Chemical derivatization

Derivatization in liquid chromatography and mass spectrometry

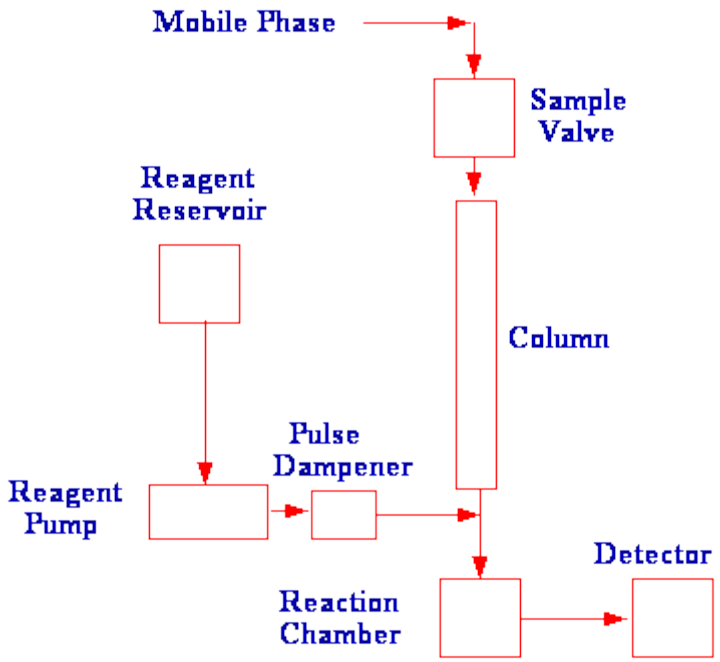
- Aims:
    - increase analyte stability
    - increase solubility
    - improve chromatographic properties
    - increase detection sensitivity
    - increase selectivity
    - reduce matrix effect
    - allow chiral separation in achiral systems

What we expect from the derivatization reaction:

- completion in a reasonable time frame (preferably: fast)
- should not require highly special technologies
- should yield a single product of each analyte
- should be reproducible if not quantitative
- robustness with respect to the matrix composition
- should be non-toxic
- feasible in aqueous medium

Derivatization in liquid chromatography and mass spectrometry

- pre-column derivatization: part of the preanalytical workup
- post-column derivatization: part of the analytical assay



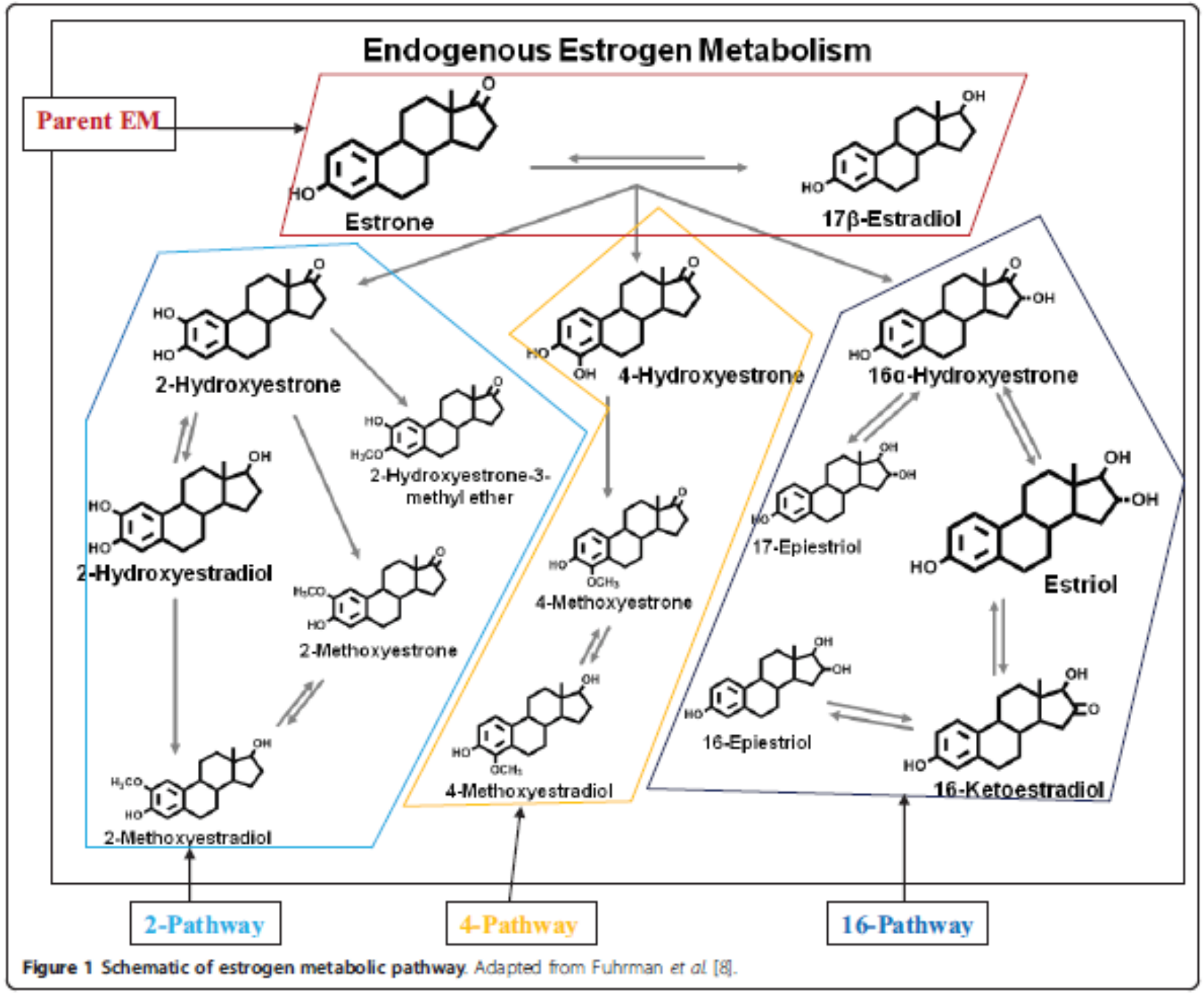
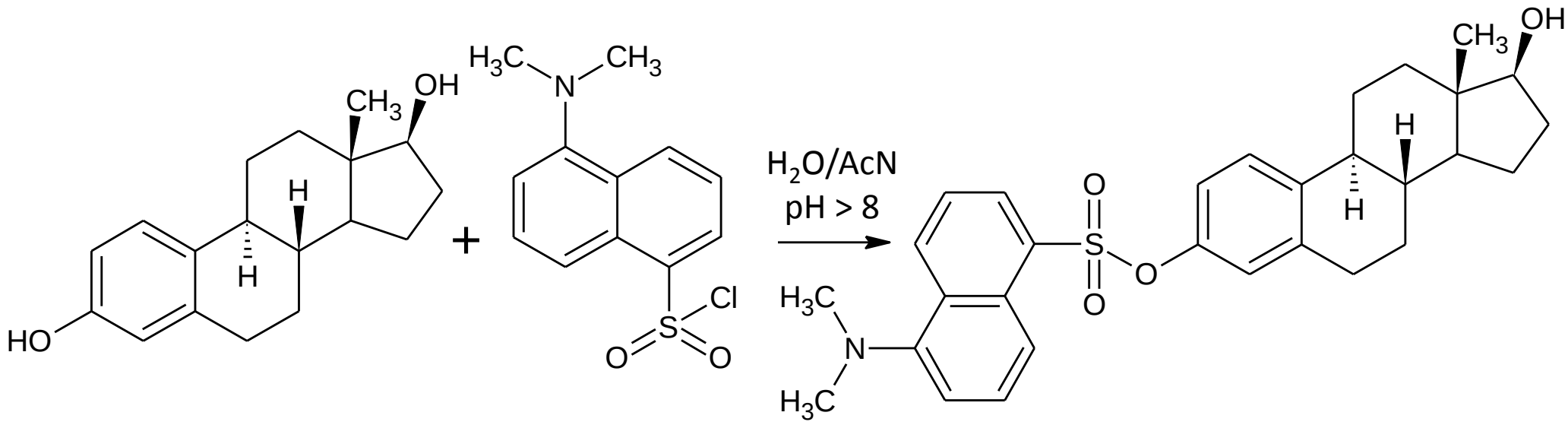
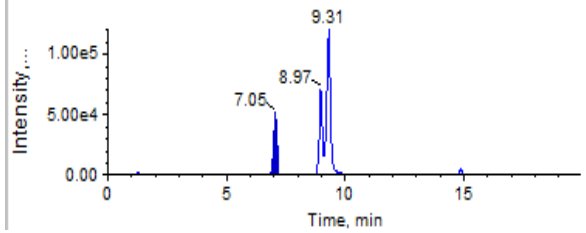


Figure 1 Schematic of estrogen metabolic pathway. Adapted from Fuhrman *et al.* [8].

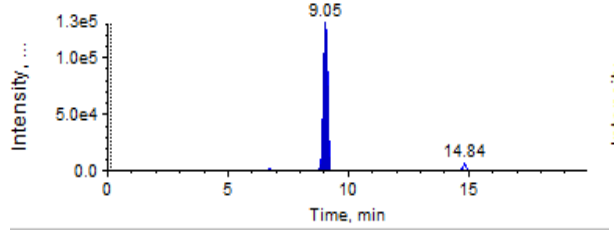


estradiol dansyl chloride

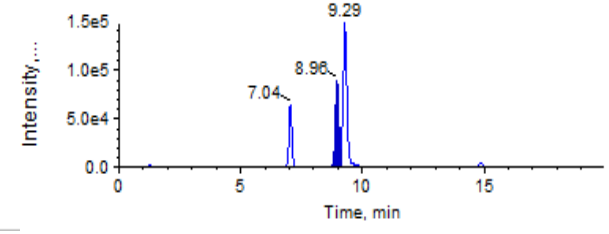
Emix-2 - estriol (Unknown) 522.000/171.000 Da - sample 10 of 12 from E..
Area: 4.47e+005 counts Height: 5.14e+004 cps RT: 7.05 min



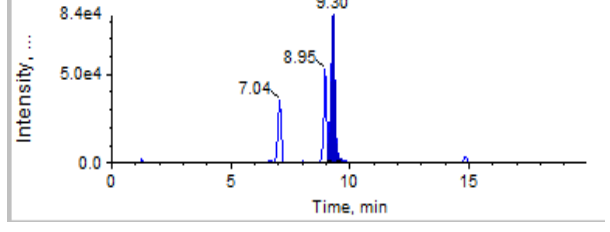
Emix-2 - 16OHE1 (Unknown) 520.500/171.000 Da - sample 10 of 1...
Area: 1.44e+006 counts Height: 1.31e+005 cps RT: 9.05 min



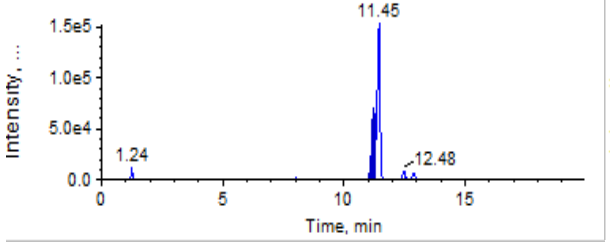
Emix-2 - 16epiE3 (Unknown) 522.100/171.100 Da - sample 10 of 12 from
Area: 8.28e+005 counts Height: 8.90e+004 cps RT: 8.96 min



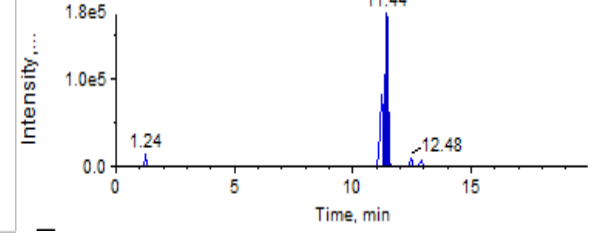
Emix-2 - 17epiE3 (Unknown) 521.900/170.900 Da - sample 10 of 12 from
Area: 8.13e+005 counts Height: 8.26e+004 cps RT: 9.30 min



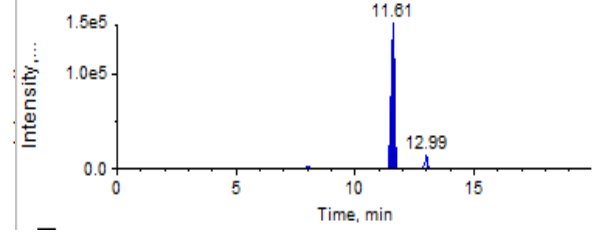
Emix-2 - 2OMeE2 (Unknown) 536.500/171.000 Da - sample 10 of 12 from
Area: 6.43e+005 counts Height: 7.05e+004 cps RT: 11.2 min



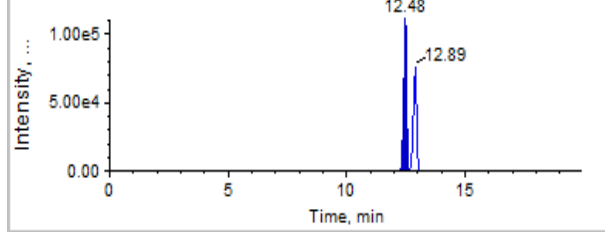
Emix-2 - 4OMeE2 (Unknown) 536.400/171.100 Da - sample 10 of 12 from.
Area: 1.51e+006 counts Height: 1.75e+005 cps RT: 11.4 min



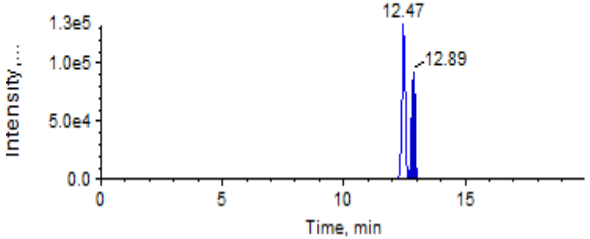
Emix-2 - estradiol (Unknown) 506.500/171.000 Da - sample 10 of 12 from
Area: 1.32e+006 counts Height: 1.52e+005 cps RT: 11.6 min



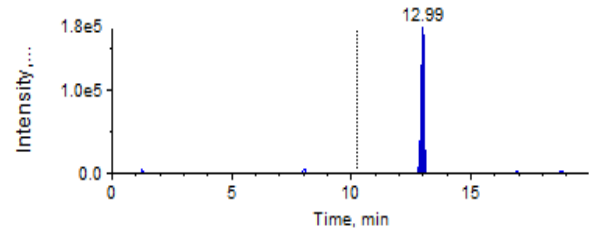
Emix-2 - 2OMeE1 (Unknown) 534.500/171.000 Da - sample 10 of 12 from.
Area: 8.99e+005 counts Height: 1.11e+005 cps RT: 12.5 min



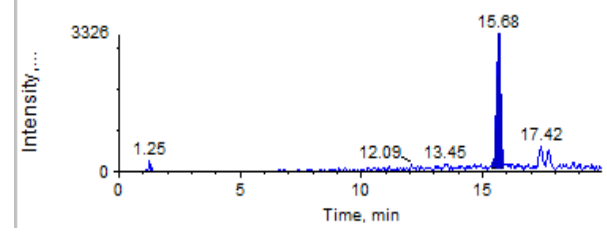
Emix-2 - 4OMeE1 (Unknown) 534.400/171.100 Da - sample 10 of 12 from
Area: 7.85e+005 counts Height: 9.21e+004 cps RT: 12.9 min



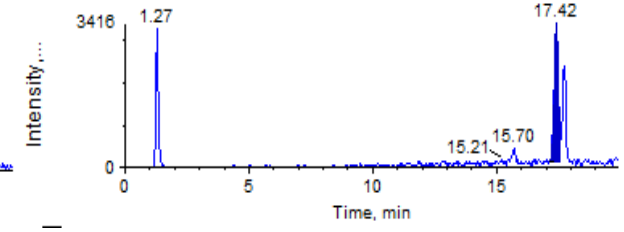
Emix-2 - estrone (Unknown) 504.500/171.000 Da - sample 10 of 12 from ..
Area: 1.45e+006 counts Height: 1.76e+005 cps RT: 13.0 min



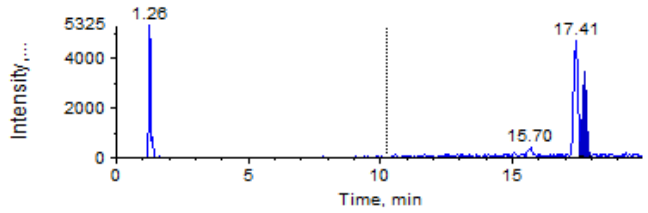
Emix-2 - 2OHE2 (Unknown) 756.000/171.000 Da - sample 10 of 12 from
Area: 3.45e+004 counts Height: 3.23e+003 cps RT: 15.7 min



Emix-2 - 2OHE1 (Unknown) 754.000/171.000 Da - sample 10 of 12 from E
Area: 3.42e+004 counts Height: 3.26e+003 cps RT: 17.4 min



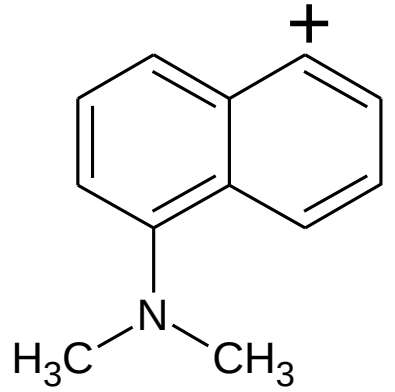
Emix-2 - 4OHE1 (Unknown) 754.100/171.100 Da - sample 10 of 12 from ..
Area: 3.41e+004 counts Height: 3.35e+003 cps RT: 17.7 min

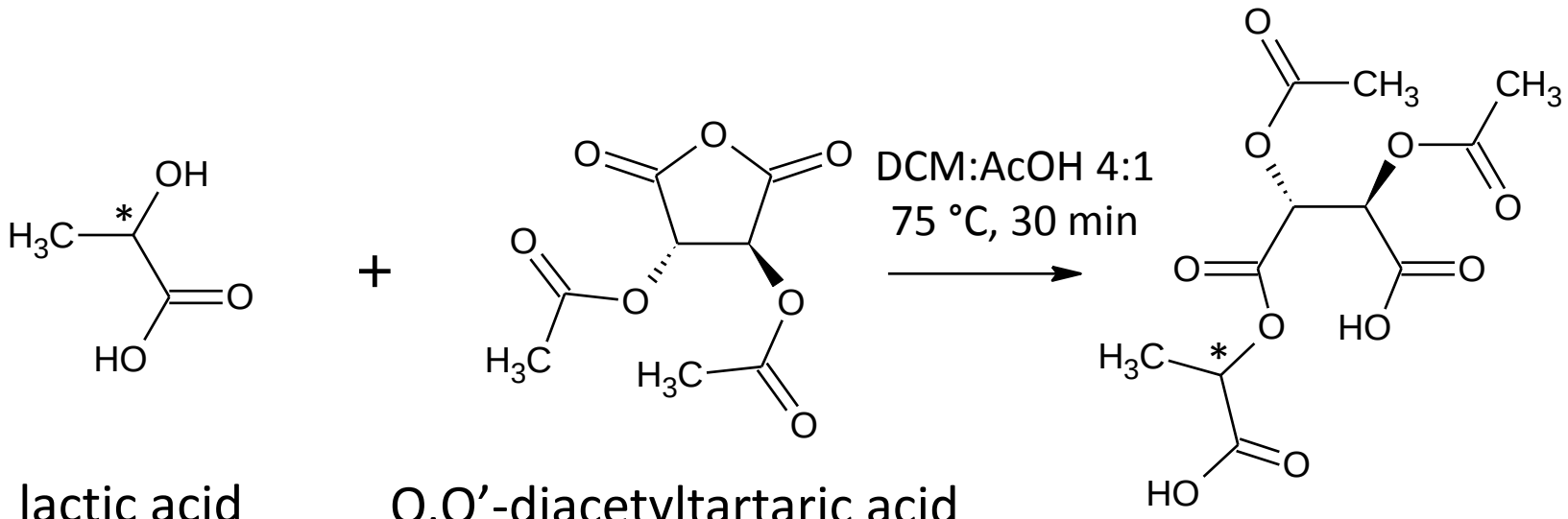


Method in plasma:

- sample preparation:
  - 500 µL plasma + 10 µL internal standard solution + 500 µL water
  - add 2 mL EtOAc, vortex 2 min, centrifuge, repeat process, combine organic phases
  - evaporate to dryness
  - add 100 µL 1 mg/mL dansyl chloride in AcN + 25 µL 0.1 M NH_4CO_3, keep at 45 °C / 15 min
  - add 20 µL 0.5 M HCl, vortex
  - add 50 µL AcN + 105 µL water

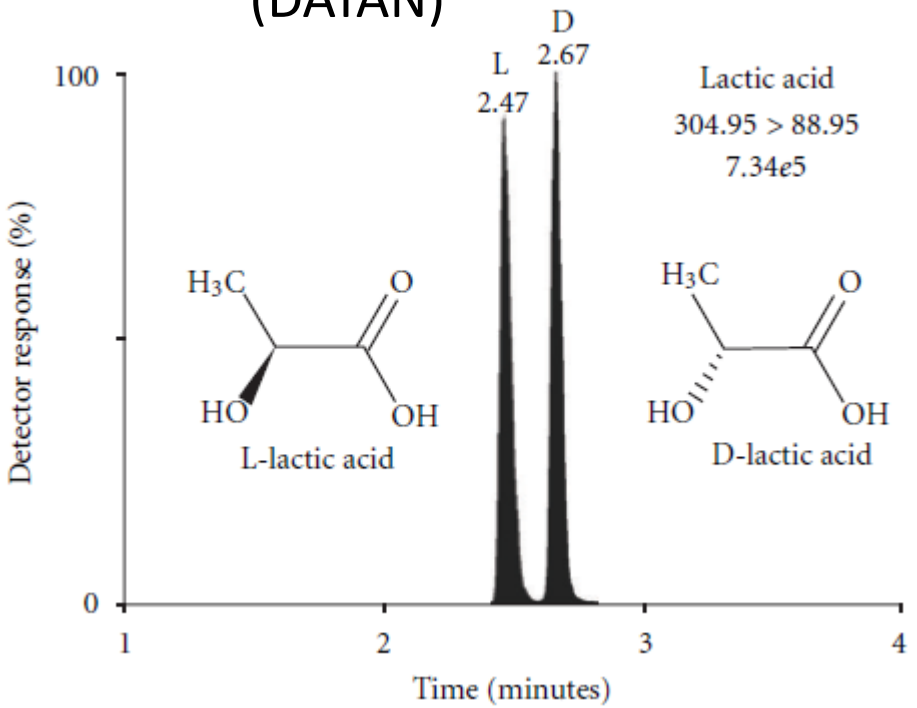
- analysis: LC-ESI(+)-MS/MS, run time: 17 min
  - SP: C18 50x2.1 mm, 1.7 µm + biphenyl 50x2.1 mm, 1.7 µm
  - MP: water (A), acetonitrile (B), both containing 0.1% formic acid
  - sample volume: 10 µL, 15 °C
  - FR: 0.2 mL/min
  - CTO: 35 °C
  - MS mode: MRM





lactic acid

O,O’-diacetyltartaric acid (DATAN)

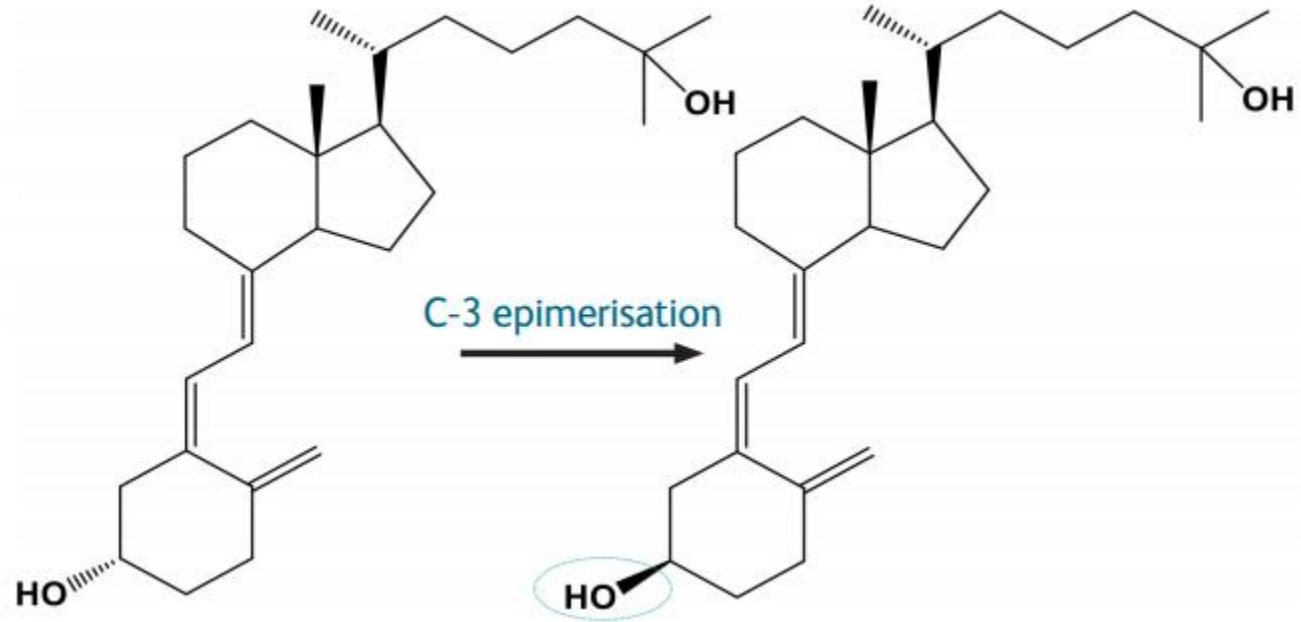


Scheijen JLJM et al. Exp Diabet Res 2012, ID234812.

Method in plasma:

- sample preparation:
  - 25 µL plasma + 25 µL internal standard solution
  - add 600 µL methanol:acetonitrile 1:1, vortex, centrifuge
  - evaporate to dryness
  - add 50 µL 50 mg/mL DATAN in DCM: AcOH 4:1, keep at 75 °C / 30 min
  - evaporate to dryness
  - reconstitute in 150 µL water:AcN 2:1.

- analysis: LC-ESI(-)-MS/MS, run time: 6 min
  - SP: C18 100x2.1 mm, 1.7 µm
  - MP: water/0.0015 M NH_4OOC, pH=3.6 (A), acetonitrile (B)
    - gradient: 0.5% B, 3 min – 3% B, 3.01 min – 40% B, 5 min – 0.5% B
  - sample volume: 2 µL, 6 °C
  - FR: 0.5 mL/min
  - CTO: 31 °C
  - MS mode: MRM



25-hydroxyvitamin D3

3-epi-25-hydroxyvitamin D3

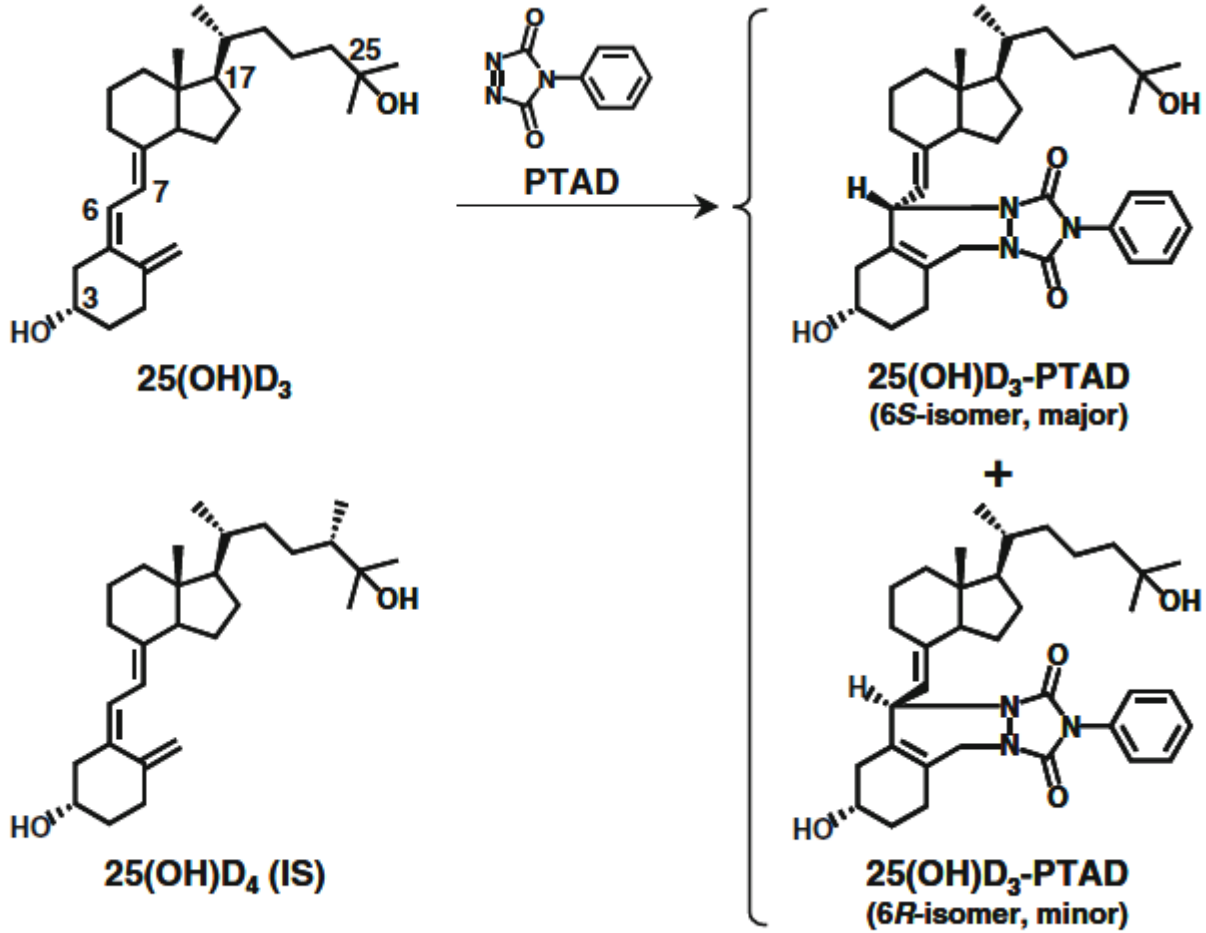
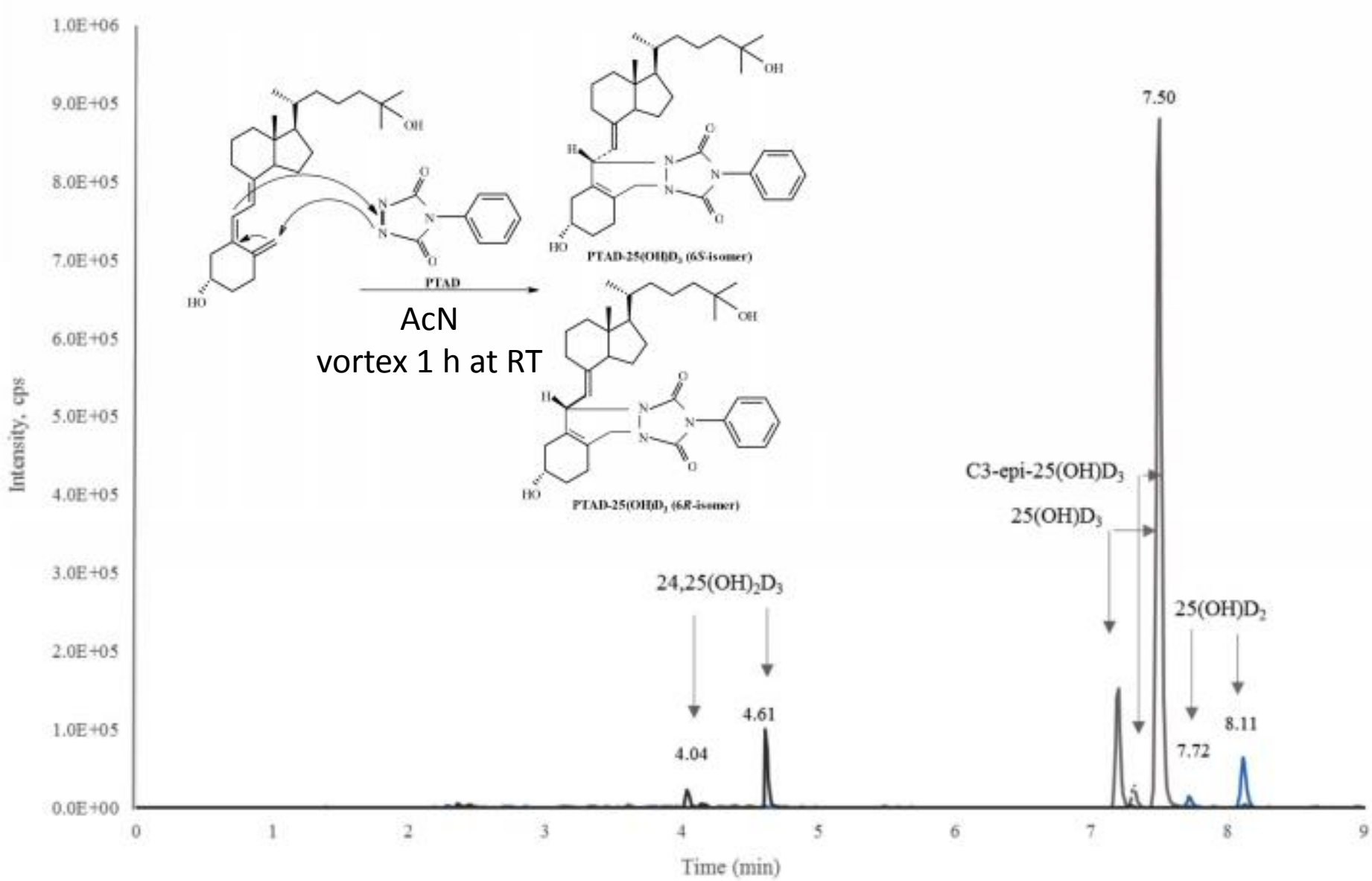


Fig. 1 Derivatization of 25(OH)D_3 with PTAD and the chemical structure of 25(OH)D_4 (IS)

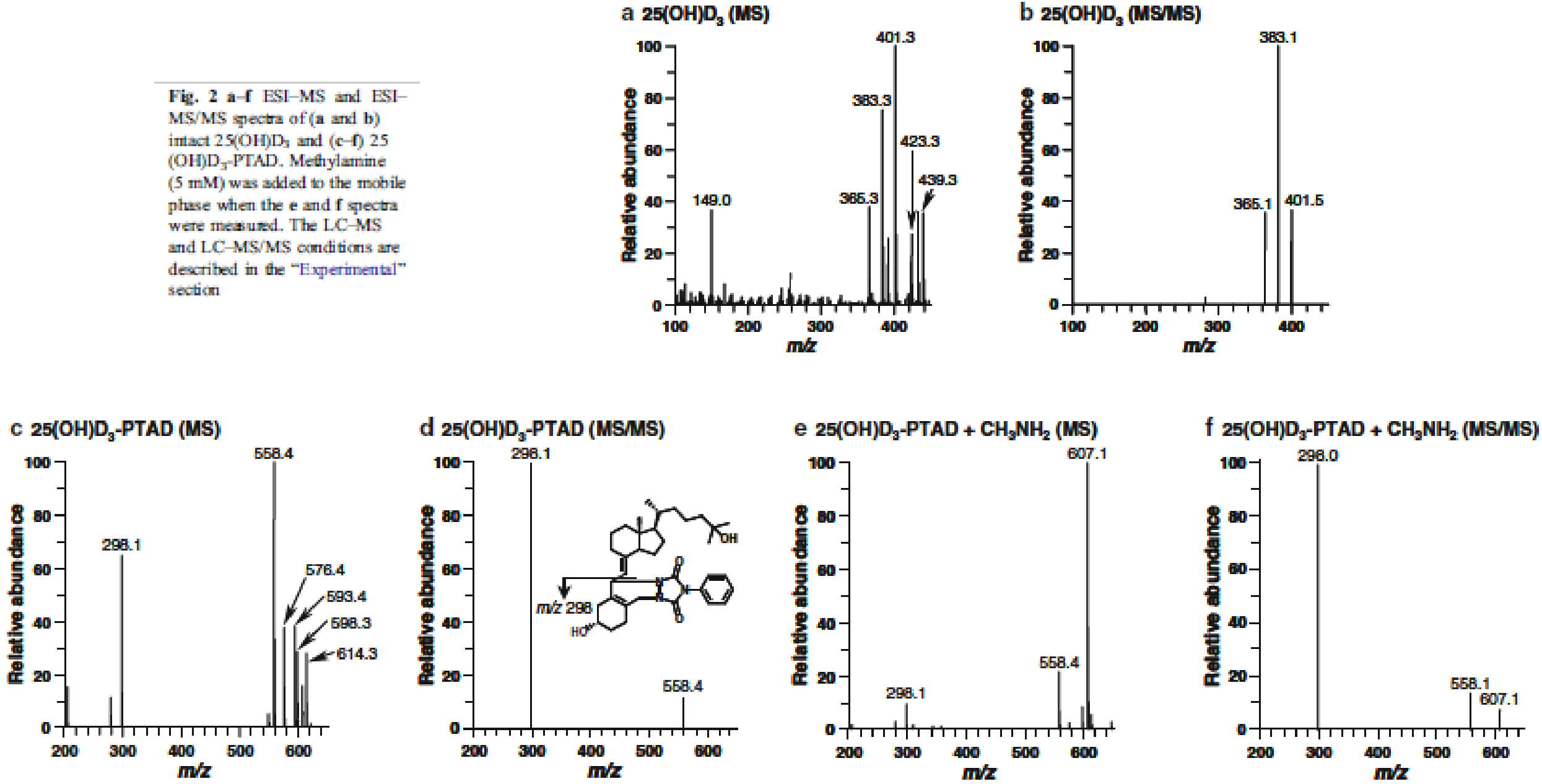
PTAD: 4-phenyl-1,2,4-triazoline-3,5-dione

Higashi T et al. Anal Bioanal Chem 2008;391:229.



PTAD: 4-phenyl-1,2,4-triazoline-3,5-dione

Fig. 2 a–f ESI–MS and ESI–MS/MS spectra of (a and b) intact 25(OH)D_3 and (e–f) 25(OH)D_3-PTAD. Methylamine (5 mM) was added to the mobile phase when the e and f spectra were measured. The LC–MS and LC–MS/MS conditions are described in the "Experimental" section



Higashi T et al. Anal Bioanal Chem 2008;391:229.

Method in saliva:

- sample preparation:
  - 1.0 mL saliva + 20 pg internal standard solution
  - add 2.0 mL acetonitrile vortex, centrifuge
  - add 3.0 mL water to supernatant, apply to Strata-X → wash: 2 mL water, 2 mL water:MeOH 3:7. Elute: 1 mL EtOAc.
  - add 25 µL 0.1 mg/mL PTAD in EtOAc, keep at RT / 30 min, add 25 µL PTAD/EtOAc, keep at RT / 30 min.
  - add 40 µL EtOH, evaporate to dryness
  - reconstitute in 30 µL MP.

- analysis: LC-ESI(+)-MS/MS, run time: 6.5 min
  - SP: C18 150x2 mm, 5 µm
  - MP: methanol/0.01 M NH_4OOC (A)
  - sample volume: 10 µL
  - FR: 0.2 mL/min
  - CTO: 40 °C
  - MS mode: MRM

Review

DOI: 10.5582/ddt.2013.v7.1.9

Derivatization in liquid chromatography for mass spectrometric detection

Tomofumi Santa*

Graduate School of Pharmaceutical Sciences, The University of Tokyo, Tokyo, Japan.

Qualitative and quantitative analysis using HPLC-QQQ-MS/MS

Remember:
when you use MS, you increase the number of analytical dimensions. That will NOT make you life easier.

Identification of target substances in MRM mode

1. retention time: set appropriate retention time window!

2. acquire an amount of data points that are enough for obtaining smooth gaussians

3. use qualifier ion transitions and always check ion ratios. Limits of toleration are typically ±20% or ±30%.

Basic rules of quantitation using a mass spectrometer

- check selectivity, sensitivity and the matrix effect when selecting your ion transitions for quantitation
- use a calibration curve containing enough data points and covering the range of expected analyte concentrations
- perform calibration at the beginning of each sample batch
- find the proper fitting algorithm (linear or quadratic)
- use matrix calibrators if possible

Using weighted regression for generating calibration curves

$$a = \frac{n\sum_{i=1}^{n} x_i y_i - \sum_{i=1}^{n} x_i \sum_{i=1}^{n} y_i}{n\sum_{i=1}^{n} x_i^2 - (\sum_{i=1}^{n} x_i)^2}$$

$$b = \frac{1}{n}(\sum_{i=1}^{n} y_i - a\sum_{i=1}^{n} x_i)$$

ordinary least squares

Slope of weighted regression: $$m = \frac{\left(\sum w_i X_i Y_i - \frac{(\sum w_i X_i)(\sum w_i Y_i)}{\sum w_i}\right)}{\left(\sum w_i X_i^2 - \frac{(\sum w_i X_i)^2}{\sum w_i}\right)}$$

Intercept of weighted regression: $$b = \frac{\sum w_i Y_i}{\sum w_i} - \left[\frac{\sum w_i X_i}{\sum w_i}\right] \times \frac{\left(\sum w_i X_i Y_i - \frac{(\sum w_i X_i)(\sum w_i Y_i)}{\sum w_i}\right)}{\left(\sum w_i X_i^2 - \frac{(\sum w_i X_i)^2}{\sum w_i}\right)}$$

weighted least squares

Weighted fits for calibration

Anal Chem. 2014 Sep 16;86(18):8959-66. doi: 10.1021/ac5018265. Epub 2014 Sep 4.

Selecting the correct weighting factors for linear and quadratic calibration curves with least-squares regression algorithm in bioanalytical LC-MS/MS assays and impacts of using incorrect weighting factors on curve stability, data quality, and assay performance.

Gu H[1], Liu G, Wang J, Aubry AF, Arnold ME.

Author information

Abstract

A simple procedure for selecting the correct weighting factors for linear and quadratic calibration curves with least-squares regression algorithm in bioanalytical LC-MS/MS assays is reported. The correct weighting factor is determined by the relationship between the standard deviation of instrument responses (σ) and the concentrations (x). The weighting factor of 1, 1/x, or 1/x(2) should be selected if, over the entire concentration range, σ is a constant, σ(2) is proportional to x, or σ is proportional to x, respectively. For the first time, we demonstrated with detailed scientific reasoning, solid historical data, and convincing justification that 1/x(2) should always be used as the weighting factor for all bioanalytical LC-MS/MS assays. The impacts of using incorrect weighting factors on curve stability, data quality, and assay performance were thoroughly investigated. It was found that the most stable curve could be obtained when the correct weighting factor was used, whereas other curves using incorrect weighting factors were unstable. It was also found that there was a very insignificant impact on the concentrations reported with calibration curves using incorrect weighting factors as the concentrations were always reported with the passing curves which actually overlapped with or were very close to the curves using the correct weighting factor. However, the use of incorrect weighting factors did impact the assay performance significantly. Finally, the difference between the weighting factors of 1/x(2) and 1/y(2) was discussed. All of the findings can be generalized and applied into other quantitative analysis techniques using calibration curves with weighted least-squares regression algorithm.

PMID: 25157966 DOI: 10.1021/ac5018265

Quality control of assays – why is it important?

- The MS is not a stable detector → various ion transitions are affected in various manners!

- Autosampler tray stability of analytes and internal standards may not be 100% over the run.

- QC is a fundamental requirement for interlaboratory comparisons.

- misquantitation may be a result of:
  - chemical degradation
  - contamination of the ion optics
  - appearance of interferences in the ionchromatograms

What sort of quality control do you need?

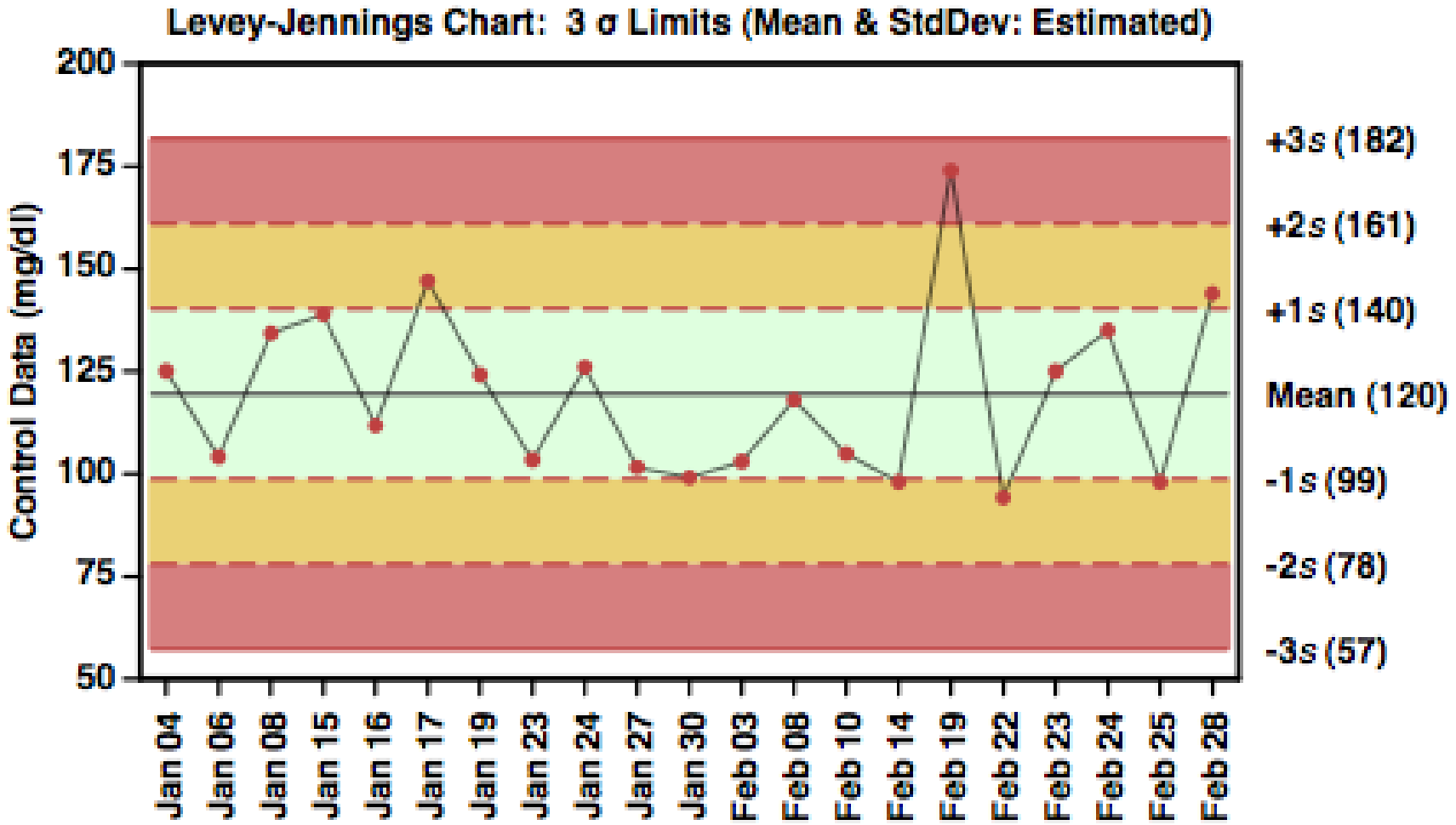
… That depends on the type and quality of information you would like to attain.

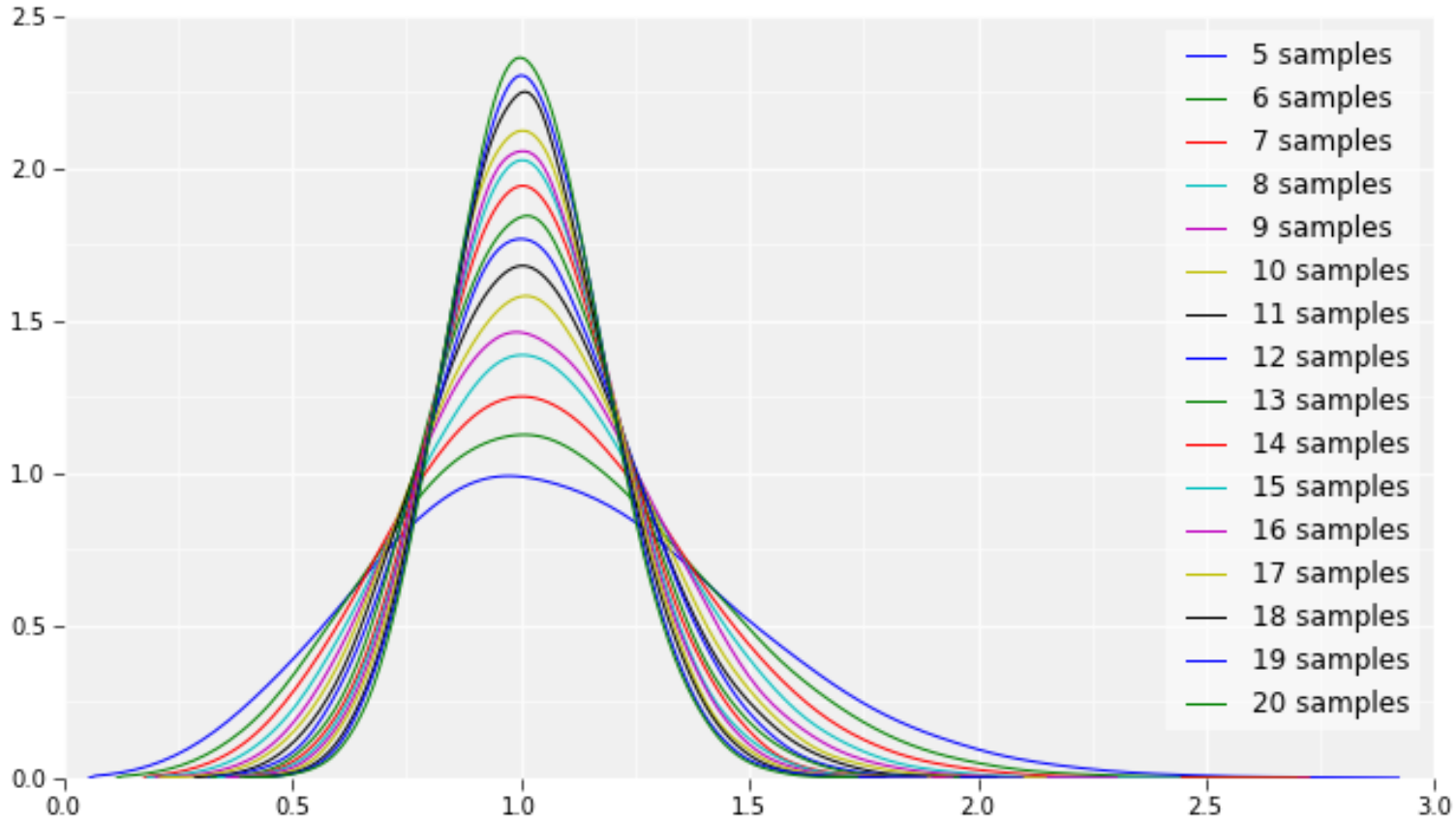
… And the regulations you are required to stick to.

Quality control of assays – approaches

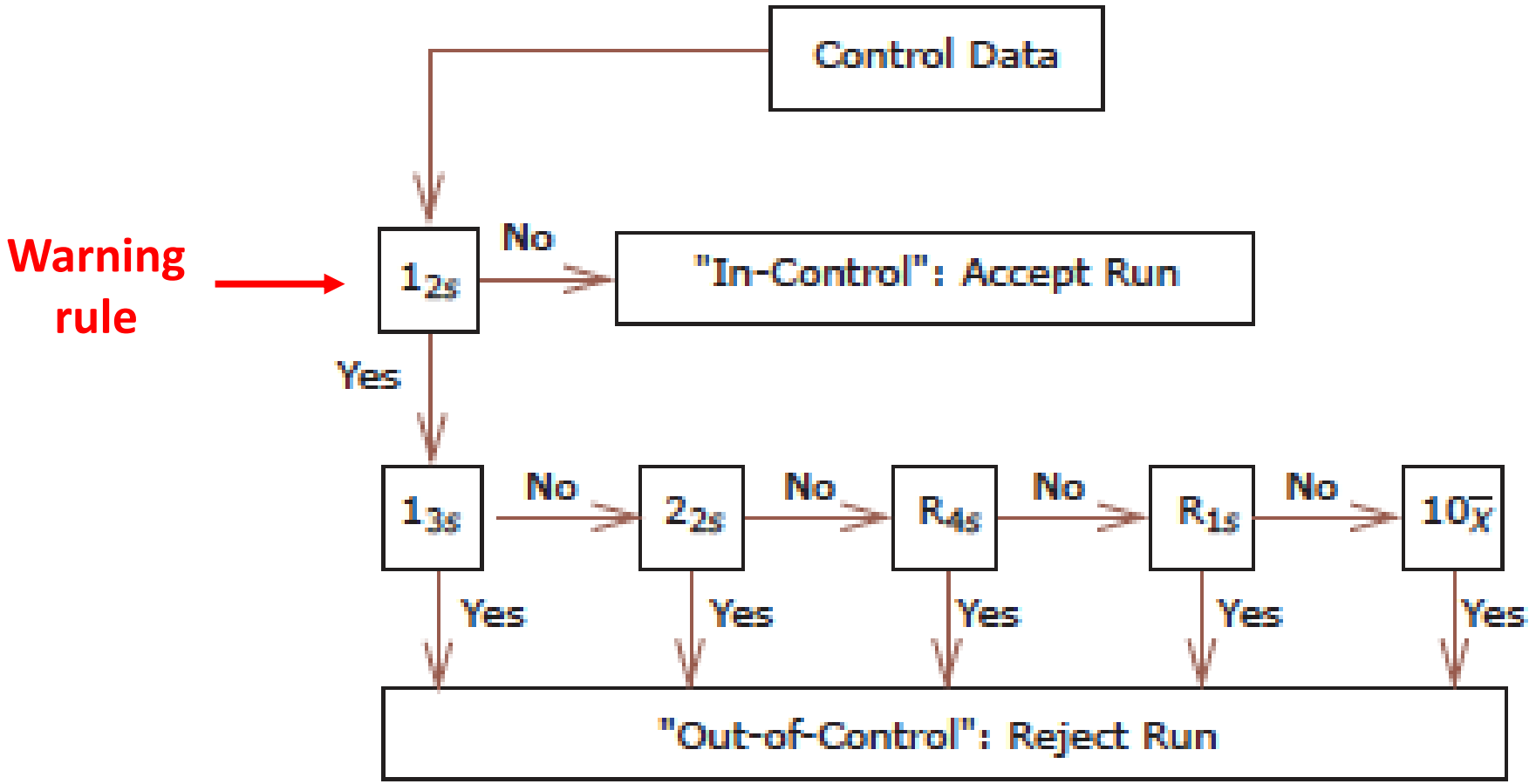
Approach	Identifited assay errors
multilevel matrix controls run at least at the beginning and at the end of the batch	loss of the validity of calibration due to contamination of the ion optics
spiked matrix samples	matrix-specific contamination of the ion optics
repeat analysis	if prepared sample is reassayed: lack of system stability if collected sample is reassayed: lack of reproducibility
incurred sample reanalysis	lack of system stability
external quality assessment scheme	suboptimal method performance

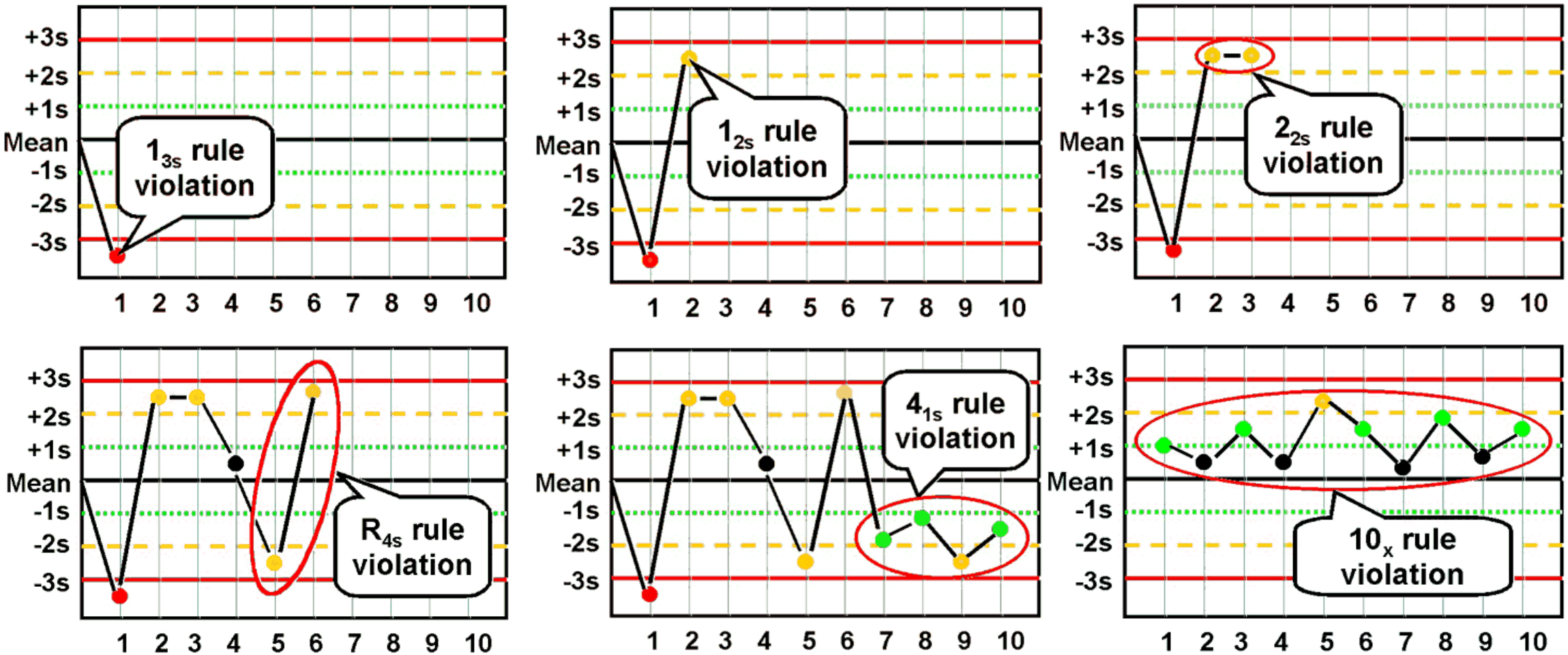
Interpretation of internal QC results: Levey-Jennings curves




2.5
2.0
1.5
1.0
0.5
0.0
0.0
0.5
1.0
1.5
2.0
2.5
3.0
5 samples
6 samples
7 samples
8 samples
9 samples
10 samples
11 samples
12 samples
13 samples
14 samples
15 samples
16 samples
17 samples
18 samples
19 samples
20 samples

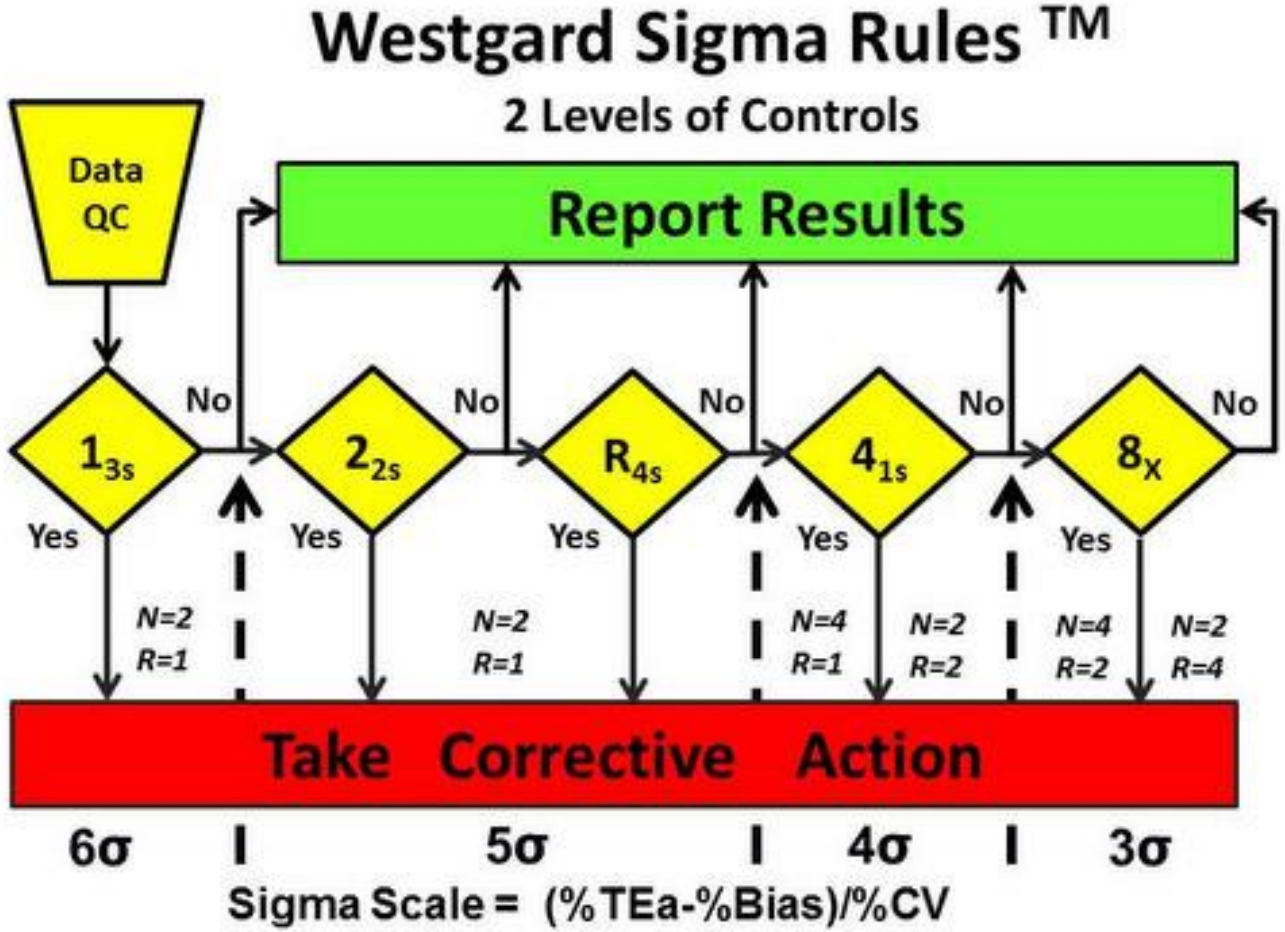
Interpretation of Levey-Jennings charts: the Westgard multirule quality control approach




+3s
+2s
+1s
Mean
-1s
-2s
-3s
1 2 3 4 5 6 7 8 9 10
1 3s rule violation
1 2s rule violation
2 2s rule violation
R 4s rule violation
4 1s rule violation
10 x rule violation

Within-run!

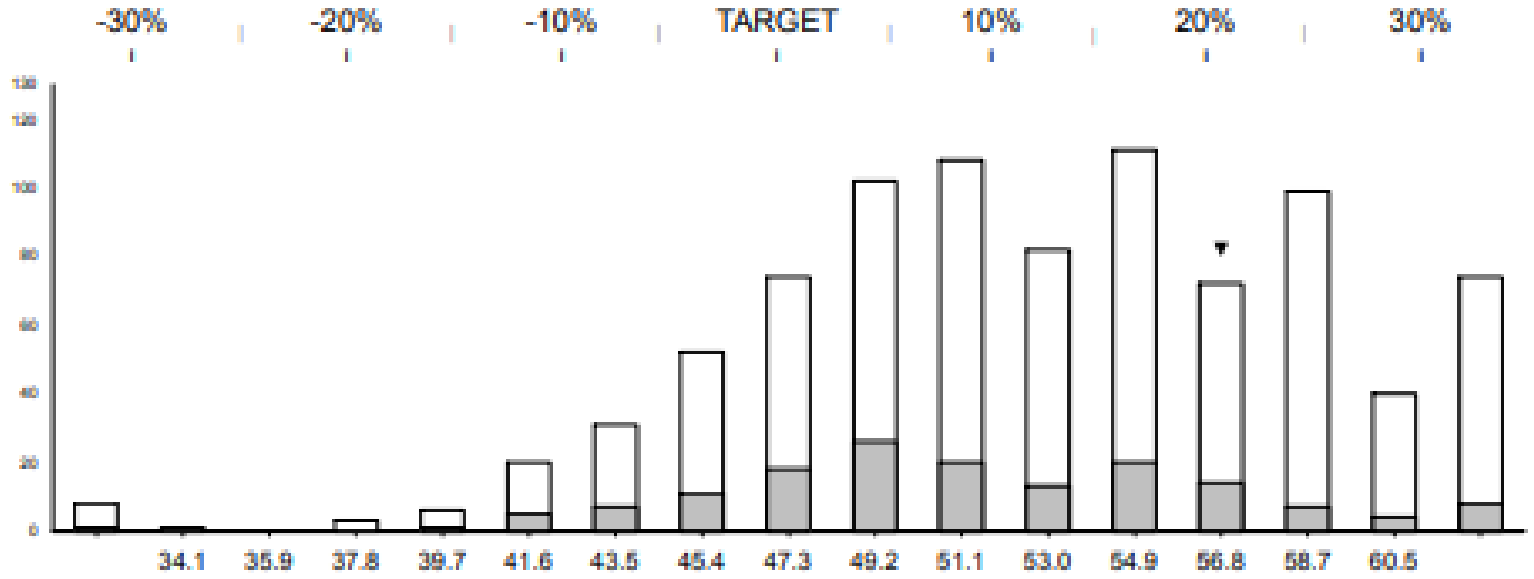
Interpretation of internal QC results: the Westgard Sigma Rules



Interpretation of the reports of external quality assessment schemes

25 Hydroxyvitamin D **January 2018** **Laboratory 2178**

Histograms



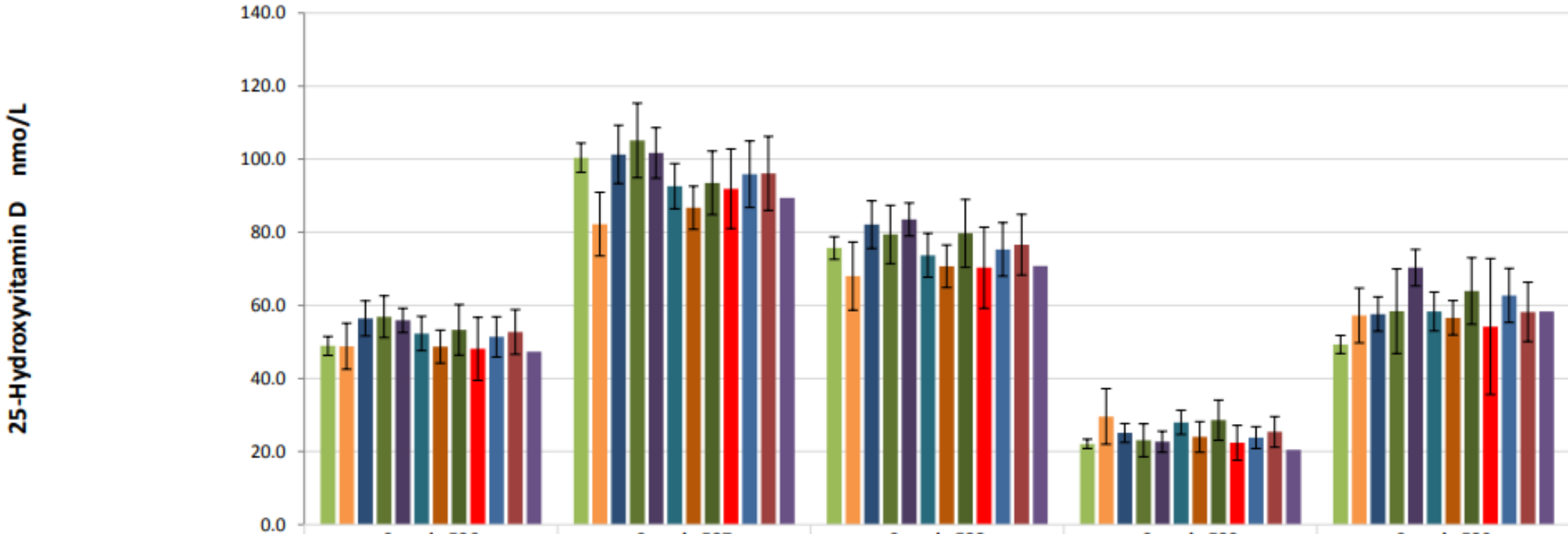
Sample: 526 (n=842)

Target Value :	47.3 nmol/L
ALTM	52.7 nmol/L
Your Method Mean (MM) :	51.4 nmol/L
Your result :	56.3 nmol/L
Bias from Target Value :	19.0 %
Bias from ALTM :	6.8 %
Bias from MM:	9.6 %

All Methods

Your method (LC-MS-MS)

DEQAS January 2018 - 25-OHD Method Means (+/-1SD) for Major Method Groups*



	Sample 526	Sample 527	Sample 528	Sample 529	Sample 530
Abbott Architect New Kit (n=70)	48.9	100.4	75.7	22.1	49.3
Beckman Unicel (n=32)	48.8	82.2	68.0	29.6	57.2
DiaSorin Liaison (n=197)	56.4	101.3	82.1	25.1	57.6
IDS iSYS (n=51)	56.9	105.1	79.4	23.1	58.4
IDS-iSYS New (n=14)	55.9	101.7	83.5	22.7	70.3
Roche Total (n=152)	52.3	92.6	73.7	28.0	58.3
Roche Vitamin D total II (n=25)	48.7	86.7	70.7	24.0	56.6
Siemens (n=65)	53.3	93.5	79.7	28.6	63.9
HPLC (n=16)	48.1	91.9	70.3	22.4	54.2
LC-MS/MS (n=154)	51.4	95.9	75.3	23.8	62.7
ALTM (n=842)	52.7	96.1	76.6	25.4	58.2
TARGET VALUE	47.3	89.4	70.8	20.5	58.3

Results from the NIST Reference Measurement Procedure for the October 2016 to January 2018 25-hydroxyvitamin D EQA Samples

Distribution	Sample No.	NIST 3-epi-25-OHD3 nmo/L	NIST 25-OHD2 nmol/L	NIST 25-OHD3 nmol/L	NIST 'Total' 25-OHD (25-OHD3 + 25-OHD2) nmol/L	DEQAS ALTM nmol/L	% Difference *
October 2016	**501**	6.5	2.8	93.4	96.2	100.4	4.4
	502	1.4	1.2	38.8	40.0	41.5	3.8
	503	5.5	1.4	79.2	80.6	87.1	8.1
	504	2.9	2.0	55.5	57.6	64.1	11.3
	505	0.9	0.7	21.0	21.7	23.5	8.3
January 2017	**506**	2.5	1.3	54.5	55.8	52.4	-6.1
	507	4.1	1.5	73.1	74.6	73.1	-2.1
	508	n/a	1.9	29.5	31.4	29.4	-6.4
	509	n/a	1.1	70.4	71.6	67.9	-5.1
	510	12.1	0.5	134.1	134.6	133.6	-0.8
April 2017	**511**	(4.3)	1.5	65.7	67.2	72.5	7.9
	512	(2.7)	1.9	44.9	46.8	49.9	6.6
	513	(6.8)	0.8	102.8	103.7	104.4	0.7
	514	(1.5)	0.6	27.1	27.7	29.6	6.9
	515* **	(3.0)	18.5	47.7	66.2	66.3	0.2
July 2017	**516**	2.9	1.3	45.2	46.5	47.3	1.7
	517	7.1	0.8	67.5	68.3	70.5	3.4
	518	8.7	1.2	103.7	105.0	110.3	5.1
	519	2.7	1.1	32.1	33.2	33.1	-0.3
	520	8.3	1.2	102.9	104.1	110.0	5.7
October 2017	**521**	2.1	1.0	39.6	40.5	41.2	1.7
	522	9.1	1.0	83.9	84.9	89.3	5.2
	523	1.2	3.5	22.5	25.9	25.7	-0.8
	524	14.1	0.9	107.9	108.8	124.8	14.7
	525	3.8	0.8	55.6	56.3	61.5	9.2
January 2018	**526**	3.0	0.9	46.5	47.3	52.7	11.3
	527	5.5	1.0	88.4	89.4	96.1	7.5
	528	4.5	1.9	68.9	70.8	76.6	8.2
	529	1.4	0.7	19.9	20.5	25.4	23.7
	530* **	2.8	21.5	36.8	58.3	58.2	-0.1

BIO-RAD

Lab 145728

SE LABORATORIUMI MEDICINA INTEZET
NAGYVARAD TER 4.
BUDAPEST 1083
HUNGARY

Sample Summary Report
Immunoassay (Monthly) Program

Cycle 15
Dec 2017 - Dec 2018
Sample No: 4
Sample Date: 09 Apr 18
Lot No: 231400

EQAS
External Quality Assurance Services

Instrument: Shimadzu LC-MS

	Analyte	Unit	Result	Mean	Z-score	RMZ	Comparator
✔	11-Deoxycortisol	ng/mL	0,04	0,158	-0,96	-1,05	Mode

Instrument: Tandem Mass Spectrometer

	Analyte	Unit	Result	Mean	Z-score	RMZ	Comparator
✔	17-a-OH-Progesterone	ng/mL	5,82	5,79	0,04	-0,64	Mode
✔	Aldosterone	pg/mL	253,7	286	-1,07	-0,68	Mode
✔	Androstenedione	ng/mL	1,78	1,89	-0,17	-0,09	Mode
✔	Cortisol	ng/mL	87,7	104	-1,64	-3,16	Mode
✔	DHEA	ng/mL	0,67	2,03	-1,79	-1,93	All
✔	Progesterone	ng/mL	9,68	11,4	-1,19	-1,24	Mode
✔	Testosterone	ng/mL	4,18	3,26	1,77	2,03	Mode

Instrument: Waters Mass Spectrometer

	Analyte	Unit	Result	Mean	Z-score	RMZ	Comparator
✔	DHEA-Sulfate	ng/mL	1815	1969	-0,71	-0,51	Mode

Legend: ✔ No Warnings Missing Result 1 Late Results ▼ 2,0 ≤ |Z-score| < 3,0 ✘ |Z-score| ≥ 3,0

* Amended Result (per participant's request) ¤ Non-robust determination of Mean and SD

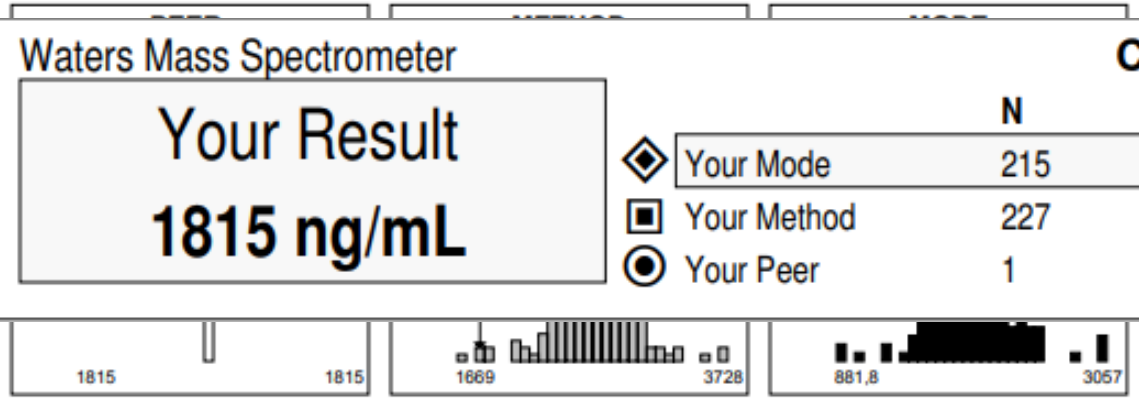
Waters Mass Spectrometer

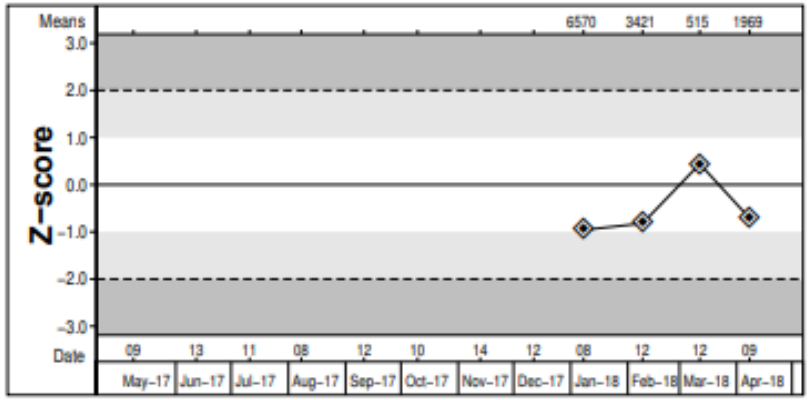
Your Result
1815 ng/mL

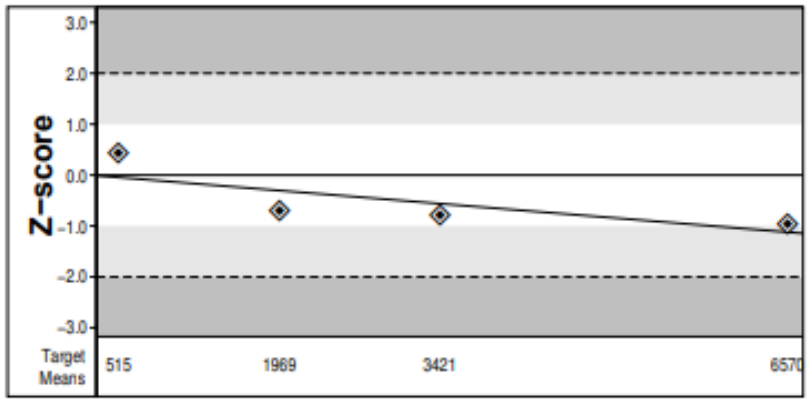
	N	Mean	SD	CV	U¹	Z-score	RMZ	%
Your Mode	215	1969	218	11,0	37,1	−0,71	−0,51	−7,84
Your Method	227	2698	206	7,63	34,2	−4,29	−4,41	−32,7
Your Peer	1	1815	0	0	0	0	0	0

Comparative Statistics

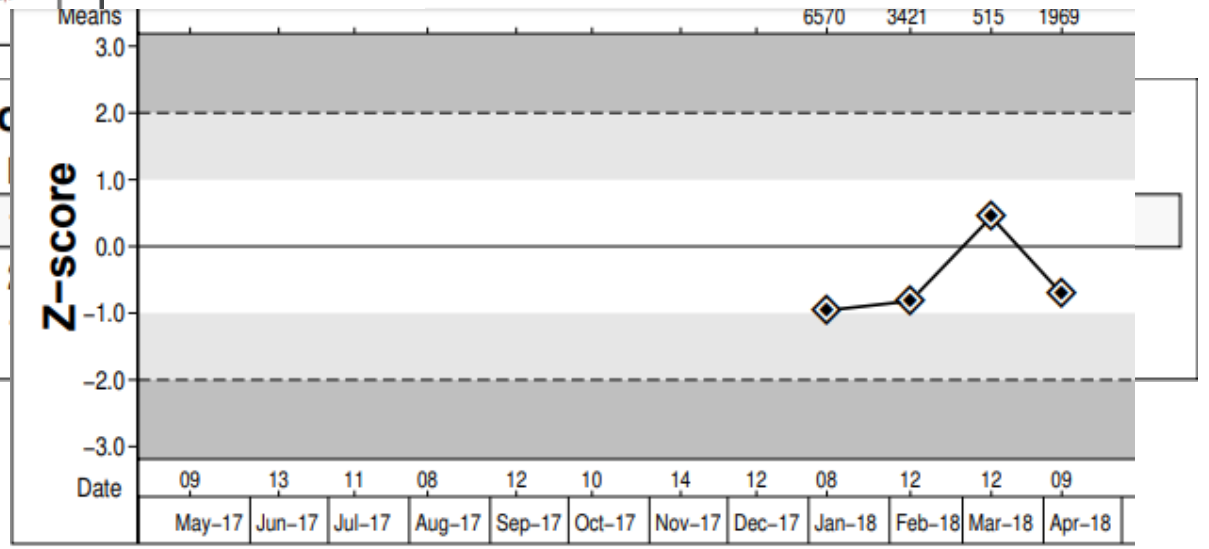
Your Deviation

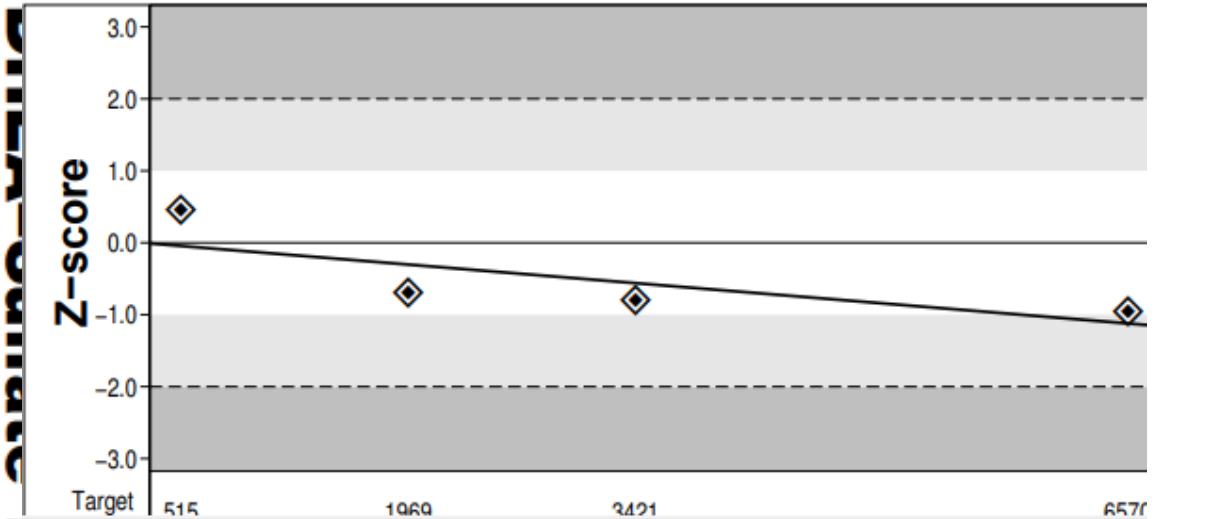






	N	Mean	CV	U¹
Roche cobas 6000/8000/c 311 Electrochemiluminescence (ECL) Dedicated Reagent	123	2702	6,46	39,3
Abbott AEROSET/ARCHITECT (c, i, ci ... Chemiluminescence Dedicated Reagent	110	2520	5,47	32,8
Roche Elecsys / cobas e 411 Series Electrochemiluminescence (ECL) Dedicated Reagent	94	2694	8,65	60,1
Siemens IMMULITE 2000/2500/XPi Chemiluminescence Dedicated Reagent	69	1908	11,7	67,2
Beckman Coulter UniCel DxI Series Chemiluminescence Dedicated Reagent	48	1979	8,96	64,0
Siemens ADVIA Centaur Systems Chemiluminescence Dedicated Reagent	39	2040	8,62	70,3
Beckman Coulter Access, LXi 725, Dx... Chemiluminescence Dedicated Reagent	22	2130	11,3	129
Siemens IMMULITE/1000 Chemiluminescence Dedicated Reagent	17	1927	9,00	105
DiaSorin LIAISON/XL Chemiluminescence Dedicated Reagent	9	1663	10,5	145





	17	1927	9,00	105
DiaSorin LIAISON/XL Chemiluminescence Dedicated Reagent	9	1663	10,5	145

Yundt chart