Chemical Primary Reference Materials: from Valine to C-peptide

Robert Wielgosz

Bureau International des Poids et Mesures

Outline

- Establishing traceability for organic measurements
- Mass Balance Method for Small Organics
- qNMR for purity measurements
- Peptide purity measurement methods

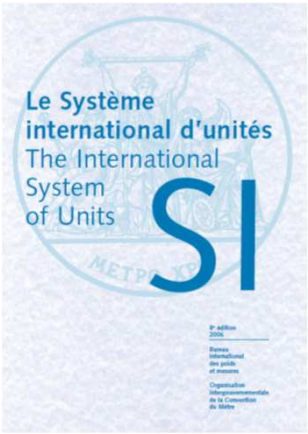
Questions you will be able to answer after this lecture:

1) How can Traceability be applied to Chemical Measurement?
2) What methods can be used to quantify Chemical Purity?
3) What instrumentation is required for a Mass Balance Approach?
4) What relative uncertainty is achievable with Mass Balance Methods?
5) What are common sources of bias for Mass Balance Methods?
6) How can NMR be used to quantify Chemical purity?
7) What factors limit the performance of NMR for purity measurement?
8) How can you measure the purity of a peptide?
9) How can you identify a peptide from its high-res mass spectrum?
10) How can Amino Acid analysis be used for peptide purity?

Realising the mole

Realising the mole:

(2006)



1. For a pure sample the amount of substance n in the sample may be measured by determining the mass m of the sample and dividing by the molar mass M using the relation:

$$n = m / M \qquad (1)$$

procedure. The mole may easily be realised with a relative standard uncertainty of less than 1×10^{-6} by this method. However it is important to note that this procedure depends on having a pure sample of the material, which implies having a precise chemical analysis of the sample, and this will often be the limiting factor in an uncertainty evaluation.

Draft for Appendix 2 of the SI Brochure for the <<New SI>>

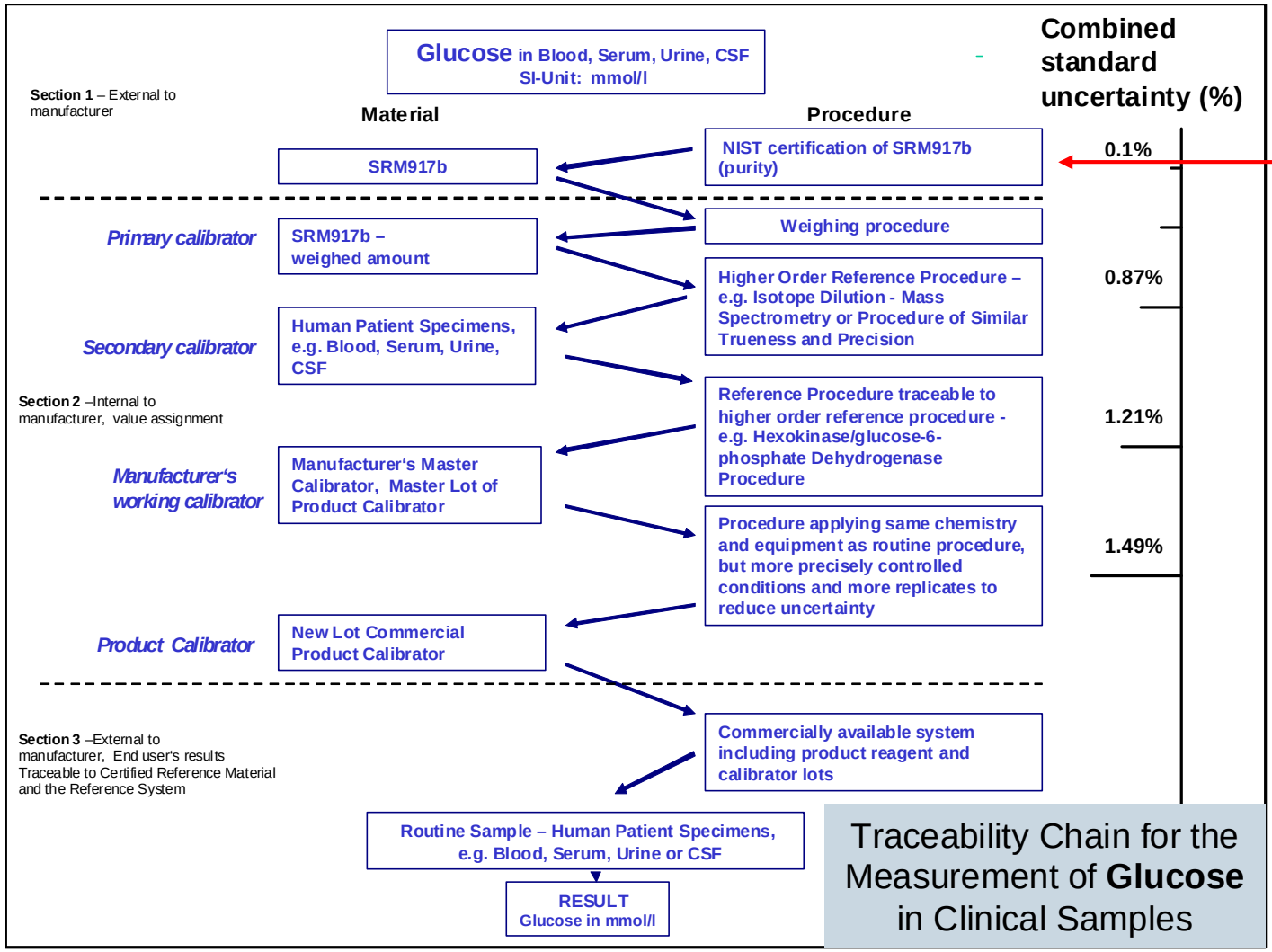
(2012)

1. For a compound X the amount of substance n in a sample may be measured by determining the product of the mass fraction of X in the sample (w_X) and the mass m of the sample and dividing by the molar mass $M(\mathrm{X})$ according to the formula

$$n = \frac{w_X m}{M(X)} \qquad (4)$$

A realisation of the mole for a pure organic compound will usually be limited by the uncertainty of the mass fraction assignment of the compound rather than the uncertainty of gravimetric operations. As there are very few organic compounds whose mass fraction purity is assigned with relative standard uncertainty below 1×10^{-4}, achieving a relative standard uncertainty of less than 1×10^{-4} for a realisation of the mole based on a pure organic compound would be the feasible limit in most cases.

Metrological Traceability and Primary Calibrators



SRM917
Glucose mass fraction
997 ± 2 mg/g

Traceability Chain for the Measurement of **Glucose** in Clinical Samples

(Adapted from model in ISO 17511)

Approaches to Organic Purity Assignment

- **Mass Balance (summation of impurities)**
  - Robust, wide applicability
  - Small uncertainties
  - Labor & equipment intensive

- **Direct Assay of main component**
  - **titrimetry, gravimetry, qNMR**
  - Simple, “Primary” method
  - Generally larger uncertainty
  - Potential for bias

- **Direct Assay of total impurities**
  - **calorimetry (differential or adiabatic)**
  - **phase solubility**
  - Very small uncertainties possible
  - Important but limited applicability

Mass Balance Method

Mass Balance Purity – Measurement equation

$$w_A = 1000 - (w_{RS} + w_W + w_{VOC} + w_{NV}) \qquad \textbf{(units = mg/g)}$$

w_{RS} = mass fraction of related structure impurities in the material

w_W = mass fraction of water in the material

w_{VOC} = mass fraction of residual solvent (volatile organics) in the material

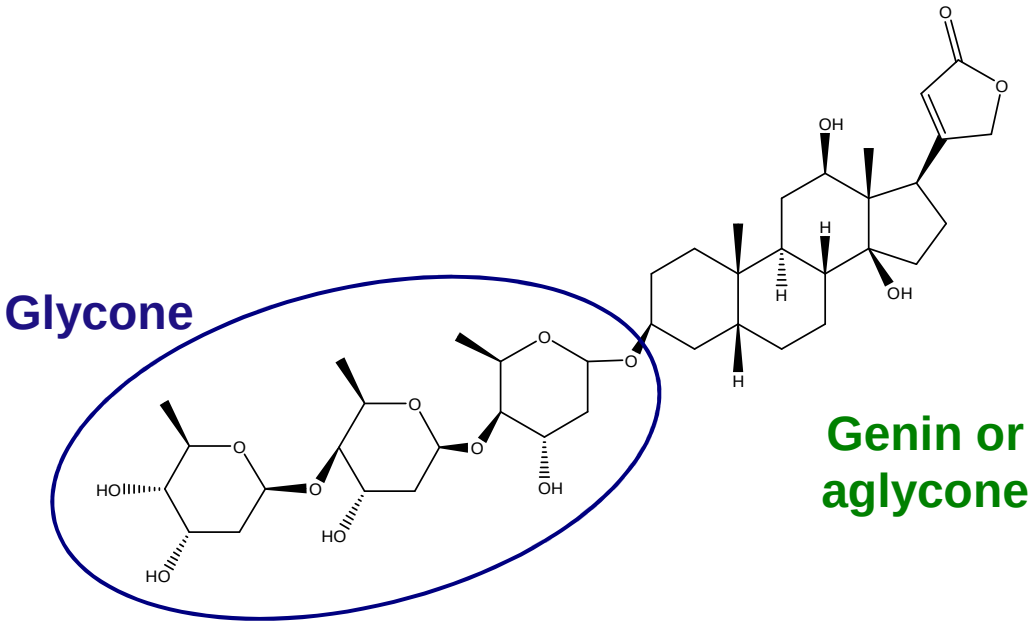
w_{NV} = mass fraction of non-volatile compounds in the material

- **Comprehensive coverage with orthogonal relation between impurity classes**
- **SI traceability (calibration chain, MU) required for <u>each</u> contributor**

$$u(w_A) = \sqrt{u(w_{RS})^2 + u(w_W)^2 + u(w_{VOC})^2 + u(w_{NV})^2}$$

Digoxin: Example of Mass Balance Approach

‘There are no poisons, only poisonous doses’



Impurity Quantification

- Digoxigenin-tetra-digitoxosid, digitoxin, gitoxin and ß-acetyldigoxin by **LC-MS/MS**
- Other unknown UV active impurities by **LC-UV**
- Ethanol, dichloromethane and toluene by **GC-MS**
- Water by **KFT**

Mass Balance: Measurement Equation

$$w_{Dg} = \frac{m_{Dg}}{m_{P20.f}} = \frac{m_{Dg}}{m_{Dg} + \sum m_i + \sum m_{other}} = \frac{1}{1 + \left(\sum \frac{A_i}{R_i} \cdot \frac{1}{A_{Dg}} \right) + \left(\sum \frac{m_{other}}{m_{Dg}} \right)}$$

w_{Dg} = mass fraction (g/g) of digoxin (Dg) in P20.f sample

m_{Dg} = mass (g) of Dg in a P20.f test sample

$m_{P20.f}$ = mass (g) of a P20.f test sample

m_i = mass (g) of individual LC-UV detectable impurities in a P20.f test sample

m_{other} = mass (g) of components in test sample not quantified by LC-UV including related impurities quantified by LC-MS/MS (m_{cg}), water content by KFT (m_{H_2O}) and organic solvents quantified by GC-MS ($m_{solvents}$)

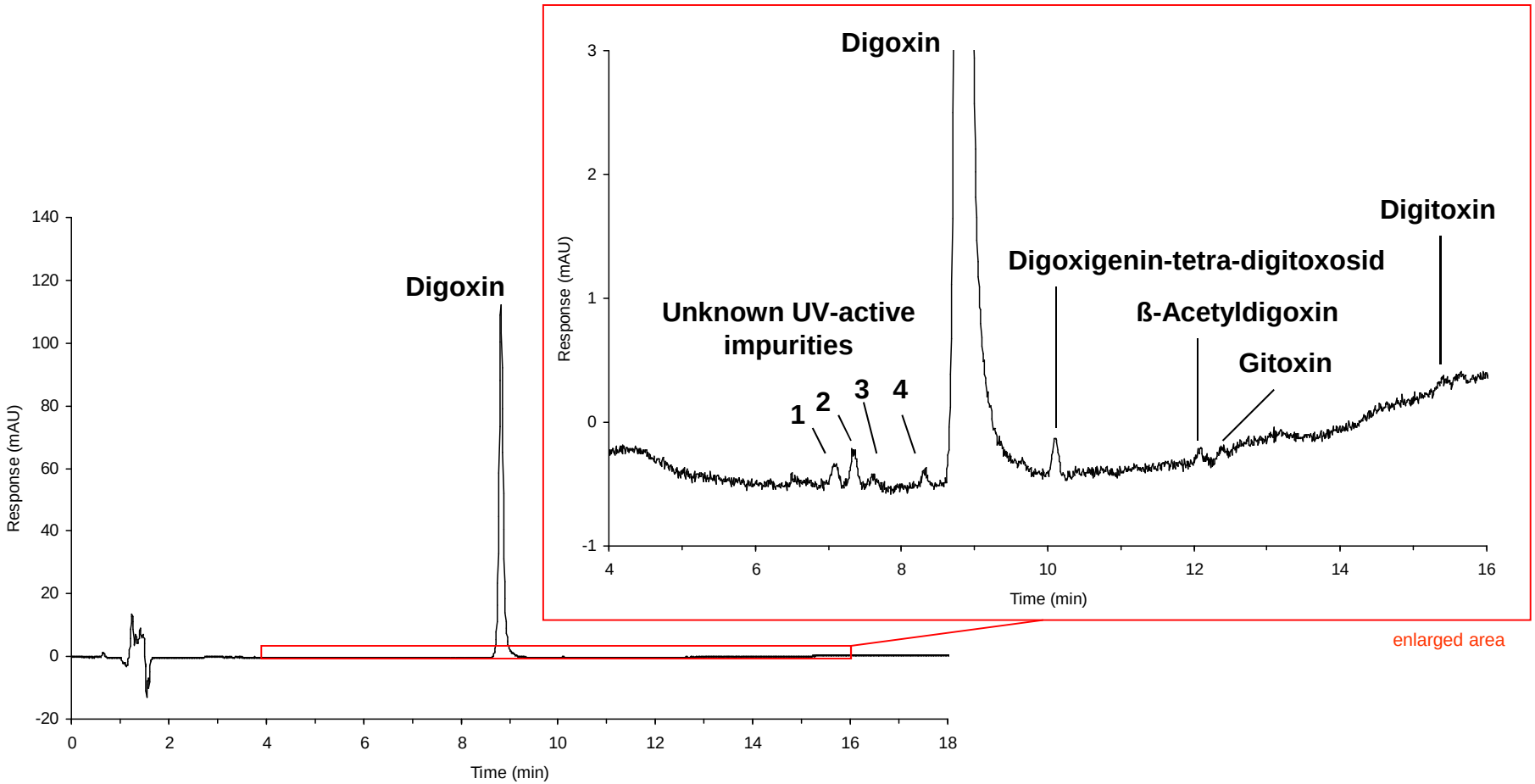
A_{Dg} = normalised area response for Dg

A_i = normalised area response for impurity i

R_i = LC-UV response factor for impurity i to relative to Dg

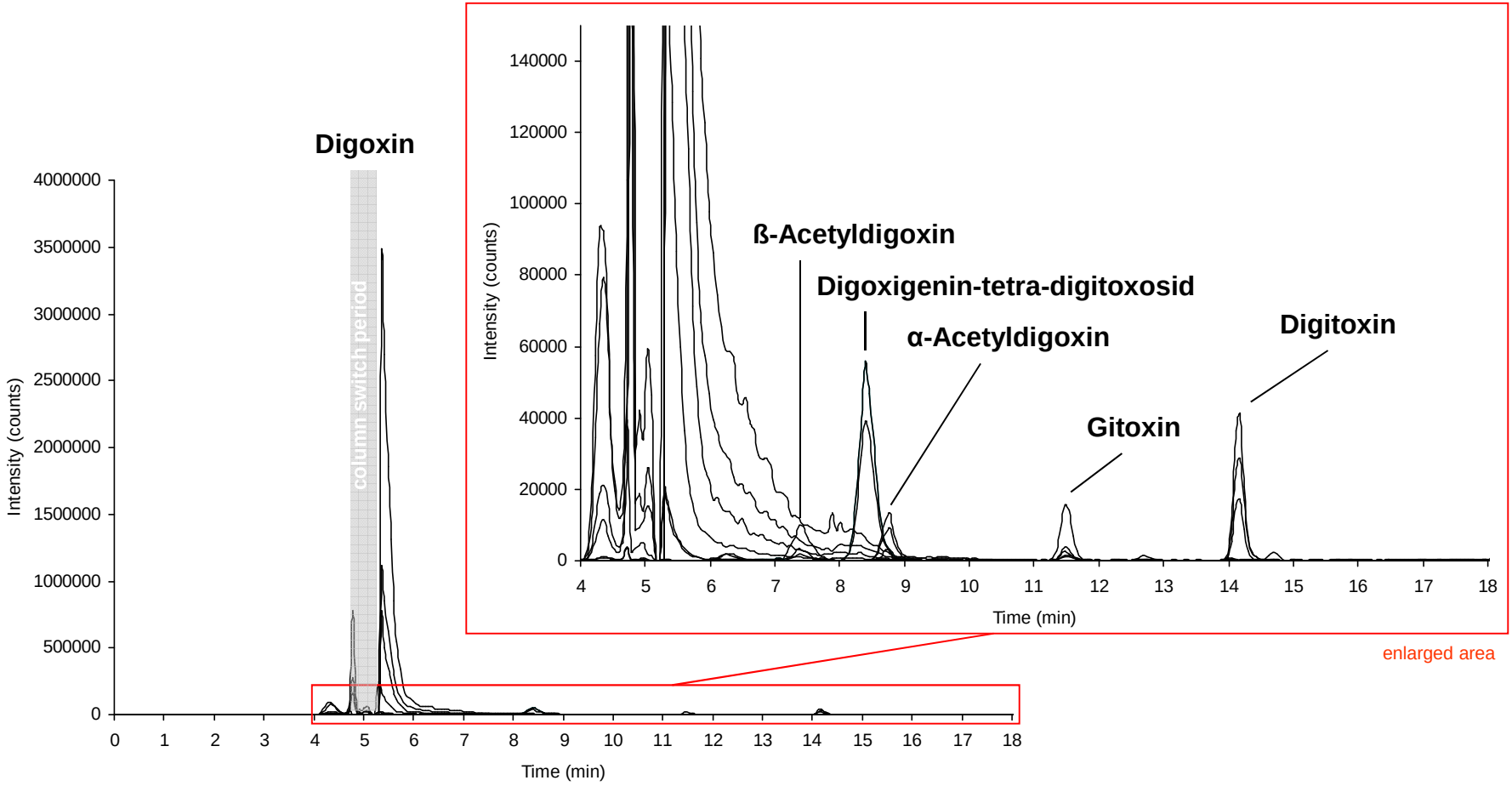
Digoxin Purity: Related Substance by LC-UV

Full scale and enlarged UV chromatograms at 220 nm



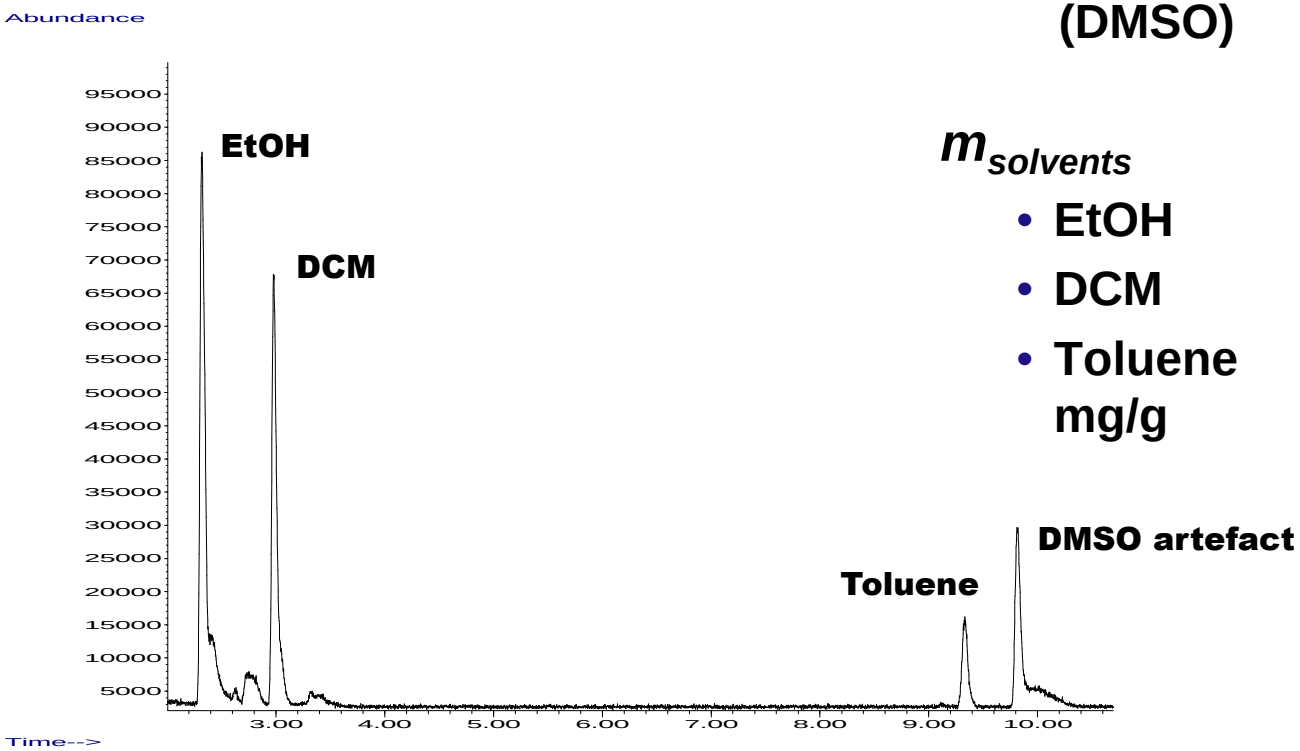
Digoxin Purity: Related Substances by LC-MS/MS

Full scale and enlarged XICs overlays



Digoxin Purity: VOCs by GC-MS

Sample and DMSO (blank) TICs



Column: Carbowax (DB-624 column, 30 x 250 x 1.4 µm)

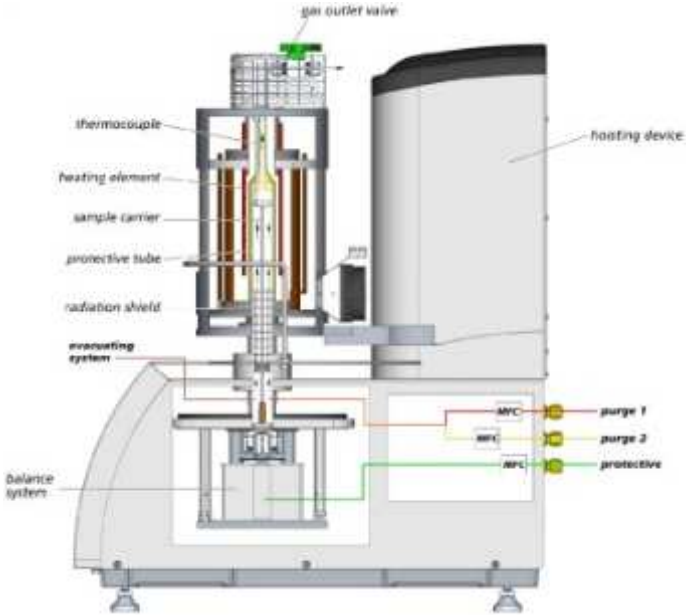
- **Direct injection of solutions of P20.f in dimethyl sulfoxide (DMSO)**

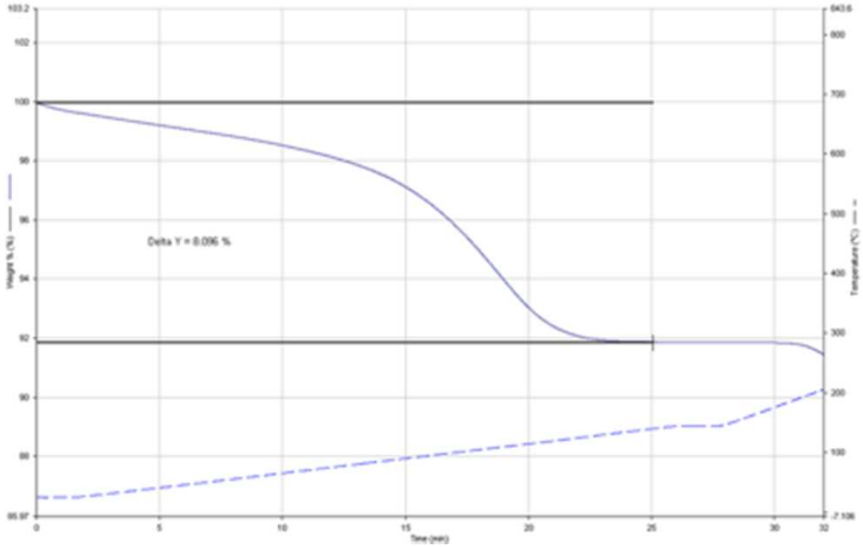
$m_{solvents}$

- **EtOH** **2.5 ± 0.15 mg/g**
- **DCM** **1.0 ± 0.1 mg/g**
- **Toluene** **0.10 ± 0.02 mg/g**

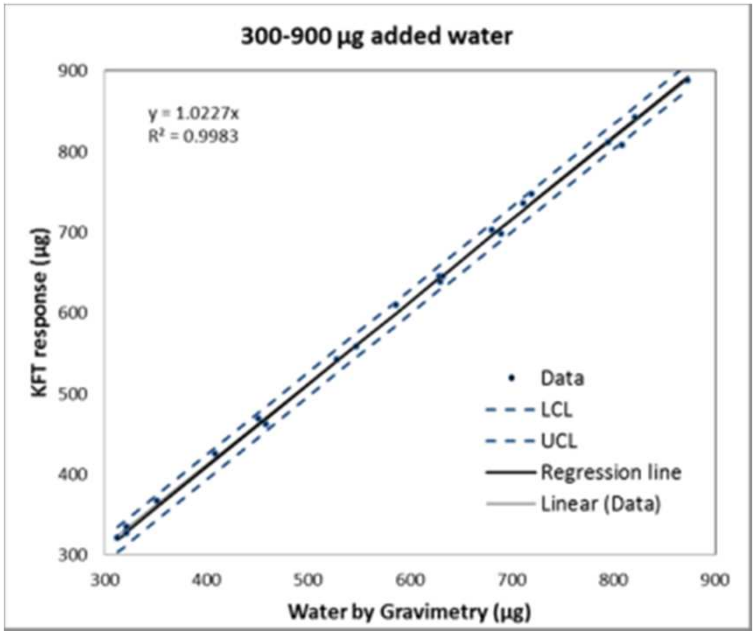
Digoxin Purity: Water Content

Thermal Gravimetric Analysis

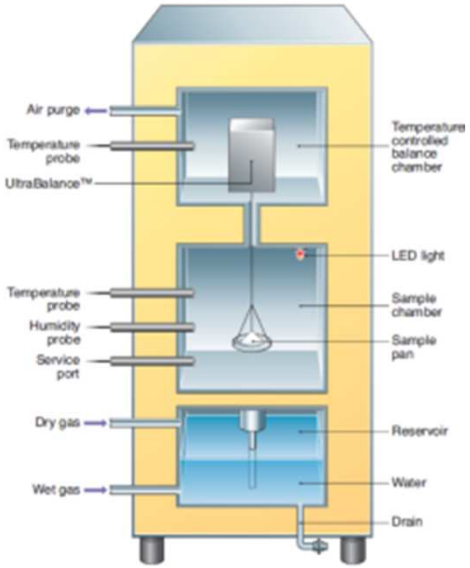




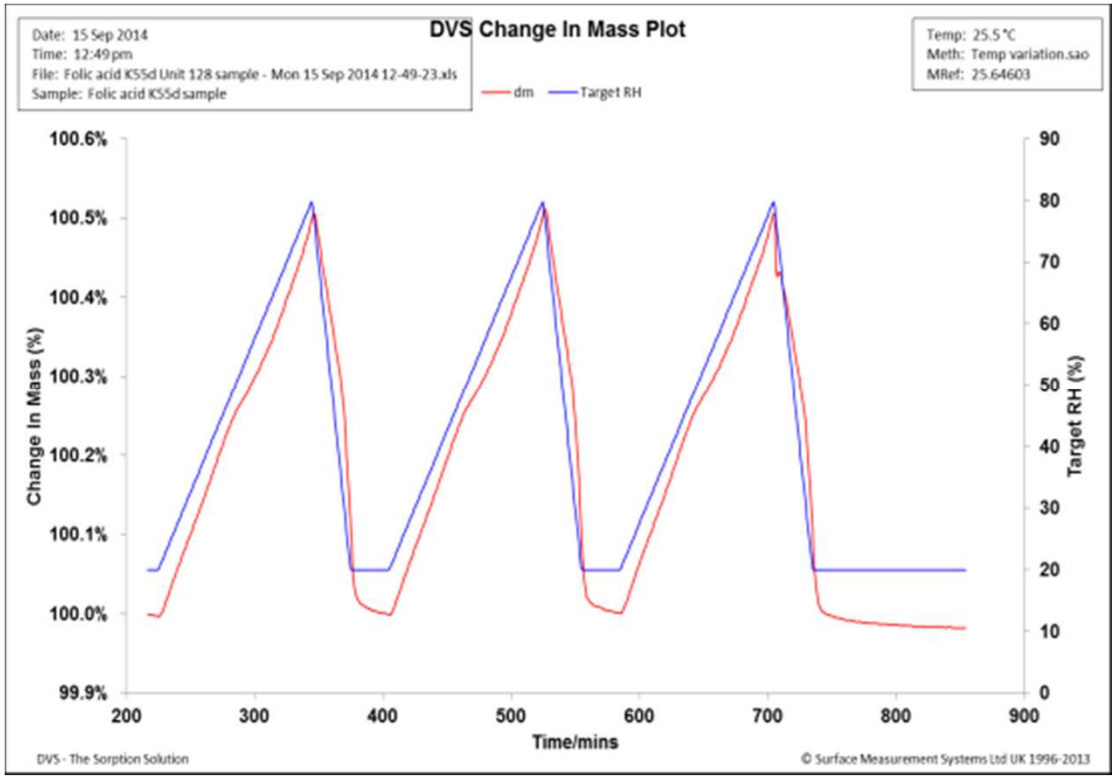
Coulometric Karl Fischer Titration



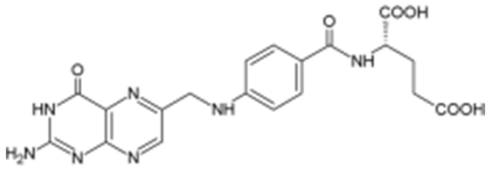
Digoxin Purity: Water Content – Sorption Effects



Schematic of the main components of the DVS Intrinsic



For folic acid, RH a significant influence on water content (Mass fraction ± 2 mg/g in range RH 45 % ± 25 %)



Digoxin Purity: Elemental Analysis

Combustion

Reduction

Separation

Detection

$C \rightarrow CO_2$

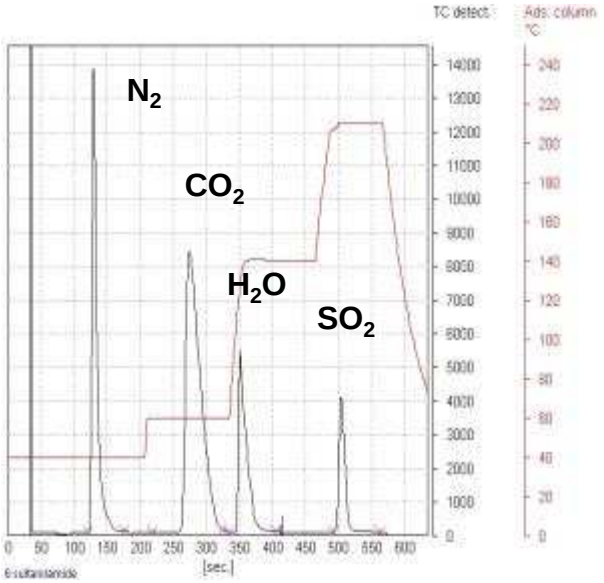
$H \rightarrow H_2O$

$N \rightarrow N_2 \ \& \ NO_x$

$S \rightarrow SO_2 \ \& \ SO_3$

$NO_x + Cu \rightarrow N_2 + CuO$

$SO_3 + Cu \rightarrow SO_2 + CuO$



Digoxin Purity: Mass Balance Result

Component i	x_i (mg/g)	$u(x_i)$ (mg/g)	Contribution (%)
Water	1.1	0.18	10
Ethanol	2.5	0.15	9
Dichloromethane	1.0	0.1	6
Toluene	0.10	0.02	1
Digoxigenin-tetra-digitoxide	3.16	0.05	3
Digitoxin	0.63	0.01	1
Gitoxin	0.63	0.02	1
ß-Acetyldigoxin	0.53	0.03	2
Unidentified UV-active impurity 1	2.37	0.28	16
Unidentified UV-active impurity 2	3.63	0.42	24
Unidentified UV-active impurity 3	1.81	0.22	12
Unidentified UV-active impurity 4	1.92	0.23	13
Combined minor UV-active impurities	1.0	0.03	2
Digoxin	979.6	0.65	
Expanded uncertainty (k = 2)		1.3	

NMI Services for IVD Industry: CRMs

digoxin in high purity digoxin

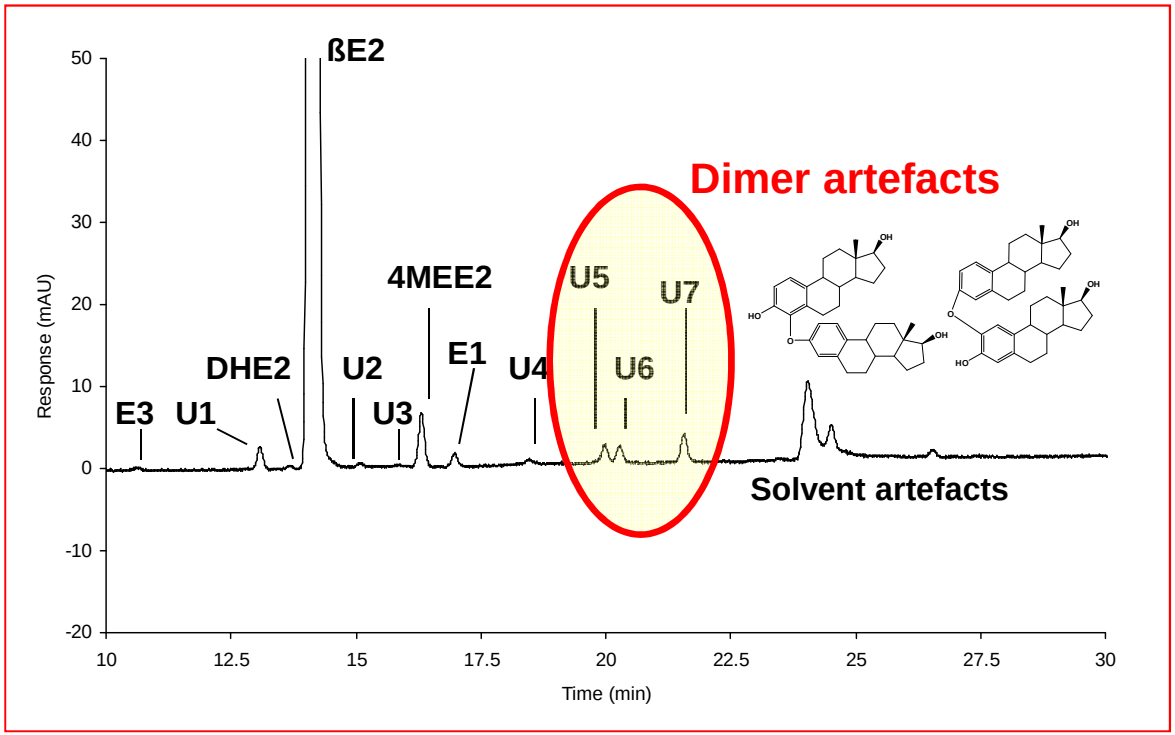
LGC Limited (LGC), United Kingdom

Phone : +44 (0)20 8943 8480
Fax : +44 (0)20 8943 7554
Email : uksales@lgcstandards.com
Web : http://www.lgc.co.uk

Name of the reference material	ERM-AC200a, Digoxin
Quantity	Mass fraction
Analyte certified/assigned value	98 %
Expanded uncertainty (level of confidence 95 %)	0.5 %
Reference(s) on commutability	Not applicable: a high-purity material used as a primary calibrator for higher order reference methods
Traceability	SI
CRM listing	List I

Potential Biases in Mass Balance: Artefacts

RP-LC-UV chromatogram at 225 nm



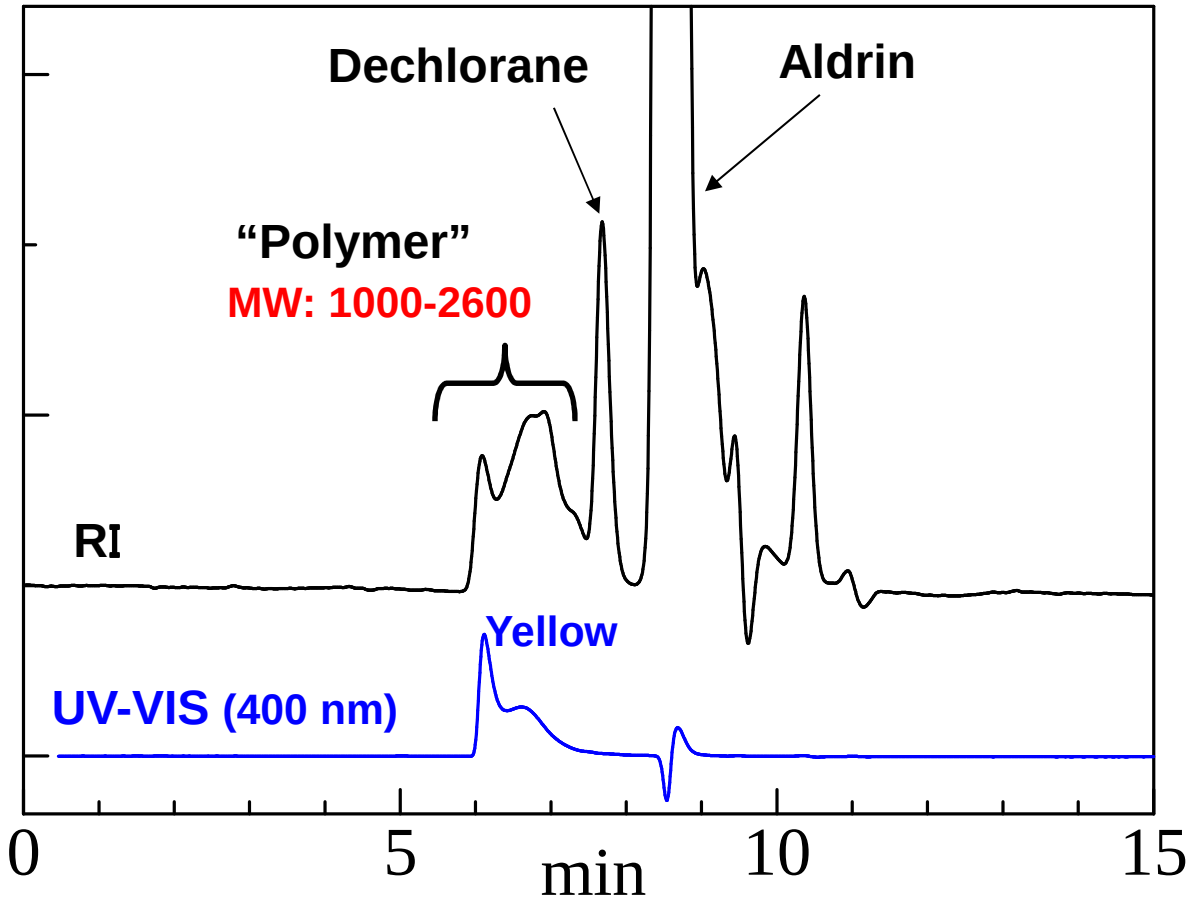
enlarged area

Quantification

- 4-Methylestradiol (4MEE2), 9,11-didehydroestradiol (DHE2), estrone (E1) and estriol (E3) by LC-MS/MS and LC-UV
- Seven unknown UV active impurities by LC-UV (U1-U7)

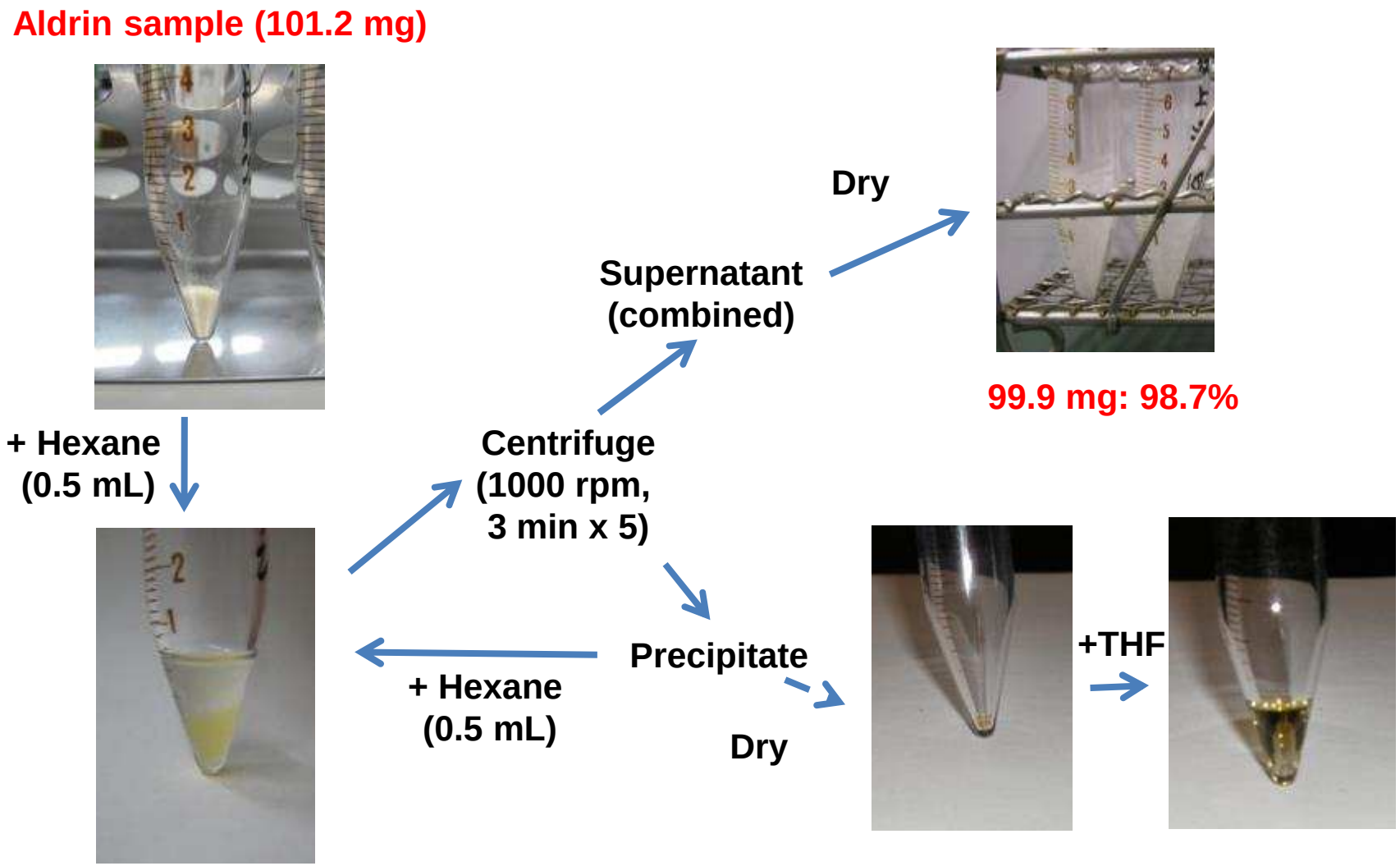
Estradiol dimers can form *in situ – leading to a bias in the measurement*

Potential Biases in Mass Balance: Undetected Impurities



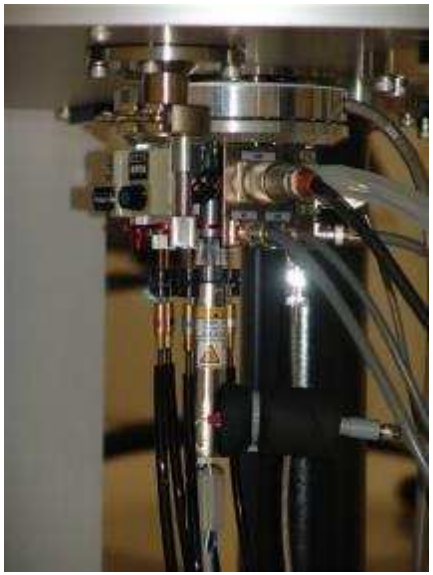
Courtesy of NMIJ

Potential Biases in Mass Balance: Undetected Impurities



Courtesy of NMIJ

Instrumentation for (quantitative) NMR Spectroscopy at BIPM



JEOL JNM-ECS 400 NMR system:

400 MHz superconducting **magnet** (field strength: 9.39T), **Royal Autotune Probe**, 24 positions **autosampler**, system control & data processing **software** “Delta” and “Mnova”. The instrument was kindly donated by JEOL France in 2014.

NMR- the basics

Nuclear Magnetic Resonance (NMR)

- **property of any atom with odd number of protons and/or neutrons**
- **simplest case nuclear spin (I) = ½**
- **main applications use ^{1}H and/or ^{13}C**

Isotope with I = ½	Natural abundance (%)
^{1}H	99.98
^{13}C	1.11
^{15}N	0.37
^{19}F	100
^{31}P	100

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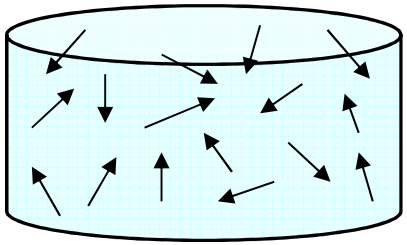
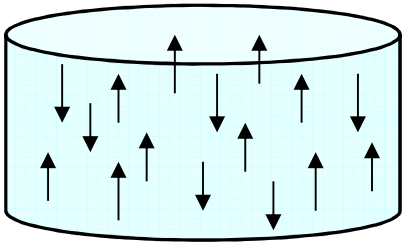
NMR – the basics

I = ½ nuclei have 2 energy states

- equivalent in absence of external field (B_o)
- differ in presence of B_o

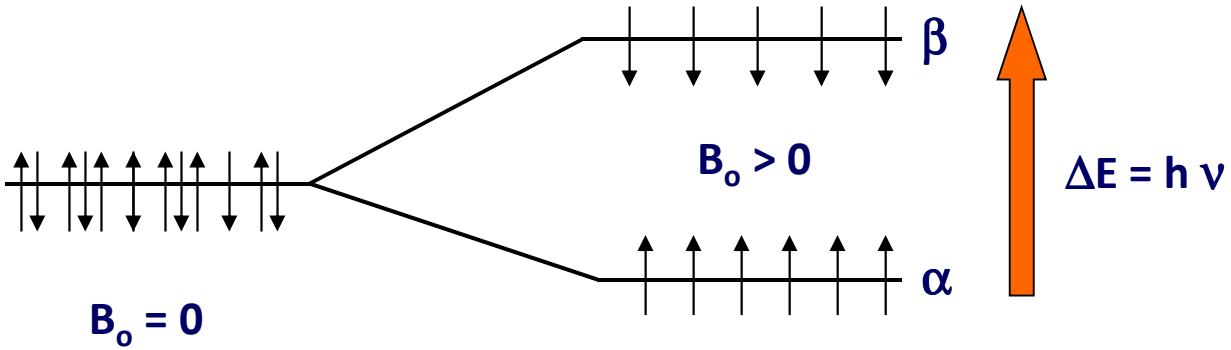


Bo = 0; energetically equivalent



$B_o > 0$; can align with (low energy) or against (high energy) external field

- ΔE corresponds to radiofrequency radiation
- magnitude a function of nucleus and B_o
- For B_o = 9.4 T, $\Delta E \Rightarrow \nu$ = 400 MHz



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Information from the NMR experiment

^{1}H NMR frequency depends on applied field AND molecular environment

- **<u>local</u> magnetic fields differ from B_0 at ppm level**
- **Separate NMR signals for non-equivalent hydrogens**

<u>Observation</u>	**<u>Name</u>**	**<u>Quantity</u>**	**<u>Information</u>**
Peak position	Chemical shifts (δ) (relative to applied field)	δ (ppm)	Chemical (electronic) environment of nuclei
Peak splitting	Coupling constant (J) Hz (absolute)	Peak fine structure	Number and arrangement of neighboring nuclei
			2D- and 3D- molecular structure
Peak shape	Line width	Peak half-height	Molecular motion & chemical exchange
Peak intensity	Integral	Ratio of integrals	Number of equivalent nuclei
			Purity by qNMR

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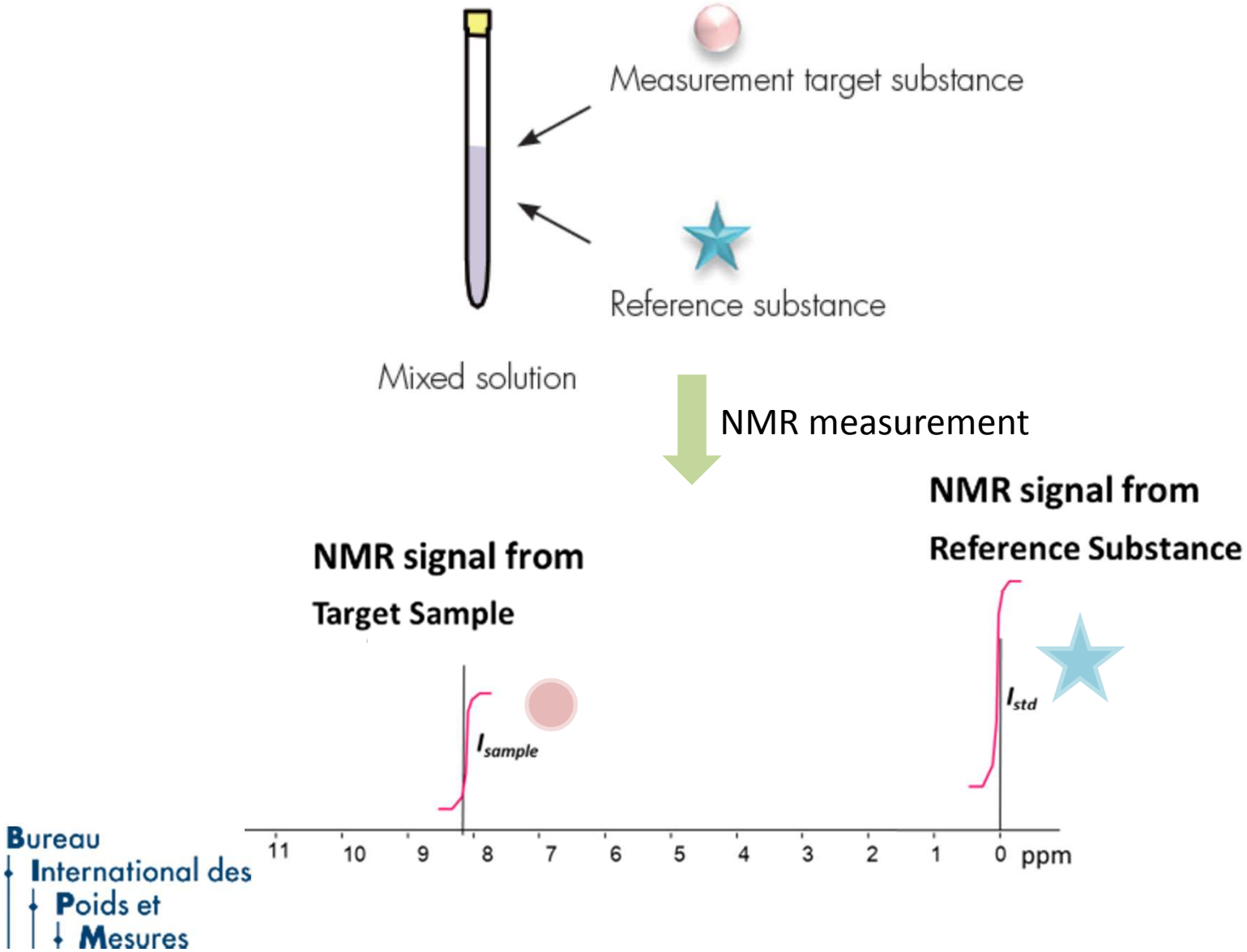
Quantitative NMR (qNMR)



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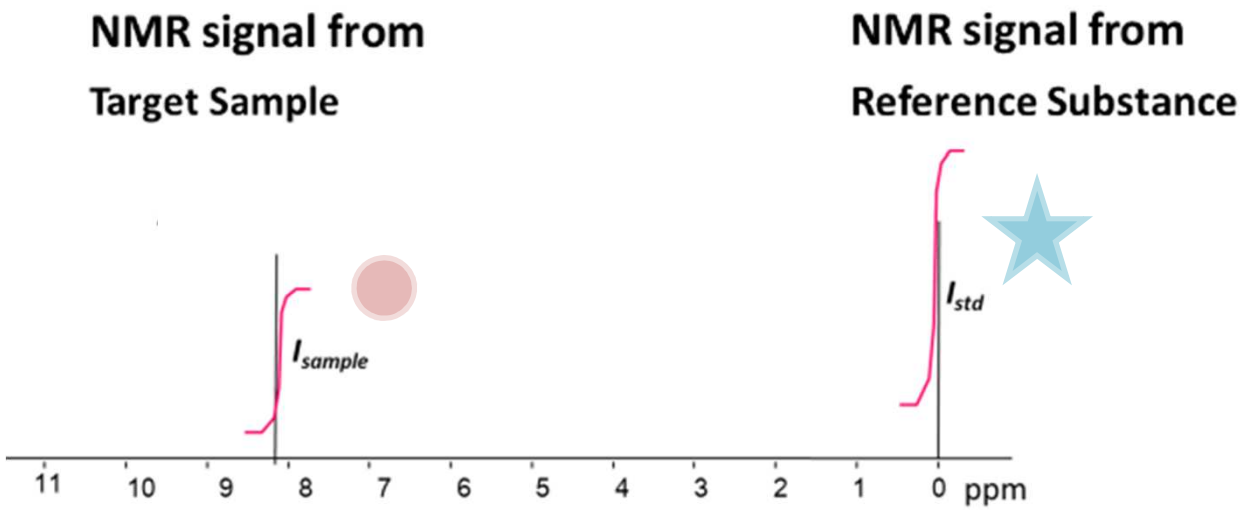
qNMR: Measurement Equation



$$\frac{I_{sample}}{I_{Std}} = \frac{N_{sample}}{N_{Std}} \times \frac{n_{sample}}{n_{Std}} = \frac{N_{sample}}{N_{Std}} \times \frac{m_{sample} * Psample}{m_{Std} * PStd} \times \frac{M_{std}}{M_{Sample}}$$

(I = integral, N = No. equivalent hydrogens, n = moles, m = sample mass, M = molar mass, P = mass fraction purity

$$P_{sample} = \frac{I_{sample}}{I_{Std}} \times \frac{N_{Std}}{N_{sample}} \times \frac{m_{Std}}{m_{sample}} \times \frac{M_{sample}}{M_{Std}} \times P_{Std}$$

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Universal Calibrators for qNMR

REQUIREMENT 1: WIDER RANGE of SUITABLE HIGH-PURITY CRMs

Identify a suite of potential qNMR Primary RMs providing:

- 3^+ compounds for any deuterated solvent
- 3^+ signals in range 0 – 10 ppm in any solvent
- ready integration with high precision

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Universal Calibrators for qNMR

Characteristics for a qNMR Primary RM

- **Stable crystalline solid;**
- **Suitable for accurate gravimetry;**
  - Non-volatile
  - Low hygroscopiscity
  - Non-deliquescent
  - Not subject to electrostatic influence
- **Available at high purity in multigram amounts;**
- **Soluble at > 5 mg/g per suitable solvent;**
- **NMR resonances suitable for qNMR**
  - Narrow, fully resolved
  - Readily integrated
  - Separate from residual solvent/water peaks

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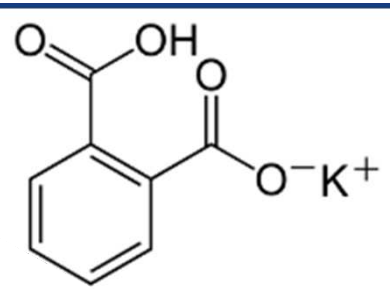
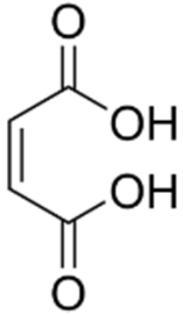
Universal Calibrators for qNMR

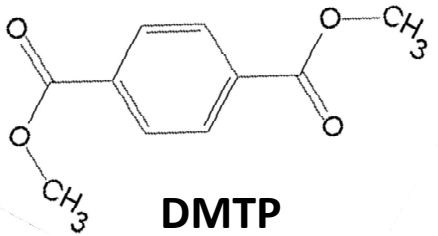


KHP
(Potassium hydrogen phthalate)

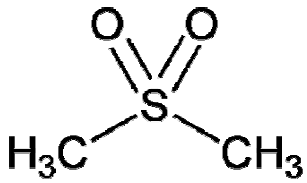


MA
(Maleic Acid)

δ > 6

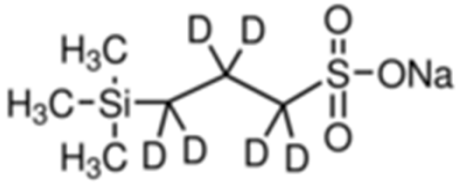


DMTP
(Dimethyl terephthalate)

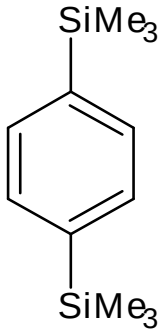


$DMSO_2$
(Dimethyl sulfone)

4 > δ > 3



DSS-d6
(Dimethylsilapentane sulfonate, d-6)



BTMSB
(Bis-TMS Benzene)

δ < 0.2

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Universal Calibrators for qNMR in different Solvents

	KHP	MA	DMTP	$DMSO_2$	DSS-d_6	BTMSB
D_2O	☑	☑	☒	☑	☑	☒
$CDCl_3$	☒	☒	☑	☑	☒	☑
d_6-DMSO	☑	☑	☑	☑	☑	☑
CD_3OD	☒	☑	☑	☑	☑	☑

☒ **Insufficient solubility**

☑ **Solubility > 5 mg/g**

☑ **Soluble but obscured by solvent or water peaks**

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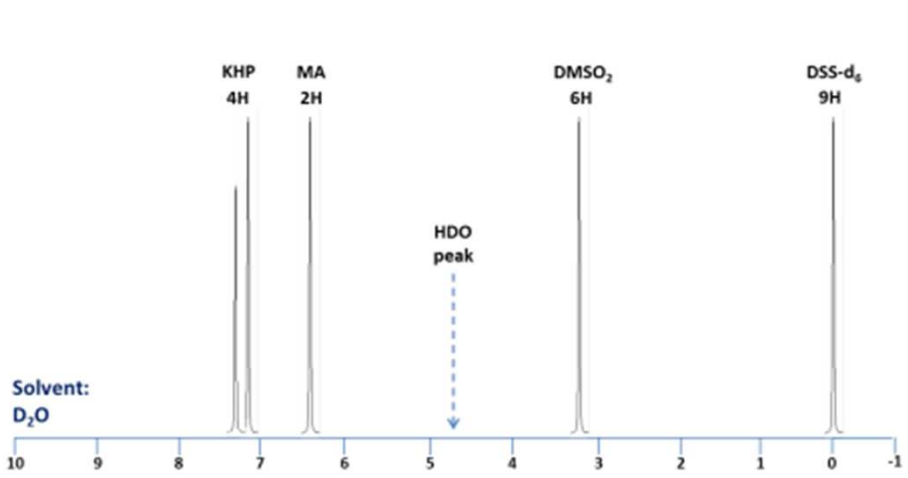
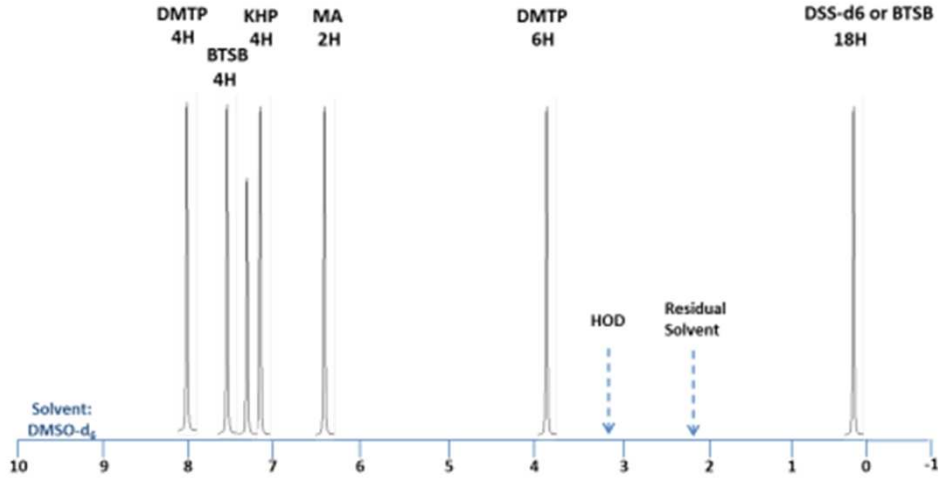
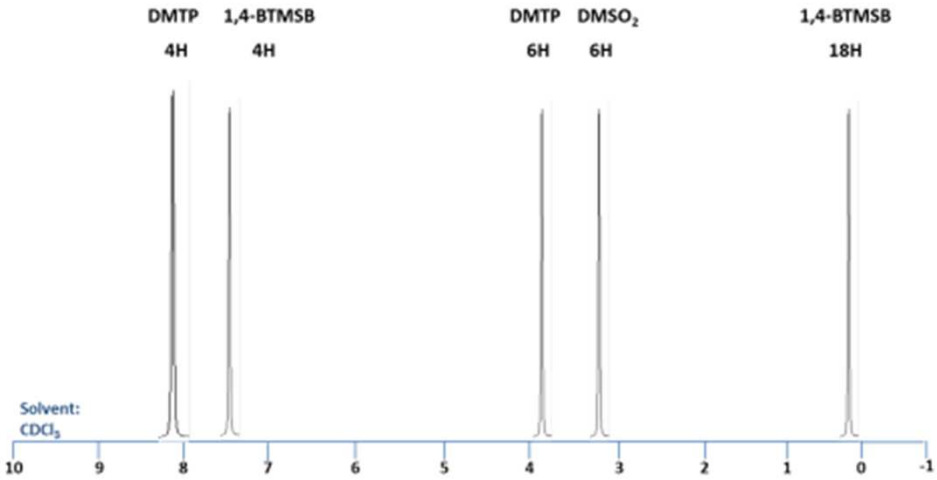
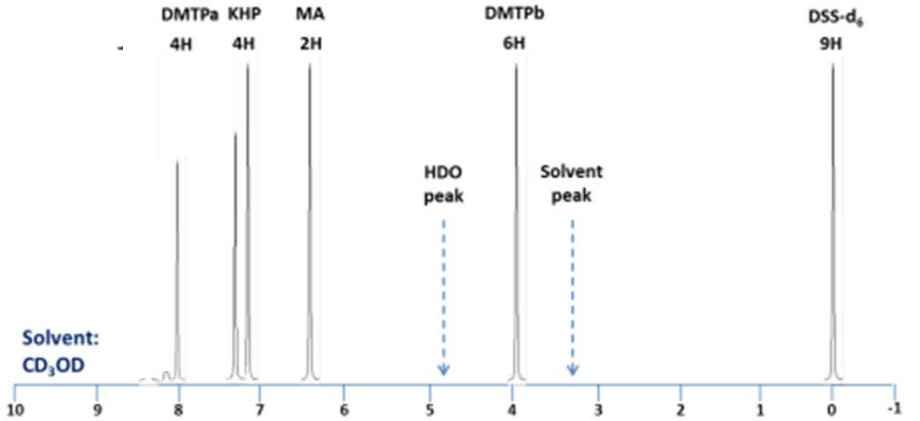
Universal Calibrators: NMR Spectra (4 different Solvents)





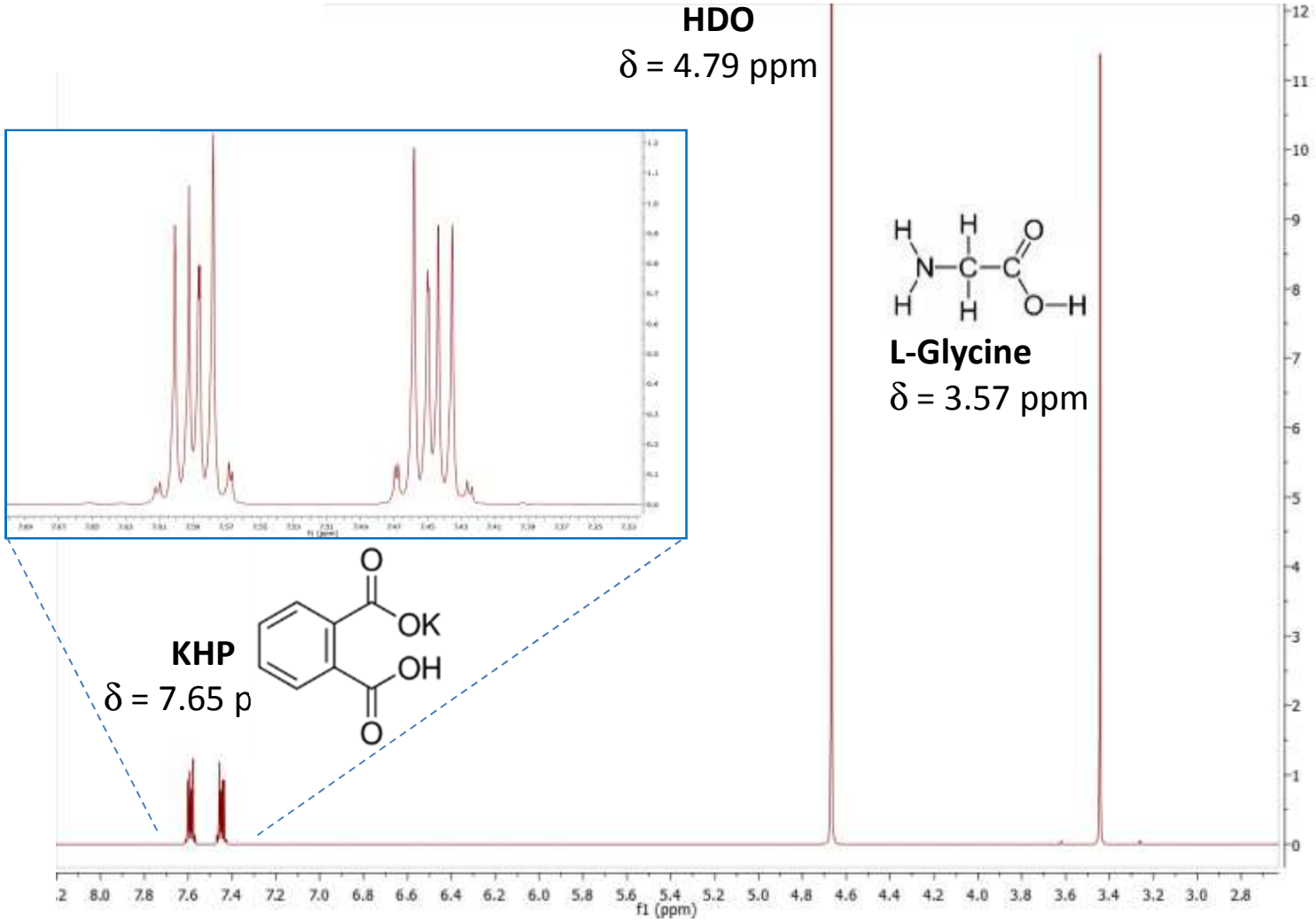




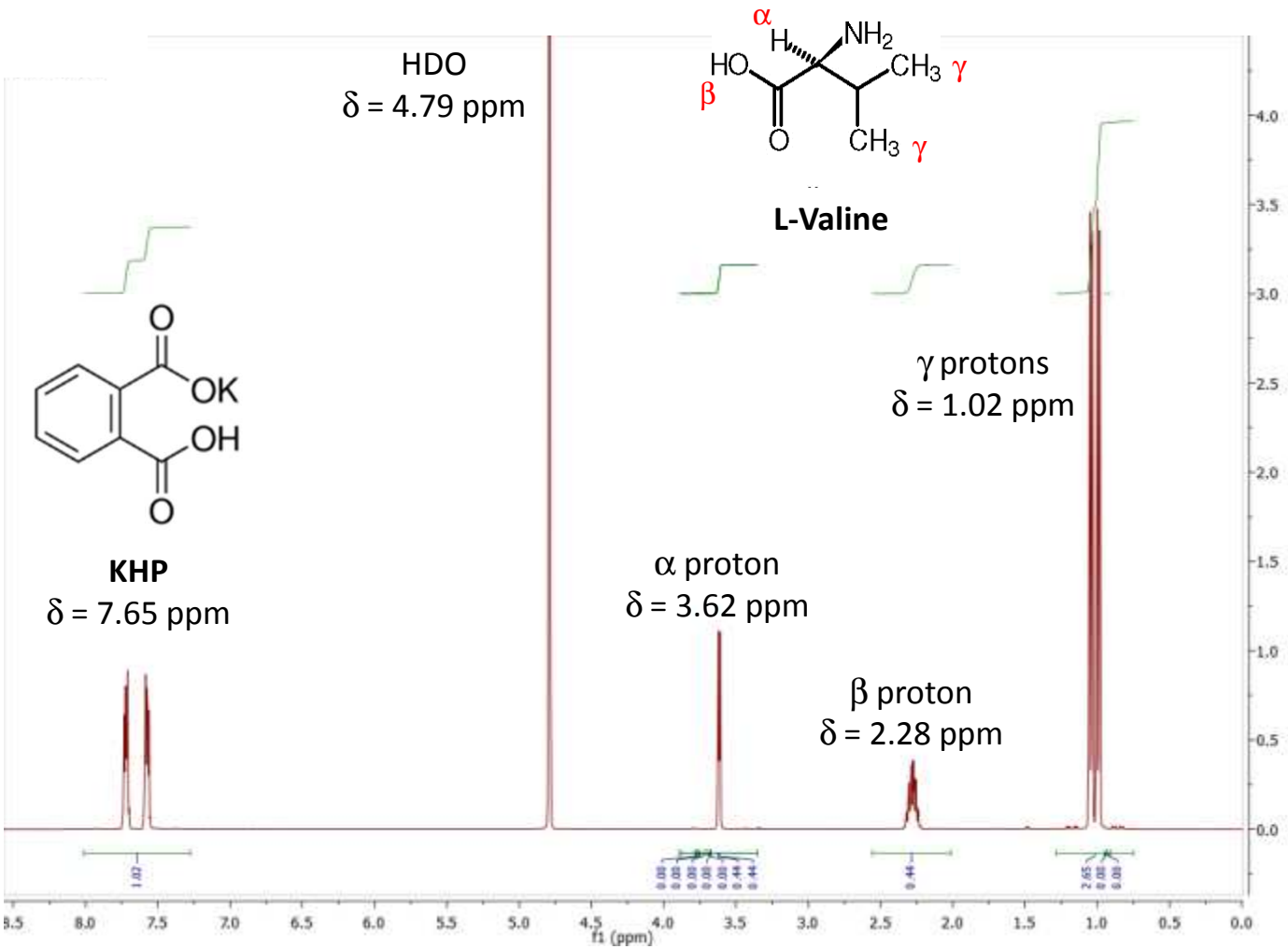
Bureau International des Poids et Mesures

- **Relative peak heights not to scale!**
- **Open to other proposals meeting criteria**

^{1}H-NMR spectrum of analyte L-glycine and internal standard potassium hydrogen phthalate (KHP), dissolved in D_2O



^{1}H-NMR spectrum of L-Valine and internal standard potassium hydrogen phthalate (KHP), dissolved in D_2O

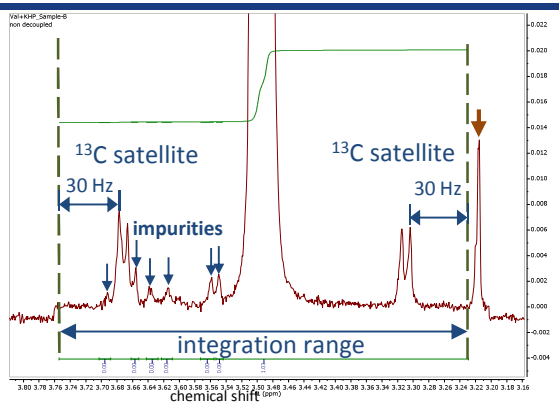


Relevant for purity calculations:

Areas of peaks at δ = 7.65 ppm for KHP and 3.62 (α), 2.28 (β) or 1.02 ppm (γ) for Val

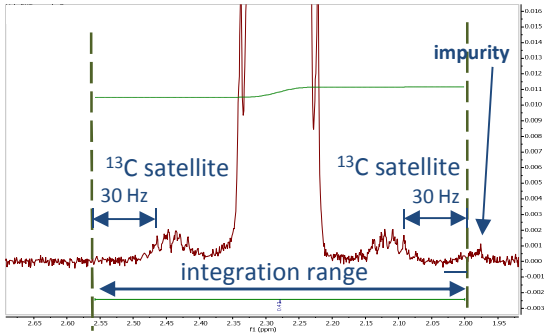
Influence of impurities on the quantification of qNMR signals: Example: L-Valine

α



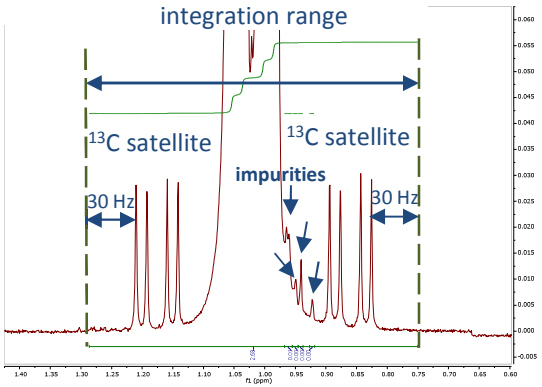
SIGNAL α 3.62 ppm	Purity	Standard deviation
Before correction	99.64 %	0.15 %
Sum of all impurities (6)	0.81 %	0.17 %
After correction	98.83 %	0.32 %

β



SIGNAL β 2.28 ppm	Purity	Standard deviation
Before correction	98.55 %	0.07 %
1 impurity	0.10 %	0.01 %
After correction	98.45 %	0.07 %

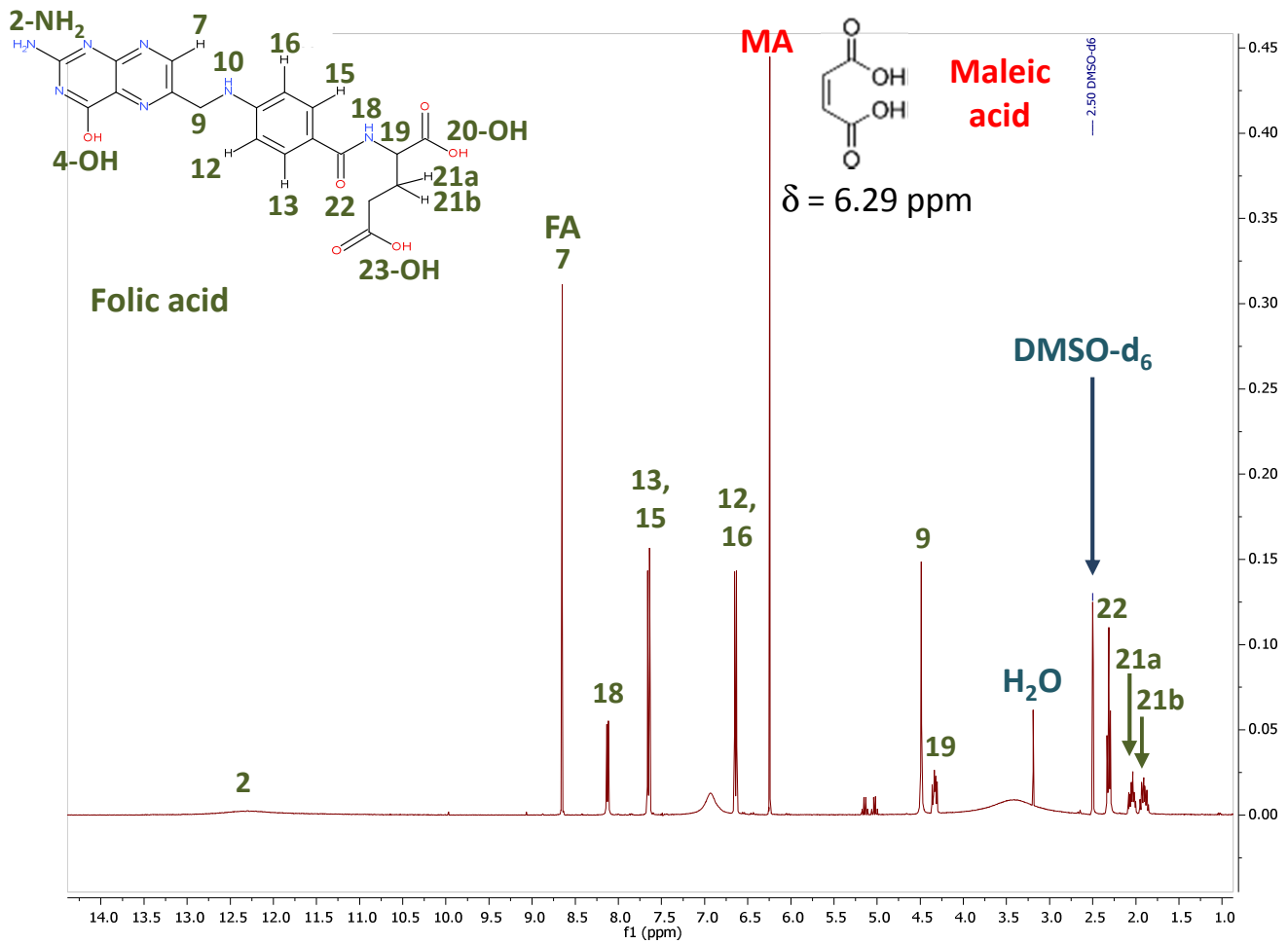
γ



SIGNAL γ 1.02 ppm	Purity	Standard deviation
Before correction	99.02 %	0.10 %
Sum of all impurities (4)	2.99 %	0.05 %
After correction	98.66 %	0.13 %

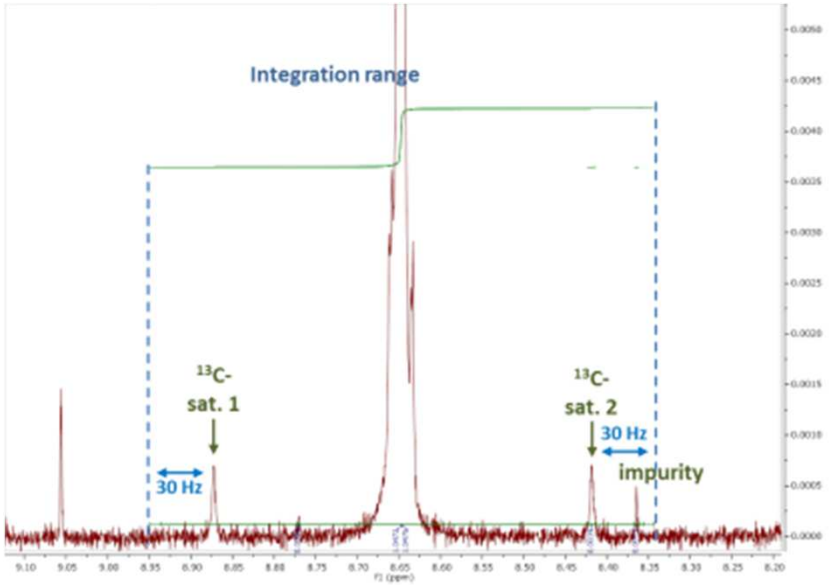
Values represent averages of 3 qNMR measurements of 3 independent identical samples each.

^{1}H-NMR spectrum of folic acid (FA) and maleic acid (MA), dissolved in DMSO-d_6

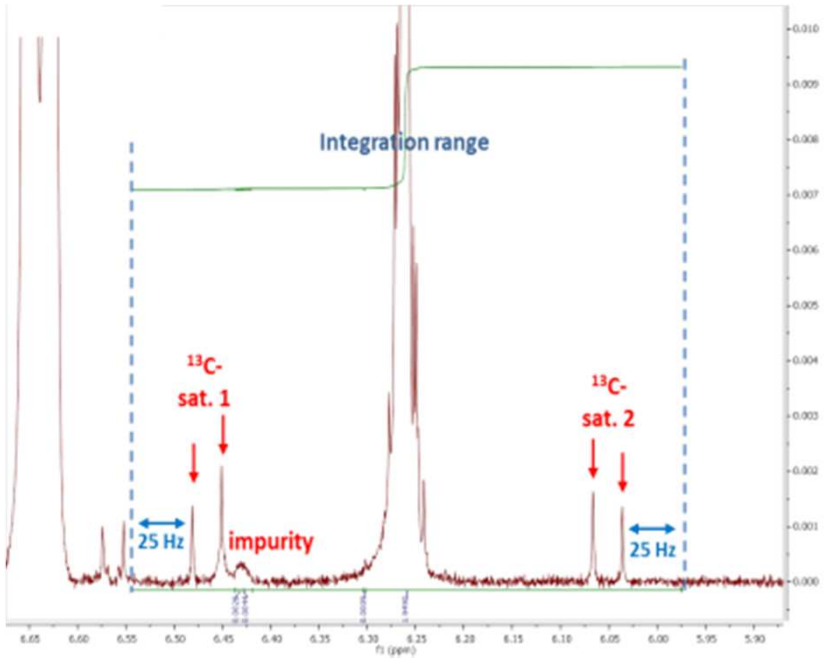


The FA and MA peaks at δ = 8.65 ppm and δ = 6.24 ppm were used for qNMR data evaluation.

qNMR peak integration ranges for folic acid and maleic acid



Folic acid



Maleic acid

qNMR purity and uncertainty calculations

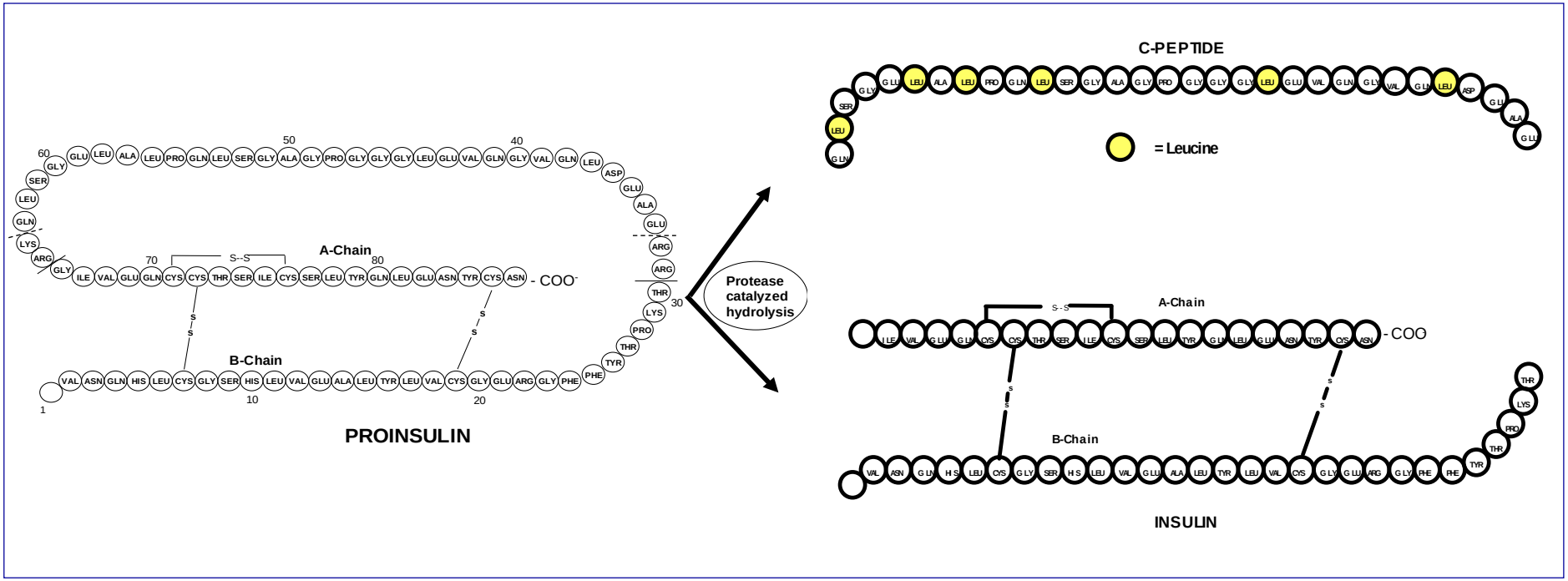
Sample A

Term	Abbreviation	Unit	values	uncertainties	rel u
Purity of the Internal Standard (MA)	P_{IS}	[mg/g]	999.9	0.145	0.000145
Number of 1H nuclei of Internal Standard (MA)	N_{IS}	-	2	0	
Number of nuclei of analyte (FA)	N_A	-	1	0	
Peak area ratio Analyte / Internal Standard	S_A / S_{IS}	-	0.314031	0.000810	0.002579
Molecular weight of Internal Standard (MA)	M_{IS}	[g/mol]	116.0719	0.002150	1.8519E-05
Molecular weight of Analyte (FA)	M_A	[g/mol]	441.3973	0.004629	1.0488E-05
Weighed amount of Analyte (FA)	m_A	[mg]	5.4948	0.0012	0.000226
Weighed amount of Internal Standard (MA)	m_{IS}	[mg]	2.1105	0.0012	0.000588
Purity of Analyte (FA)	P_A	[mg/g]	917.2507		
Combined uncertainty for the purity of analyte	$u(P_A)$	[mg/g]	2.4383		0.266%

Comparison of qNMR versus Mass Balance Approach

qNMR +	qNMR -	Mass Balance +	Mass Balance -
Short method development time (< 2 weeks)			Long method development time (> 2 months)
Small amount of material (< 5 mg per experiment)			Large amount of material (> 50 mg per experiment)
	Uncertainties of 0.3%	Uncertainties of 0.1%	
Small number of “universal” standards			Standards for each impurity
	Poor resolution and sensitivity for related structure impurities	High resolution and sensitivity for related structure impurities	
Direct assay of main component			Indirect assay, potential bias by undetected impurities
	Best practice to be benchmarked	Methods understood and documented	
	High capital cost of equipment		High capital cost of equipment

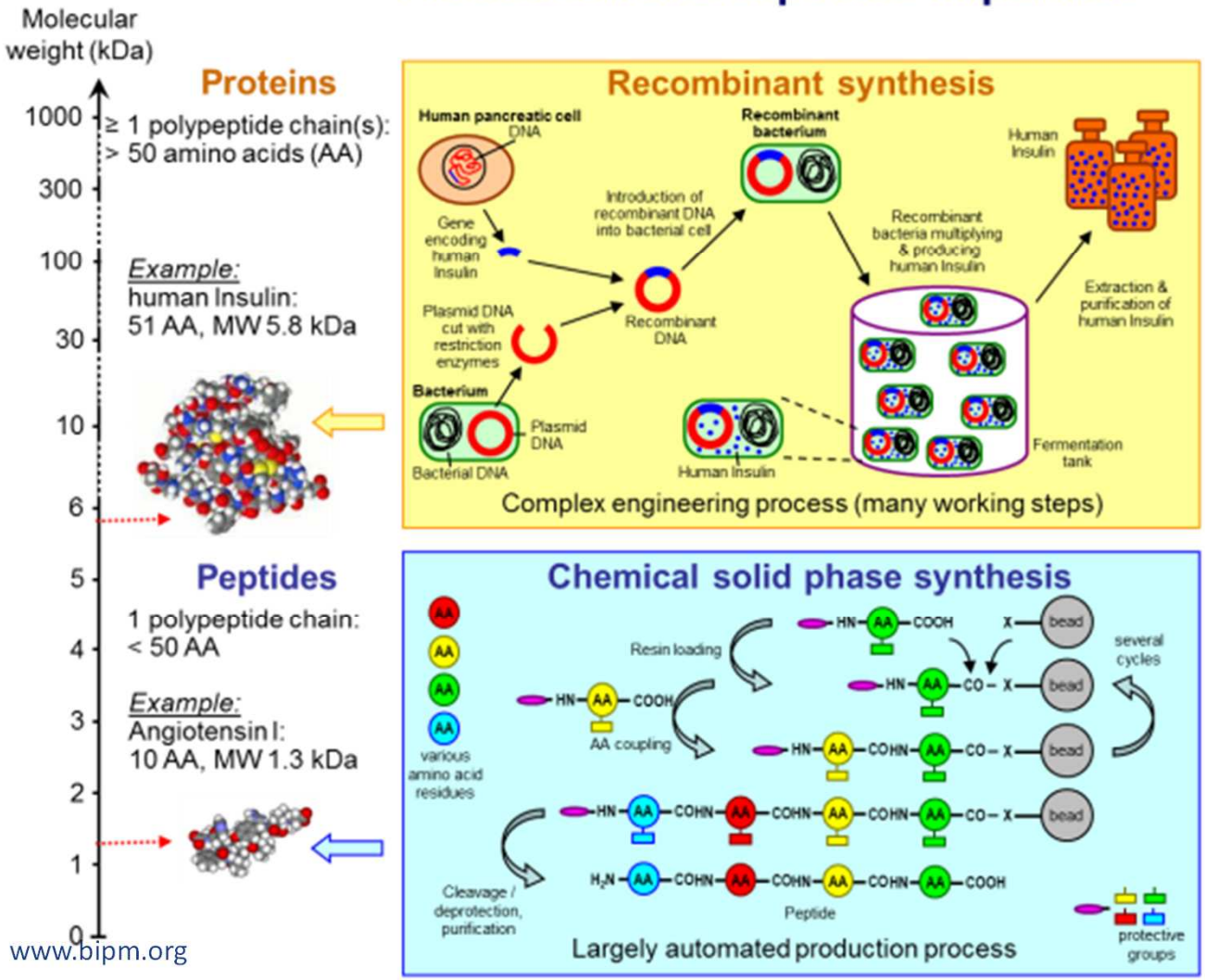
C-peptide and Insulin Measurements and Standards



- Pro-insulin is synthesized in the pancreatic beta cells
- Pro-insulin is packaged into granules and cleaved to insulin and C-peptide.
- Insulin and C-peptide are secreted in a 1:1 molar ratio.
- Insulin (but not C-peptide) is cleared by the liver; C-peptide remains in the circulation longer than insulin
- C-peptide is the best marker of insulin secretion

Organic Large Molecules: Peptide Purity

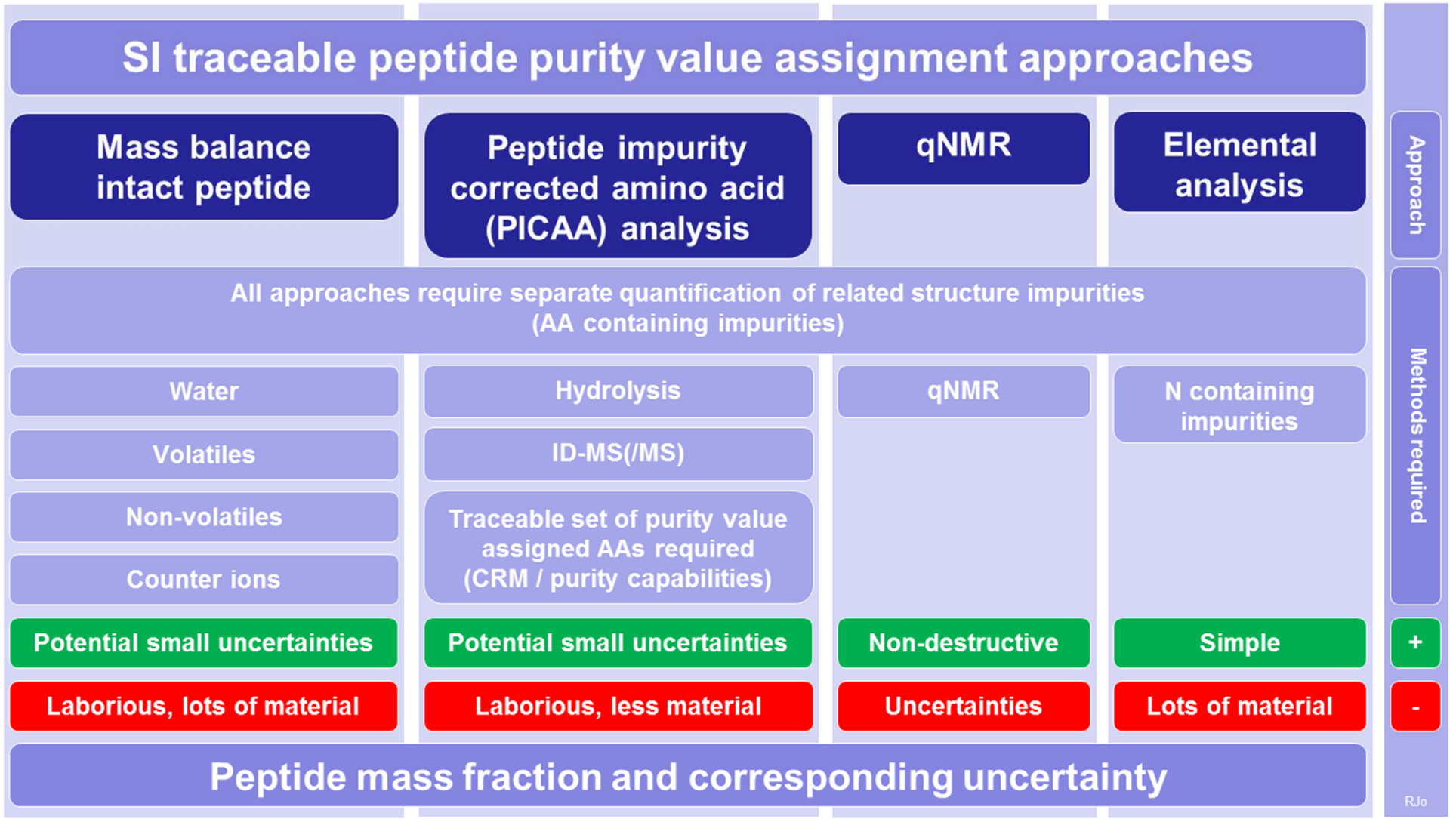
Large Molecule Primary Calibrator Standards: Production and expected impurities



Possible impurities

- Degradation products of protein of interest
- Residues of ...
  - culture medium ingredients
  - culture cells' debris (e.g. LPS)
  - DNA, RNA
  - enzymes used in processing of product
  - reagents
  - solvents
- Degradation products of peptide
- Residues of ...
  - reagents
  - solvents

Organic Large Molecules: Peptide Purity



Instrumentation for Peptide Characterization
Liquid Chromatography – Mass Spectrometry

For analyses at high mass resolution (hr) & quantification:

Series 1200 HPLC (Agilent)

+

LTQ-Orbitrap XL mass spectrometer with
MS^n capability & ESI source (Thermo Scientific)

For screens, characterisation & quantification:

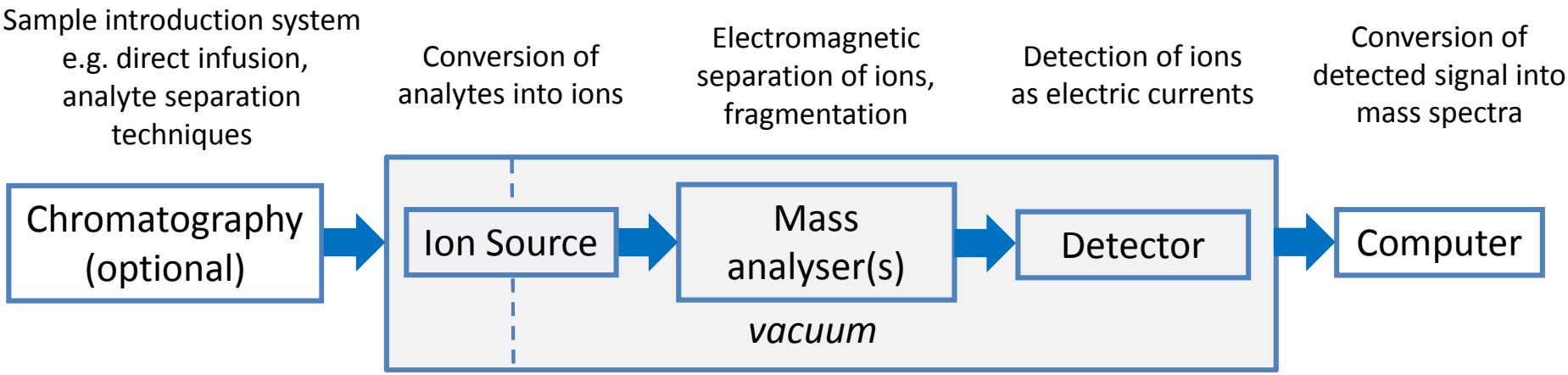
Series 1100 HPLC (Agilent)

+

QTrap 4000 LC/MS/MS System
with ESI source (AB Sciex)

For both systems: Column: Jupiter C_{18}, 150 x 2,1 mm, 5 µm, 300 Å (Phenomenex)
Eluents: acetonitrile, water & formic acid

Typical configuration of a mass spectrometer



For some components shown above there exist numerous possibilities:

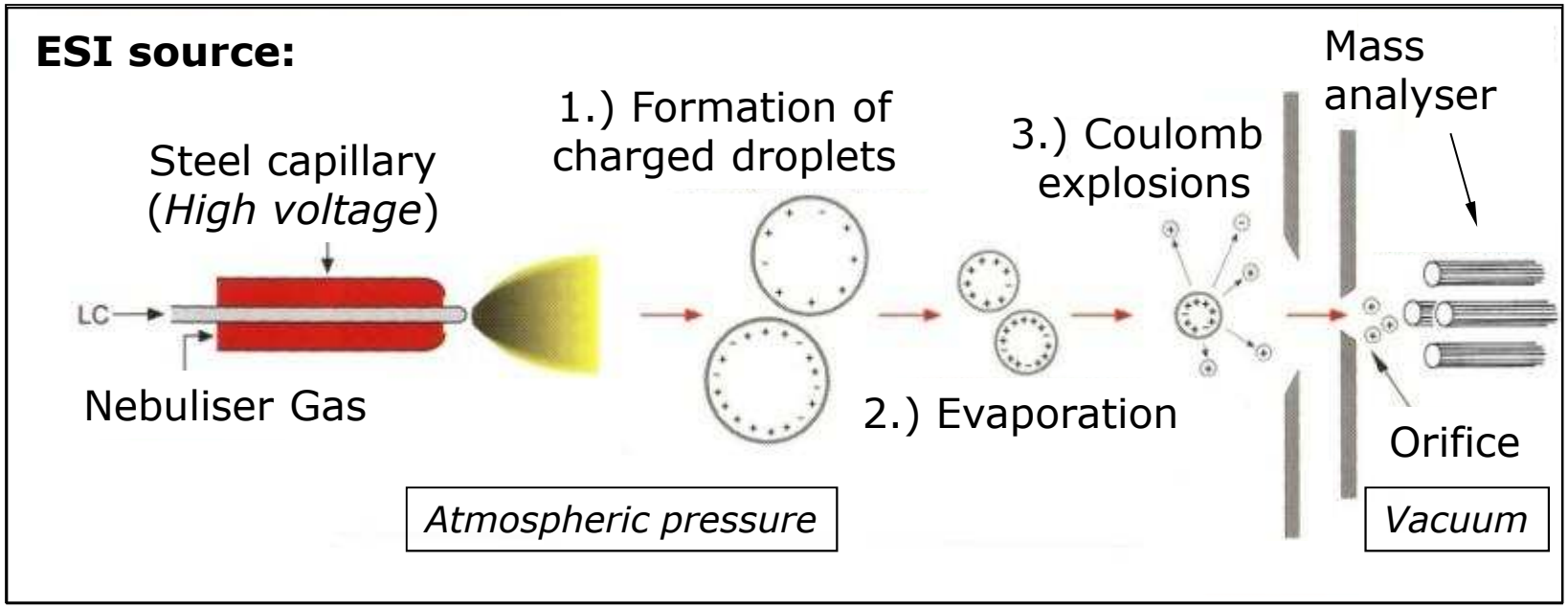
Chromatography: HPLC, GC, …

Ion source: ESI, APCI, MALDI, …

Mass analyser(s): in case of 2 mass analysers = "Tandem Mass Spec"
e.g. Orbitrap, (triple) quadrupole, time-of-flight (TOF), …

The MS instrument parts including the mass analyser(s), the detector (and in some cases the ion source) is kept under high vacuum (10^{-2} – 10^{-6} Pascal), to minimise unwanted collision of ions with residual atmospheric gas molecules.

The Electrospray Ion Source



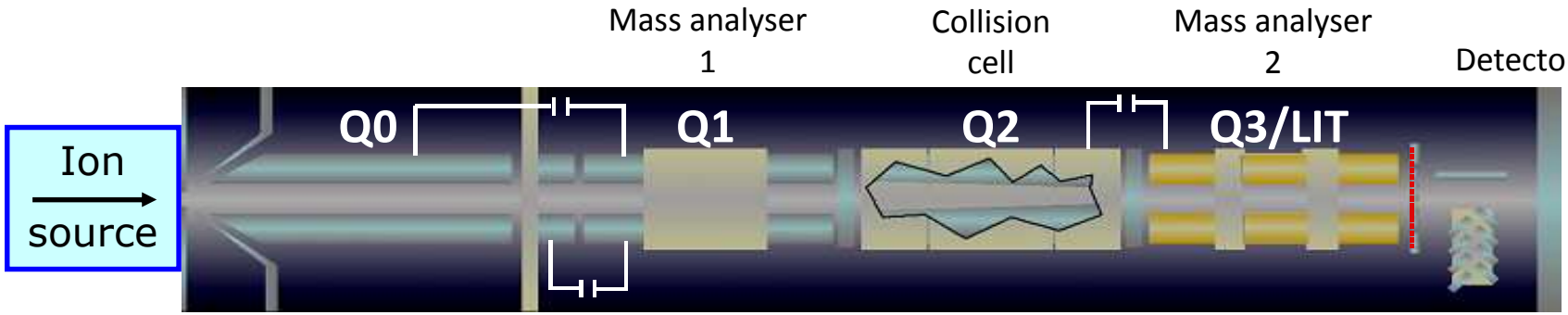
… one of the most widespread ion sources in mass spectrometers coupled to HPLC

Organic samples: often complex mixtures of various ion types of different substances present at same time

➔ Possibility of ion suppression / enhancement.

➔ **Chromatographic separation:** of mixture constituents prior to MS advantageous.

Mass Specs with different Mass Analysers (I)



Triple-Quadrupole (QqQ): e.g. in instruments from AB Sciex ("QTrap" series). Features: Scanning & filtering of ions by using specific DC and RF combinations for ions with particular *m/z* ratios. Fragmentation of selected ions in the collision cell and analysis of product ions.

Advantage: very fast analysis possible => ideal for quantifications

Limitation: low - medium mass resolution

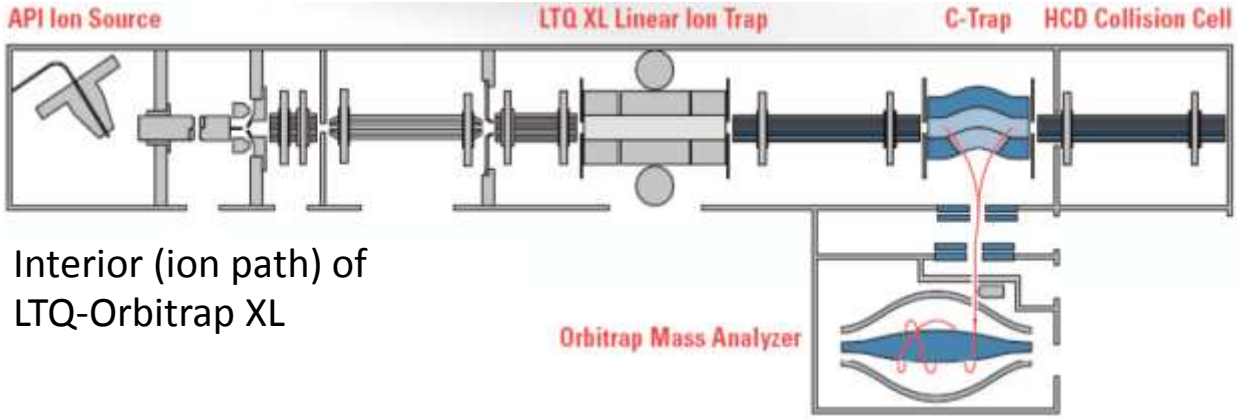
QTrap4000 LC-MS/MS system at the BIPM

Mass Specs with different Mass Analysers (II)

Orbitrap: an electrostatic mass analyser, inside which ions are trapped and circulating at different paths, depending on their *m/z* ratios. Image currents from the ions are detected by the electrodes and converted via Fourier transformation of the frequency signal into a mass spectrum. Manufacturer: Thermo Fisher Scientific.

Advantage: hrMS and hrMS/MS analysis at high mass resolution => ideal for structural characterisation of small and large organic molecules (especially peptides and proteins).

Limitation: Scan speed: high speed & low resolution vs. low speed & high resolution



Interior (ion path) of LTQ-Orbitrap XL

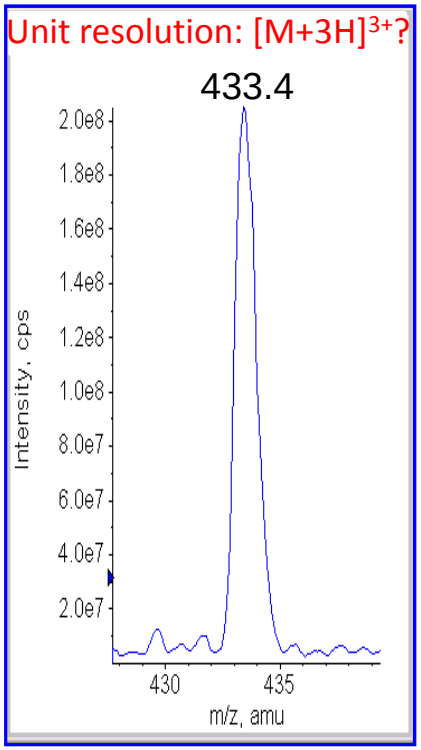
LTQ-Orbitrap XL
LC-hrMS/MS at the BIPM

Mass resolving power

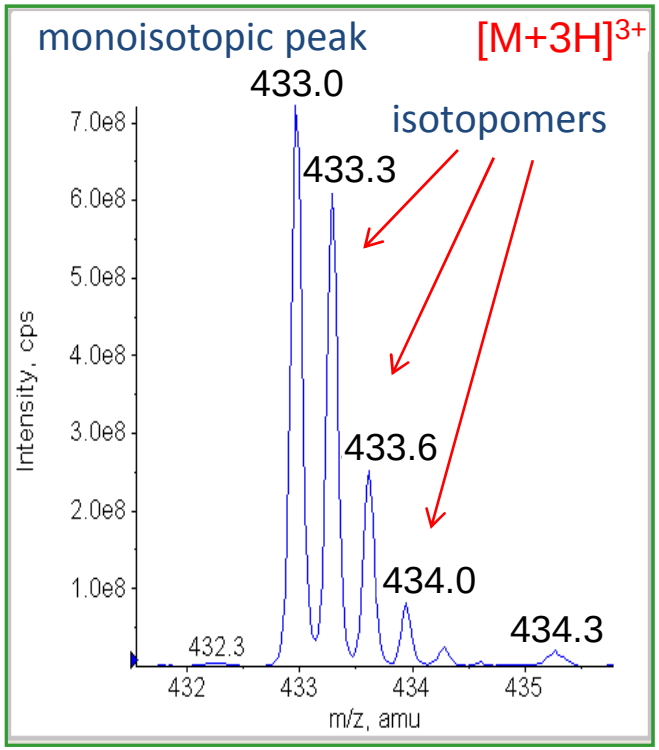
QTrap4000 vs. LTQ-Orbitrap XL

DC-RF mass analyser type "linear quadrupole"

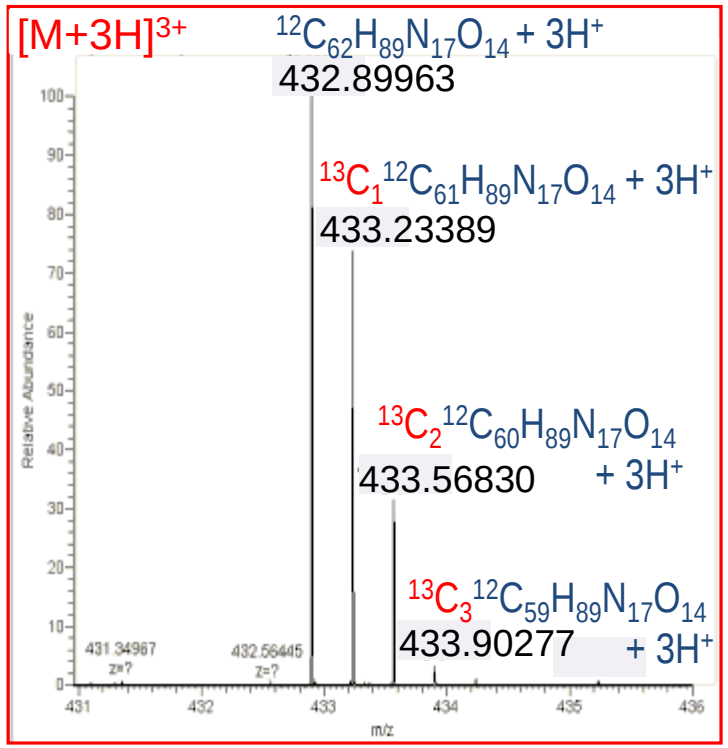
Electrostatic mass analyser "Orbitrap"



Fullscan mode Q1-MS, for wide mass range



Enhanced Resolution Scan mode "ER" for narrow mass range



Fullscan at a Resolution of 60,000 (FWHM)

Comparison of mass resolution for the $[M+3H]^{3+}$ ion of Angiotensin I
High Resolution-MS enables the assignment of a molecular formula to an ion of a compound in the MS spectrum.

Some terms in high resolution Mass Spectrometry

- **Monoisotopic mass:** molecule mass, calculated with the masses of the most abundant isotopes only of its constituting chemical elements (^{12}C, ^{14}N, ^{16}O, …). Used for MS.
- **Average mass:** molecule mass, calculated with the average atomic masses of its constituting chemical elements. This is usually stated as "Molecular weight (MW)".
- **Nominal mass:** molecule mass, calculated with the integer mass values of the most abundant isotopes of its constituting chemical elements

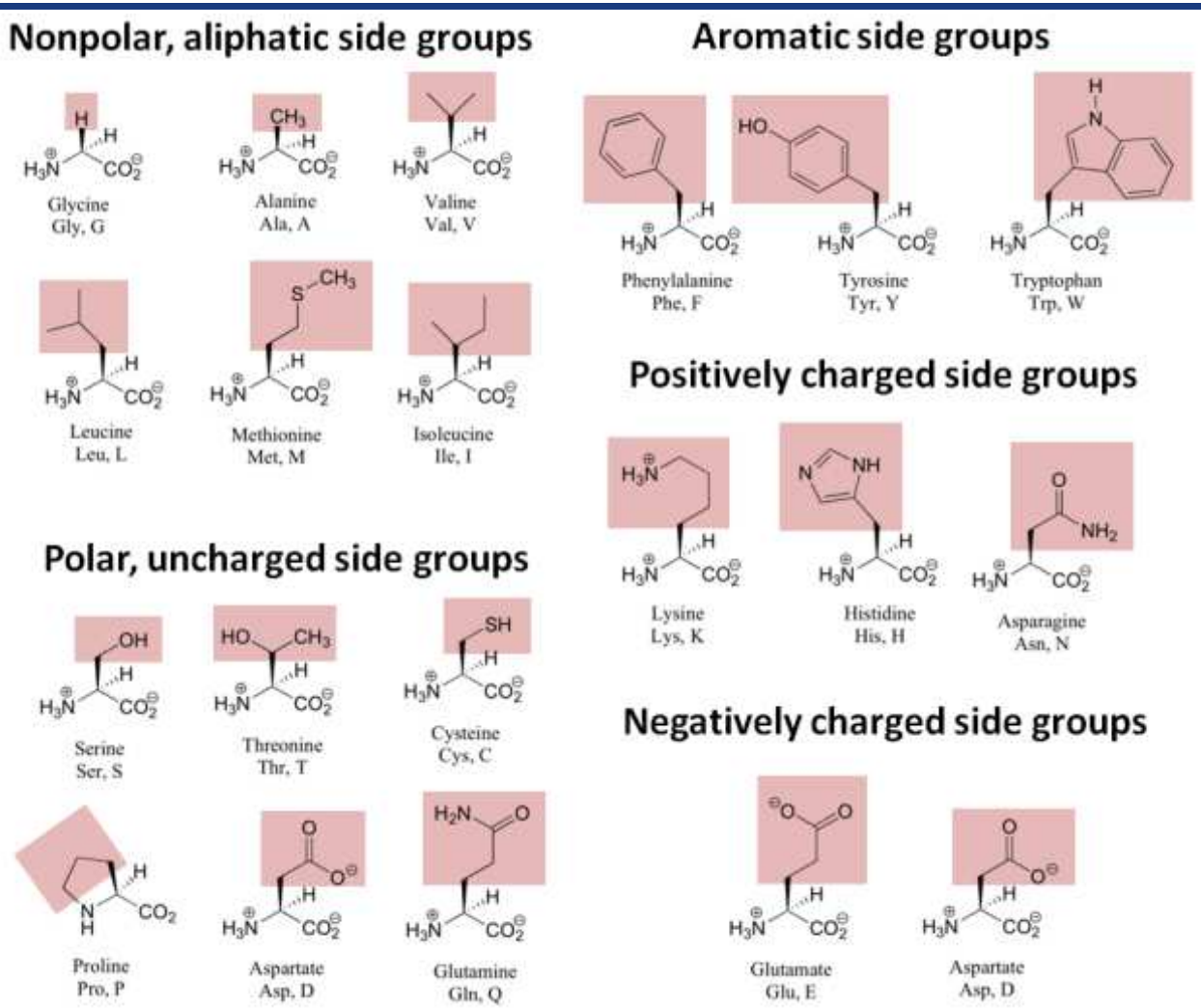
Example: Peptide Angiotensin I: molecular formula: $C_{62}\ H_{89}\ N_{17}\ O_{14}$

nominal mass: 1295 Da, monoisotopic m: 1295.677 Da, average m: 1296.477 g/mole

Abundance table for natural isotopes of selected chemical elements:

Element	Symbol	Nominal mass	Monoisotopic mass	Average atomic mass	Abundance [%]
Hydrogen	^{1}H ^{2}H or D	1 2	1.0078 2.0141	1.0079	99.985 0.015
Carbon	^{12}C ^{13}C	12 13	12.0000 13.0034	12.0110	98.91 1.11
Nitrogen	^{14}N ^{15}N	14 15	14.0031 15.0001	14.0070	99.63 0.37
Oxygen	^{16}O ^{17}O ^{18}O	16 17 18	15.9949 16.9991 17.9992	15.9990	99.759 0.037 0.204
Sulfur	^{32}S ^{33}S ^{34}S	32 33 34	31.9721 32.9715 33.9679	32.0600	95.002 0.76 4.22

Some facts about Amino acids

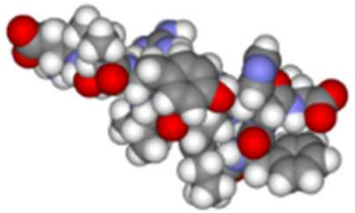


Amino acid	molecular weight [Da]		
	nominal	average	monoisotopic
Gly	75	75.07	75.0320
Ala	89	89.09	89.0477
Ser	105	105.09	105.0426
Pro	115	115.13	115.0633
Val	117	117.15	117.0790
Thr	119	119.12	119.0582
Cys	121	121.16	121.0197
Ile	131	131.17	131.0946
Leu	131	131.17	131.0946
Asn	132	132.12	132.0535
Asp	133	133.10	133.0375
Gln	146	146.14	146.0691
Lys	146	146.19	146.1055
Glu	147	147.13	147.0532
Met	149	149.21	149.0510
His	155	155.15	155.0695
Phe	165	165.19	165.0790
Arg	174	174.20	174.1117
Tyr	181	181.19	181.0739
Trp	204	204.23	204.0899

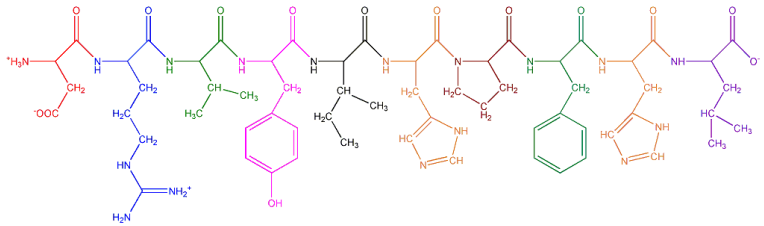
Building blocks of the peptides and proteins

Some facts about Angiotensin I

Angiotensin I (ANG I)



A linear oligopeptide consisting of 10 amino acids:
Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu (DRVYIHPFHL)

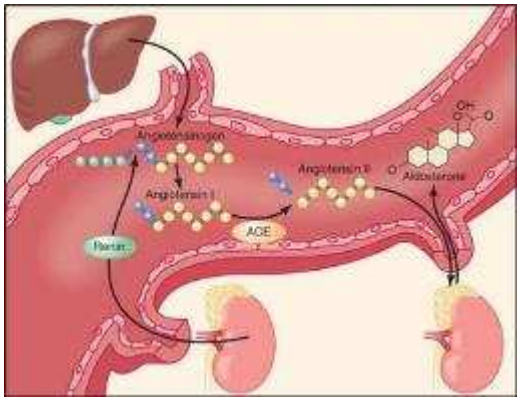


Elemental formula: $C_{62}H_{89}N_{17}O_{14}$
Average mass (MW): 1296.47762 g/mole
Monoisotopic mass: 1295.67749 Da

Biological role:

An inactive prohormone in the Renin-Angiotensin-Aldosterone System (RAAS)

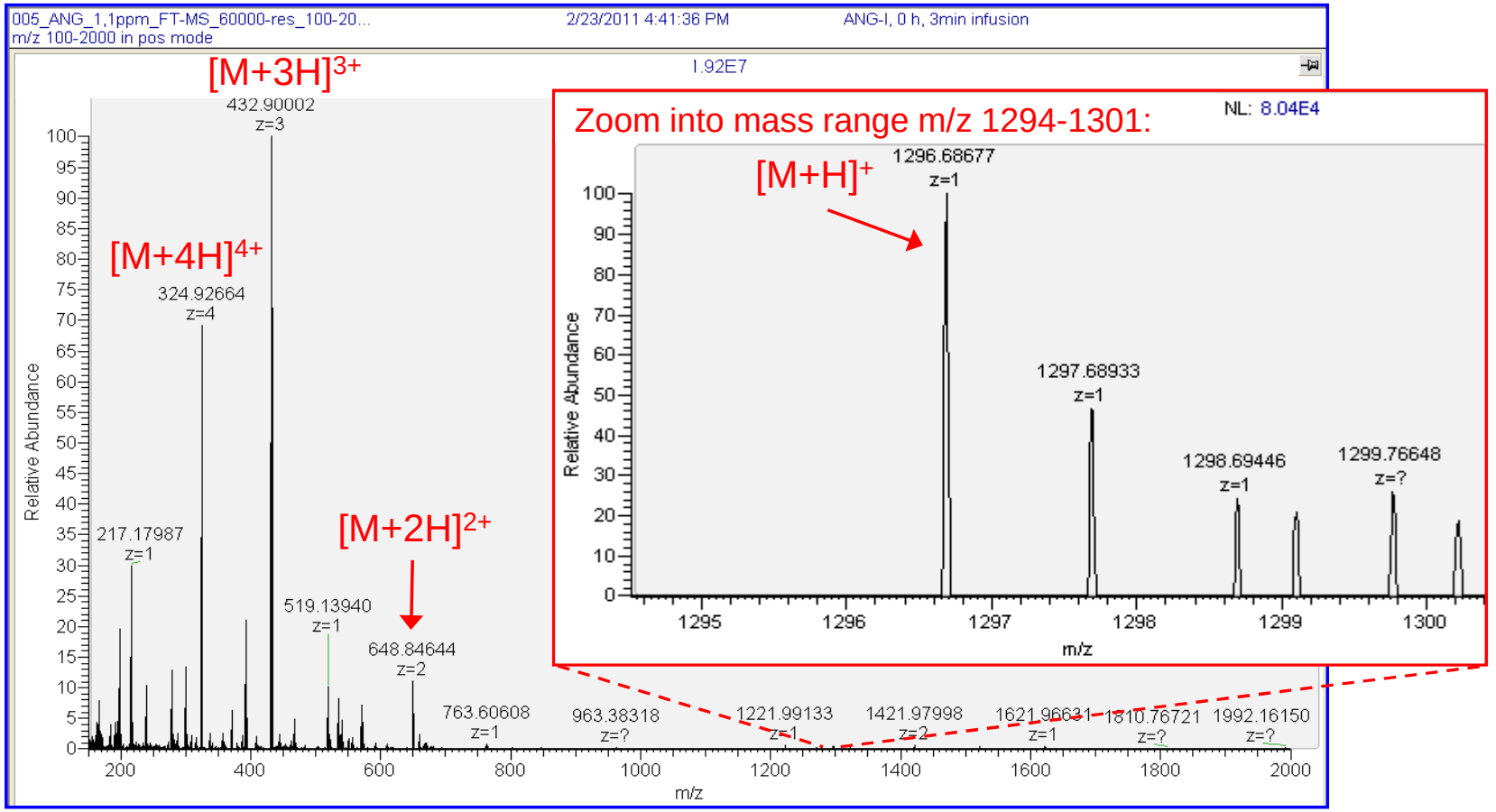
Generation of biologically active shortened Angiotensin peptides ANG II, III, IV, ...



<u>Angiotensin II</u> with a variety of biological effects:

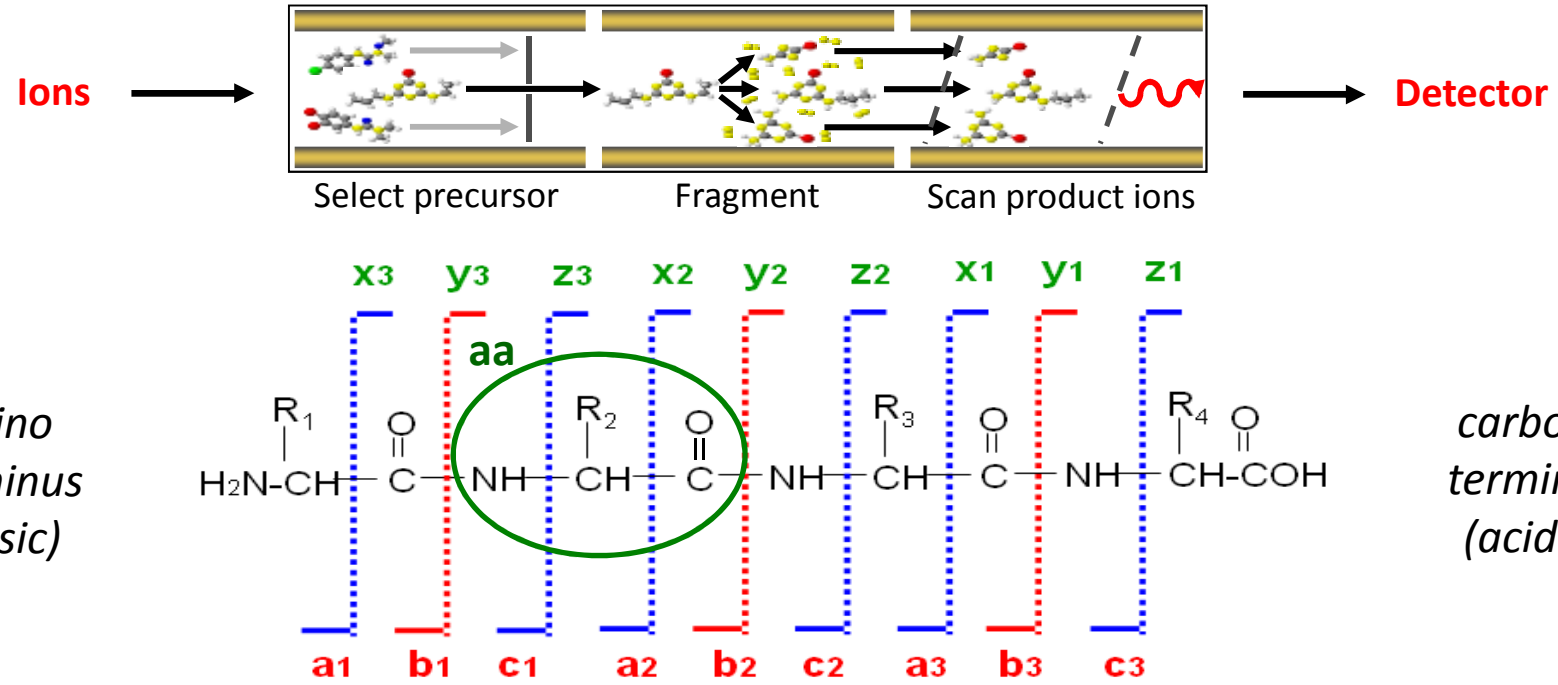
- Absorption/release of ions, retention of water & salt → Increase of circulating volume
- Vasoconstriction → Increase of the blood pressure

hrMS analysis of the peptide ANG I by LTQ-Orbitrap XL



Different ion species (charge states) of Angiotensin I are visible; fullscan MS in the high-resolution FT-MS mode.

Sequencing of a peptide using MS/MS



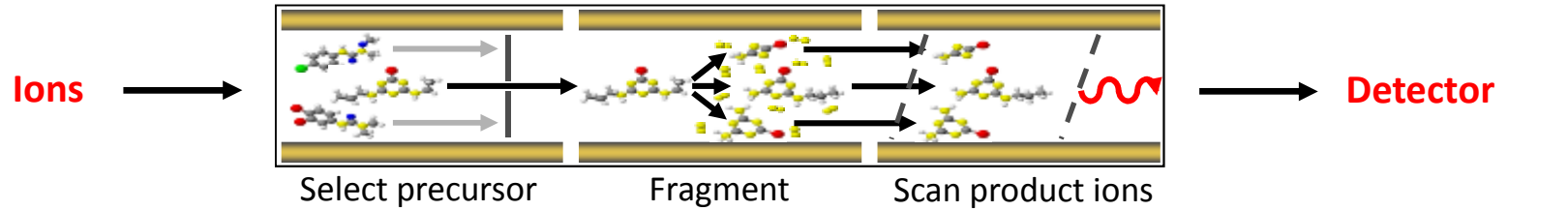
Example: H_2N - **Ala** ——— **Leu** ——— **Met** ——— **Tyr** - COOH

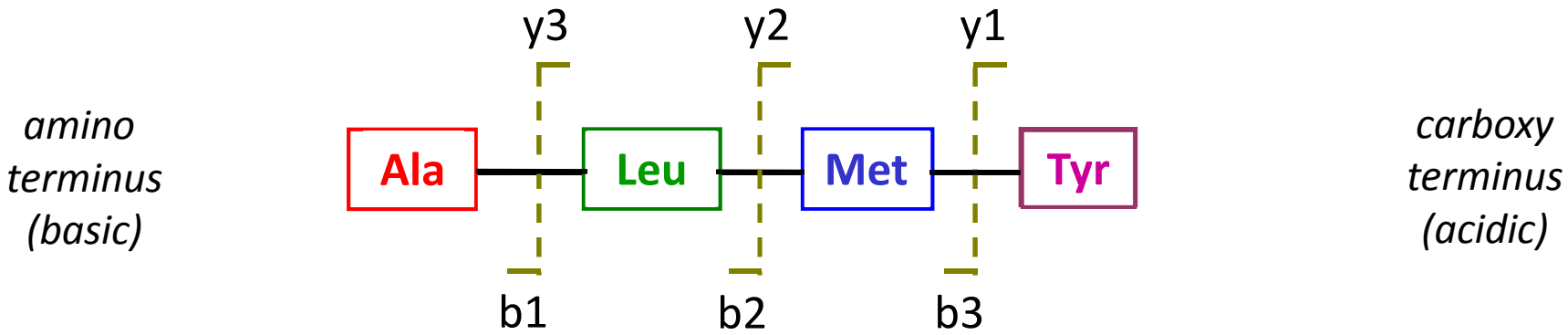
b1	H_2N – **Ala**	**Leu** – **Met** – **Tyr** – COOH	y3
b2	H_2N – **Ala** – **Leu**	**Met** – **Tyr** – COOH	y2
b3	H_2N – **Ala** – **Leu** – **Met**	**Tyr** – COOH	y1

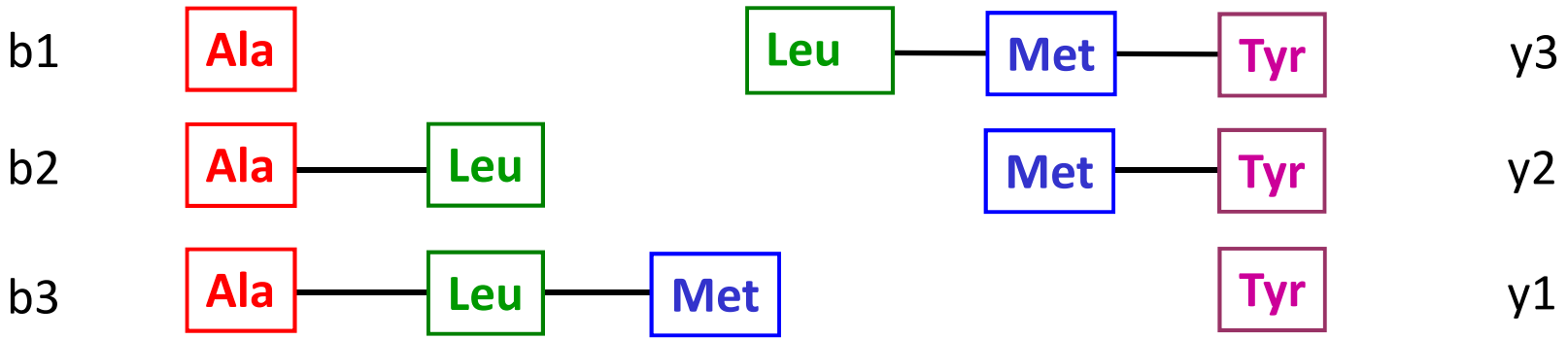
b ion series — y ion series

Roepstorff & Fohlman (1984), Biemann (1992)

Sequencing of a peptide using MS/MS

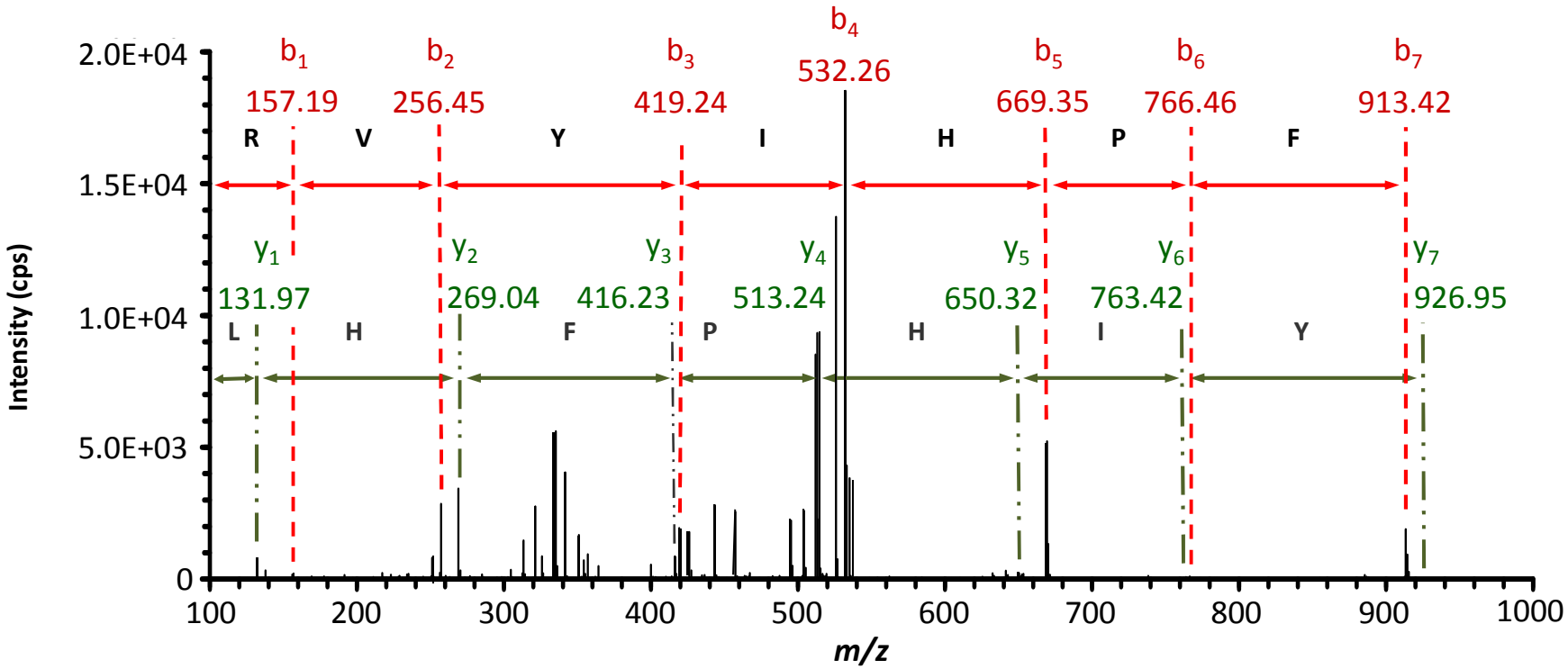






Roepstorff & Fohlman (1984), Biemann (1992)

MS/MS sequencing of ANG I peptide



Sequence deduced from MS/MS spectrum:

N-Terminus D – R – V – Y – I – H – P – F – H – L C-Terminus

Heat degradation experiments

Peptide	Alias	1 Asp	2 Arg	3 Val	4 Tyr	5 Ile	6 His	7 Pro	8 Phe	9 His	10 Leu	MW (g/mole)	Remark
ANG I	ANG (1-10)	D	R	V	Y	I	H	P	F	H	L	1295.8	√
ANG III	ANG (2-8)		R	V	Y	I	H	P	F			930.5	√
	ANG (2-7)		R	V	Y	I	H	P				783.4	√
ANG IV	ANG (3-8)			V	Y	I	H	P	F			774.4	n.d.
	ANG (2-10)		R	V	Y	I	H	P	F	H	L	1180.7	√
	ANG (3-10)			V	Y	I	H	P	F	H	L	1024.6	√
	ANG (4-10)				Y	I	H	P	F	H	L	925.5	√
	ANG (5-10)					I	H	P	F	H	L	762.4	√
	ANG (6-10)						H	P	F	H	L	649.3	√
	ANG (7-10)							P	F	H	L	512.3	√
	ANG (8-10)								F	H	L	415.2	√
	ANG (9-10)									H	L	268.2	√
	ANG (1-9)	D	R	V	Y	I	H	P	F	H		1182.6	√
ANG II	ANG (1-8)	D	R	V	Y	I	H	P	F			1045.5	√
	ANG (1-7)	D	R	V	Y	I	H	P				898.5	n.d.
	ANG (1-6)	D	R	V	Y	I	H					801.4	√
ANG V	ANG (1-5)	D	R	V	Y	I						664.4	n.d.
	ANG (1-4)	D	R	V	Y							551.3	n.d.
	ANG (1-3)	D	R	V								388.2	√
	ANG (1-2)	D	R									289.1	n.d.

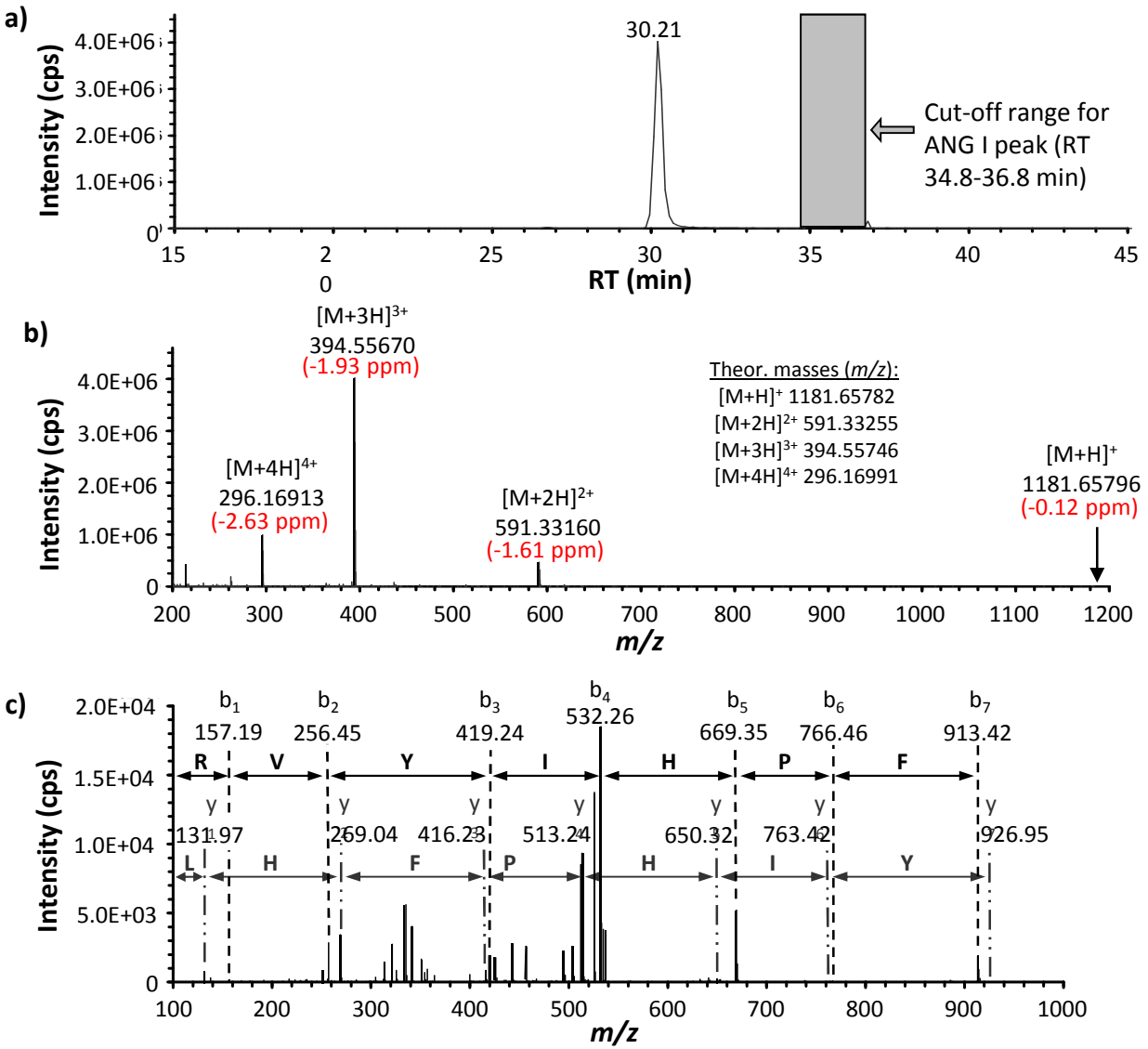
LC-hrMS/MS results for an altered ANG I material (143h at 90° C/363 K), & the potential impact on a value assignment by amino acid analysis using isoleucine, phenylalanine, proline, and valine. **Abbr.:** n.d. = not detected

Identification of an impurity in ANG I peptide material

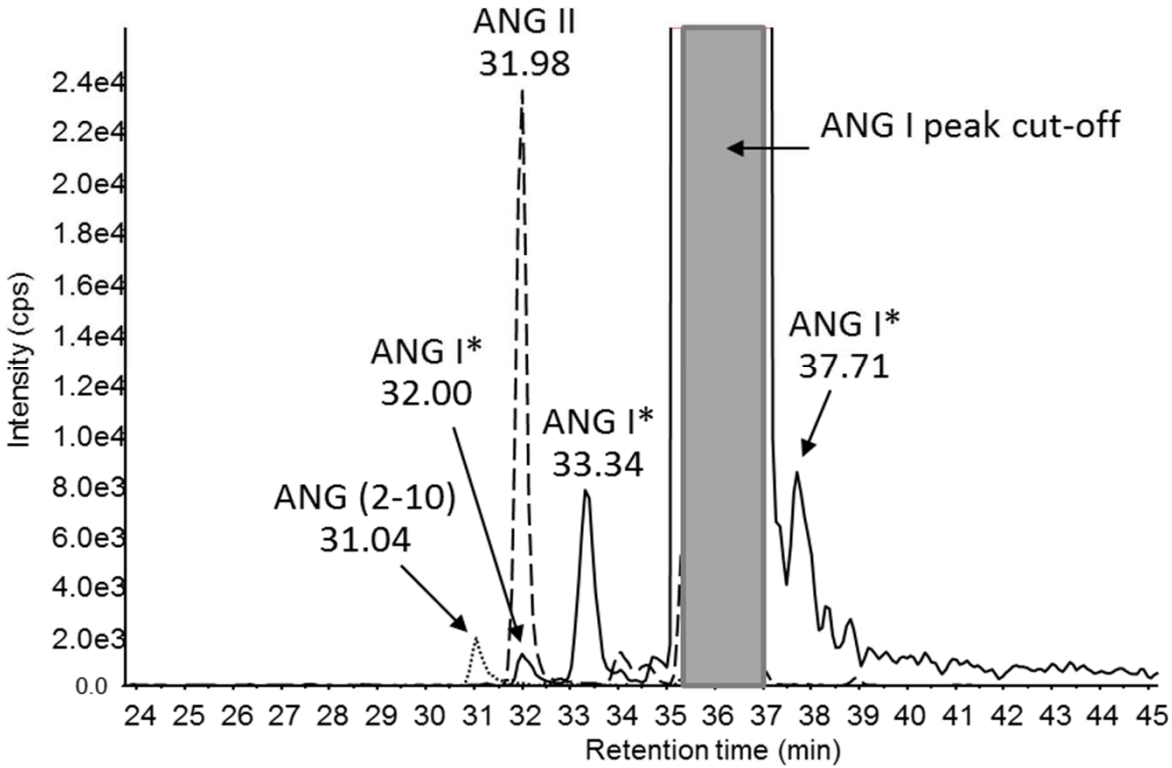
Extracted Ion Current (XIC) chromatogram of **impurity ANG (2-10)** in ANG I standard: $[M+3H]^{3+}$ ion at *m/z* 394.55746, injected at 100 µg/g

hrMS spectrum with 4 charge states of peptide ANG (2-10), under an LC peak at RT 30.21 min. Deviations (ppm) to theor. mass values are given in brackets.

MS/MS spectrum of ANG (2-10) at the same RT, showing peptide sequence RVYIHPFHL



Impurities in an ANG I material, detected by Orbitrap



Overlay of 4 extracted ion current chromatograms of diagnostic molecular ions of ANG I and an ANG I isomer (ANG I*) ($[M+3H]^{3+}$ at *m/z* 432.89977, grey line), ANG II ($[M+2H]^{2+}$ at *m/z* 523.77453, red line), ANG III ($[M+2H]^{2+}$ at *m/z* 466.26106, black line), ANG (2–10) ($[M+2H]^{2+}$ at *m/z* 591.33255, green line, obtained from replicate measurements of a candidate reference material. The mass tolerance was ± 5 ppm.

Impurities in an ANG I material, detected by Orbitrap

ANG I material isobaric impurities

ANG I	D	R	V	Y	I	H	P	F	H	L
ANG II	D	R	V	Y	I	H	P	F		
ANG (2-10)		R	V	Y	I	H	P	F	H	L
ANG I isobar	D	R	V	Y	L	H	P	F	H	I
ANG I isobar	D	R	V	Y	L	H	P	F	H	L
ANG I isobar	D	R	V	Y	I	H	P	F	H	I

Isobaric ANG I impurities - different Leu-Ile-isomers of ANG I

Material chemical formula

$[ANG\ I+3H]^{3+}\ AcO_{2.2}TFA_{0.8}.\ 4H_2O$

ANG I material is a salt and mass fraction of peptide component reported

Angiotensin I - Purity by Amino Acid Analysis

- **Peptide impurity corrected amino acid (PICAA) analysis**
  - **Microwave-assisted vapor-phase hydrolysis**
    - **AA calibrator/13C-spike blend**
    - **ANG I/13C-spike sample**
  - **Exact matching double LC-IDMS/MS**

$$W_x = W_z \cdot \frac{m_z}{m_{yc}} \cdot \frac{m_y}{m_x} \cdot \frac{R'_B}{R'_{Bc}}$$

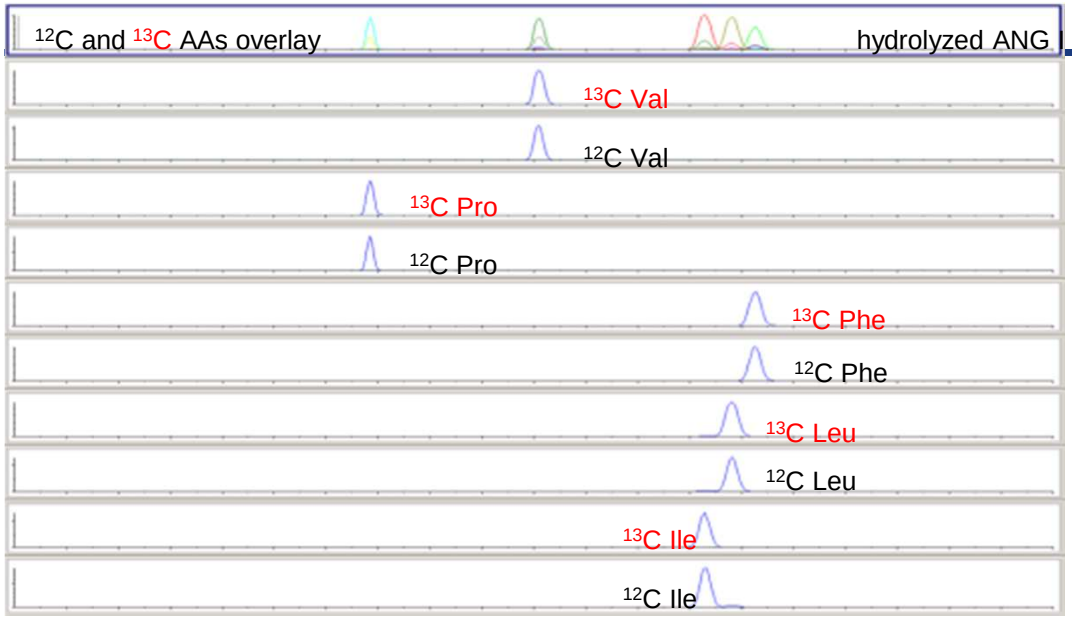
W_x: mass fraction of AA in sample
W_z: mass fraction of original AA in calibration blend
m_z: mass of original AA solution in calibration blend
m_{yc}: mass of the labelled AA solution in calibration blend
m_y: mass of the labelled AA solution in sample blend
m_x: mass of sample used
R'_B: sample ratio
R'_{Bc}: calibration ratio

  - **Correction for amino acids from impurities**

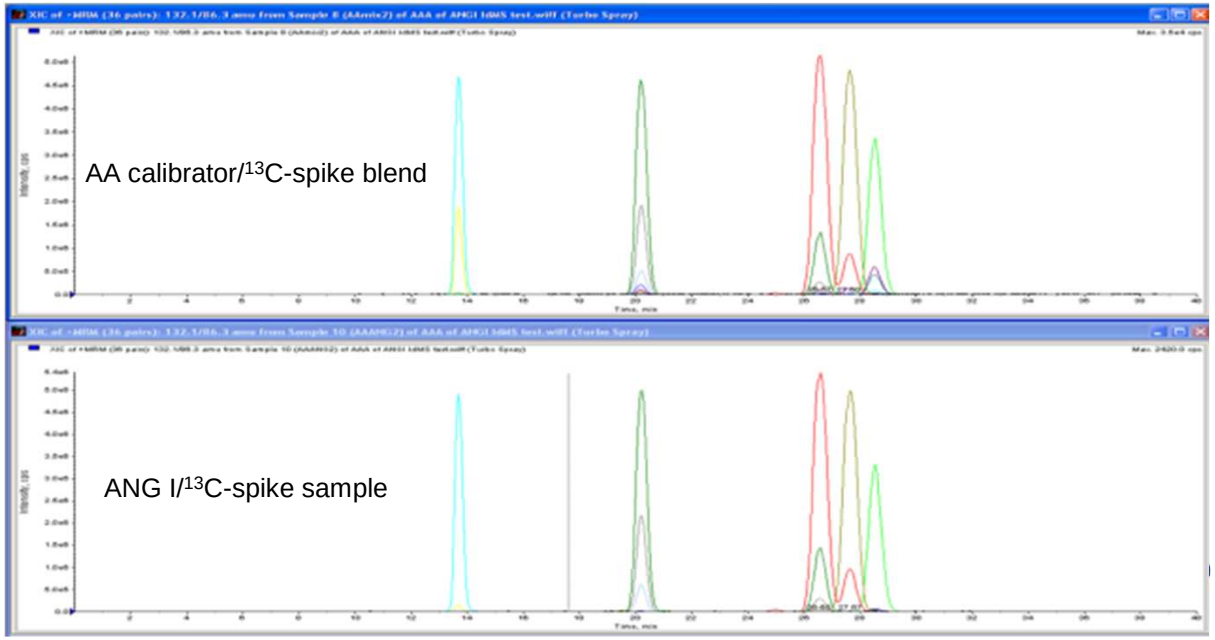
$$x_{\mathrm{P}} = \left(\frac{M_{\mathrm{r}}(\mathrm{P})}{Z_1}\right)\left[\frac{n_{\mathrm{AA}}}{m_{\mathrm{m}}} - \sum Y_{\mathrm{IMP}_i}\frac{x_{\mathrm{IMP}_i}}{M_{\mathrm{r}}(\mathrm{IMP}_i)}\right]$$

x_{P}: mass fraction of peptide in the material
M_{m}: mass of the material analyzed
$M_{\mathrm{r}}(\mathrm{P})$: relative molecular mass of peptide
Z_1: number of molecules of the amino acid of interest per peptide molecule
n_{AA}: amount of substance of the amino acid of interest measured in the material
Y_i : number of molecules of the amino acid of interest per peptide impurity molecule (IMP_i)
$x_{\mathrm{IMP}i}$: mass fraction of the peptide impurity IMP_i
$M_r(\mathrm{IMP}_i)$: relative molecular mass of the peptide impurity IMP_i

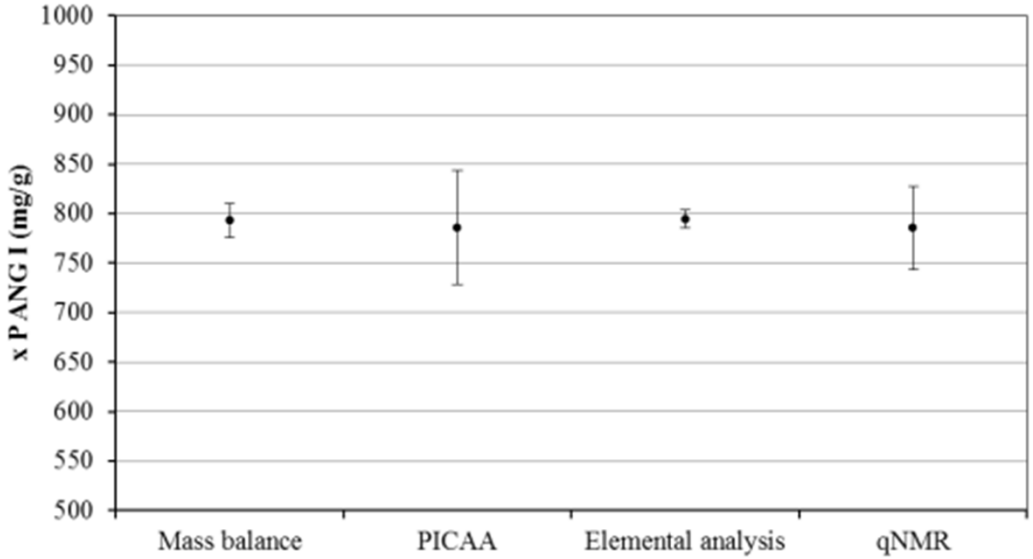
Impurities in an ANG I material, detected by Orbitrap



Exact matching double LC-IDMS of hydrolyzed ANG I



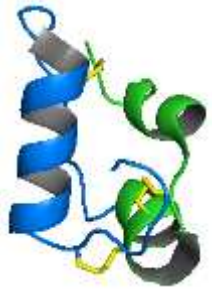
Angiotensin I - Purity by Amino Acid Analysis



	x P ANG I (mg/g)	U-(x P ANG I) (mg/g)	U+(x P ANG I) (mg/g)
Mass balance	793	17	17
PICAA	785	58	58
Elemental analysis	794	9	9
qNMR	786	42	42

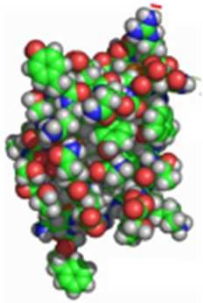
Larger peptides/ small proteins: Human Insulin (hINS)

- A hormone, produced in the pancreas, with effects on cells in the muscle, fat and liver tissue: glucose uptake from blood & storage as glycogen in liver & muscle.
- Insulin inhibits glucagon release => fat is no longer used as energy source



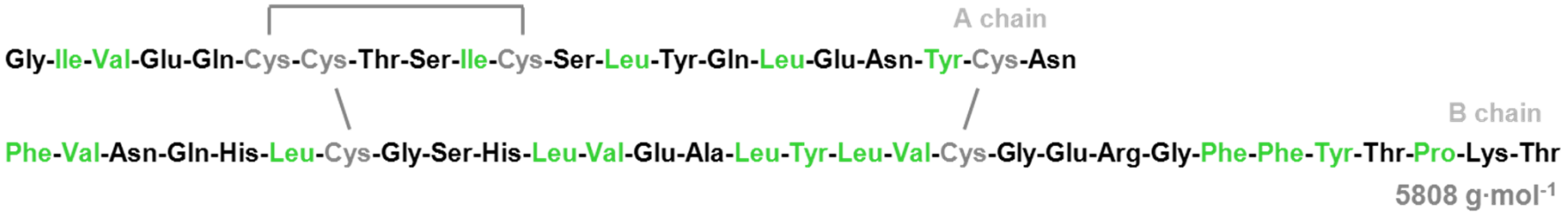
Metabolic disorders:

- Lack of insulin production of the body => **Diabetes mellitus** (Type 1 D.)
- No use of insulin by body cells (resistance) => **Type 2 Diabetes**



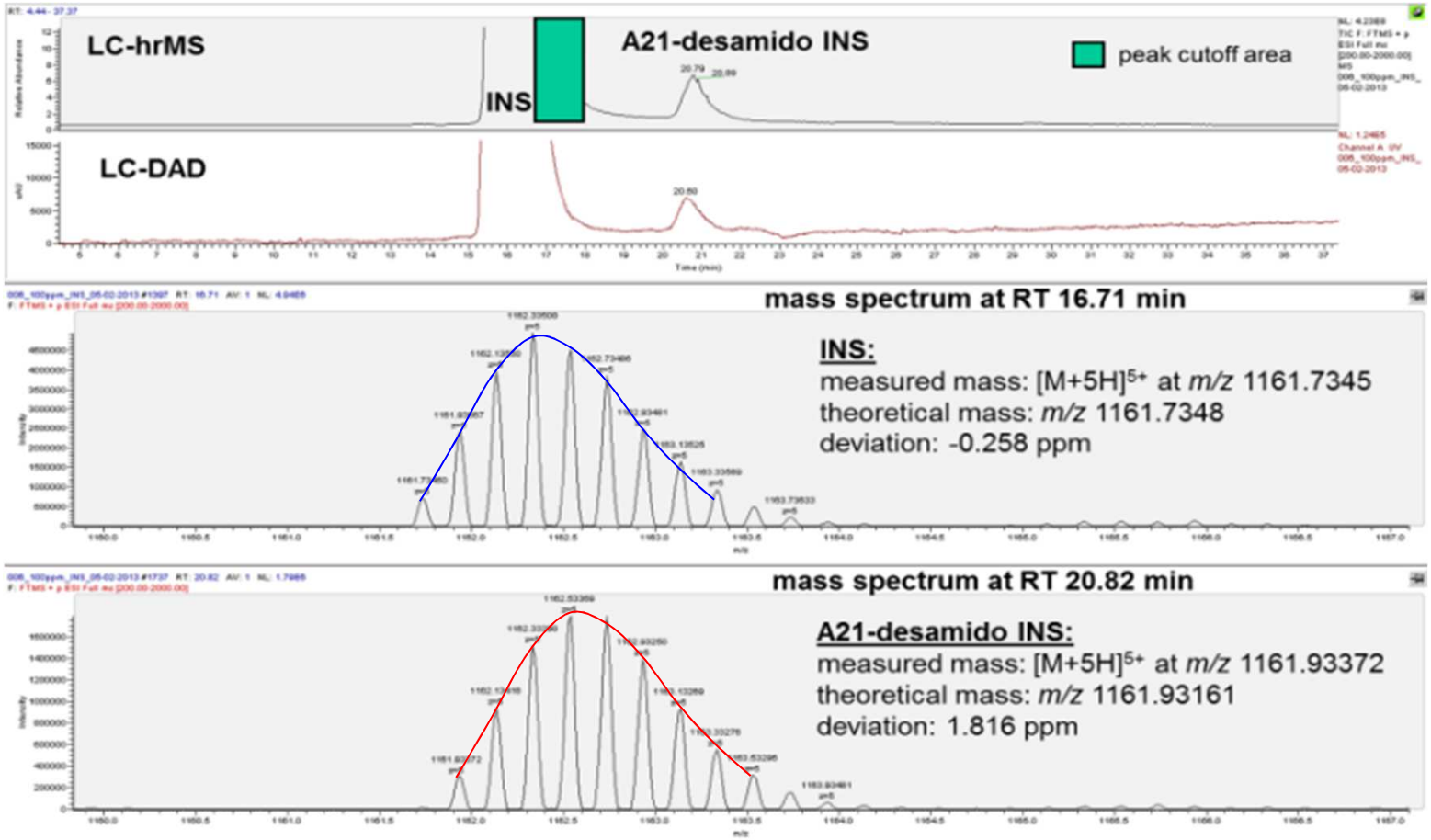
1 molecule rhINS shown in 2 different visualisation modes

Structure: Small protein of MW = 5807.57 g/mol, consists of A chain (21 amino acids) & B chain (30 AA), cross-linked by 2 inter- & 1 intramolecular disulfide bonds



Study material: Recombinant human Insulin

LC-hrMS and LC-UV analysis of INS and its impurity

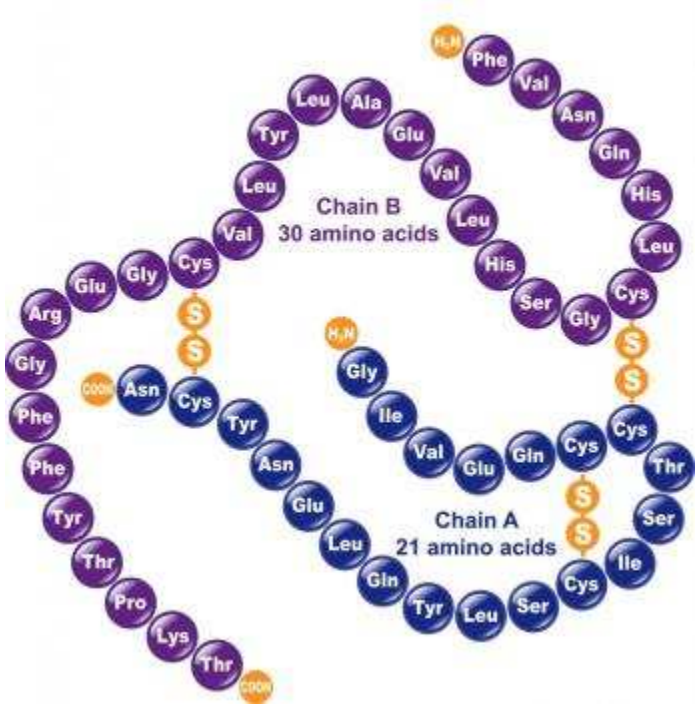


Measured masses of $[M+5H]^{5+}$ ions correspond to monoisotopic masses for neutral …
INS: 5803.63612 Da, **A21-desamido INS: 5804.63222 Da**, mass difference: 0.9961 Da

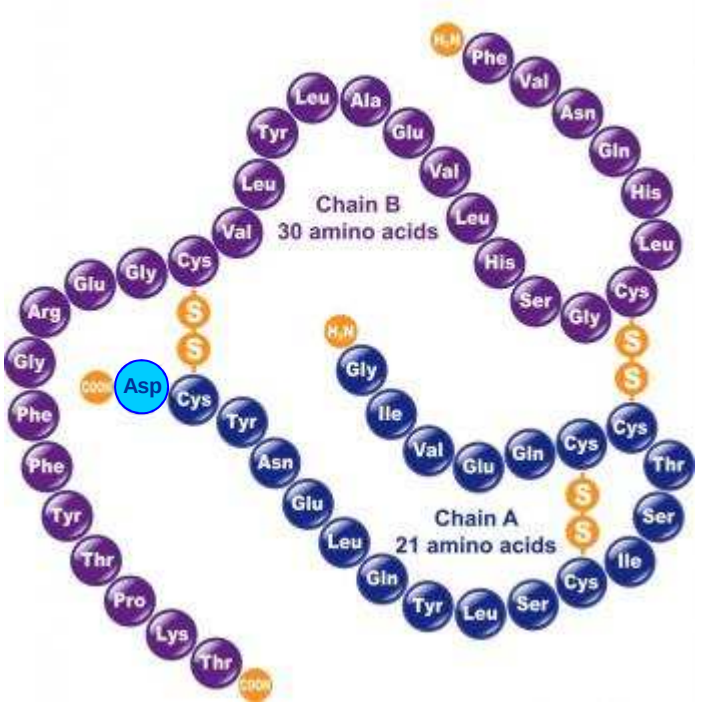
A21-desamido hINS: Amino acid #21 on the A chain is changed from Asn to Asp by deamidation (replacement of –CO-NH_2 by –COOH in the side chain), resulting in a theoretical mass change of +0.984 Da.

Chemical structures of INS and A21-desamido-INS

huINS

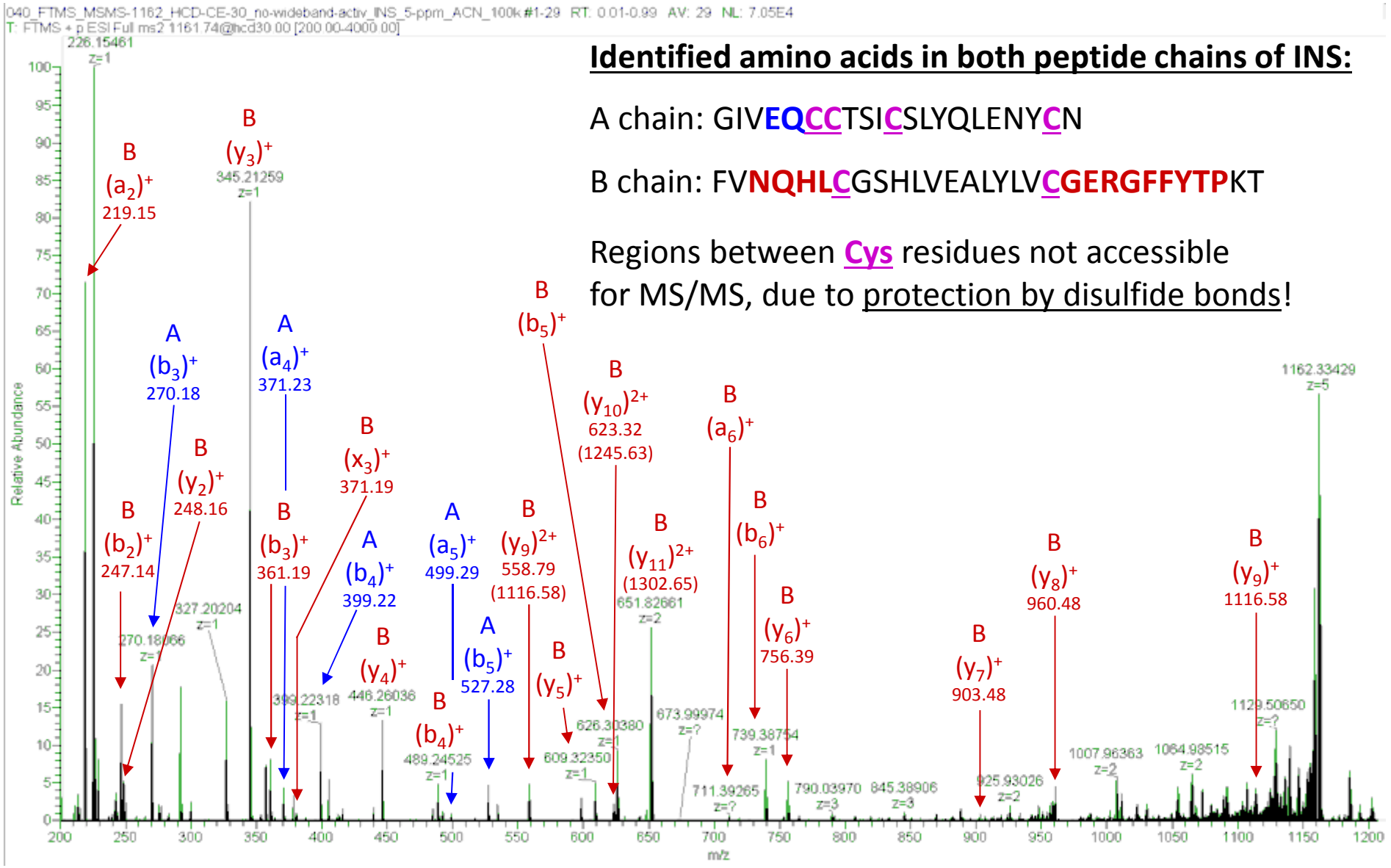


A21-desamido-INS



Amino acid Asn at position 21 on the A chain has been converted to Asp by deamidation.

Structural elucidation of hINS:
1) MS/MS sequencing of the native protein



Identified amino acids in both peptide chains of INS:

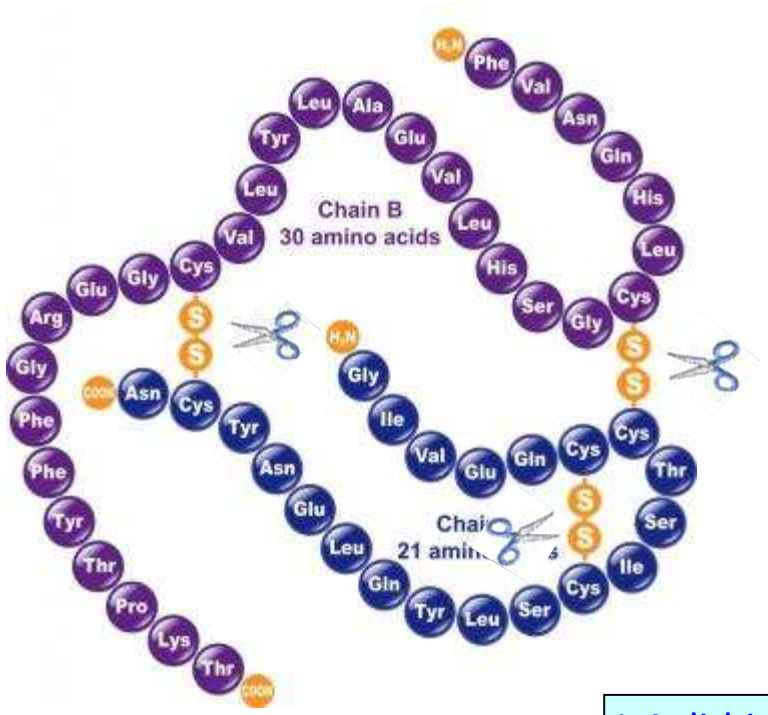
A chain: GIV**EQ**C**C**TSI**C**SLYQLENY**C**N

B chain: FV**NQHL**C**C**GSHLVEALYLV**C**G**ERGFFYTP**KT

Regions between **Cys** residues not accessible for MS/MS, due to protection by disulfide bonds!

Structural elucidation of hINS: 2) Reduction of disulfide bonds

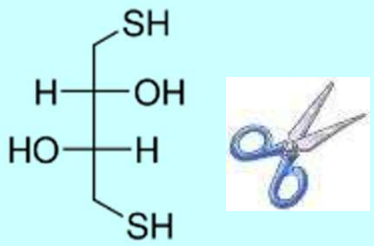
Intact (native) human Insulin



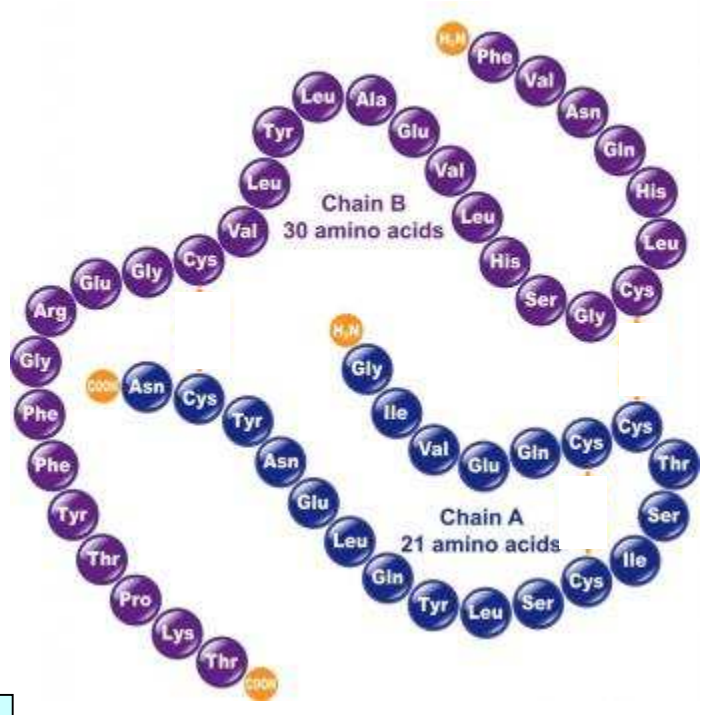
$C_{257}\ H_{383}\ N_{65}\ O_{77}\ S_6$

Theor. monoisotopic mass:
5803.63763 Da

1,4-dithiothreitol (DTT)



2 chains

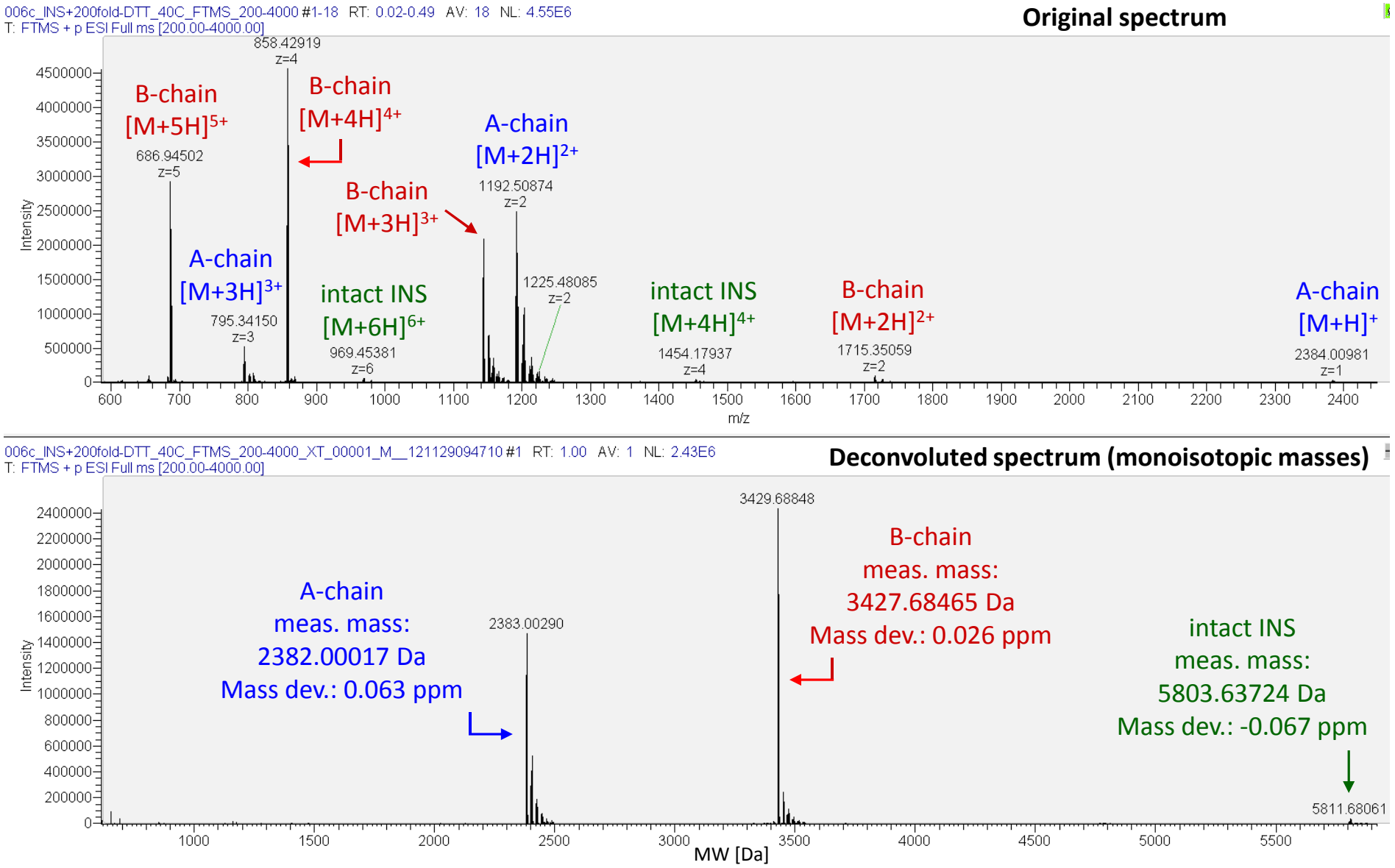


A chain: $C_{99}\ H_{155}\ N_{25}\ O_{35}\ S_4$
2382.00002 Da

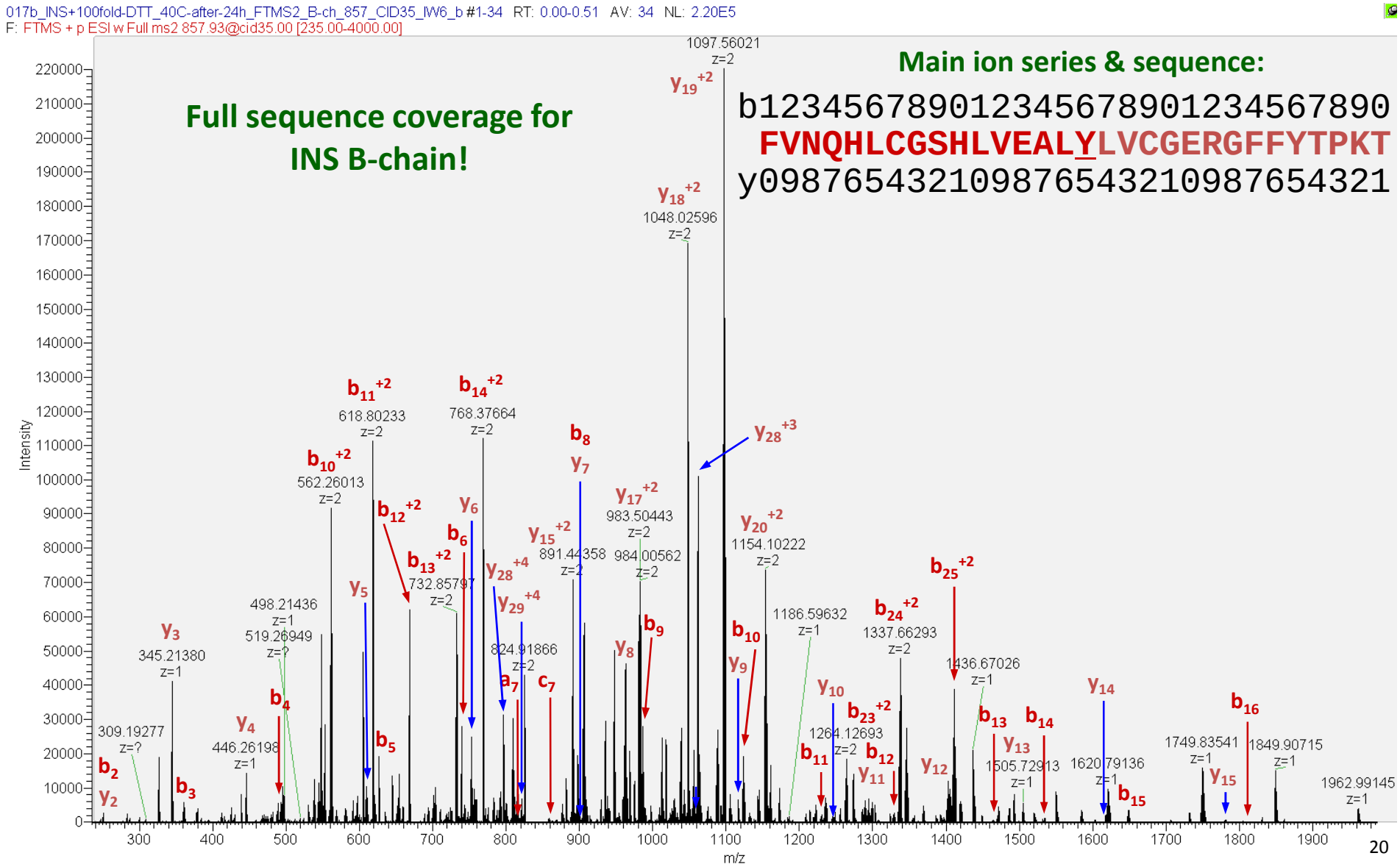
B chain: $C_{158}\ H_{234}\ N_{40}\ O_{42}\ S_2$
3427.68456 Da

Purpose: To facilitate full analysis of amino acid sequence by MS/MS

Structural elucidation of hINS:
3) Analysis of biochemically denatured protein (I)

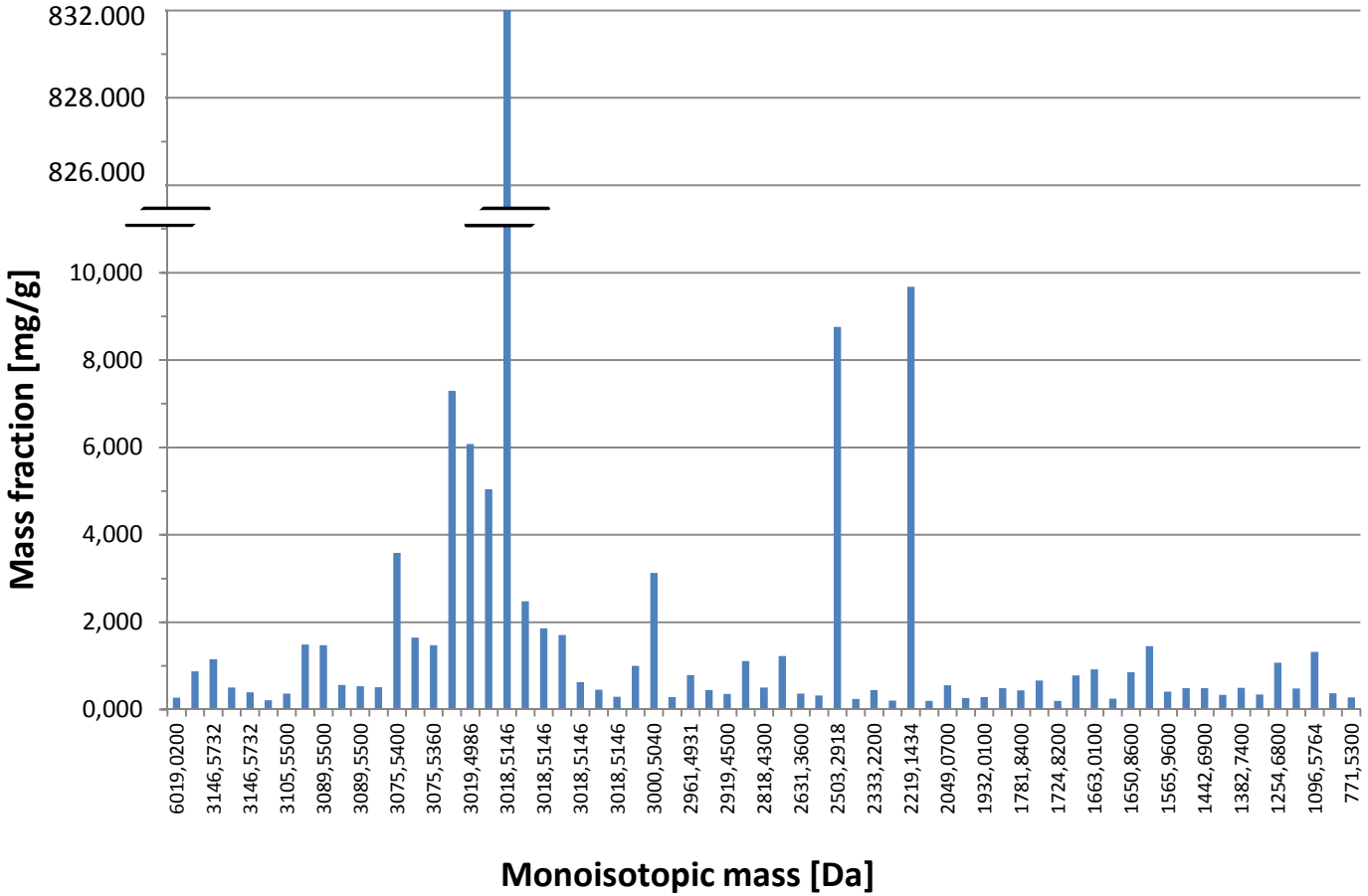


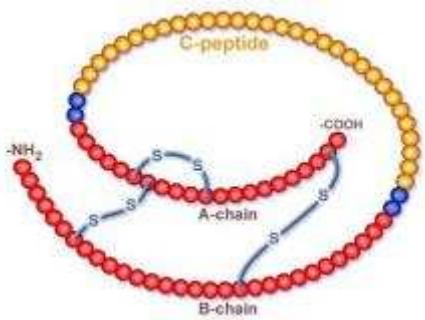
Structural elucidation (MS/MS sequencing) of hINS: 3) Analysis of biochemically denatured protein (II)



LC-hrMS/MS analysis of human C-peptide (hCP)

Mass fractions of structurally related impurities in a hCP material (mass balance approach)





Most abundant [mg/g]:

hCP: 831.32

acetyl-hCP(9-31): 9.67

acetyl-hCP(6-31): 8.76

dea9hCP: 7.30

dea6hCP: 6.08

dea22hCP: 5.04

Chemical Synthesis – over 70 impurities identified and quantified

Questions you will be able to answer after this lecture:

1) How can Traceability be applied to Chemical Measurement?
2) What methods can be used to quantify Chemical Purity?
3) What instrumentation is required for a Mass Balance Approach?
4) What relative uncertainty is achievable with Mass Balance Methods?
5) What are common sources of bias for Mass Balance Methods?
6) How can NMR be used to quantify Chemical purity?
7) What factors limit the performance of NMR for purity measurement?
8) How can you measure the purity of a peptide?
9) How can you identify a peptide from its high-res mass spectrum?
10) How can Amino Acid analysis be used for peptide purity?