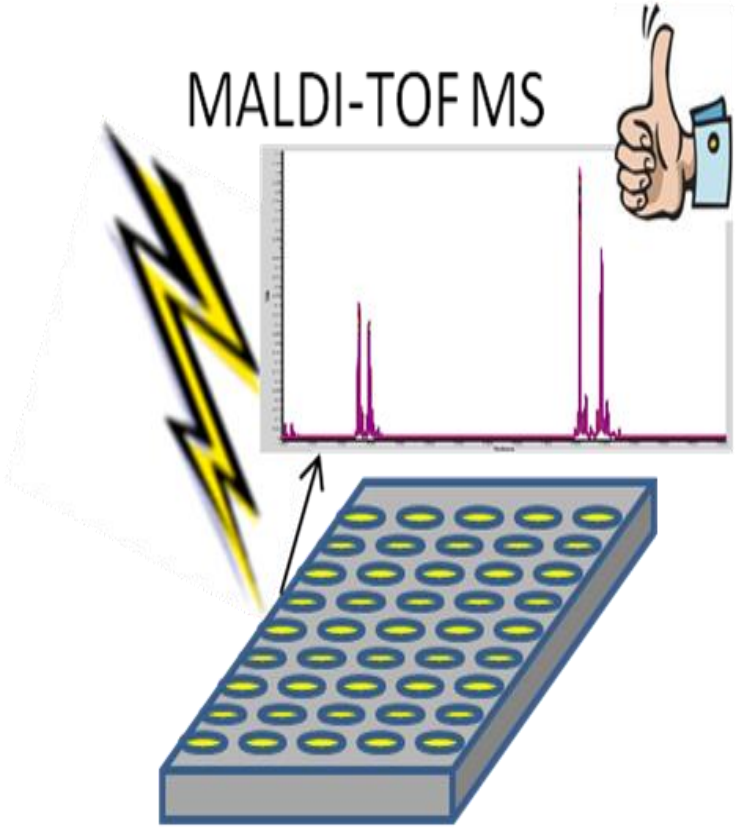
Developing an assay using MALDI-TOF MS



What to consider ?

Sample preparation

-Sample collection

-Sample storage and stability

-Need for clean-up or purification?

MALDI-TOF MS conditions

-choice of matrix

-choice of [sample]

Instrument acquisition conditions

-mass range

-laser fluence

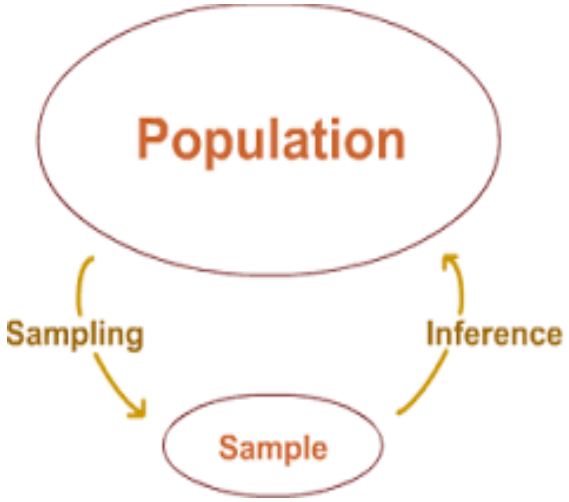
-sample scanning conditions

Data processing

-data manipulation

-information output

Sampling and Sample preparation



- How is sample obtained?
- Does it accurately represent the whole?
  - Does a cup of water represent the whole lake?
- Is the sample stable during the time frame of the analysis?
  - Does the sample change from the time of collection to the time of analysis?
- Does the sample need purification or clean-up?
  <https://en.wikibooks.org/wiki/Proteomics/Protein_Sample_Preparation>

-desalting, buffer exchange

-solid phase extraction, precipitation, size-exclusion

- enrichment

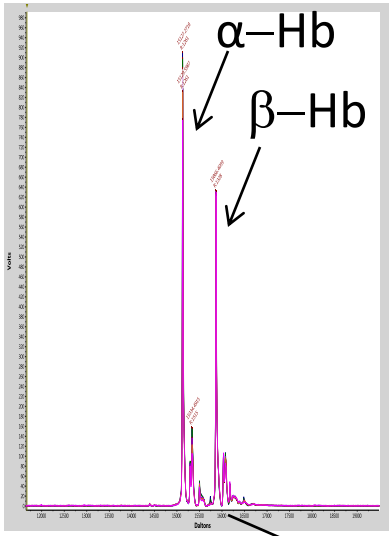
-SPE, Affinity capture,

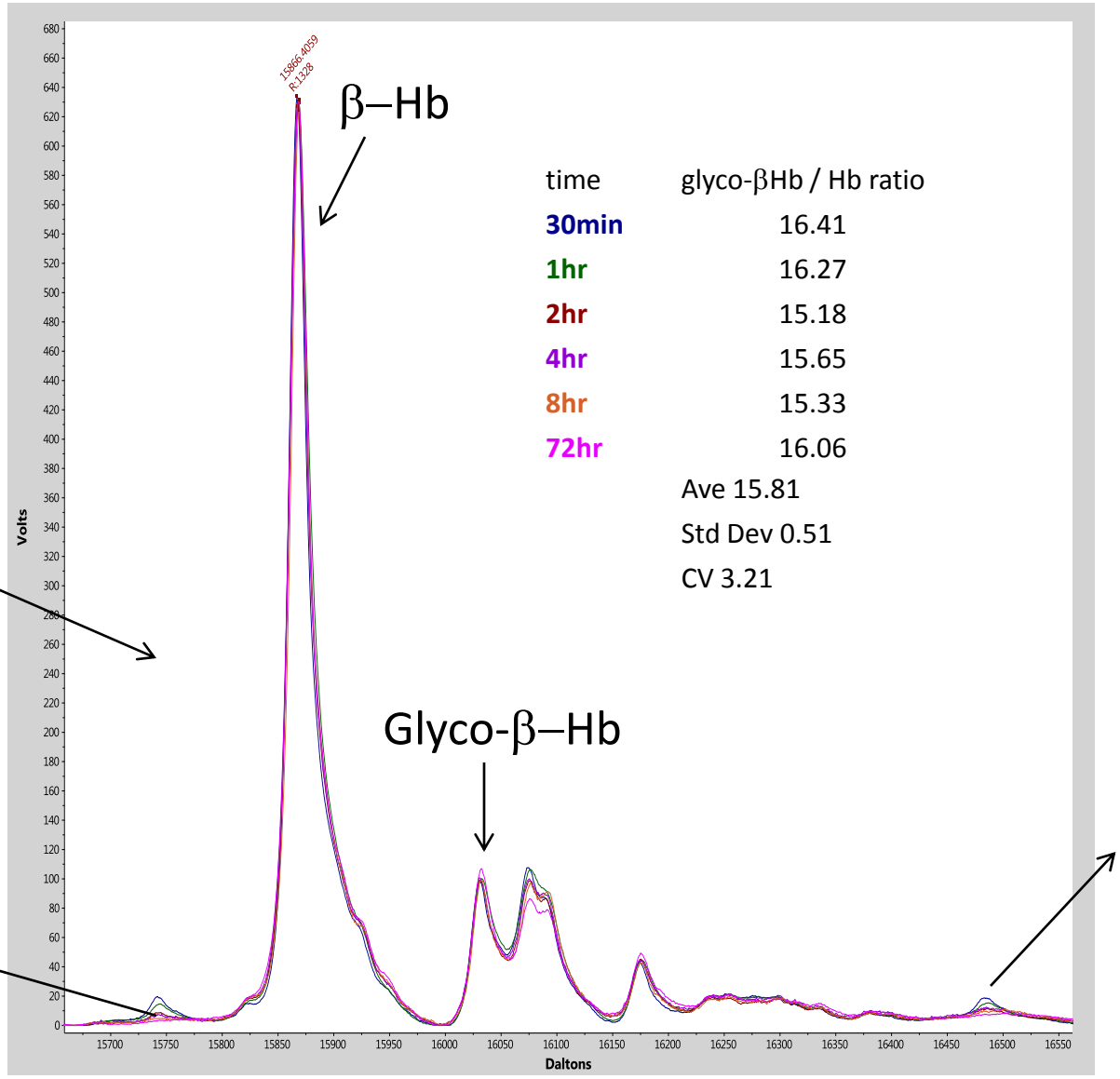
-separation

-UHPLC / HPLC, electrophoresis

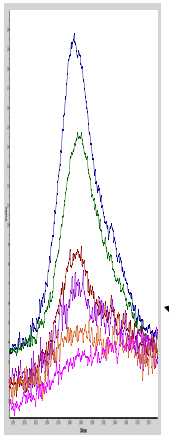
Do any of the preparation steps introduce biases or interferences into the analysis?

Stability / time course of glyco-βHb / Hb ratio

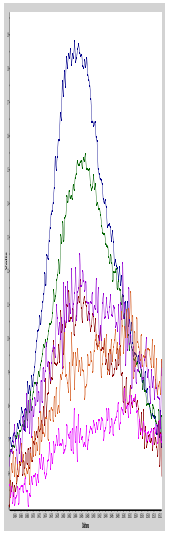




time	glyco-βHb / Hb ratio
30min	16.41
1hr	16.27
2hr	15.18
4hr	15.65
8hr	15.33
72hr	16.06
	Ave 15.81
	Std Dev 0.51
	CV 3.21



?



Some things are happening ?

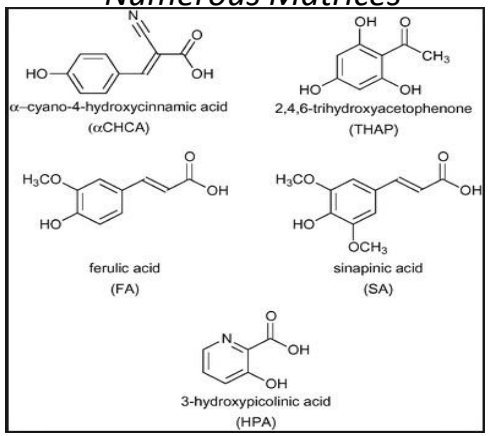
Matrix-Assisted LDI-TOF MS

Functions of the Matrix

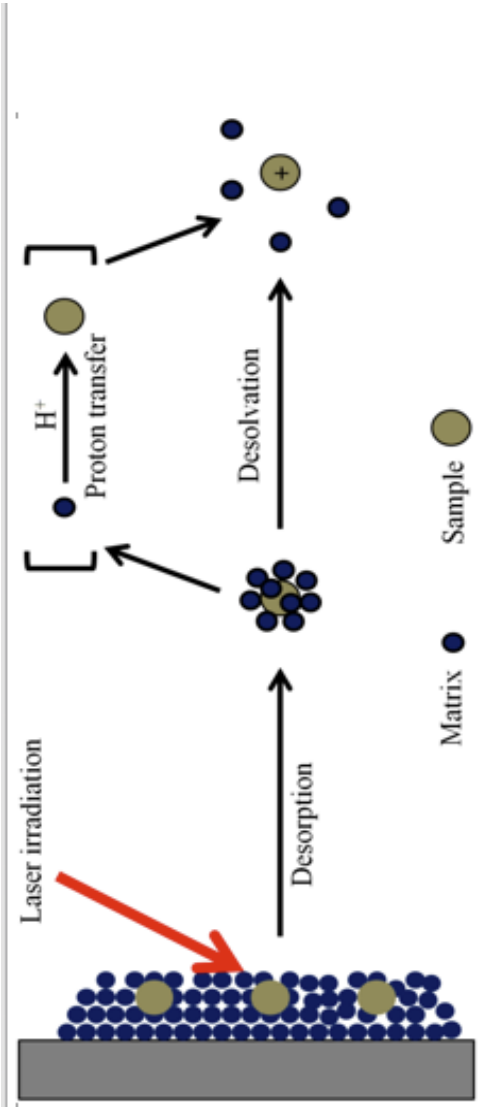
1) Soluble in a formulation of sample
2) Crystallizes on drying
   - -Analyte mixed in and co-crystallizes with matrix
   - -Analyte layered on top of crystallized matrix
3) Absorbent at λ of laser
4) Absorbs laser energy and desorbs
   - -Analyte desorbs collaterally
5) Transfers charge to analyte

Many vendors and MS Core facilities have websites with published matrix preparation protocols.
- Good starting points-

Numerous Matrices



most are small (<200 Da) aromatic acids



Two of many guides for MALDI Matrices

Sigma Aldrich
<http://www.sigmaaldrich.com/analytical-chromatography/analytical-products.html?TablePage=103431784>

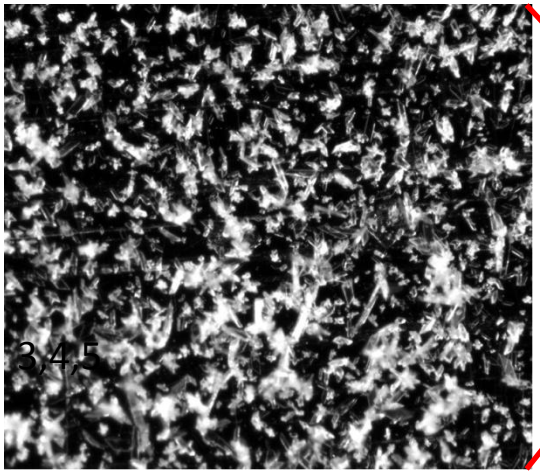
Protea Biosciences
<https://proteabio.com/media/document/Maldi_selection_guide3.pdf>

Sample preparation for glyco-βHb analysis

- Sinapinic acid (30% CH_3CN, 0.1% TFA)
- Final sample dilution of 1:2000

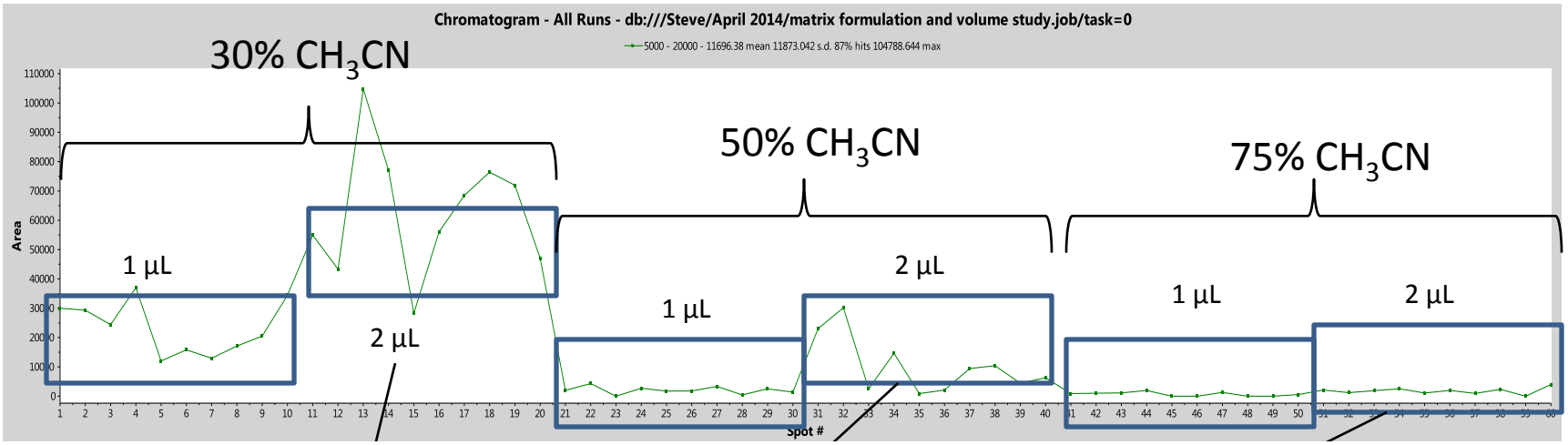
Tested several other matrices and formulations
for analyte sensitivity and resolution and interferences

Other matrices examined
Alpha-cyano
DHB
Super-DHB
HABA
3-HPA
Ferulic acid
trans 3,5-Bis (trifluoromethyl) cinnamic acid
3,4,5 trimethoxycinnamic acid

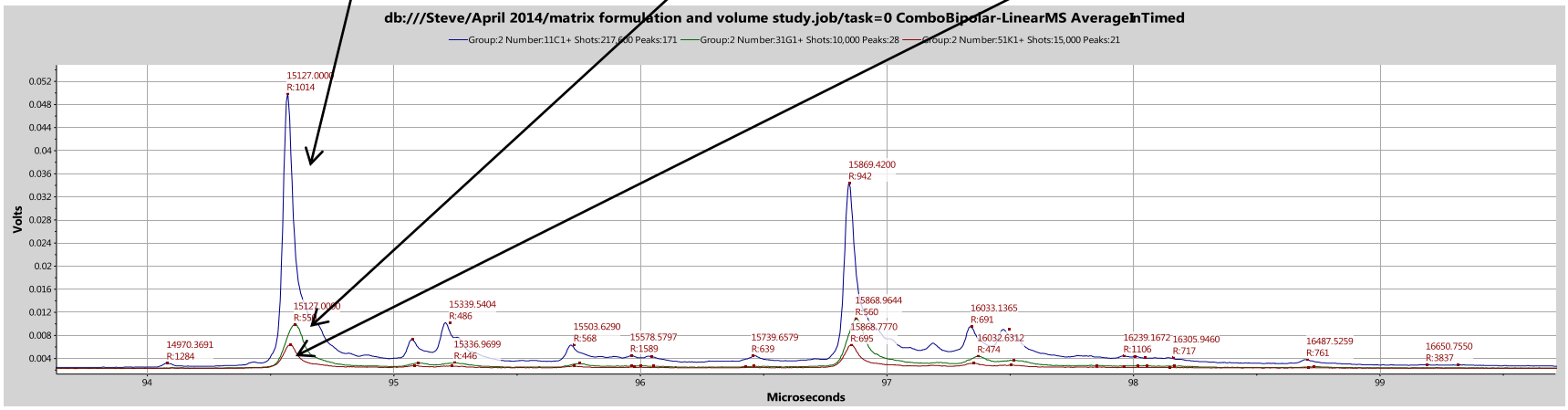


Crystals ~ 10-20 µM

Sinapinic Acid formulation and load volume

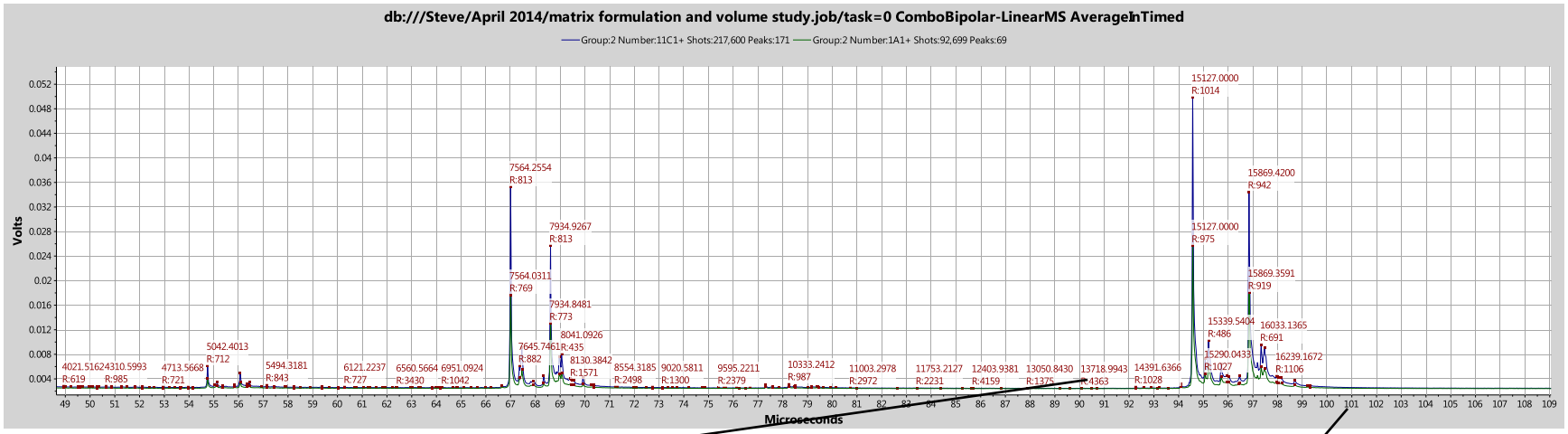


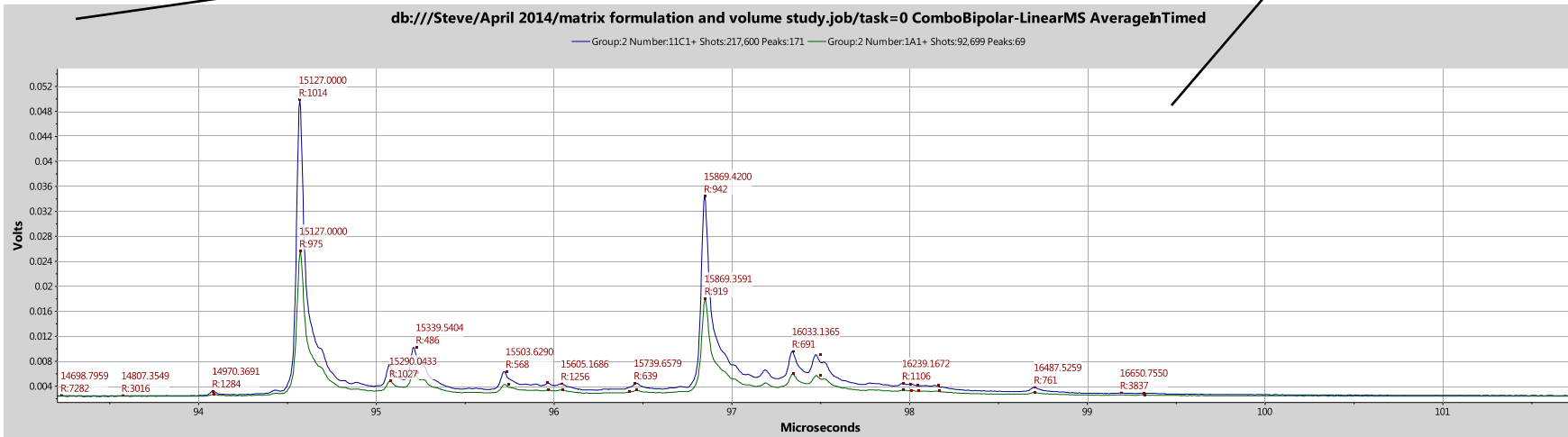
signal intensity (5000-20000 Da) for Hb spotted in sinapinic acid with using different solvent formulations (n=10 for each volume at each formulation)



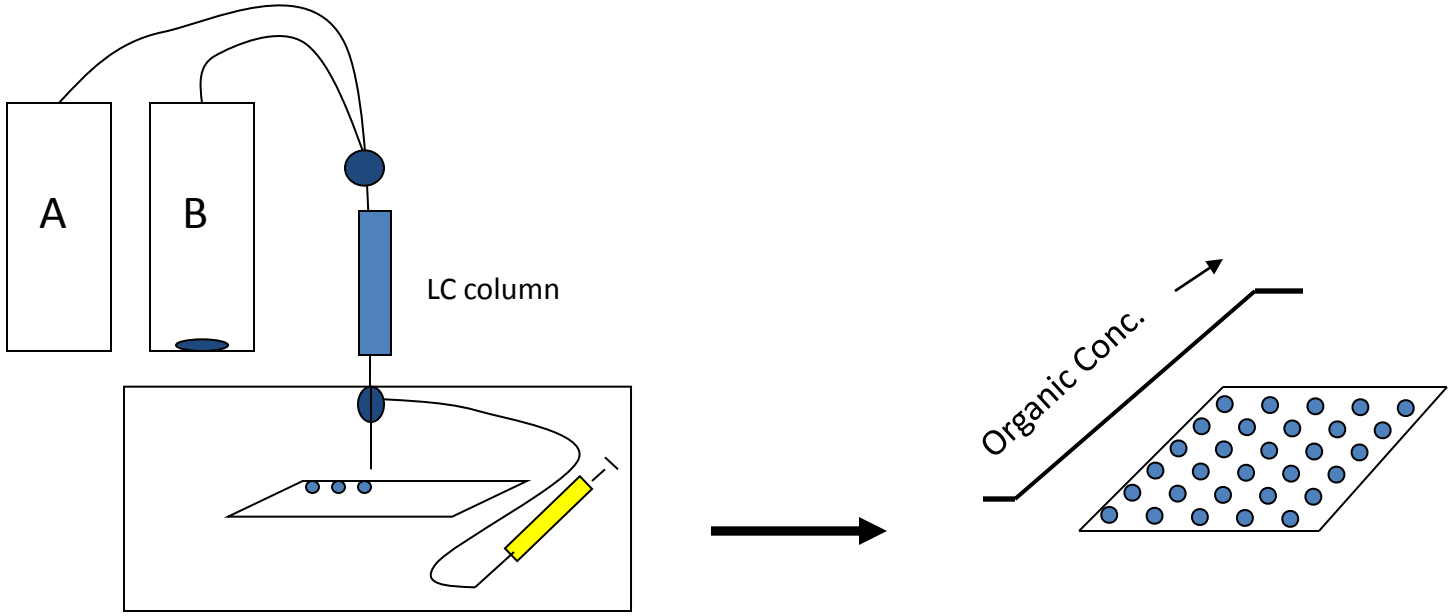
Averaged signal intensity (5000-20000 Da) of the 2µL loading for each formulation)

30% Organic formulation overlay 2 and 1 uL loadings





matrix formulation change influences signal intensity



-Internal Std added to matrix

A=2% CH_3CN, 0.1% TFA

B=85% CH_3CN, 0.1% TFA

Matrix = 7 mg/mL αCHCA, 10fmol/uL Std

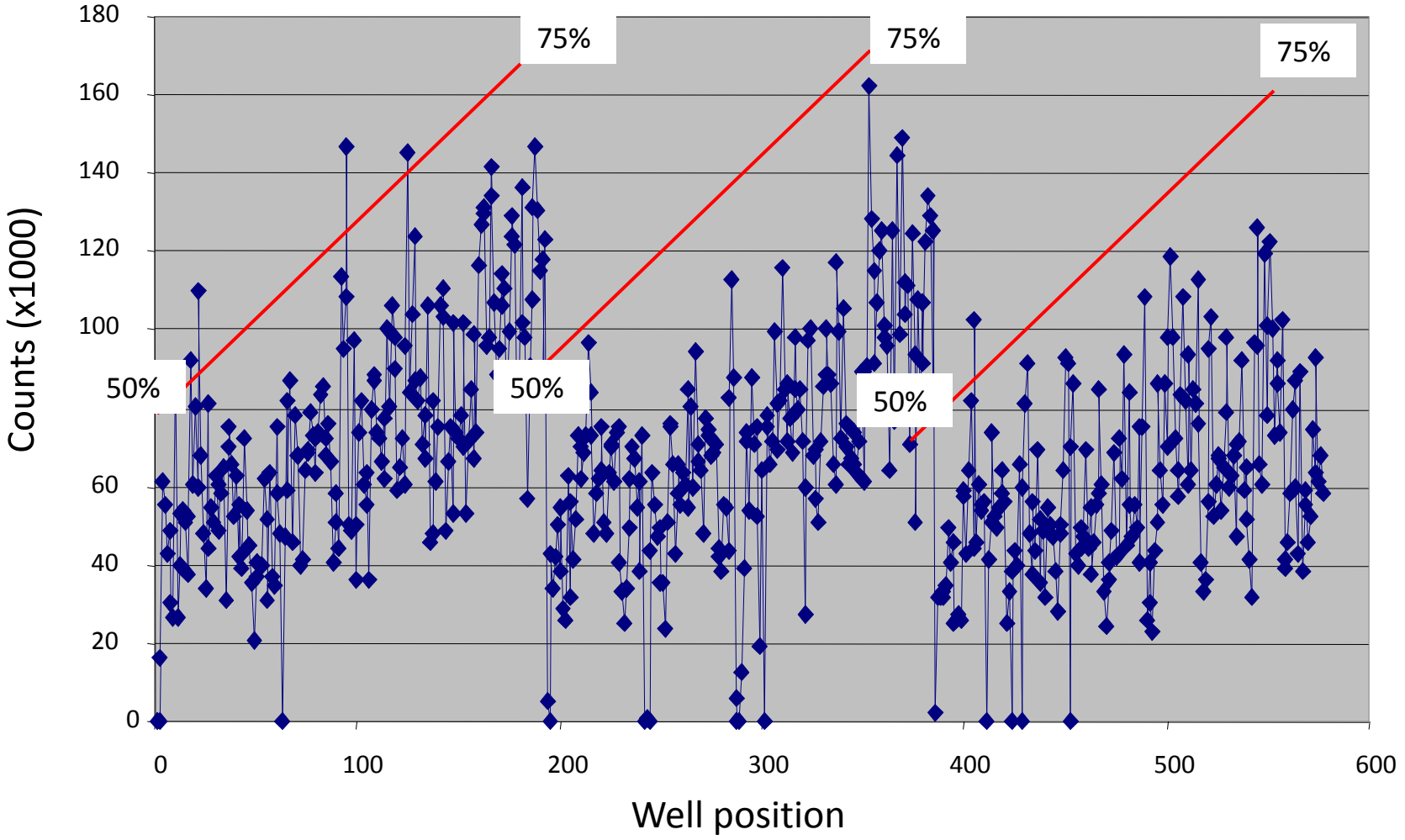
-Peptide Std concentration remains constant, organic content of formulation increases with gradient

1) Hattan SJ, Marchese J, Albertinetti M, Krishnan S, Khainovski N, Juhasz P. "*Effect of solvent composition on signal intensity in liquid chromatography-matrix-assisted laser desorption ionization experiments*." J Chromatogr A. **2004**, 1053(1-2):291-7.

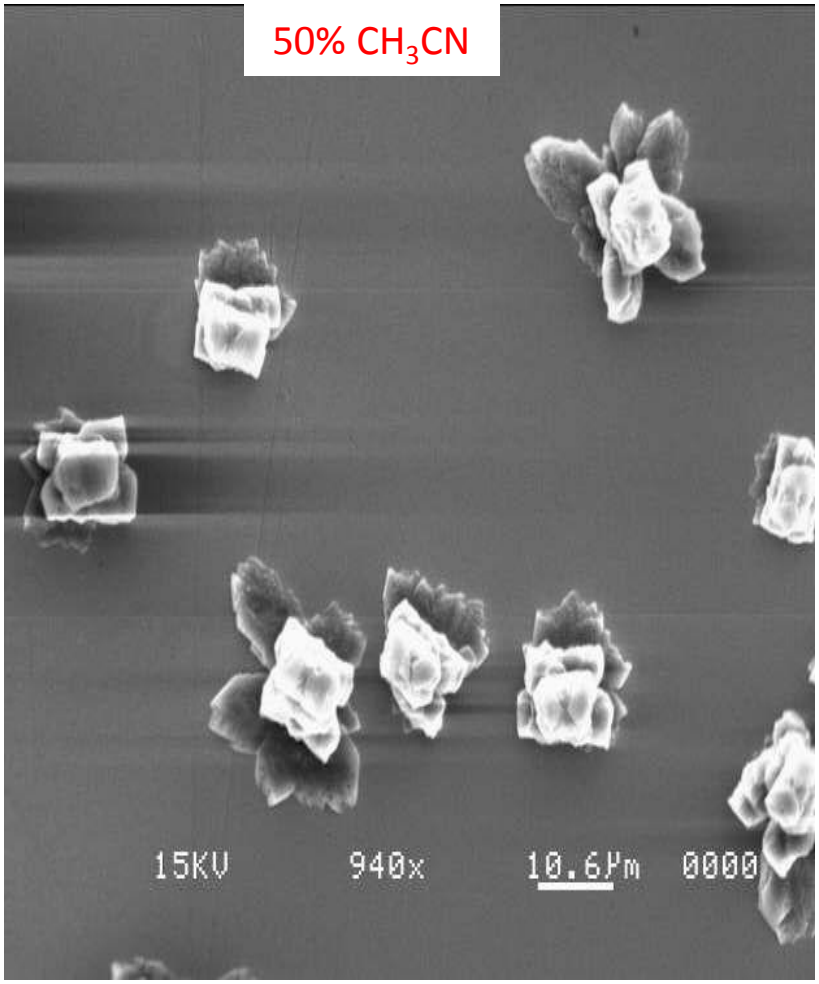
2) Steven L. Cohen and Brian T. Chait *"Influence of Matrix Solution Conditions on the MALDI-MS Analysis of Peptides and Proteins"* Anal. Chem. **1996,** 68, 31-37.

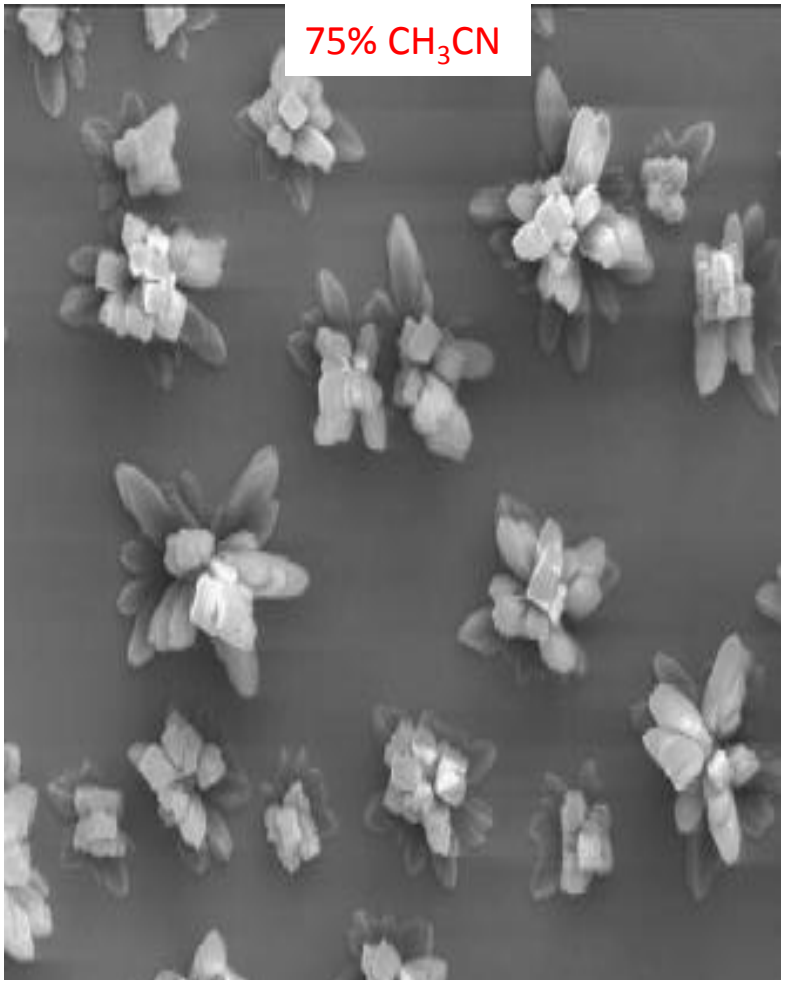
<http://prowl.rockefeller.edu/protocols/96_cohen_anal-chem.pdf>

Effect of matrix formulation on Signal Intensity

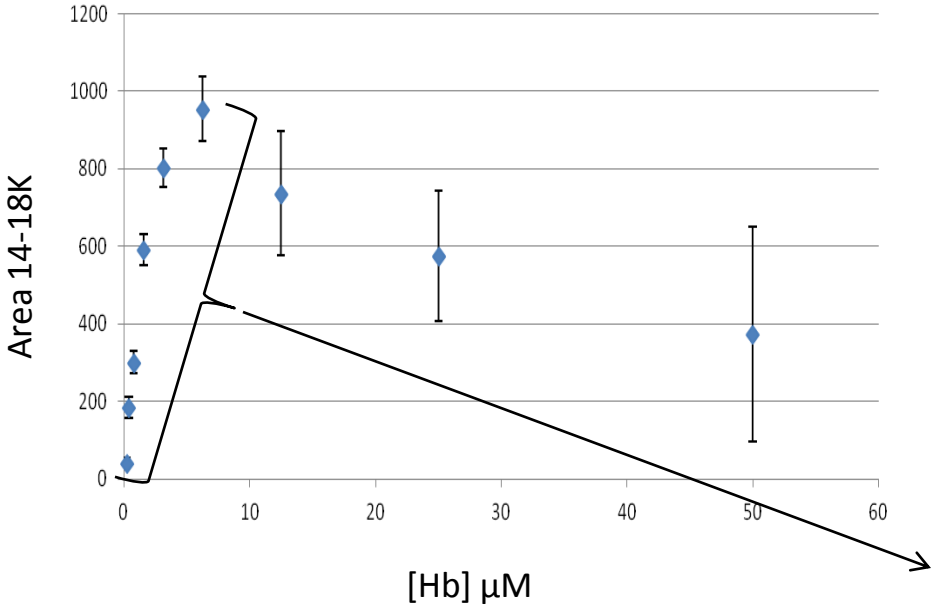


Solvent composition influences Crystal morphology

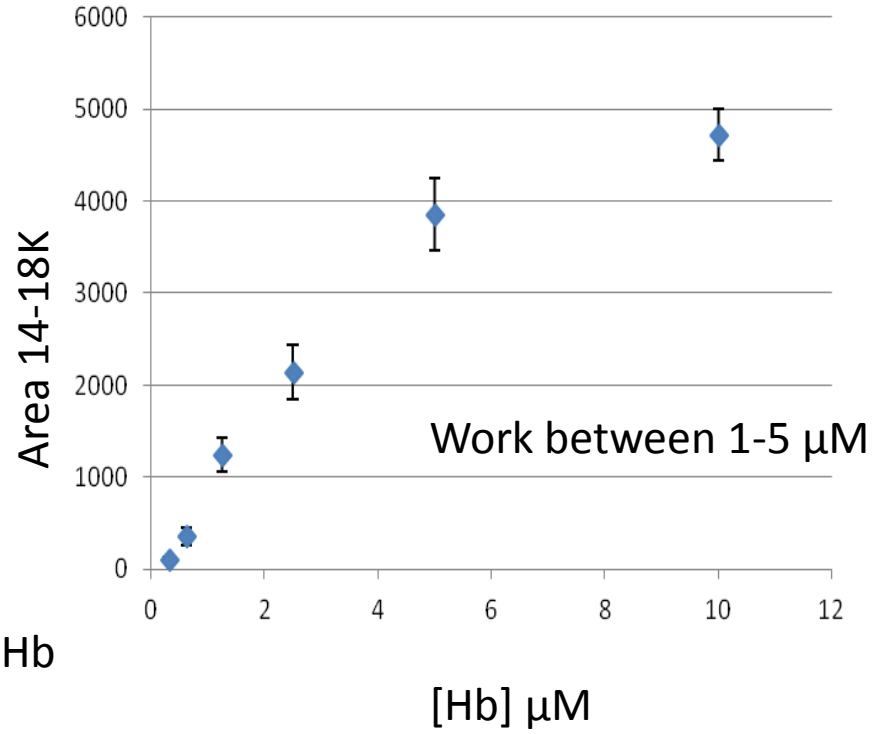




[Where] to work for quantitative response?

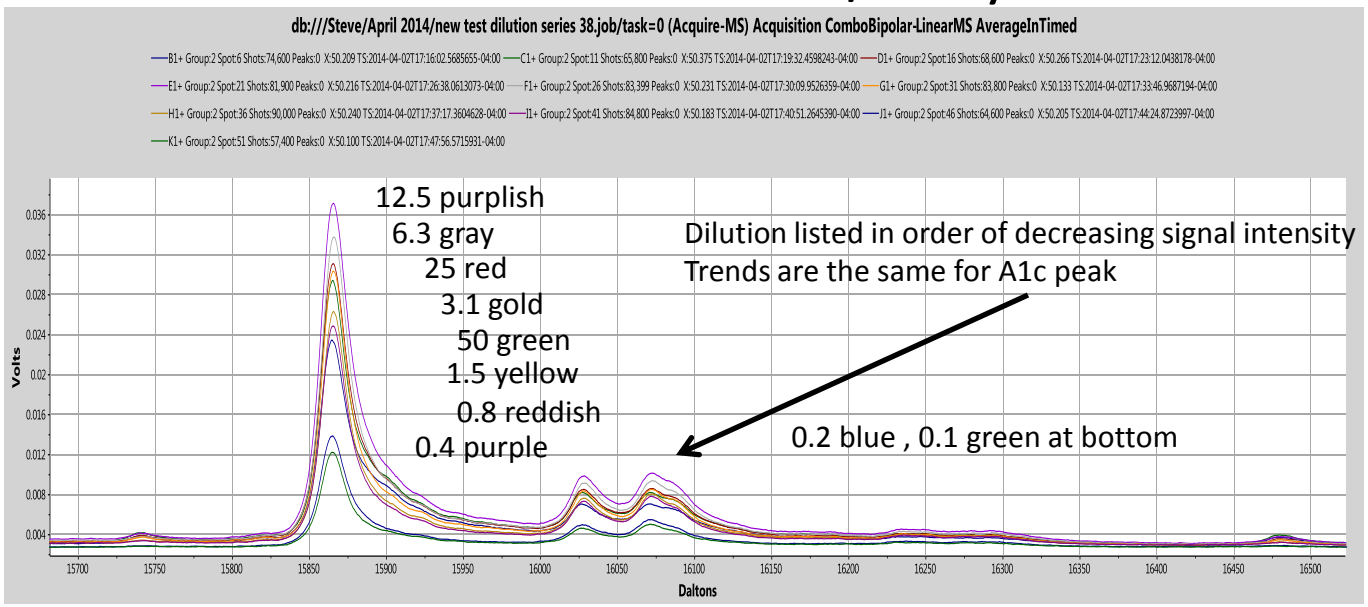


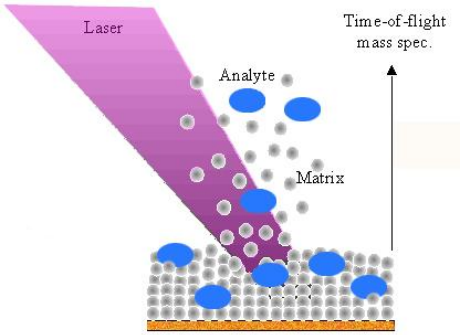
Range: 0.2 – 10 µM
Signal: 14-18 kDa
Measurements: 5x each



- Serial dilution 50.0 – 0.2 µM Hb
- Linear response 0.2- 5.0 µM
- ***1:2000 dilution of whole blood*** ~ 2.0 µM Hb

Dilution series response of βHb and glyco-βHb and matrix /analyte ratio





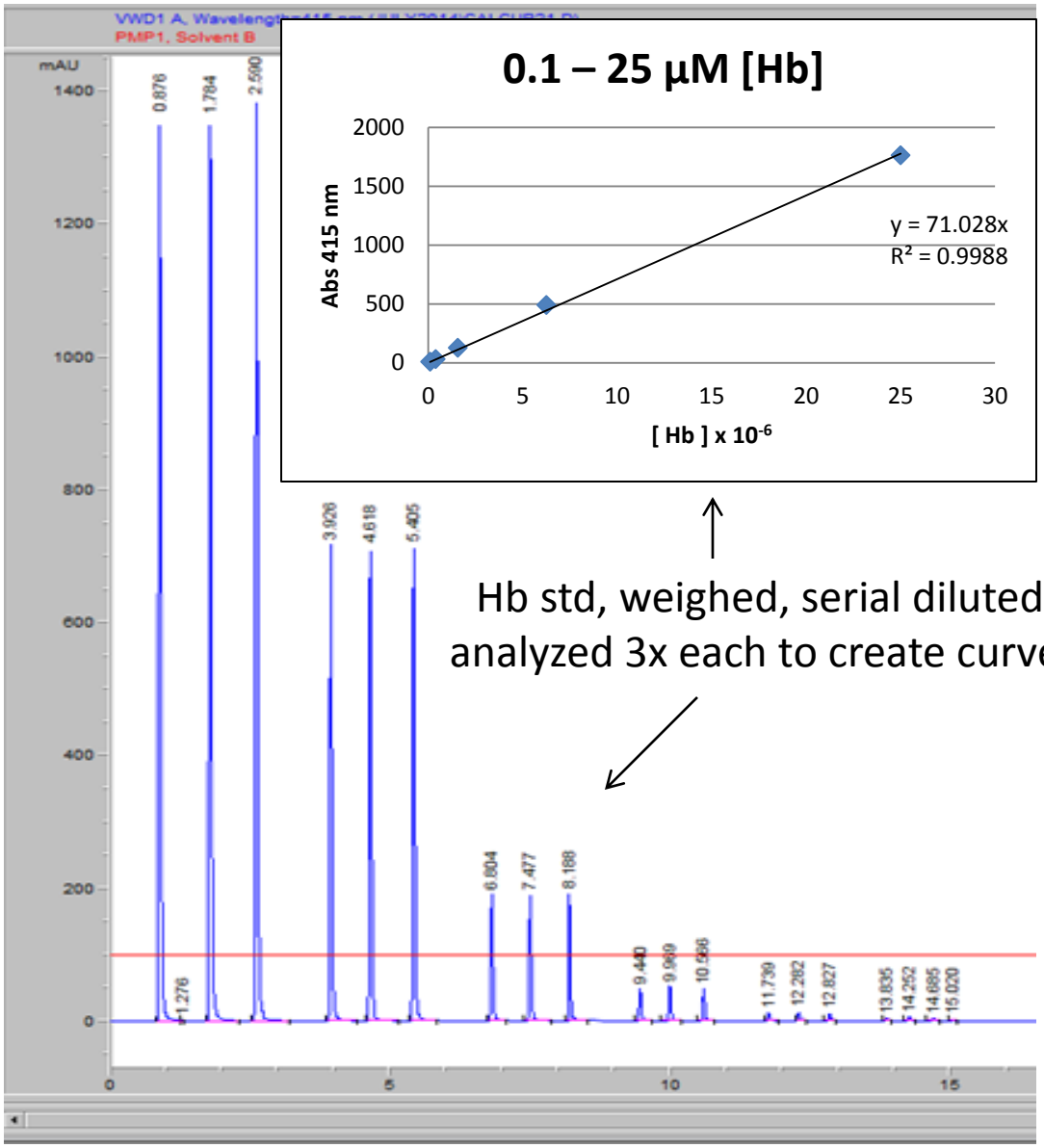
[Matrix] / [Analyte] ratio = [Sinapinic acid] 44.6 mM / [βHb] 50 µM = 450

50 µM = 450
25 µM = 900
12 µM = 1800
6.3 µM = 3200
3.1 µM = 6400
1.6 µm = 13000
0.8 µM = 26000

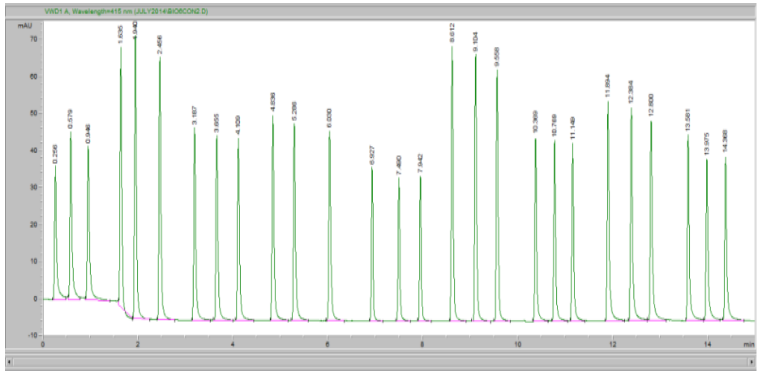
[Matrix] / [Analyte] ratio ideally ~ > 5000
(analyte, sample & matrix dependant)

1) Beavis RC, Chait BT, "*Factors affecting the ultraviolet laser desorption of proteins*" Rapid Commun Mass Spectrom. **1989** Jul;3(7):233-7.
2) Hillenkamp F, Karas M, Beavis RC, Chait BT, "*Matrix-assisted laser desorption/ionization mass spectrometry of biopolymers*" Anal. Chem. **1991**, 63, 1193A–1203A
3) Mass Spectrometry: A Textbook. Gross J.H. Ed. Chap 11, Matrix-Assisted Laser Desorption/Ionization 520–522

1:2000 dilution ~ 2 µM [Hb] by Abs 415 nm



Hb std, weighed, serial diluted analyzed 3x each to create curve

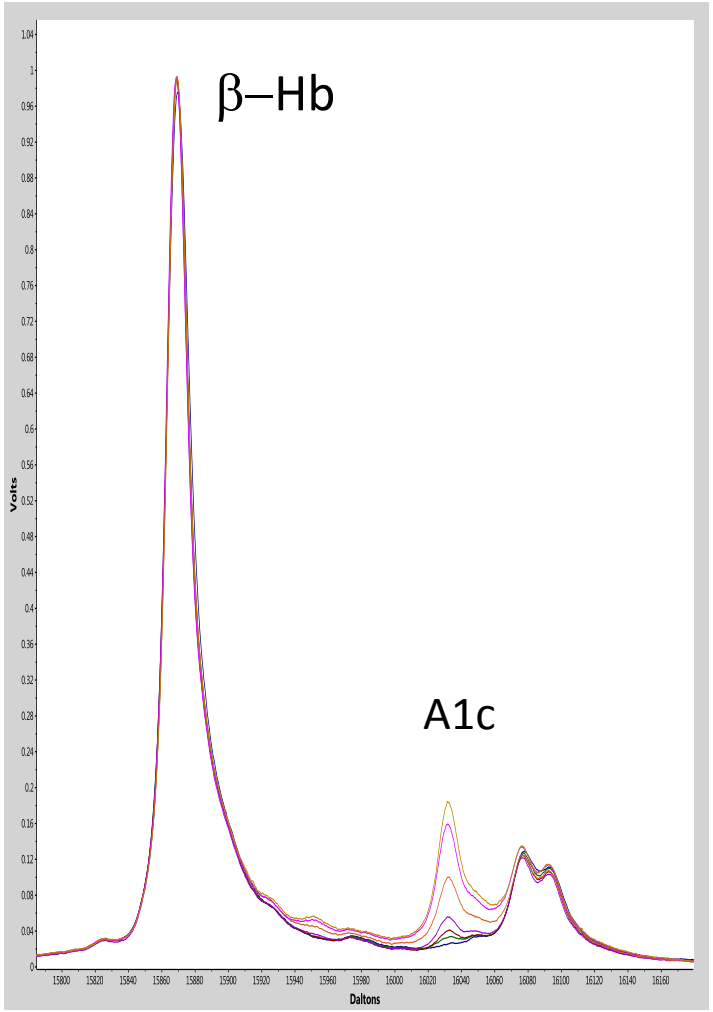


9 blood samples 3x each

Integrated peak areas blood 1/2000

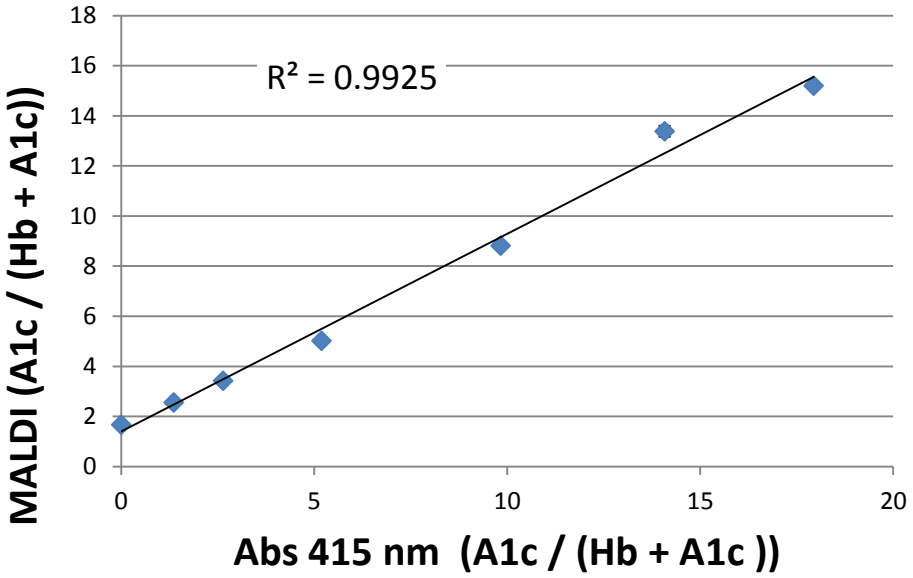
	Ave	SD	Rel SD	[Sample]	ave
1	131.93	16.28	12.34	1.86	2.20*
2	203.47	20.52	10.08	2.86	
3	156.20	2.54	1.63	2.2	
4	161.20	4.10	2.54	2.27	
5	97.70	0.53	0.54	1.38	
6	199.93	8.88	4.44	2.81	
7	136.90	1.32	0.97	1.93	
8	175.87	4.15	2.36	2.48	
9	140.90	7.62	5.40	1.98	

Ave 2.20 µM
High 2.86 – Low 1.38 µM
in appropriate [range]



Quantitative response of A1c across the clinically relevant range

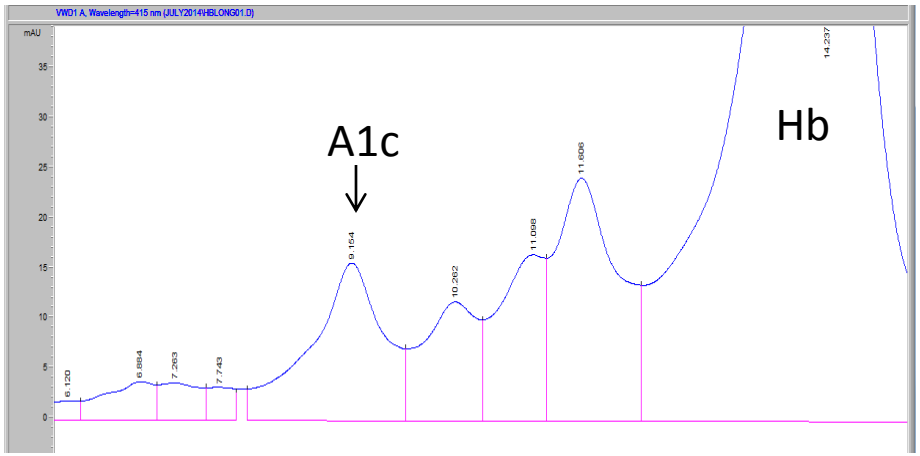
Avg	Std	CV
1.67	0.07	4.03
2.56	0.02	0.74
3.42	0.05	1.35
5.02	0.04	0.81
8.82	0.08	0.95
13.38	0.22	1.62
15.20	0.07	0.48



H_2O µL	A1c µL	Hb µL	Matirx µL	[Hb] µM	[A1c] µM	% A1c	% A1c/Hb +A1c
20	0	20	60	1.88	0		
19	1	20	60	1.88	.026	1.37	1.36
18	2	20	60	1.88	.051	2.73	2.64
16	4	20	60	1.88	.104	5.47	5.19
12	8	20	60	1.88	.208	10.93	9.83
8	12	20	60	1.88	.312	16.44	14.08
4	16	20	60	1.88	.416	21.92	17.94

Linear response across clinically relevant range

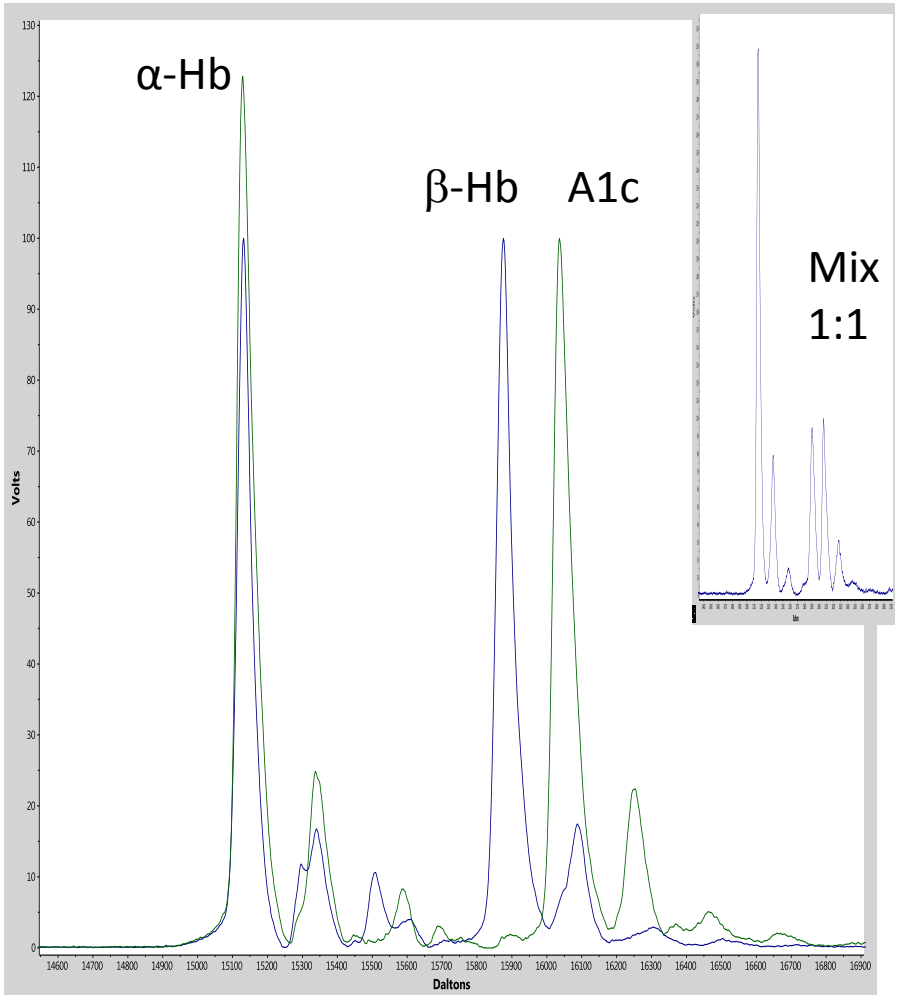
Quantitative response amongst the analytes of interest



LC time & [Li^+]

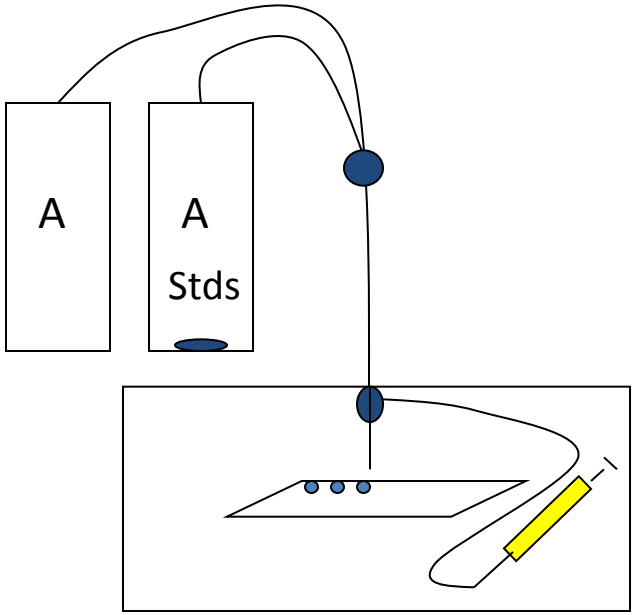
-Agilent 1100 series LC
-Mono S 5/50 GL column
Buffers
A = 10mM Na Malonate pH 5.7
B = 10mM Na Malonate pH 5.7, 0.3 M LiCl
Detection 415 nm

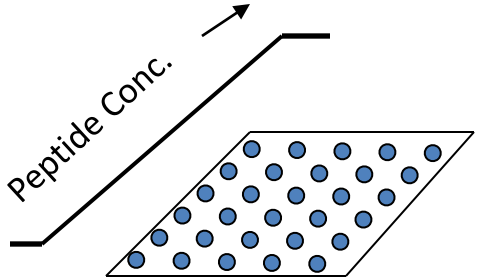
***CLIN. CHEM*. 32/10, 1867-1 872 (1986)**
Measurement of Hemoglobin A1C by a New Liquid-Chromatographic Assay: Methodology, Clinical Utility, and Relation to Glucose Tolerance Evaluated. Jan-Olof Jeppsson,' Per Jemtorp, Sundkvıst, KanEnglund, and Virve Nylunde



Spectra from purified materials

Absolute Sample Concentration affects Signal



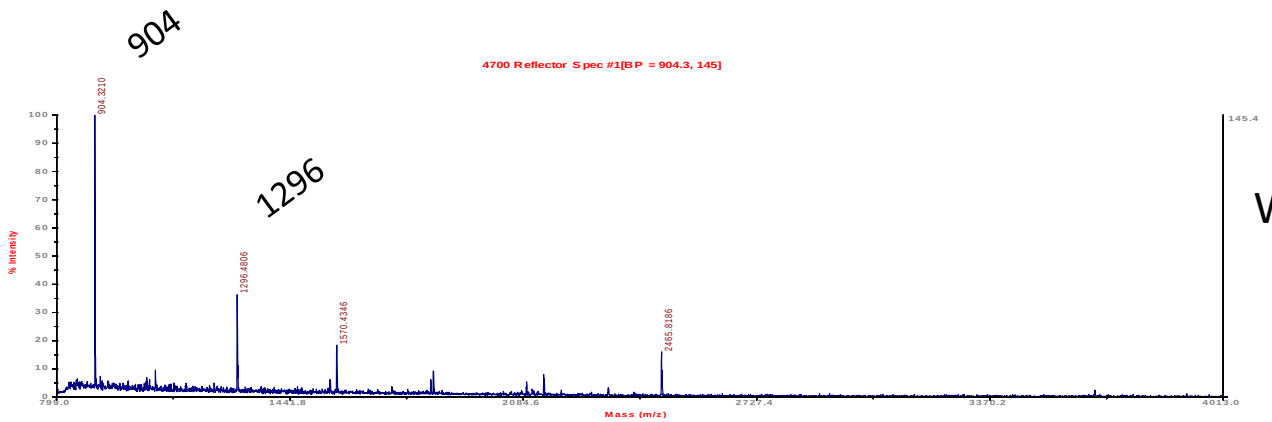


-Use LC pump to create a gradient of sample concentration

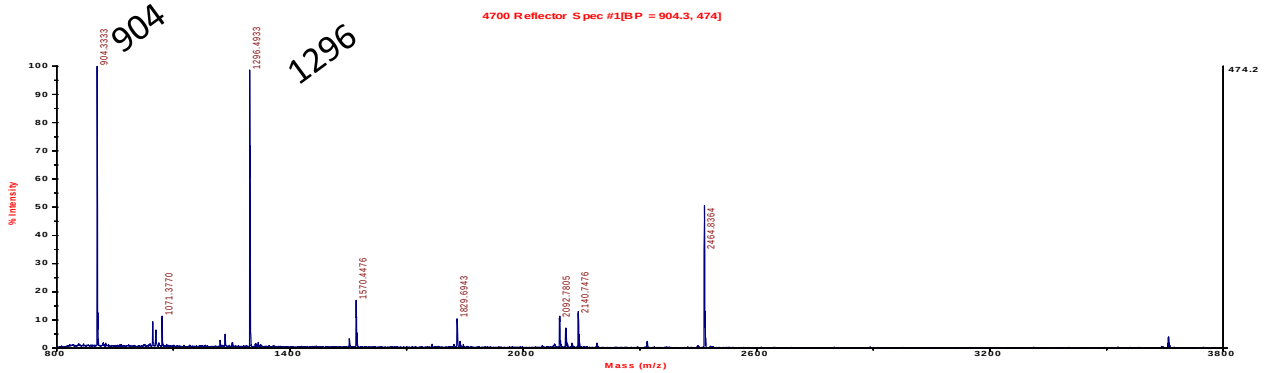
-Absolute peptide concentration increases relative peptide ratios remain constant

A = 50/50 ACN/H_20, 0.1% TFA
Matrix = HCCA 7mg/mL
No column

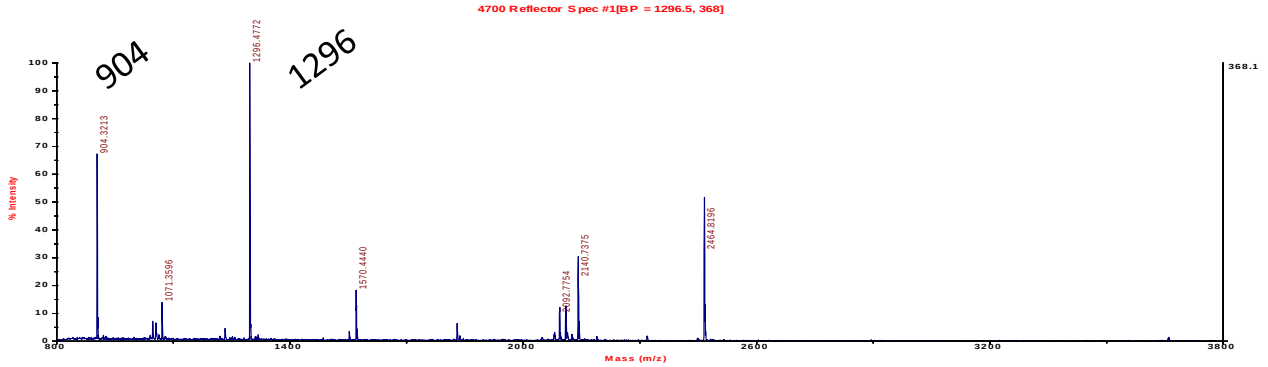
MS Pattern Varies with Absolute Concentration



Well 40



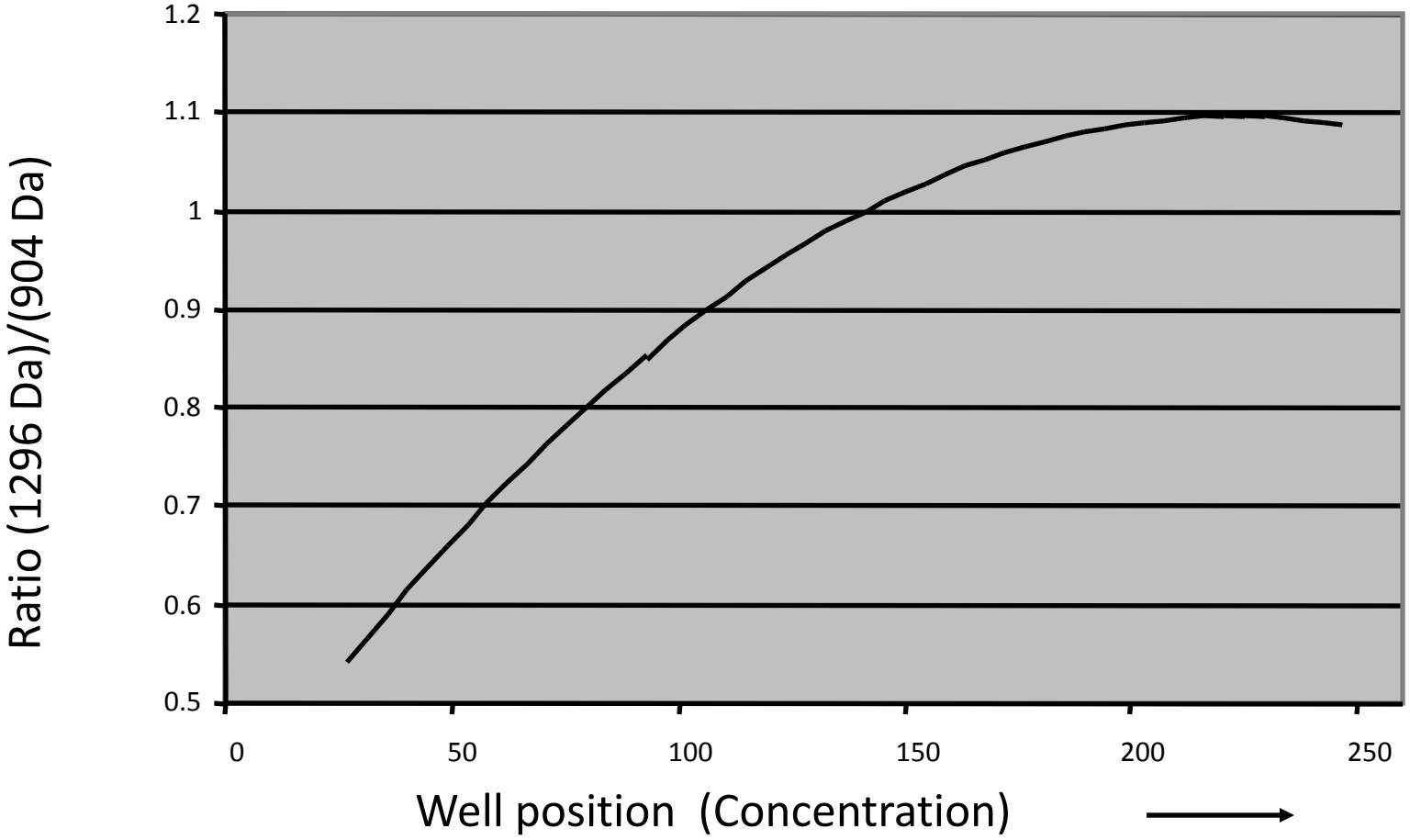
Well 140



Well 240

Increasing Concentration

Sample Concentration Influences MS signal



Sample Acquisition

Acquisition parameters

-linear mode using positive-ion polarization

-mass range 5000 – 20,000 Dalton (how is this accomplished) reasons keeping tight

-focus mass 15,000

-laser pulse frequency 1000 Hz

-laser pulse energy 12 µJ

-scan rate 1 mm/sec

-sample spot size 2.6 mm

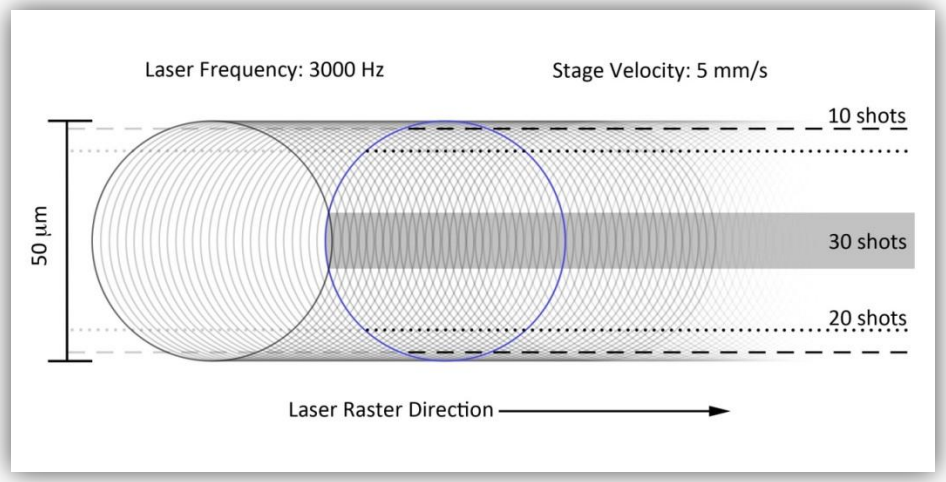
-100 µm raster to cover each sample position

Red = adjustable parameters that determine acquisition speed

MALDI Imaging Platforms

Next Generation MALDI TOF
SimulTOF ONE, TWO and THREE

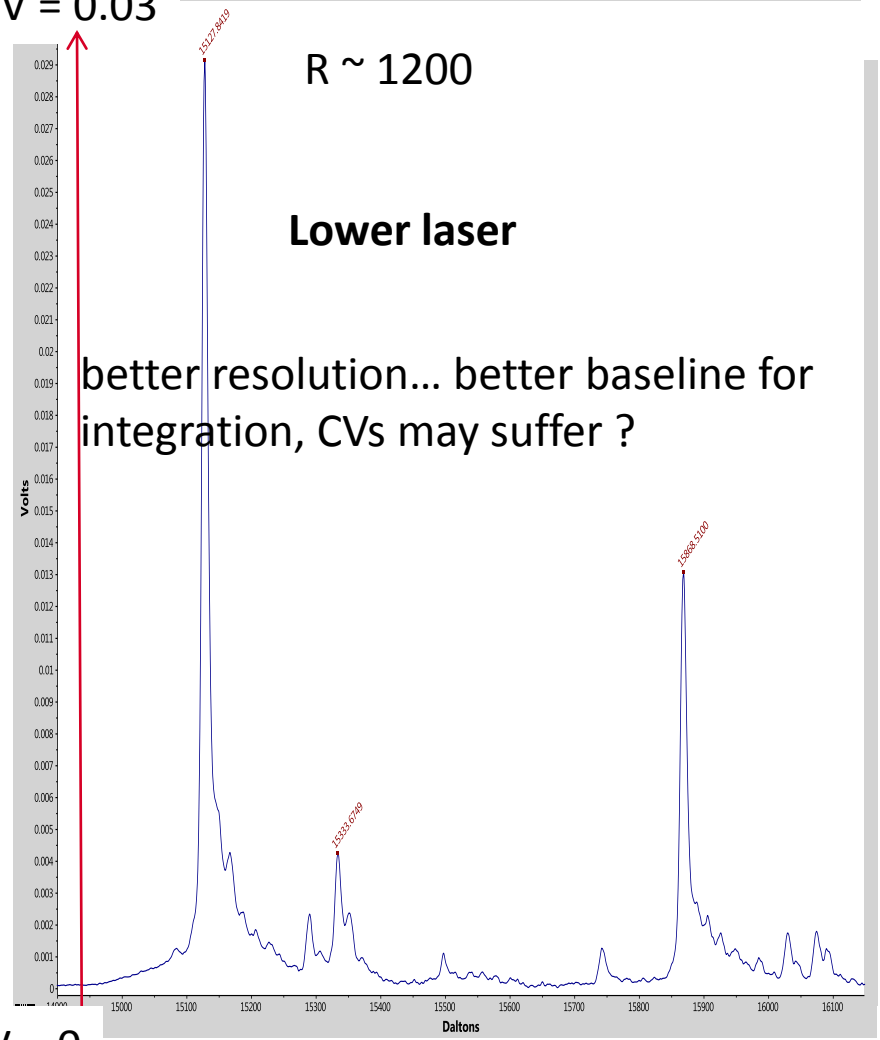
- High mass resolution
- High repetition rate laser (5 kHz Nd:YLF)
- High digitizer acquisition rate (50-100 pixels/sec)
- Continuous laser raster sampling
- MS/MS



Lateral spatial resolution = # shots / per spectrum (v stage / laser rep rate)

J.M Spraggins and R.M. Caprioli, *J. Am. Soc. Mass Spectrom.* **2011**, *22*, 1022-1031.

V = 0.03



V = 0

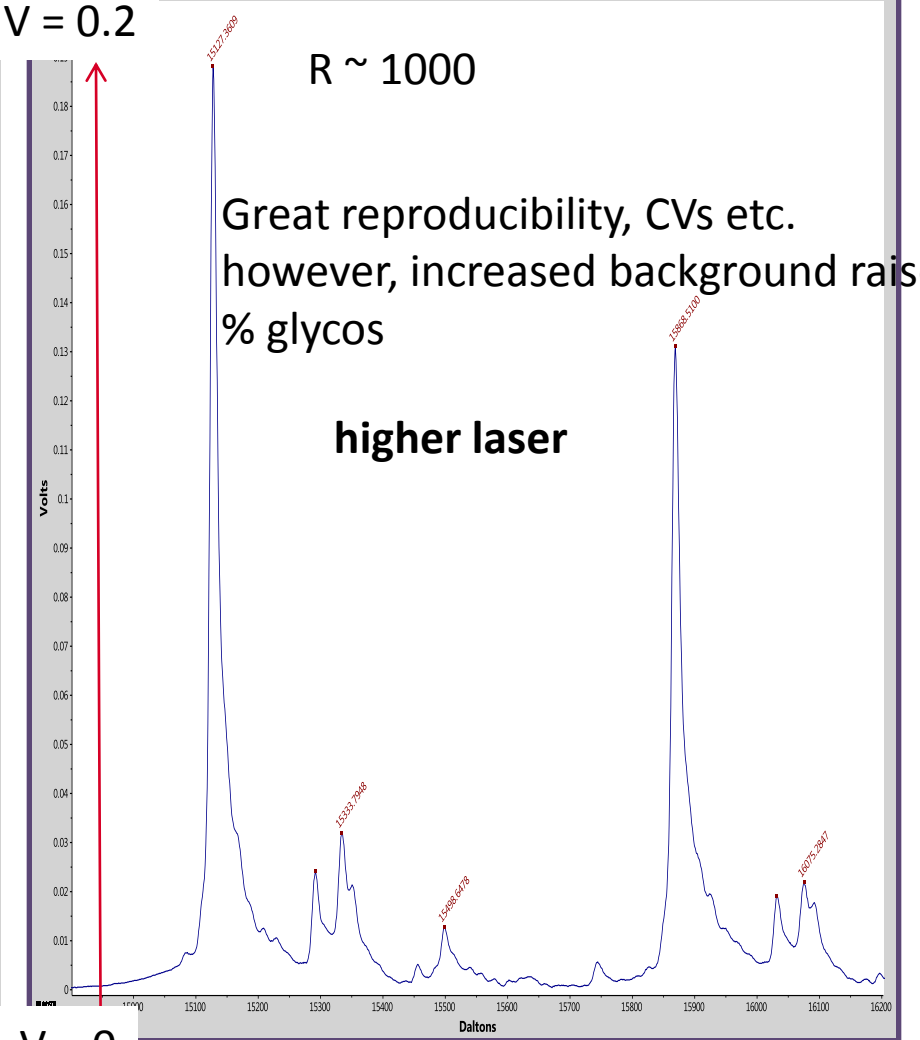
R ~ 1200

Lower laser

better resolution… better baseline for integration, CVs may suffer ?

1

V = 0.2



V = 0

R ~ 1000

Great reproducibility, CVs etc. however, increased background raises % glycos

higher laser

2

Reference Materials

Lyphochek Hemoglobin A1C Linearity Set

12000070

Human whole blood based control designed to verify linearity throughout the patient reportable range of HbA1C assays (6 x 0.5 mL, 1 of each level)

Assayed Values Typically Available for Common Analyzers

BIO-RAD ANALYZERS
- D-10™
- in2it™
- VARIANT™
- VARIANT™ II /TURBO

OTHER ANALYZERS
- Beckman Coulter® Synchron® and AU Systems
- Abbott Architect™ Series
- Roche COBAS® and Hitachi®
- Siemens ADVIA®, DCA and Dimension® Series
- TOSOH G7 and G8
- Trinity Biotech Series
- Ortho VITROS® Series
- Pointe Scientific

Expected Values for Hemoglobin A1C (%NGSP)*

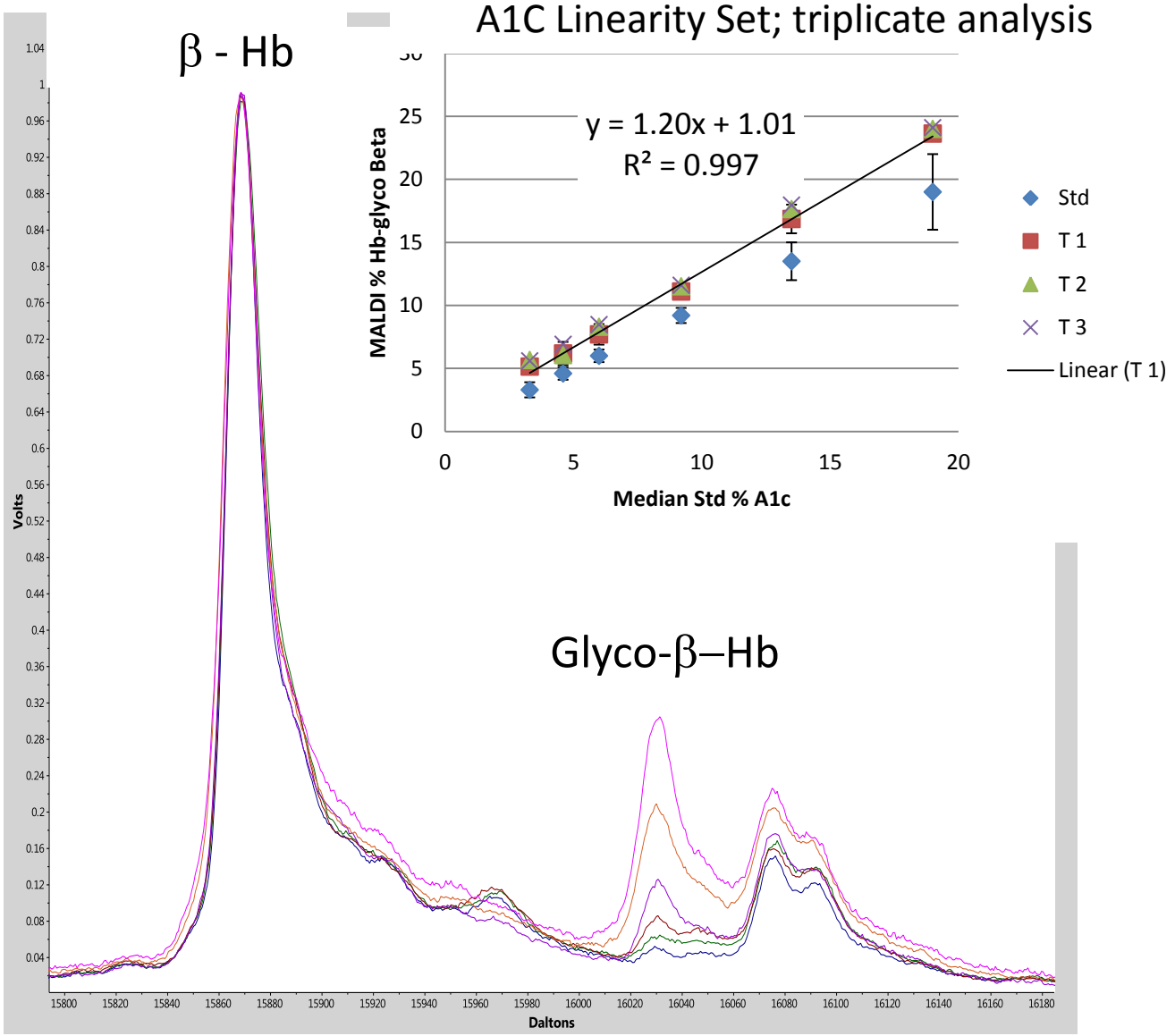
Level 1	2.7 - 3.8%
Level 2	4.1 - 5.1%
Level 3	5.5 - 6.5%
Level 4	8.4 - 10%
Level 5	12 - 15%
Level 6	16 - 22%

* Ranges reflect typical recovery values for this product. Exact values may vary depending on test/assay methodology, instruments, reagent, system calibrators or laboratory technique.

Analysis of Lyphochek® Hemoglobin A1C Linearity Set

	Expected HbA1c (%NGSP)
Level 1	2.7 - 3.8 %
Level 2	4.1 - 5.1 %
Level 3	5.5 – 6.5 %
Level 4	8.4 – 10 %
Level 5	12 – 15 %
Level 6	16 – 22 %

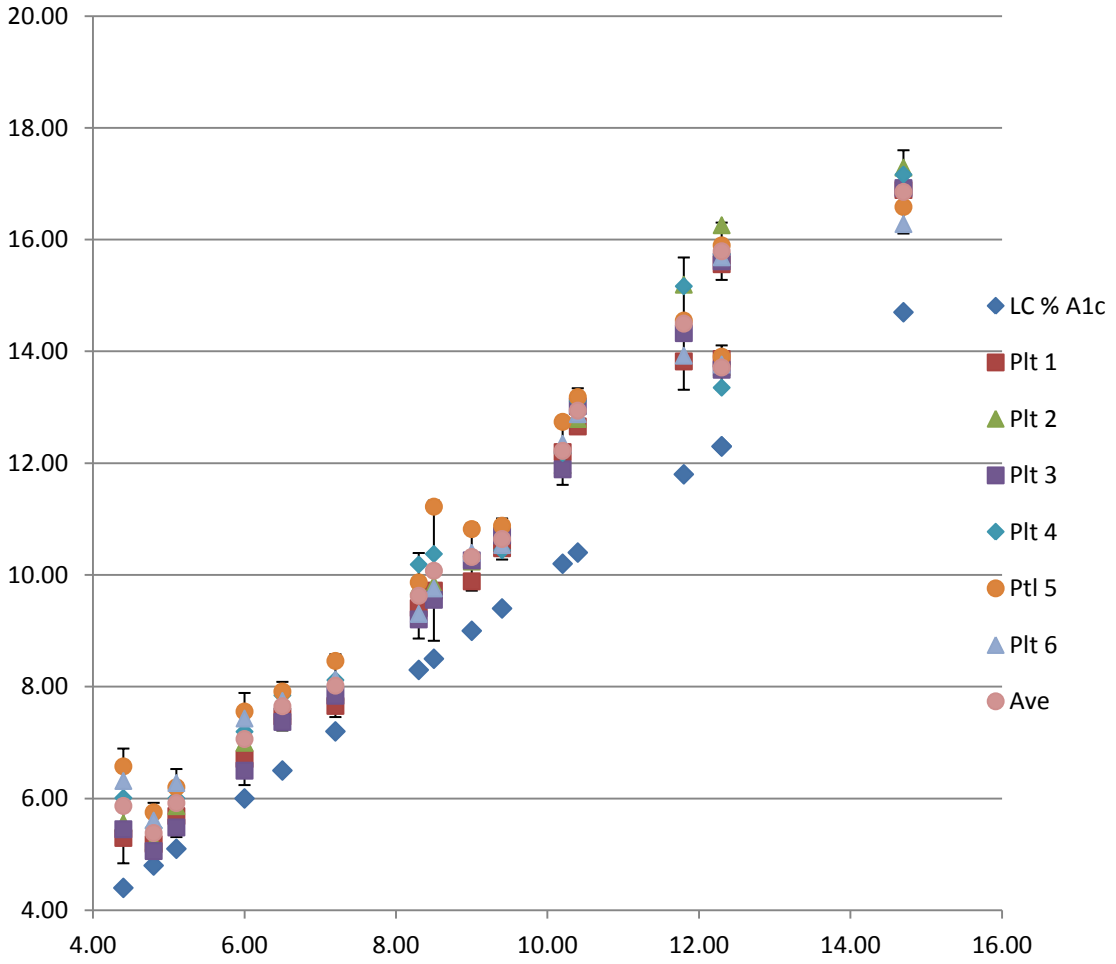
mid Std	Ave	Std Dev	CV
3.3	5.47	0.28	5.21
4.6	6.39	0.46	7.20
6	8.16	0.41	4.99
9.2	11.40	0.27	2.33
13.5	17.48	0.57	3.25
19	23.91	0.25	1.04



Power of replication

6 plates, 16 sample 5x replication
30 spots / sample
7000 – 8000 measurements / sample
8 start to finish preps
6 start to finish preparations
each data point = 8 pipette step

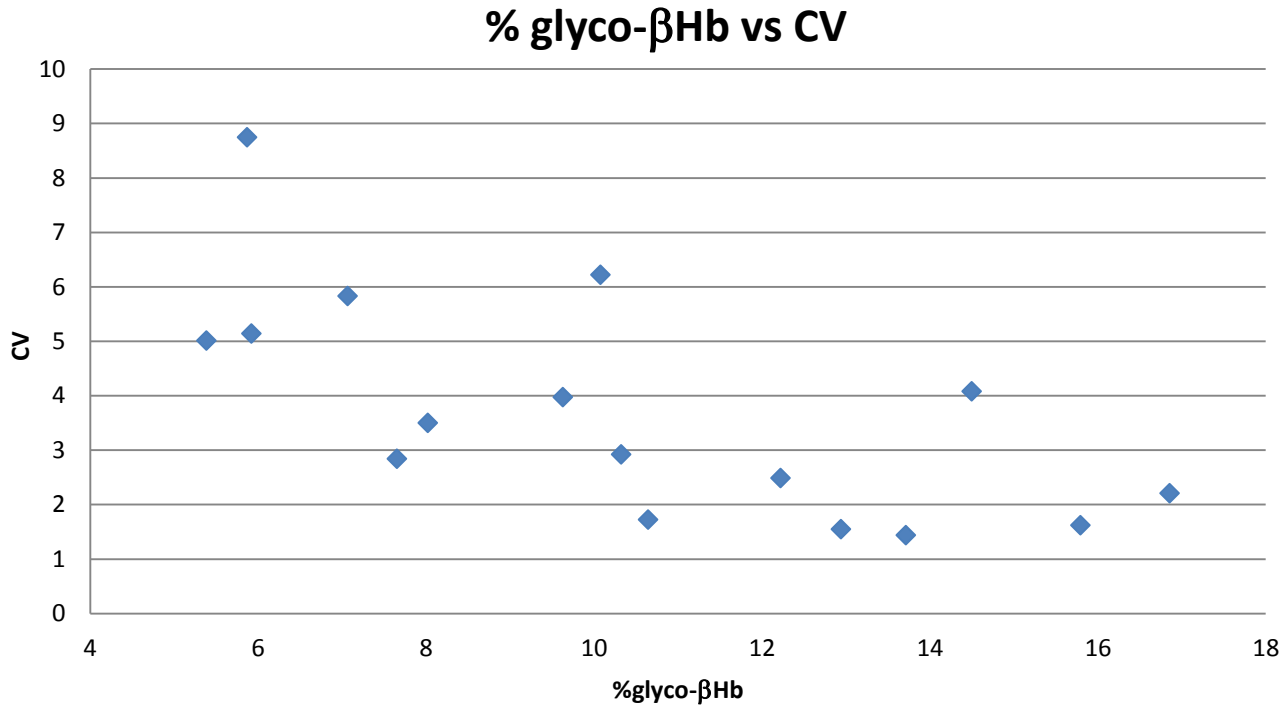
LC % A1c	Ave	Std Dev	CV
11.80	14.50	0.59	4.08
5.10	5.92	0.30	5.14
7.20	8.02	0.28	3.50
6.50	7.65	0.22	2.84
8.30	9.63	0.38	3.98
9.40	10.64	0.18	1.73
6.00	7.06	0.41	5.83
9.00	10.32	0.30	2.92
4.80	5.38	0.27	5.01
12.30	13.71	0.20	1.44
12.30	15.79	0.26	1.62
4.40	5.87	0.51	8.75
14.70	16.85	0.37	2.21
10.20	12.22	0.30	2.49
10.40	12.94	0.20	1.55
8.50	10.08	0.63	6.22
			Avg = 3.71



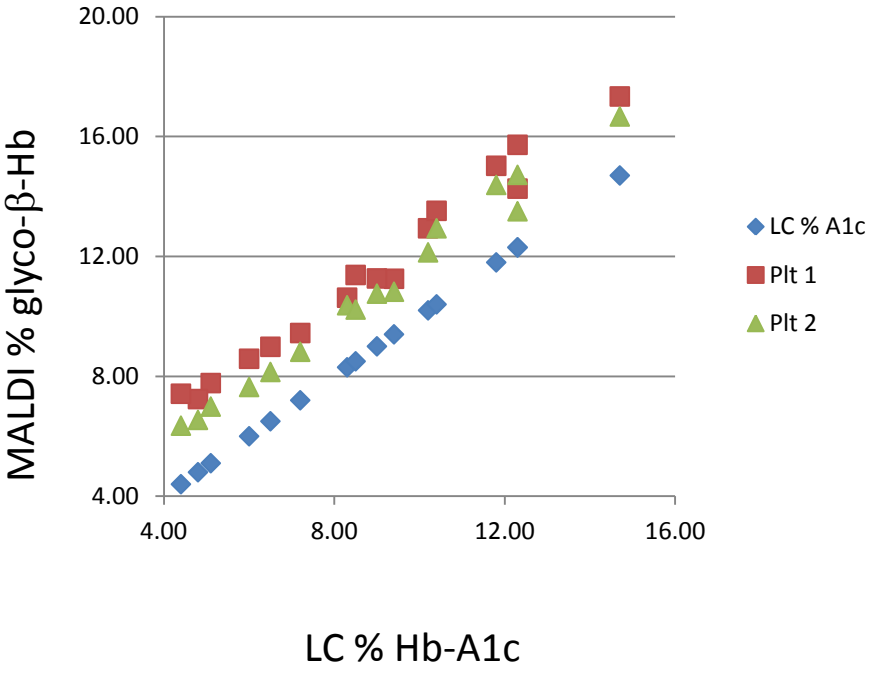
All the measurements associated with previous slide

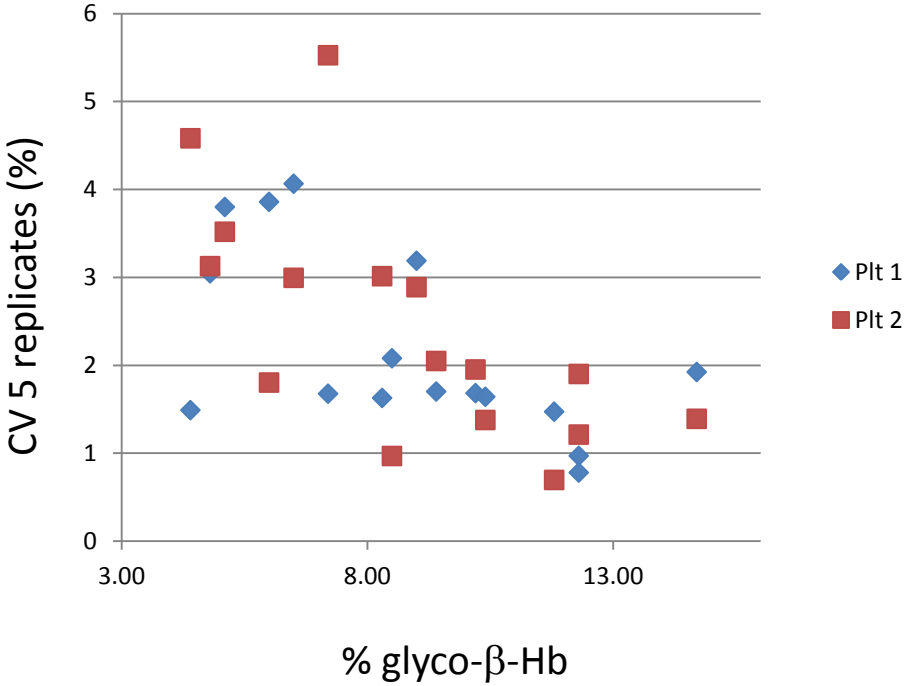
	LC % A1c	Plt 1	Plt 2	Plt 3	Plt 4	Ptl 5	Plt 6	Ave	Std Dev	CV
11.80	11.80	13.82	15.19	14.33	15.17	14.55	13.92	14.50	0.59	4.08
5.10	5.10	5.69	5.86	5.48	6.00	6.20	6.28	5.92	0.30	5.14
7.20	7.20	7.66	7.91	7.84	8.12	8.46	8.13	8.02	0.28	3.50
6.50	6.50	7.47	7.56	7.37	7.85	7.91	7.75	7.65	0.22	2.84
8.30	8.30	9.41	9.80	9.20	10.19	9.87	9.30	9.63	0.38	3.98
9.40	9.40	10.48	10.81	10.70	10.44	10.88	10.54	10.64	0.18	1.73
6.00	6.00	6.71	7.00	6.50	7.20	7.56	7.43	7.06	0.41	5.83
9.00	9.00	9.88	10.24	10.26	10.33	10.82	10.39	10.32	0.30	2.92
4.80	4.80	5.18	5.22	5.06	5.47	5.75	5.60	5.38	0.27	5.01
12.30	12.30	13.86	13.72	13.67	13.35	13.91	13.76	13.71	0.20	1.44
12.30	12.30	15.56	16.26	15.61	15.74	15.90	15.68	15.79	0.26	1.62
4.40	4.40	5.29	5.56	5.45	6.01	6.57	6.31	5.87	0.51	8.75
14.70	14.70	16.89	17.29	16.92	17.16	16.58	16.28	16.85	0.37	2.21
10.20	10.20	12.20	11.96	11.89	12.18	12.74	12.35	12.22	0.30	2.49
10.40	10.40	12.66	12.79	13.02	13.11	13.18	12.88	12.94	0.20	1.55
8.50	8.50	9.72	9.82	9.56	10.38	11.22	9.76	10.08	0.63	6.22
										Avg. = 3.71

Variance as a function of signal



Comparison of 2 sample plates with A1c estimates LC

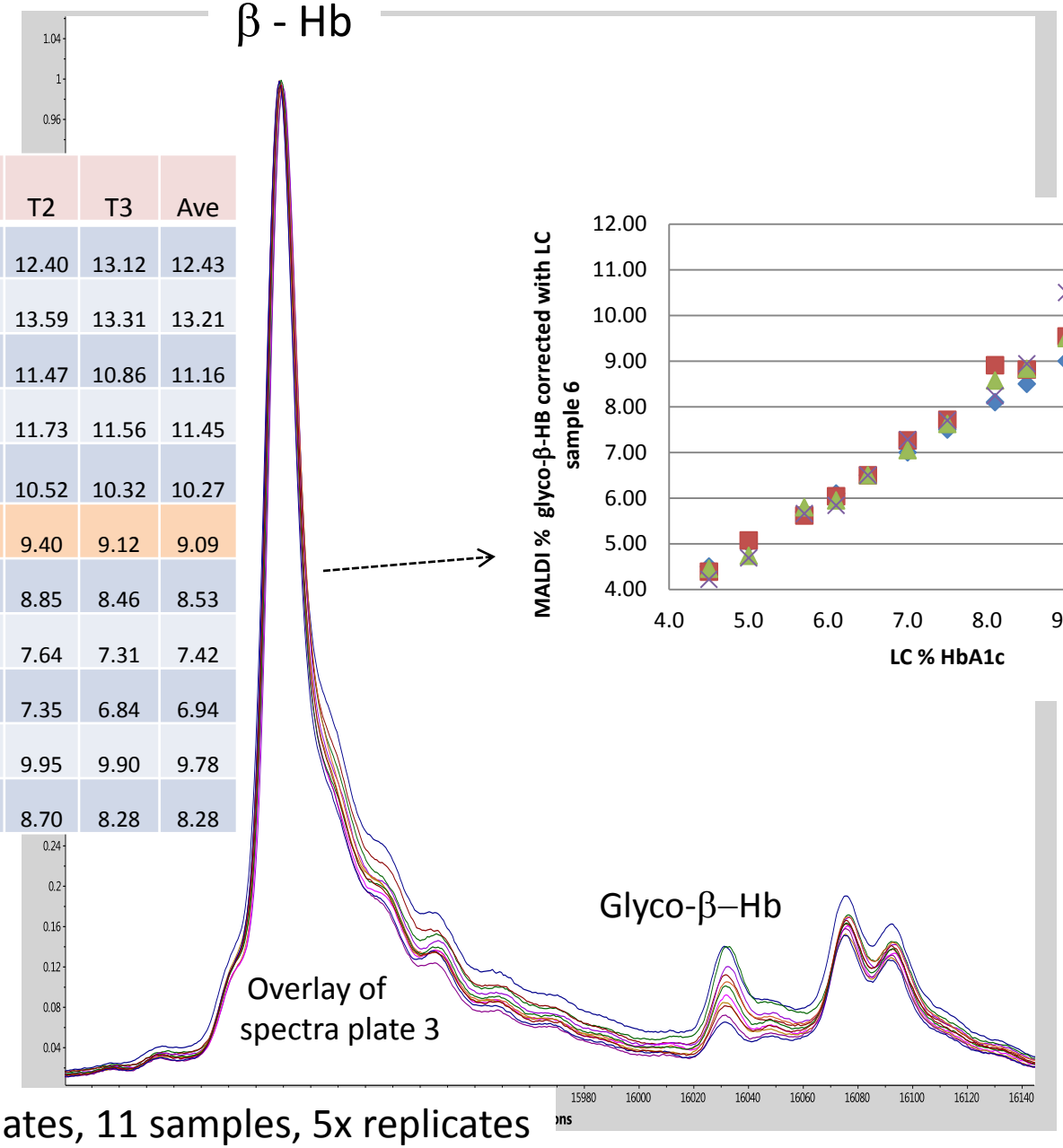




-One plate has consistently higher measurements
-Higher measurement variance with lower % gylcosylation

Blood Samples Jan. 2015; measurements high but easily corrected

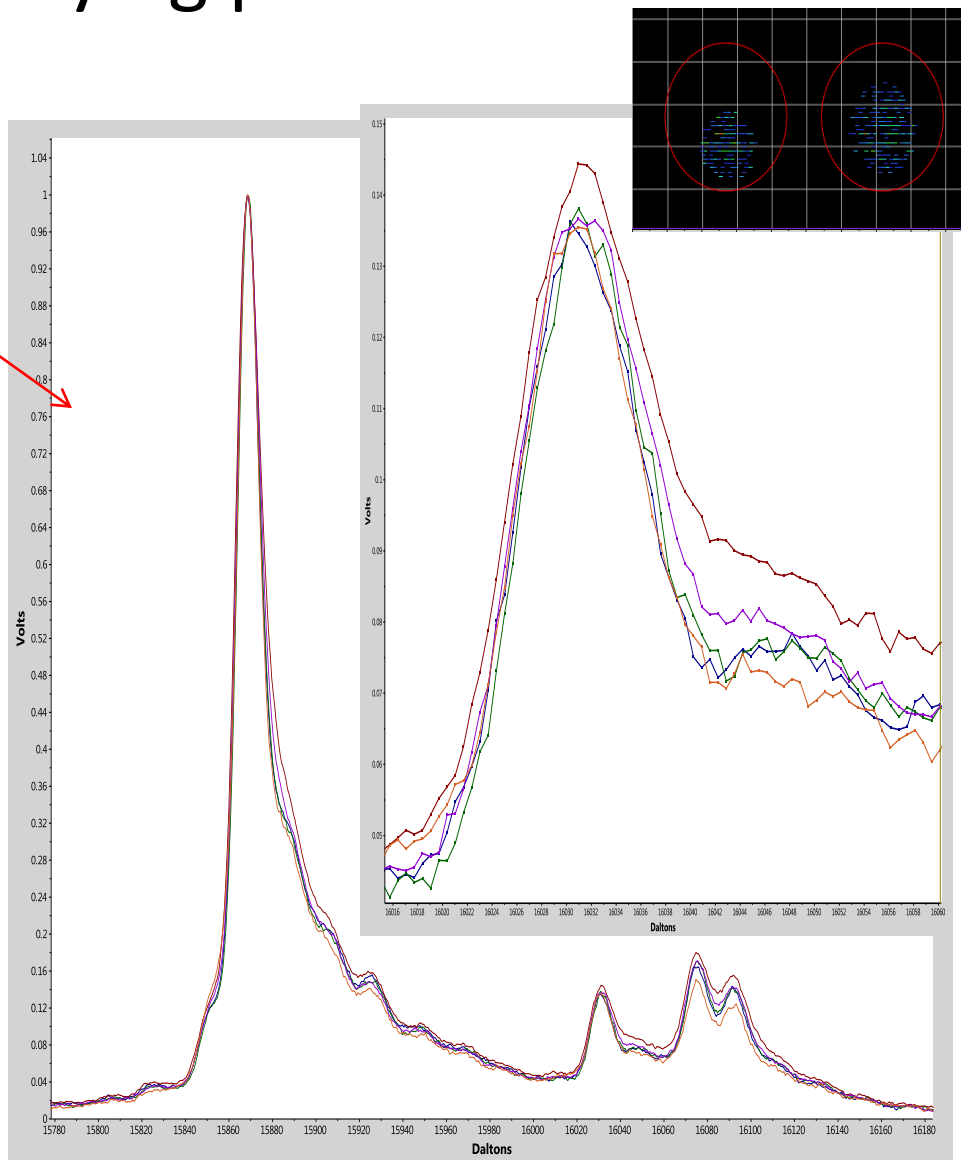
LC % A1c	T1	T2	T3	Ave
9.00	11.78	12.40	13.12	12.43
9.90	12.72	13.59	13.31	13.21
8.10	11.15	11.47	10.86	11.16
8.50	11.05	11.73	11.56	11.45
7.50	9.95	10.52	10.32	10.27
6.50	8.74	9.40	9.12	9.09
6.10	8.28	8.85	8.46	8.53
5.00	7.31	7.64	7.31	7.42
4.50	6.63	7.35	6.84	6.94
7.00	9.50	9.95	9.90	9.78
5.70	7.86	8.70	8.28	8.28

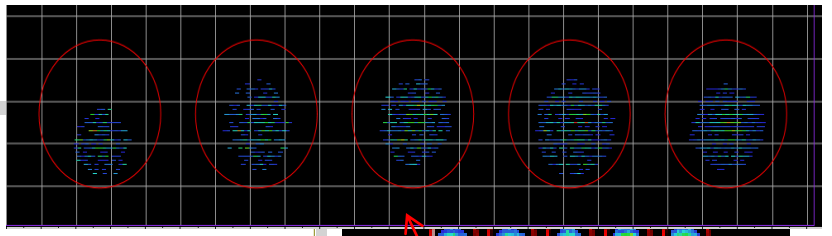


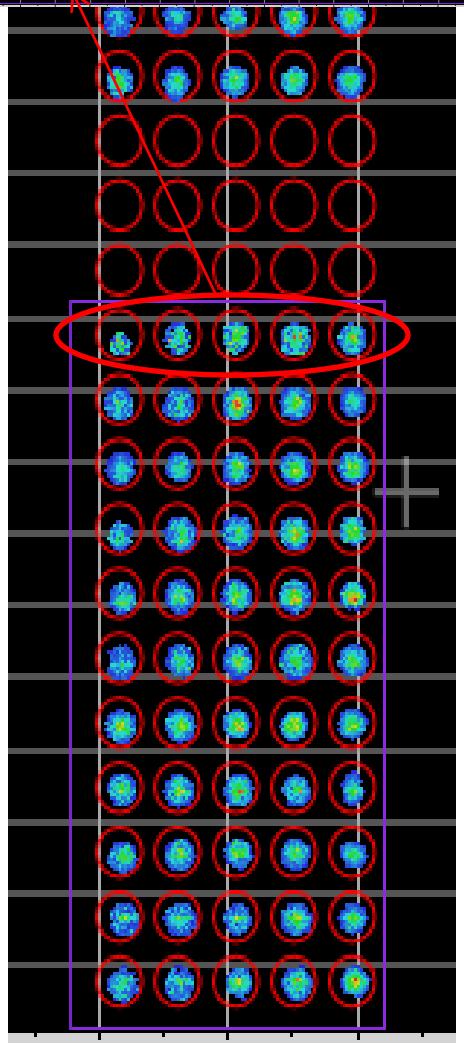
3 plates, 11 samples, 5x replicates

Plate 3; outlying point also worst CV

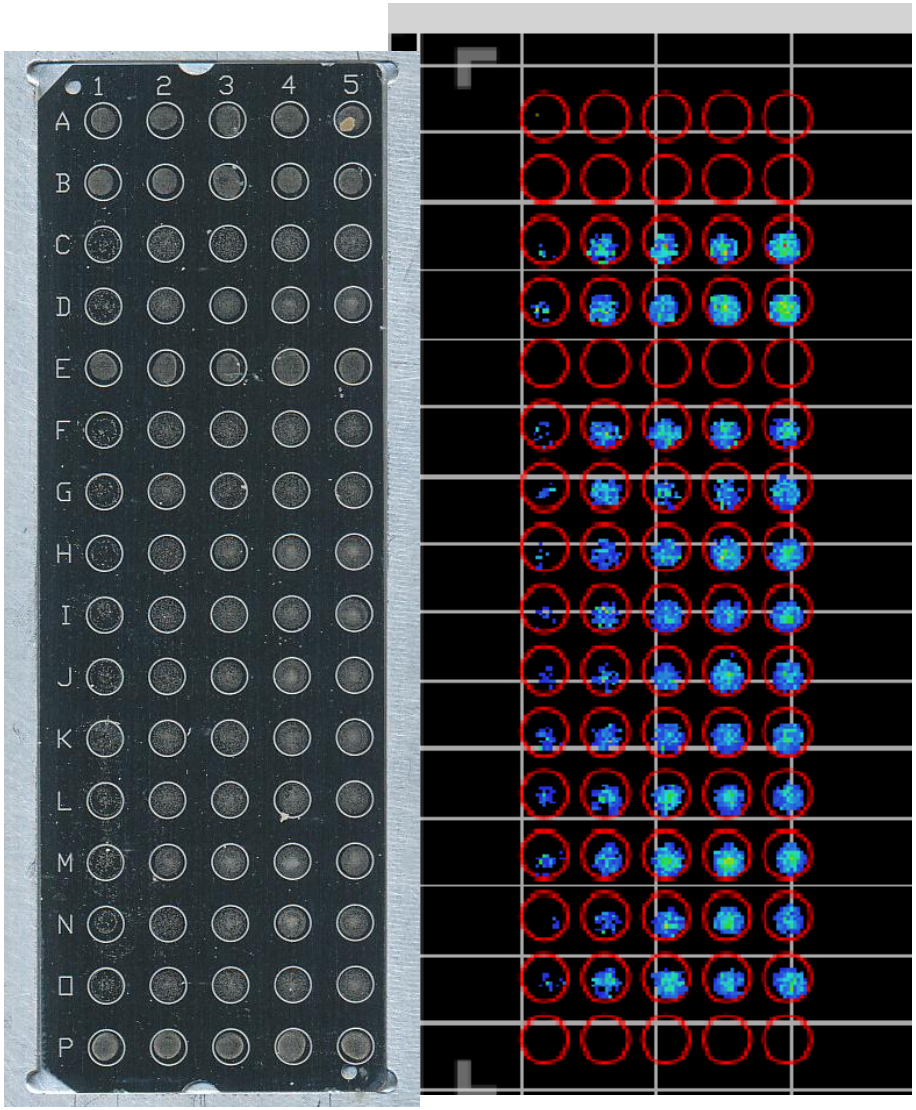
% glyco β–Hb	Std Dev	% CV
13.12	0.56	4.48
13.31	0.22	1.73
10.86	0.29	2.76
11.56	0.11	1.05
10.32	0.18	1.87
9.12	0.19	2.19
8.46	0.29	3.47
7.31	0.13	1.87
6.84	0.22	3.23
9.90	0.21	2.21
8.28	0.12	1.55

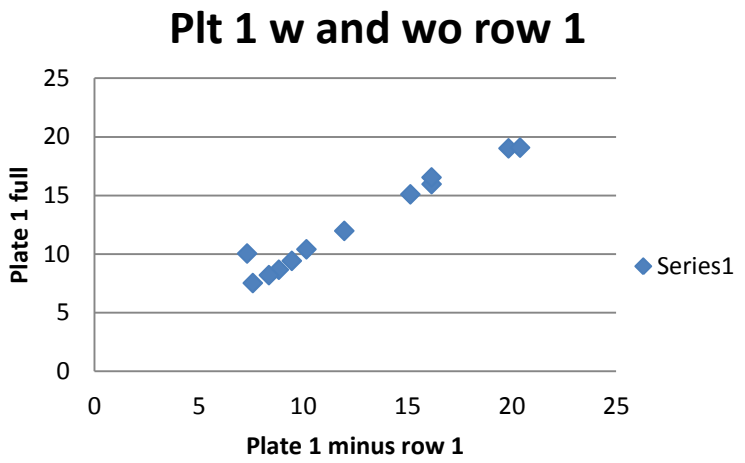






Row 1 low signal = lack of matrix





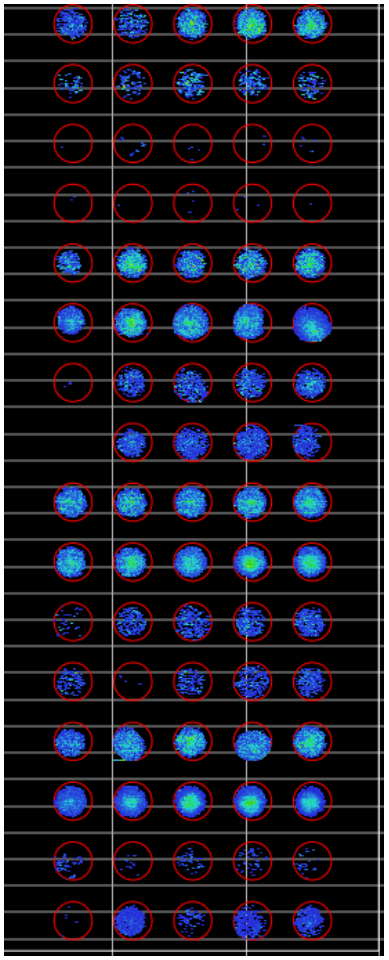
Row	CV	CV - r1
3	9.68	1.02
4	15.75	3.58
5		
6	60.96	2.39
7	3.88	4.39
8	5.54	0.80
9	1.70	1.83
10	1.71	1.97
11	3.64	3.74
12	4.77	2.42
13	5.08	3.45
14	3.30	2.33
15	5.47	1.83

<u>Remove row 1</u>

1)CV's improve

2)Ratios largely unchanged

Matrix concentration study and sample acquisition study fouled by pipette tips

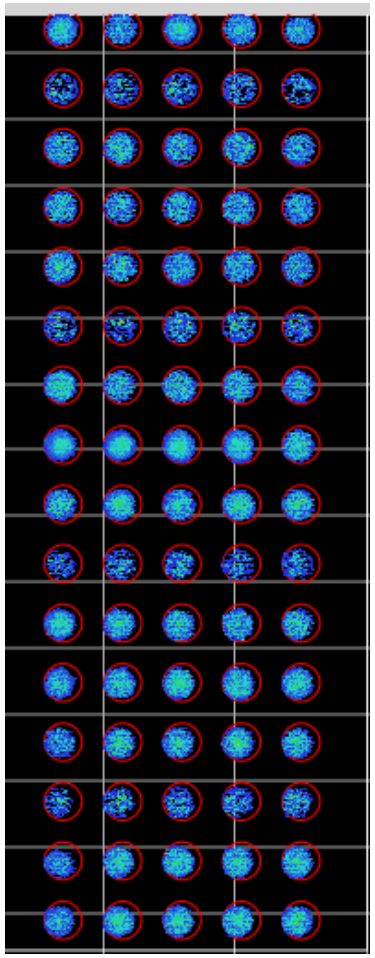


10 mg/mL

7.5 mg/mL

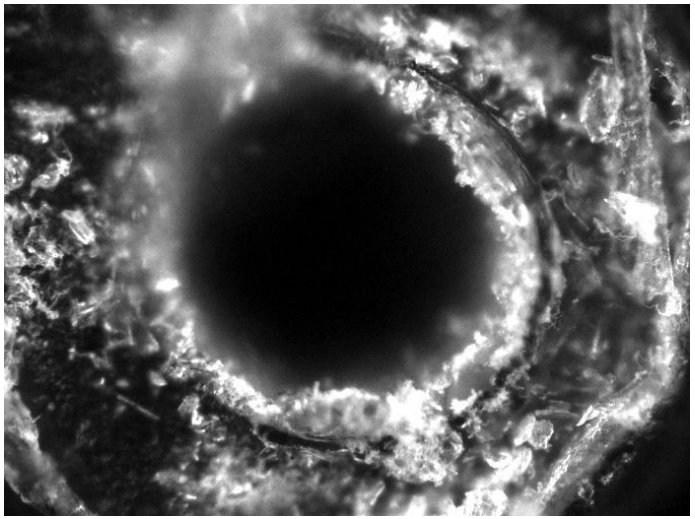
5 mg/mL

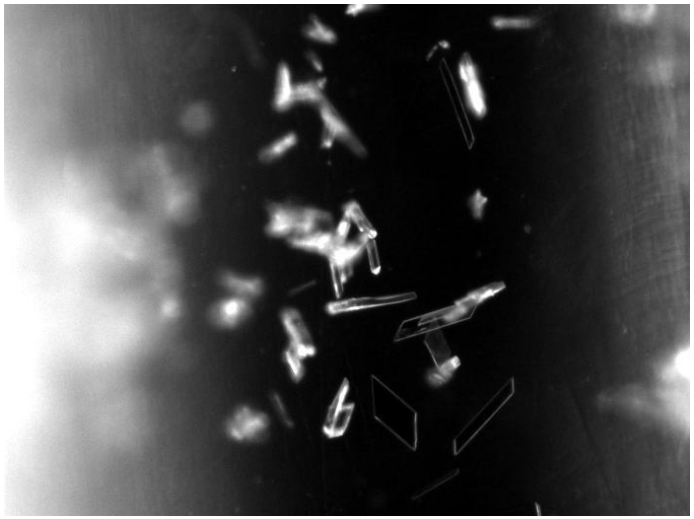
10mg/mL 10%Meth

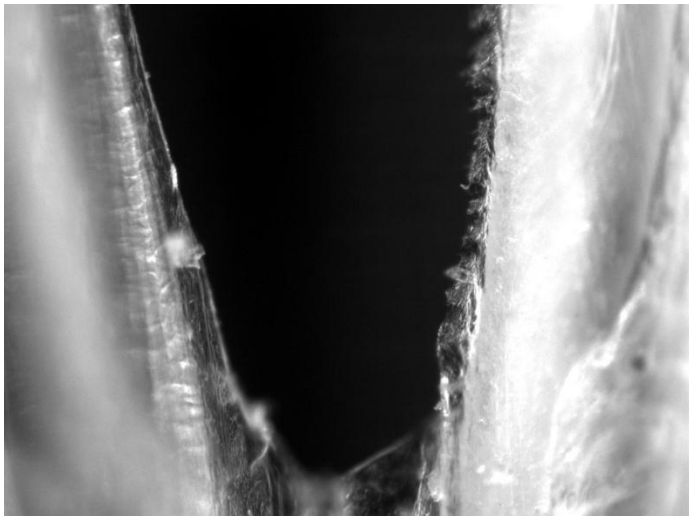


Soak tip 50:50 acetonitrile:H_2O

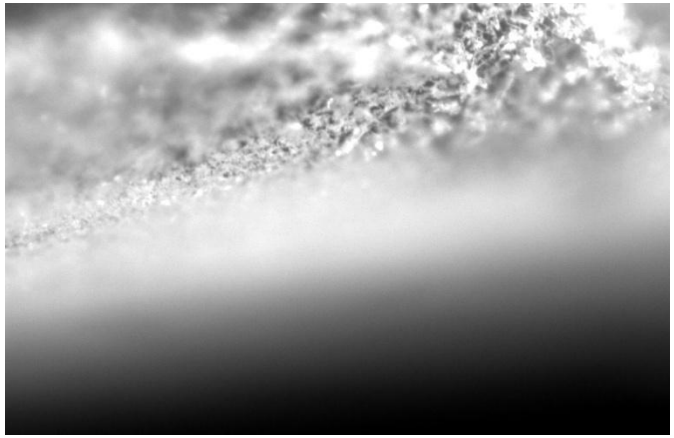
10 mg/mL - A mess at bottom and top

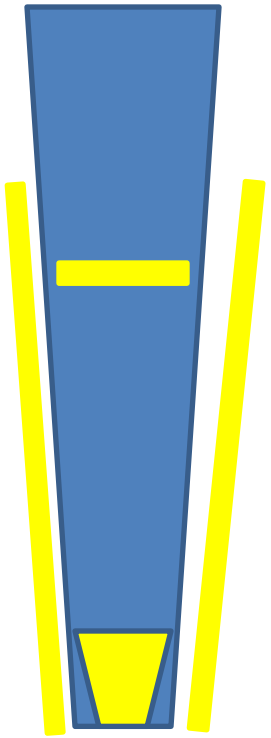






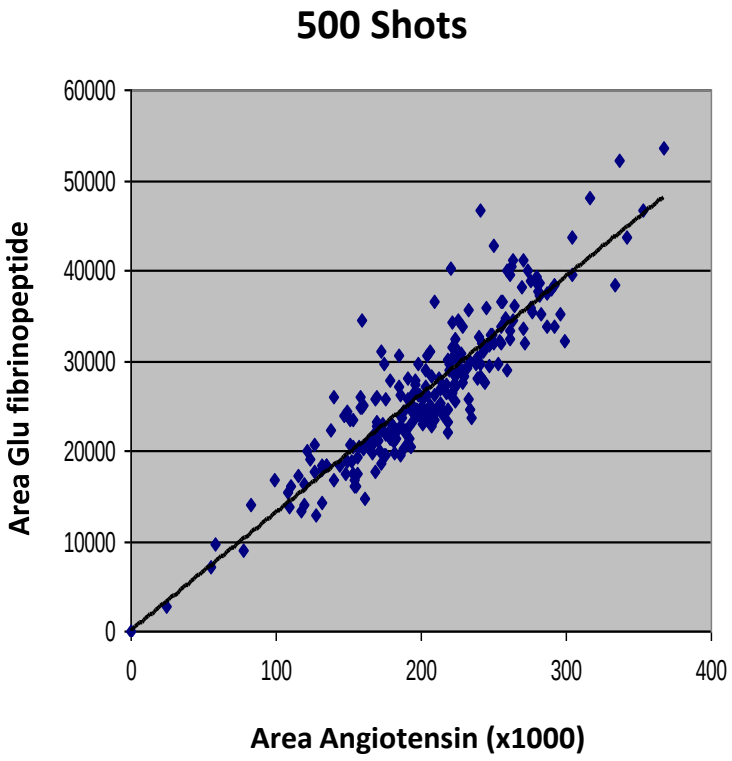
10 mg/mL outside surface

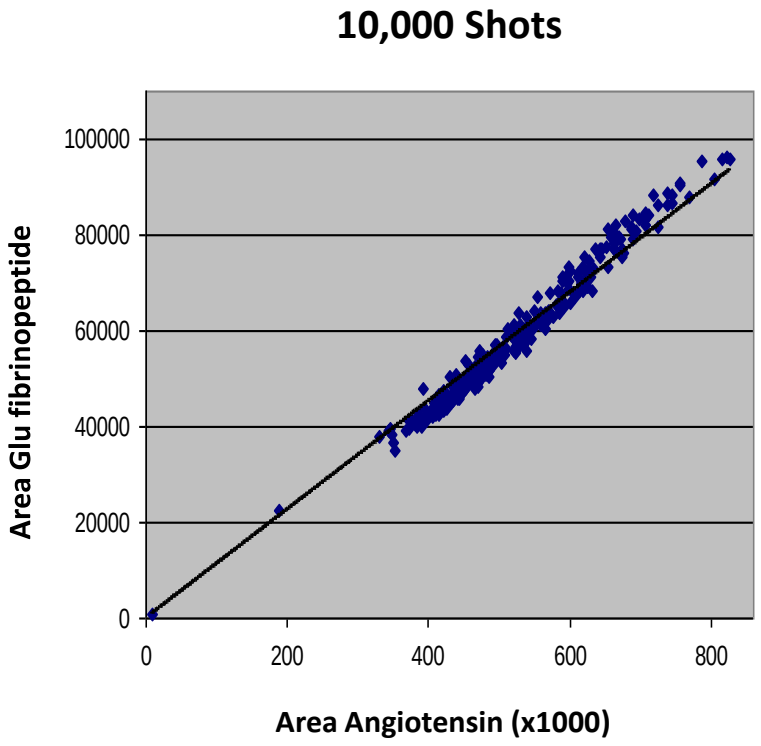




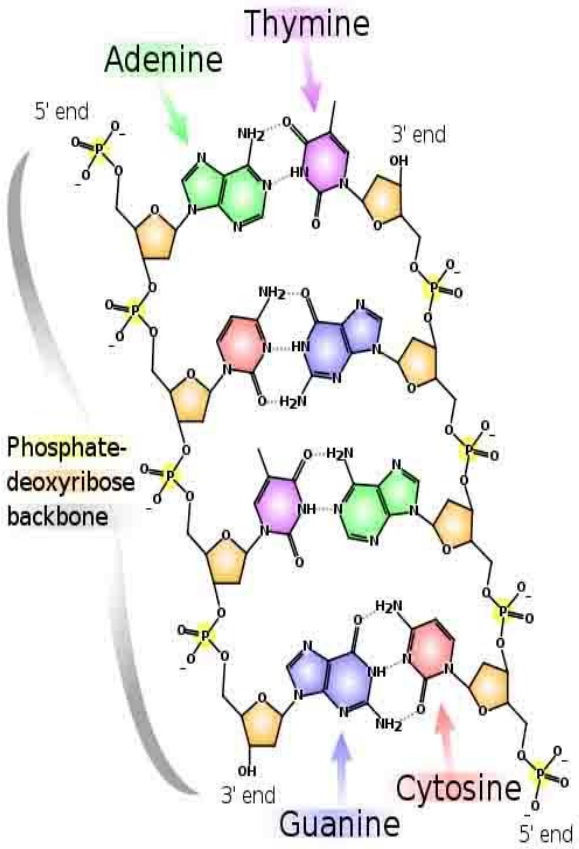
Matrix deposits at tip outlet at the upper margin and
On the outside

Shot number vs. signal reproducibility





Nucleic Acid Analyses



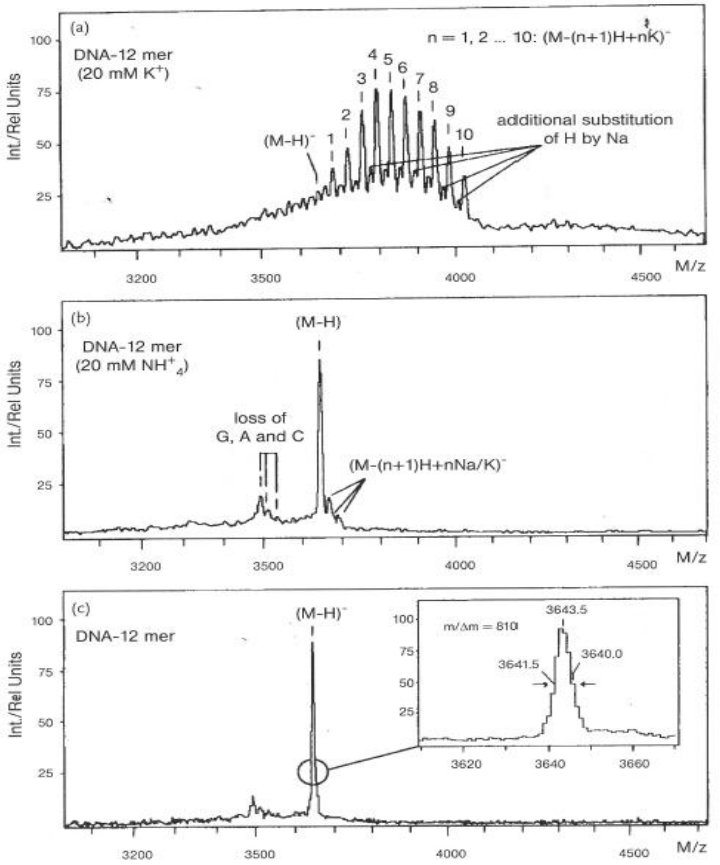
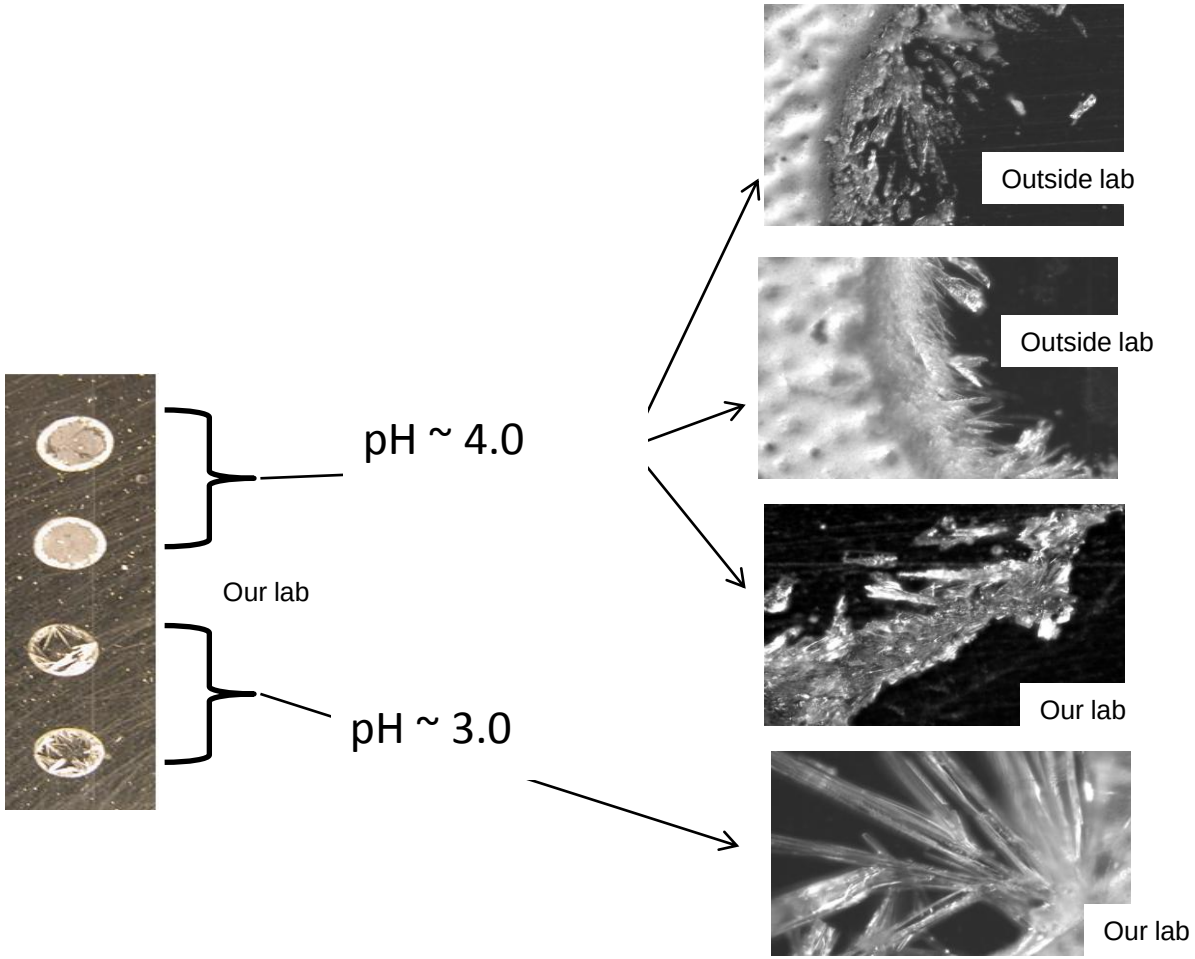


Figure 5.1 Negative-ion mode mass spectra of a 12mer DNA oligonucleotide, demonstrating the importance of careful sample preparation and conditioning of the phosphate backbone of nucleic acids for efficient analysis by MALDI-TOF-MS. (a) At concentrations of sodium or potassium similar to those conventionally used in molecular biological assays, the 12mer shows a distribution of up to 10 potassium ions attached to the phosphate backbone. Sodium and potassium can be efficiently replaced by employing ammonium-based buffers; (b) The 12mer oligonucleotide was dissolved in 20 mM ammonium acetate solution, and the Na^+ and K^+ adduct formation was significantly reduced; (c) The use of cation-exchange polymer beads further leads to a significant reduction in adduct formation and improved signal-to-noise ratios. Reprinted from Ref. [10] by permission of Oxford University Press.

Oligonucleotide analysis was finicky



Focusing plates helped this analysis

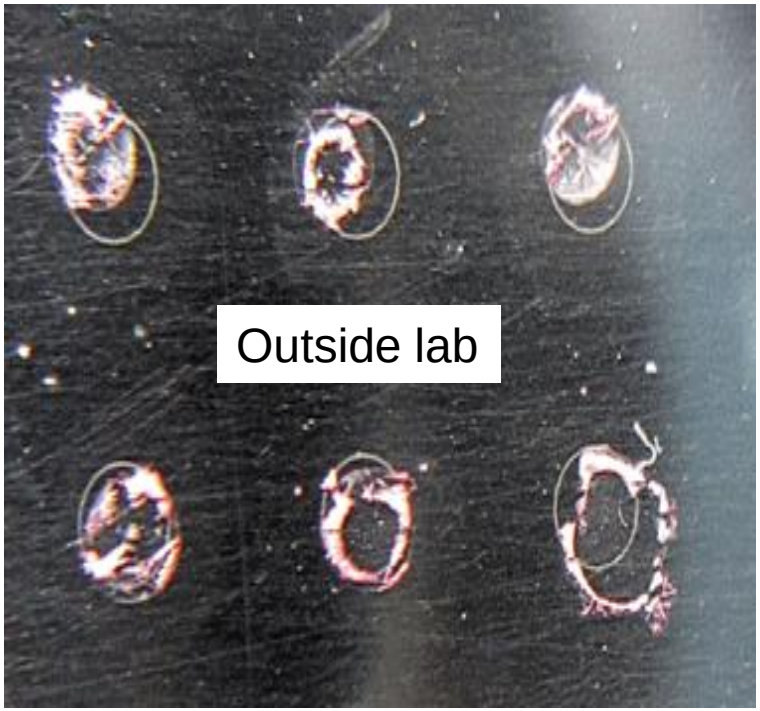


Plate not DNA but shows µfocusing

Matrices for Oligo-MALDI-TOF

- Table 1
- The typical matrix for UV-MALDI is an aromatic acid, which efficiently absorbs laser energy
- Matrix MALDI performance Comments
- 3-Hydroxypicolinic acid (3-HPA) +++ Gold standard for oligonucleotides greater than 10 bases, less suitable for smaller
- oligonucleotides due to matrix adducts but still good performance
- Picolinic acid ++ Usually a co-matrix of 3-hydroxypicolinic acid
- 6-Aza-2-thiothymine ++ Little fragmentation, rarely used in practice
- 4-Hydroxy-3-methoxycinnamic acid (ferulic acid) + Rarely used in practice, rather for oligonucleotides larger than 3500 Mr
- 2,5-Dihydroxybenzoic acid (DHB) + Rarely used for oligonucleotides
- 2,4,6-Trihydroxyacetophenone (THAP) +++ An alternative to 3-HPA, particularly for small oligonucleotides, can be mixed
- with 2,3,4-THAP
- 2,3,4-Trihydroxyacetophenone (THAP) +++ An alternative to 3-HPA, particularly for small oligonucleotides, can be mixed
- with 2,4,6-THAP
- Alpha-cyano-4-hydroxycinnamic Acid (++) Particularly for PNA, modified and small oligonucleotides with
- charge-neutral backbones
- Alpha-cyano-4-hydroxycinnamic acid methyl ester (+++) For PNA and modified oligonucleotides with charge-neutral backbones
- Anthranilic acid + Rarely used in practice, rather for oligonucleotides larger than 3500 Mr
- Salicylamide + Rarely used in practice, rather for oligonucleotides larger than 3500 Mr
- “+++” means excellent, “++” good and “+” medium-quality performance in practice. Brackets indicate a limited use of the matrix. More information can be found in
- package inserts of companies supplying these products (e.g., http://www.sigmaaldrich.com/img/assets/4242/fl_analytix6_2001_new.pdf or http://www.bdal.de/care).

Sascha Sauer “*The essence of DNA sample preparation for MALDI mass spectrometry*”
J. Biochem. Biophys. Methods 70 (2007) 311–318