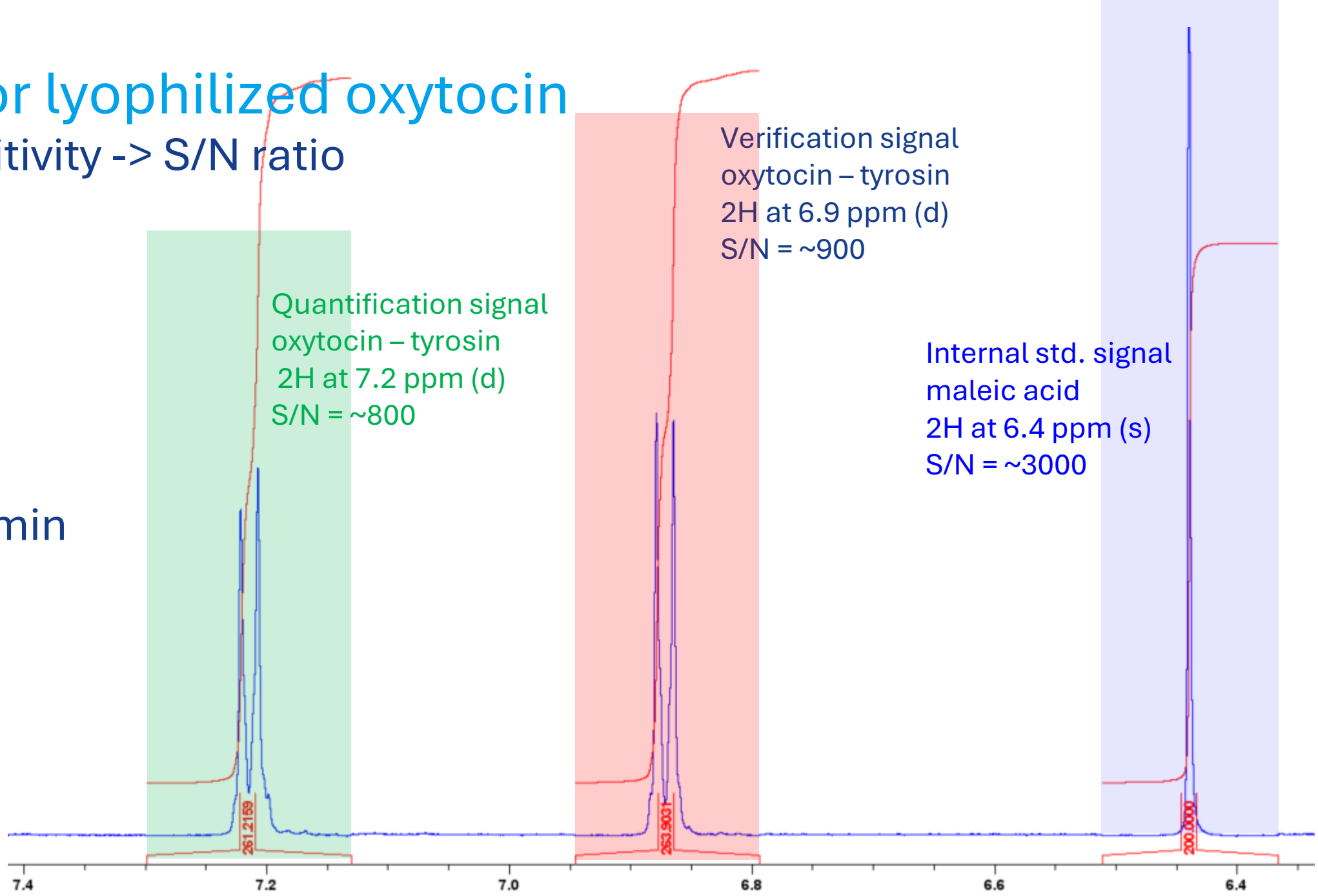


qNMR for lyophilized oxytocin

(7) Sensitivity -> S/N ratio

Oxytocin/Sucrose vial
Maleic acid
(600 MHz, 1H{13C},
D_2O/HCl/TMSP)

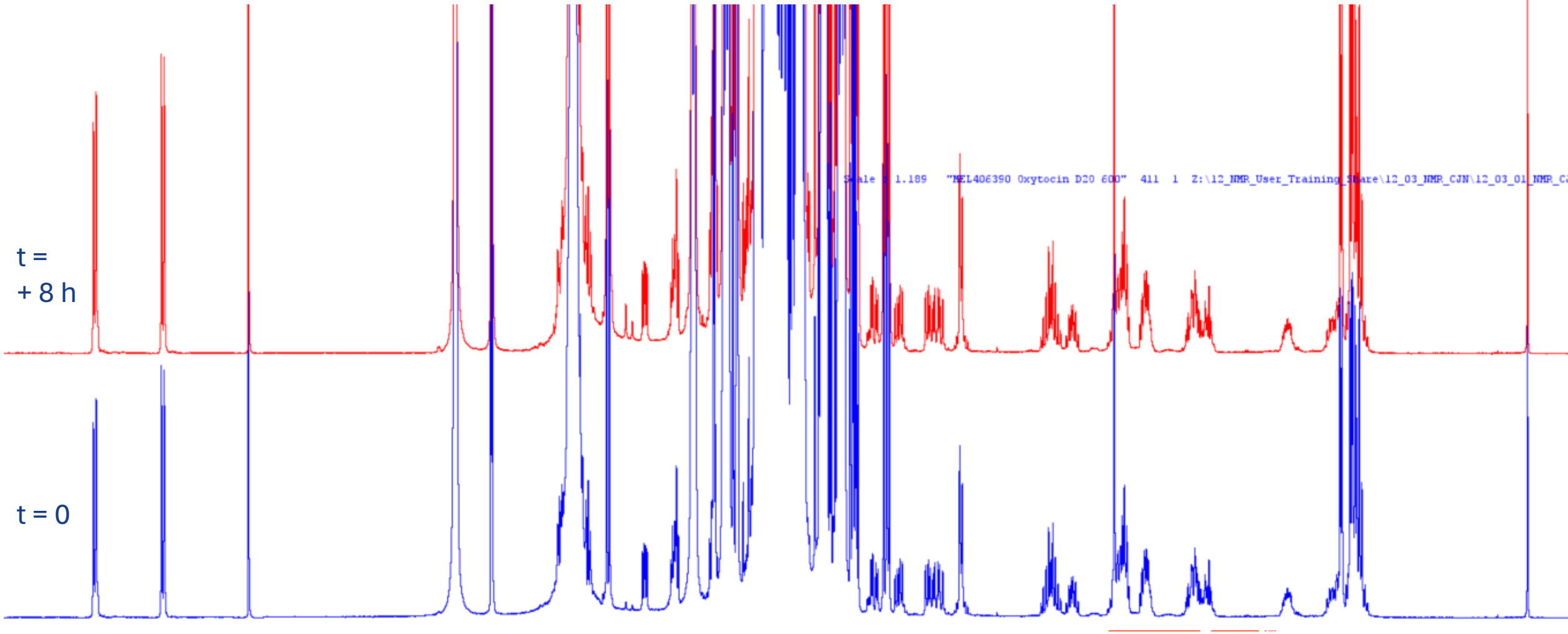
NS = 128
D1 = 60 s
Expt = 140 min



qNMR for lyophilized oxytocin

(8) Stability in solution (Expt = 140 min)

Repeat NMR experiment and compare profile and integration



qNMR for lyophilized oxytocin

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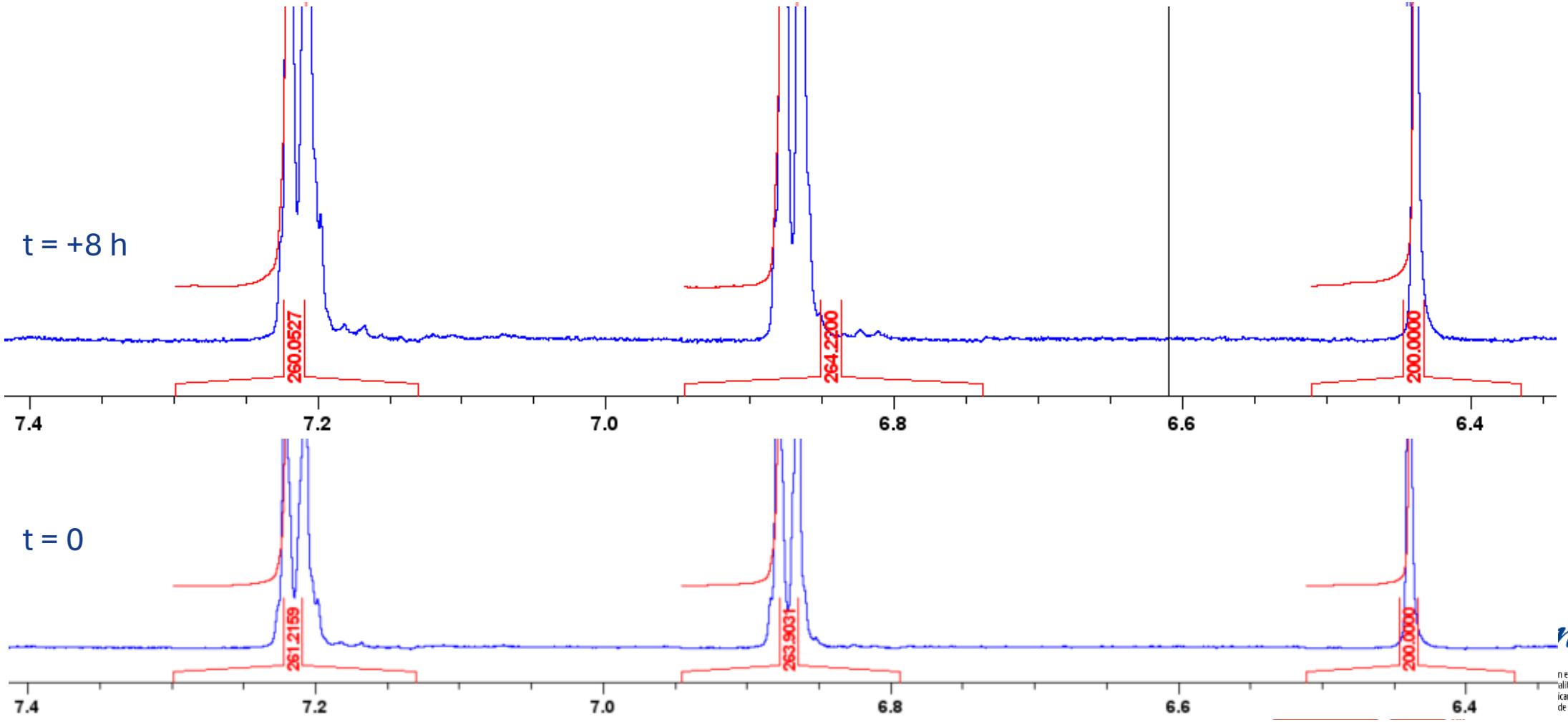

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Internal comparative qNMR study on oxytocin CRS

(9) Robustness and repeatability

Protocol

Five NMR lab technicians analysed the lyophilized vials of oxytocin (1 mg) with sucrose (40 mg) by qNMR using maleic acid as internal standard.

Each technician prepared one internal standard solution;
each technician dissolved three oxytocin vials;
each solution was analysed once.

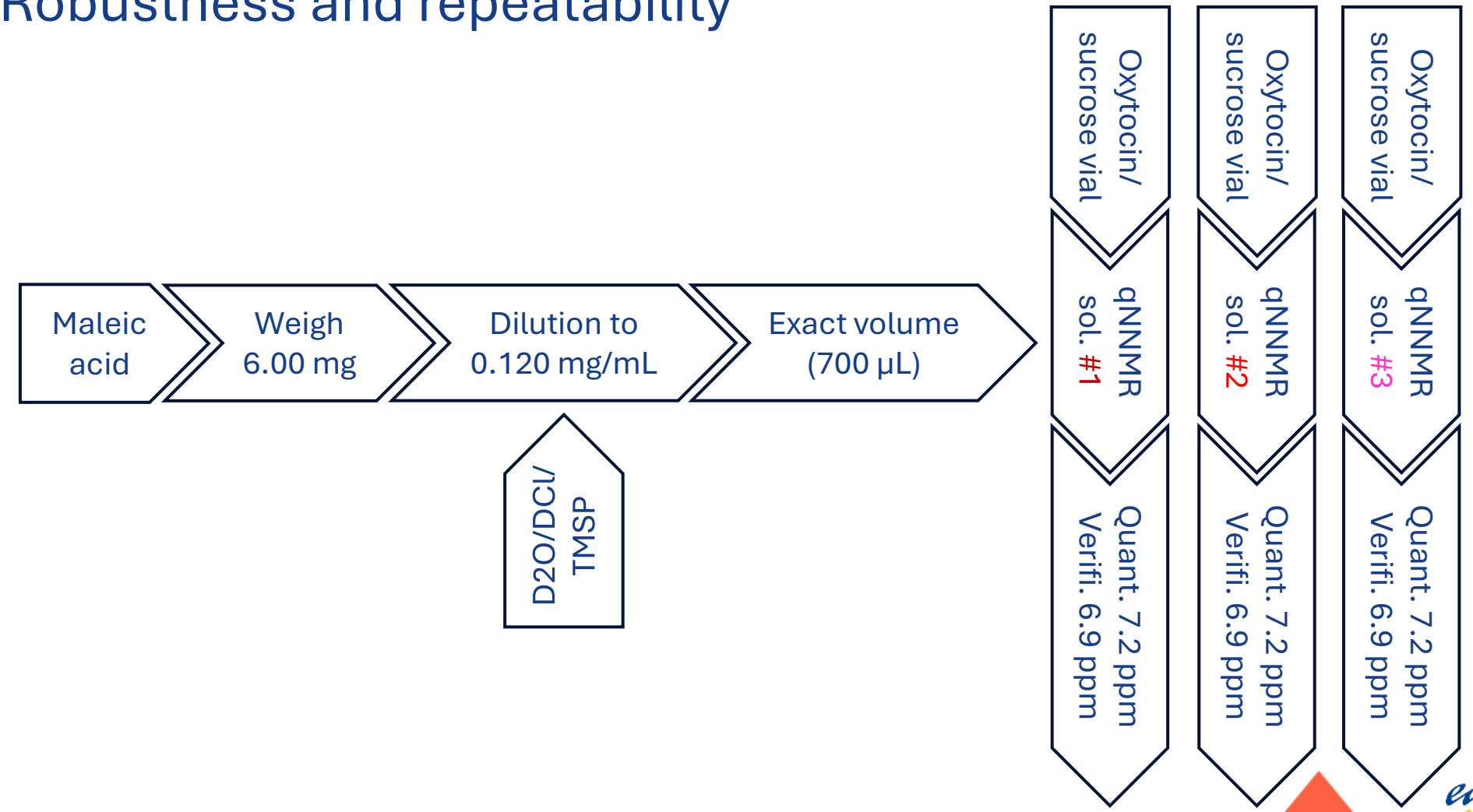
One technician repeated the test (n=5+1).

[Assumption: Same content of oxytocin in each vial.]

This resulted in 6 x 3 result sets for the quantification signal at 7.2 ppm and for the verification signal at 6.9 ppm.

Internal comparative qNMR study on oxytocin CRS

(9) Robustness and repeatability



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Results qNMR study on oxytocin CRS

Results quantification signal (7.2 ppm)

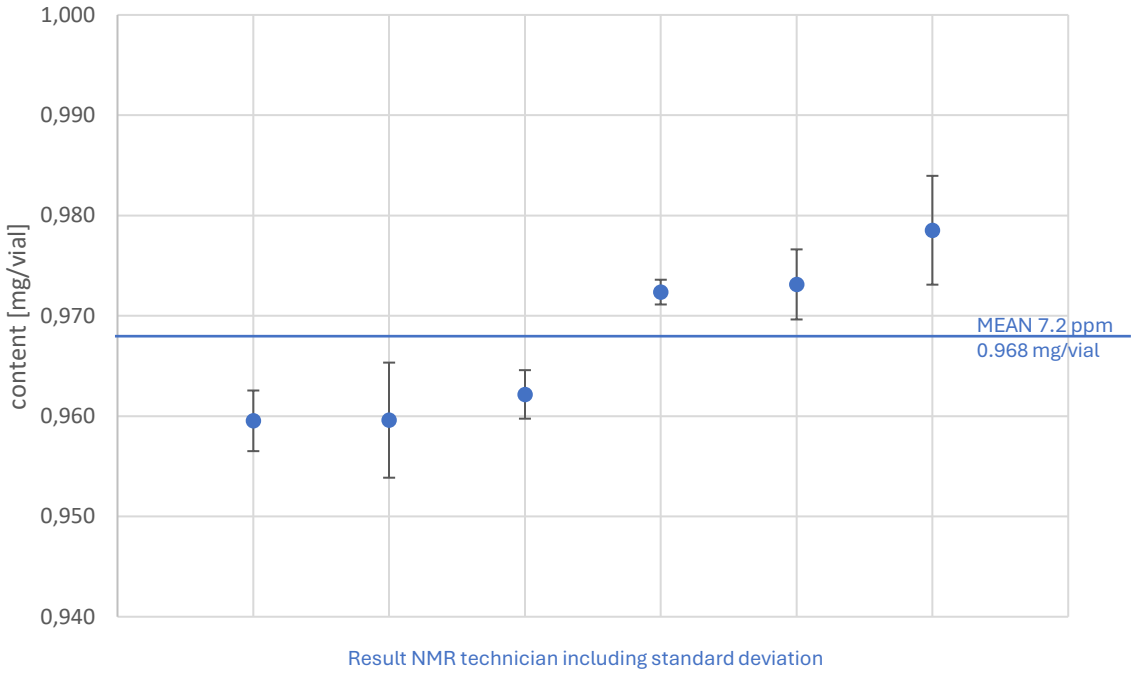
Technician	Test 1 mg/vial	Test 2 mg/vial	Test 3 mg/vial	result mg/vial	sd mg/vial	% RSD	stability %
T_1_S	0.969	0.975	0.975	**0.973**	0.003	0.36	0.04
T_2_Y	0.956	0.960	0.962	**0.960**	0.003	0.32	0.16
T_3_L	0.983	0.972	0.981	**0.979**	0.005	0.55	0.18
T_4_G	0.973	0.973	0.971	**0.972**	0.001	0.13	0.04
T_5_C_2	0.963	0.962	0.953	**0.960**	0.006	0.60	0.45
T_5_C_1	0.960	0.963	0.964	**0.962**	0.002	0.25	0.25
Intralaboratory				**0.968**	**0.008**	**0.84**	**0.19**

Results verification signal (6.9 ppm)

Technician	Test 1 mg/vial	Test 2 mg/vial	Test 3 mg/vial	result mg/vial	st. dev. mg/vial	% RSD
T_1_S	0.979	0.996	0.993	0.989	0.009	0.93
T_2_Y	0.971	0.967	0.973	0.971	0.003	0.33
T_3_L	0.989	0.984	0.978	0.984	0.005	0.56
T_4_G	0.979	0.980	0.985	0.981	0.003	0.31
T_5_C_2	0.973	0.972	0.977	0.974	0.003	0.26
T_5_C_1	0.985	0.993	0.988	0.989	0.004	0.39
Intralaboratory				**0.981**	**0.008**	**0.78**

Results qNMR study on oxytocin CRS

Results quantification signal (7.2 ppm)



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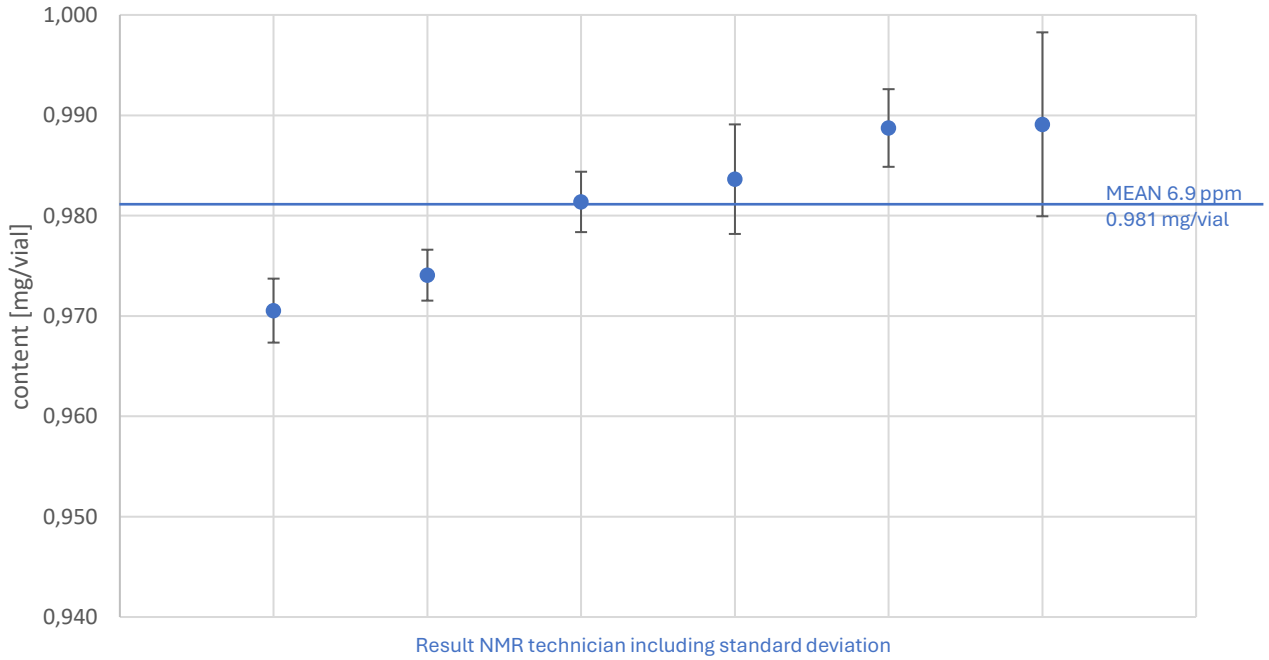


ANOVA resulted in a between-technician rsd of 0.77 % and 0.62 % respectively.

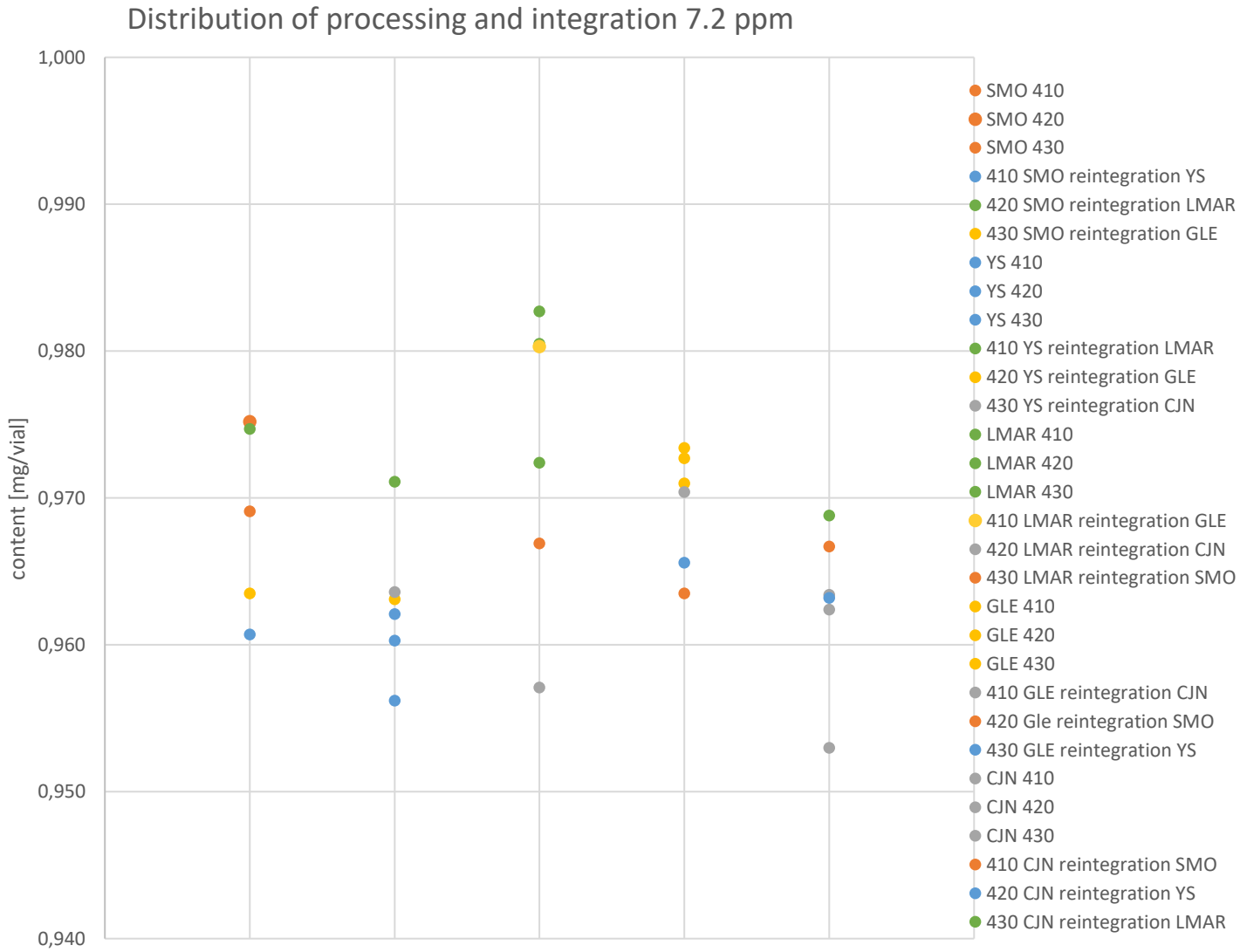
Interpretation of qNMR results on oxytocin CRS

(9) Robustness and repeatability

How to differentiate variations from the manual preparation of the solutions from differences of data treatment/integration?

→ each technician reintegrated
three results from the other technicians.

Random distribution =>
no statistically significant difference !

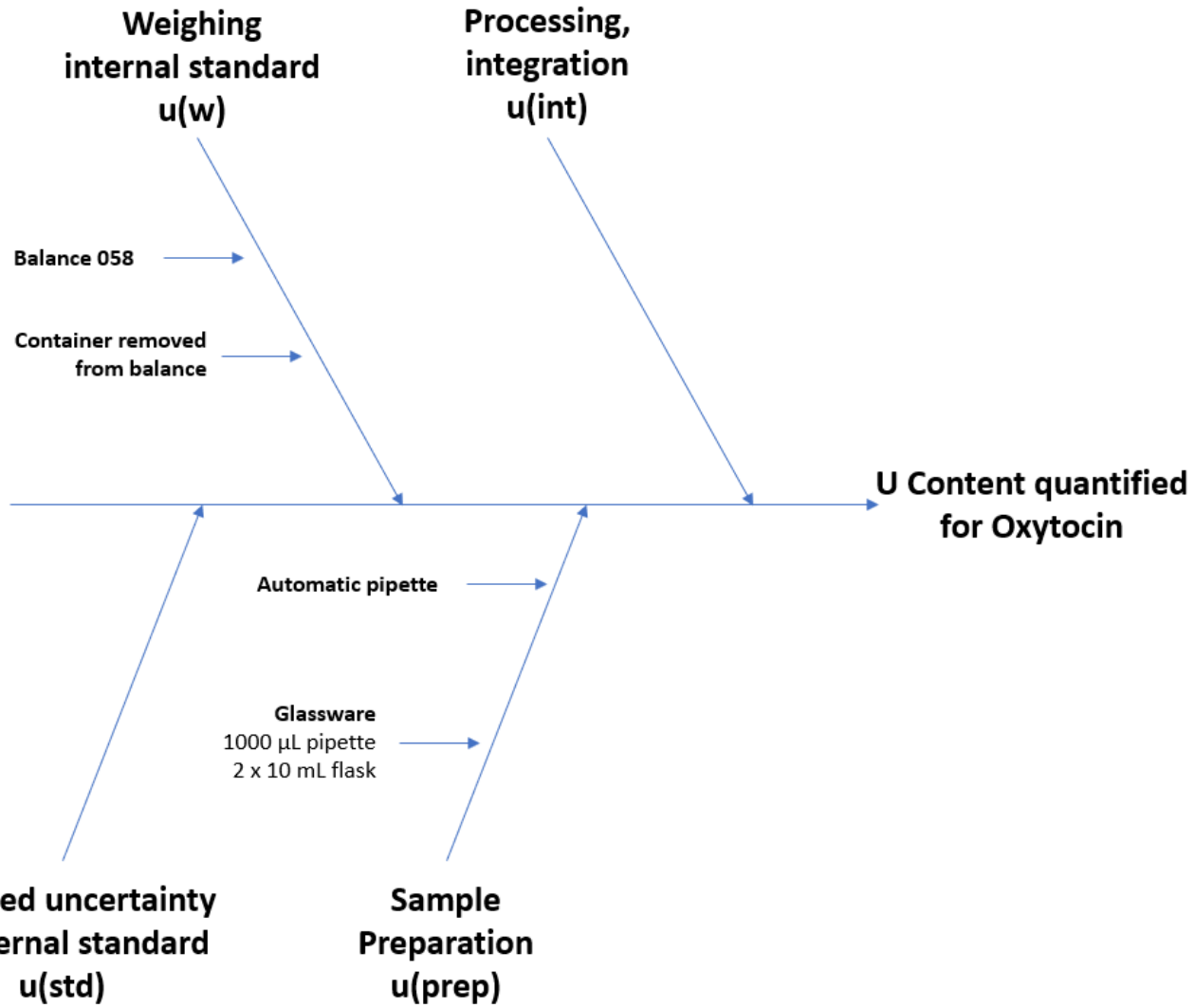


qNMR for lyophilized oxytocin CRS

(10) Estimated uncertainty

u(std) < 0.08 %
u(prep) < 0.46 %
u(w) < 0.15 % (2x)
u(int) < 0.93 %

U(k=2) ~ 2.1 %



Content oxytocin (mg/vial)

Content assignment Ph. Eur. leaflet:

- by an inter-laboratory study
- by LC assay
- against a highly characterised in-house primary standard of pure oxytocin.

0.96 mg/vial oxytocin

qNMR as a ‘selective’ analytical balance:

Quantification signal 7.2 ppm

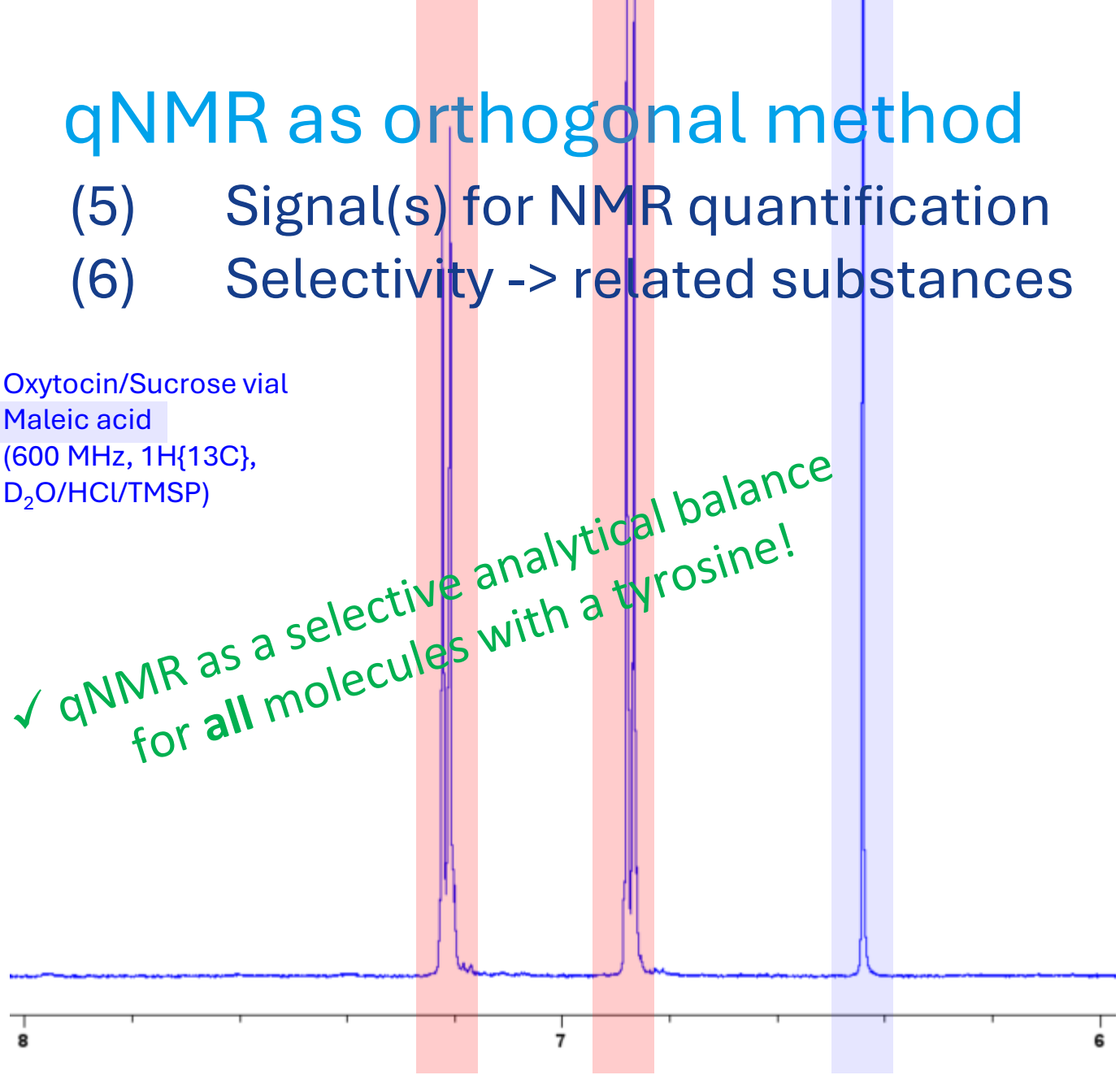
0.97 mg/vial +/- 0.02 mg

qNMR as orthogonal method

(5) Signal(s) for NMR quantification

(6) Selectivity -> related substances

Oxytocin/Sucrose vial
Maleic acid
(600 MHz, 1H{13C},
D_2O/HCl/TMSP)



✓ qNMR as a selective analytical balance for **all** molecules with a tyrosine!

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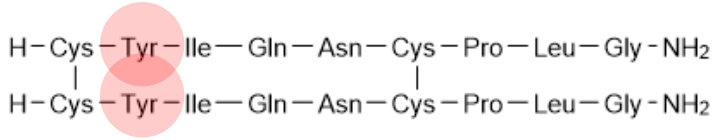

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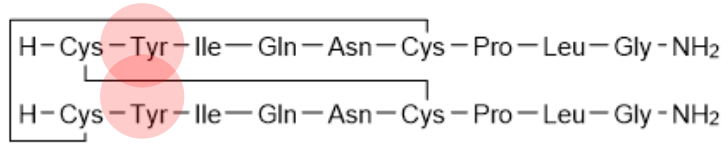

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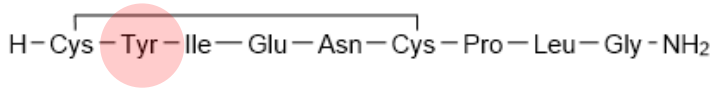

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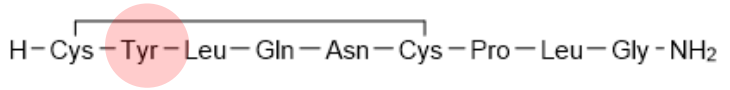

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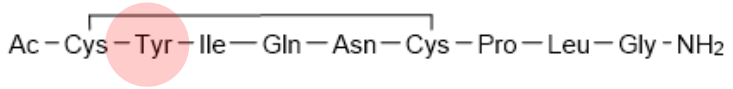

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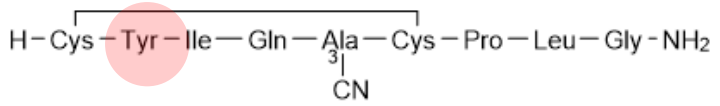

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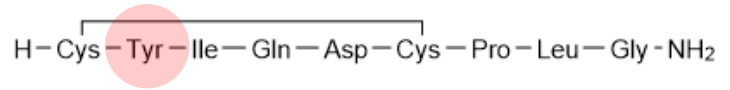

Combine qNMR and LC for oxytocin CRS

All impurities with a tyrosine amino acid are included in the qNMR result!

LC impurities: 0.95 % (n = 3, rsd 0.6 %)

Why not use the LC results according to the monograph method and combine with qNMR to *enhance* selectivity?

Combine qNMR and LC for oxytocin CRS

The qNMR result of 0.97 mg/vial includes 0.95 % of related impurities.

Assuming that all these impurities exhibit a tyrosine and have the same (or similar) molar mass and the same number of protons:

[0.97 * (100% – 0.95%)/100%] mg/mL =

0.96 mg/mL +/- 0.02 mg

(qNMR/LC combined)

Conclusion/Summary

- **qNMR can be used as a selective balance at mg level**
- a thorough and detailed processing and integration procedure is required
- very good repeatability and robustness could be achieved
- attention to selectivity for qNMR is important
- considering certain assumptions qNMR results can be combined with LC information
- the estimated expanded uncertainty was about 2 % in our laboratory
- independent quantification of the amount of oxytocin in a lyophilized vial could be done by qNMR

Merci – Thank you

Stéphanie Moneret, Cees-Jan Nap, Gilles Leclerc, Yusuf Suvay, Lina Marchetti, Elena Regourd, Jochen Pauwels

Thank you for your attention

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