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LC ESI/MS reference measurement procedure for HbA_2

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The determination of HbA_2 is accepted as the gold standard in the screening for β-thalassemia carriers.

Highly accurate and precise measurements are needed, also because the difference between normal and pathological HbA_2 values is slight.

Fetal hemoglobin: assessment of glycation and acetylation status by electrospray ionization mass spectrometry

Andrew S. Davison, Brian N. Green and Norman B. Roberts

Clin Chem Lab Med 2008;46(9):1230–1238

….Here, we describe the evaluation of ESI-MS for measurement of glycated (GHbF) and acetylated (AcHbF) fetal hemoglobin and the identification by mass of different chains of fetal hemoglobin……..

AIM: To develop a reference measurement procedure based on the quantification of intact globin chains by LC-ESI/MS

I STEP:
CHOICE OF THE ANALYTICAL CONDITIONS

II STEP:
VALIDATION OF THE METHOD

III STEP:
INTERLABORATORY COMPARISON

Sample preparation

Fresh blood in EDTA

Filtration through cellulose column → Removal of WBC, platelets, plasma

RBC

Lysis with H_2O/CCl_4 → Removal of cellular stroma

Hemolysate

Dilution with acetonitrile/ formic acid

Desalting on cation exchange resin bead → centrifugation

Samples for MS
Hb 1 mg/mL
Acetonitrile 50 %
Formic acid 0.18 %

Davison AS et al. Clin Chem Lab med 2008;46:1230 (modified)

Analytical conditions

Surveyor LC Pump Method

Eluents

A: H_20-CH_3CN 4:1+0.05% TFA

B: H_20-CH_3CN 2:3+0.05% TFA

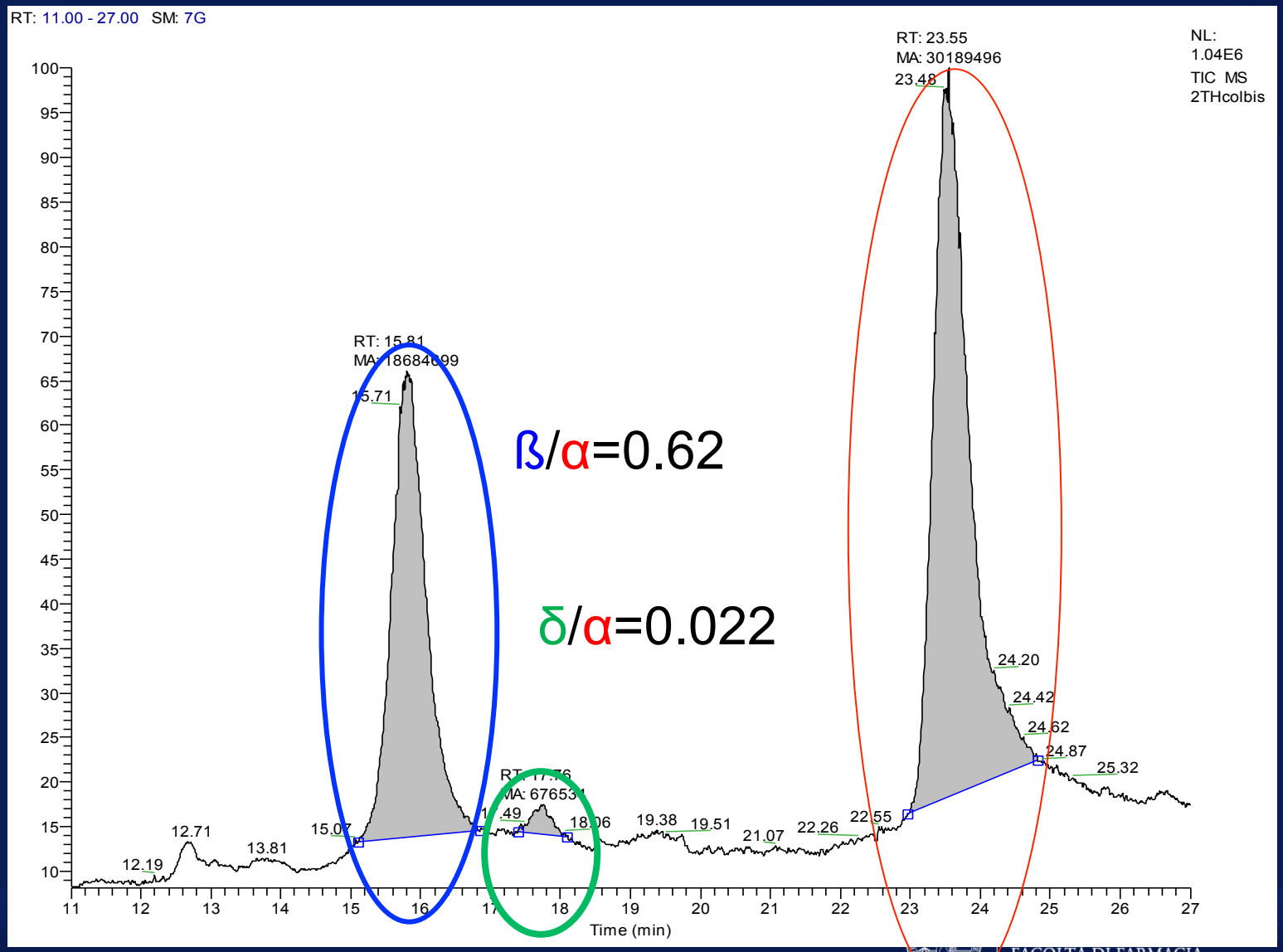
Column: Vydac C4 250x4,6mm

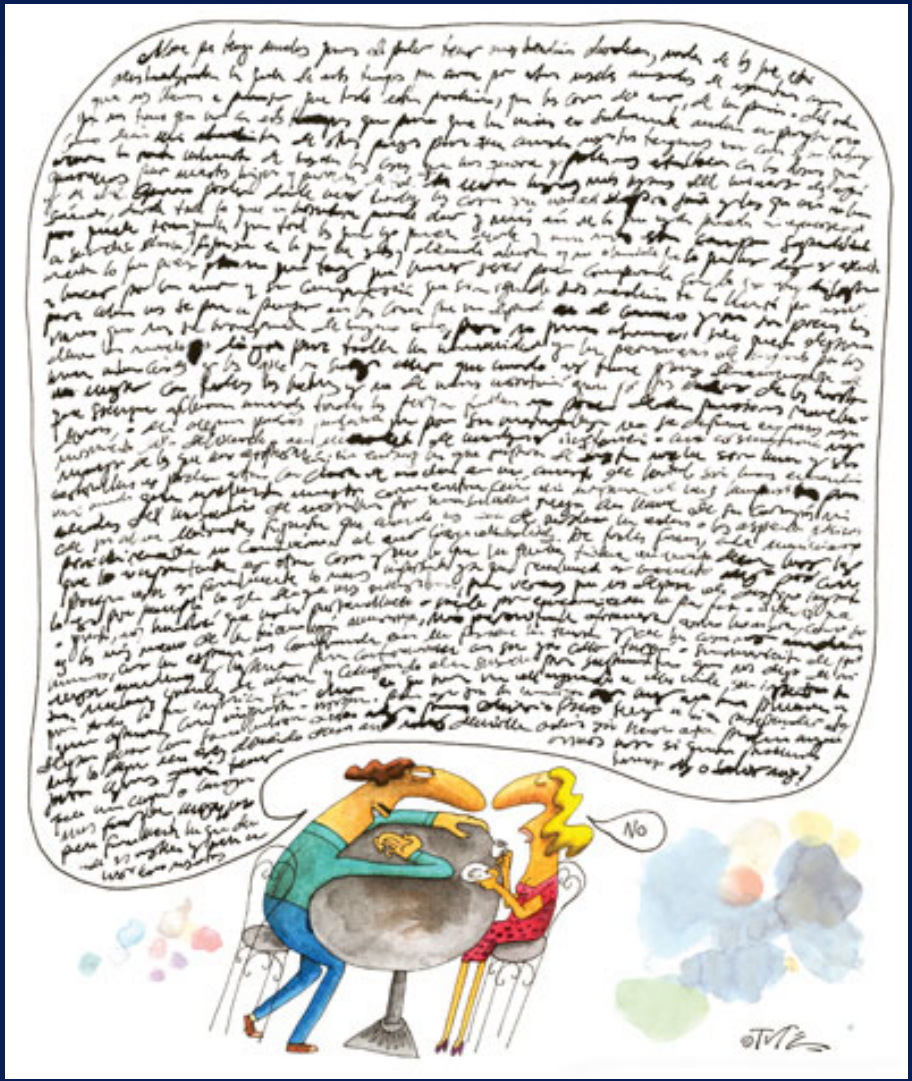
Program

Time (min)	Flow (mL/min)	A (%)	B (%)
0.00	0.800	48.0	52.0
2.00	0.800	48.0	52.0
13.00	0.800	42.0	58.0
16.00	0.800	42.0	58.0
25.00	0.800	31.0	69.0
30.00	0.800	48.0	52.0
35.00	0.800	48.0	52.0

Instrumentation: ESI-linear ion trap (LTQ, Thermofisher, USA)

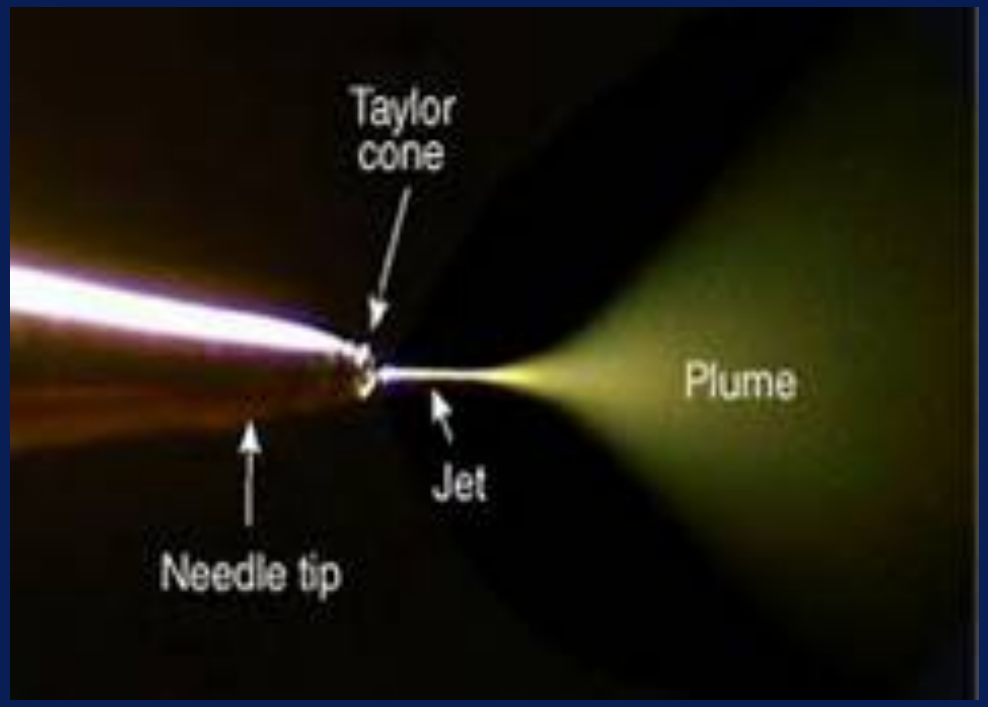
TIC trace of a β-thalassemic subject hemoglobin





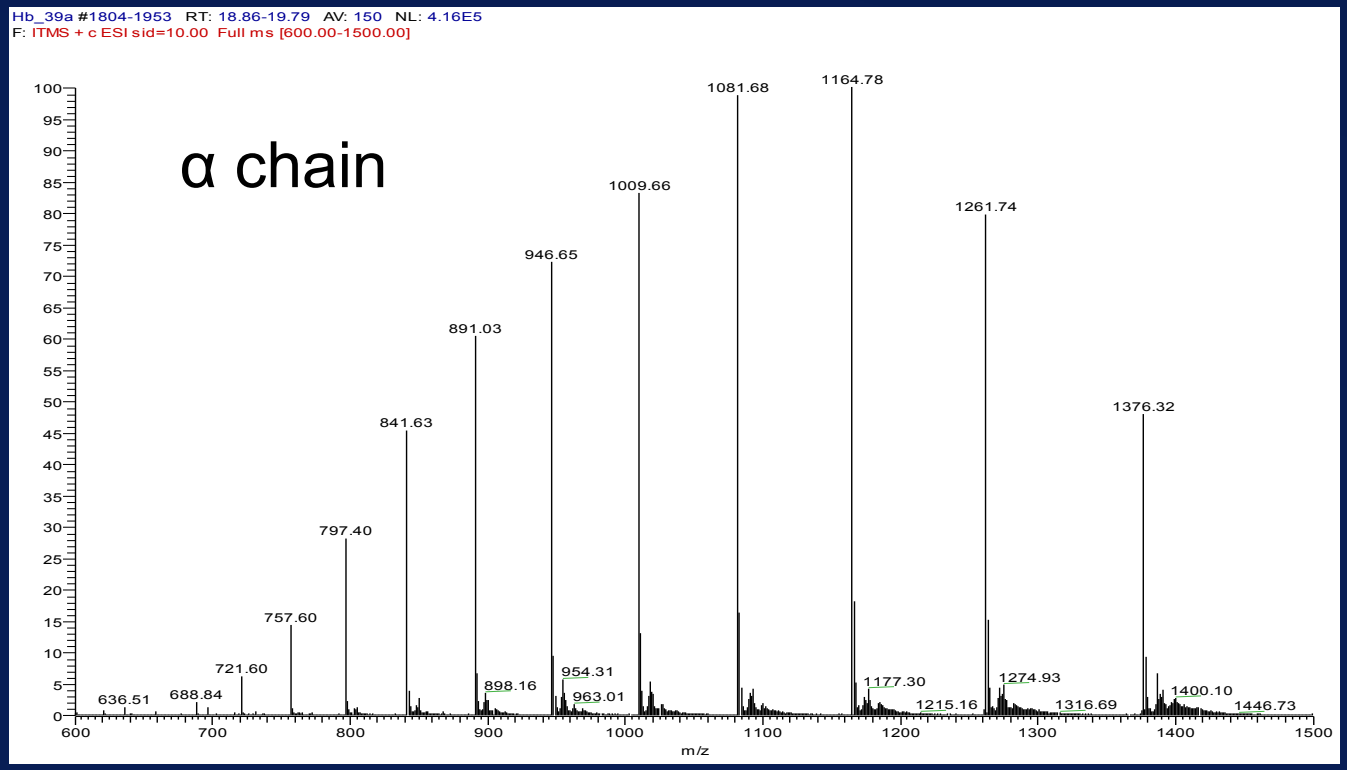
Only few words on ESI-ion trap mass spectrometry….

The general term "atmospheric pressure ionization" (API) includes the most notable technique, **electrospray ionization (ESI)**



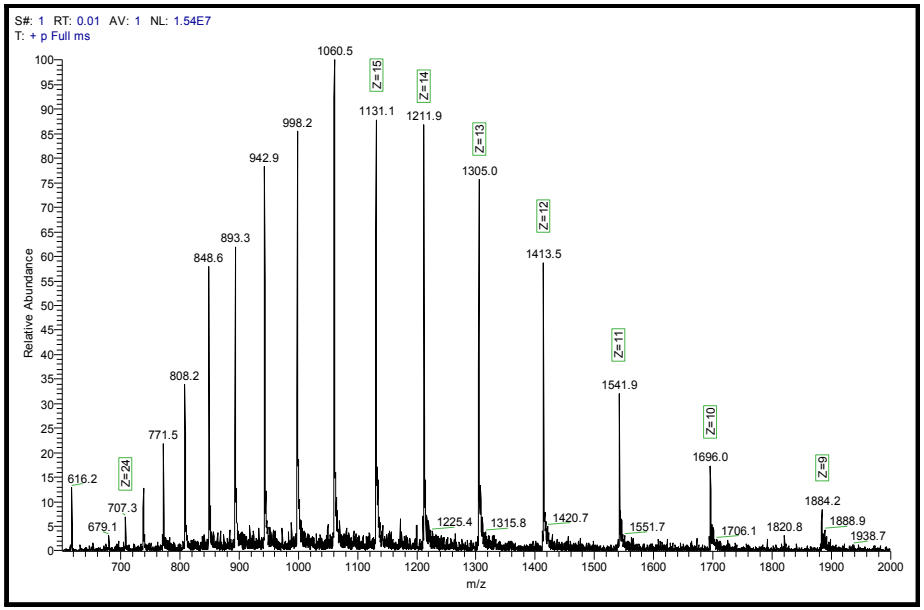
The gas phase peptide ions can be generated from the online eluate of a LC column or from direct injection

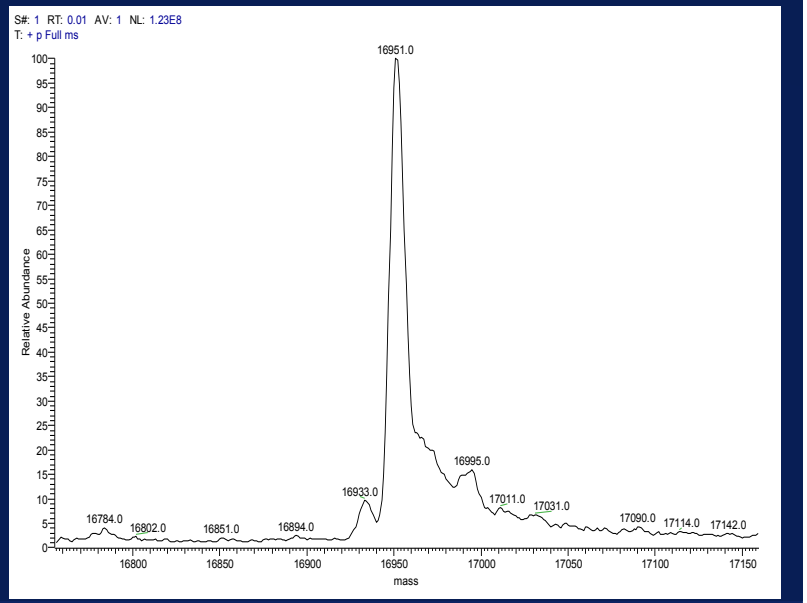
ESI is able to produce single or multiple charged ions, depending on several factors.



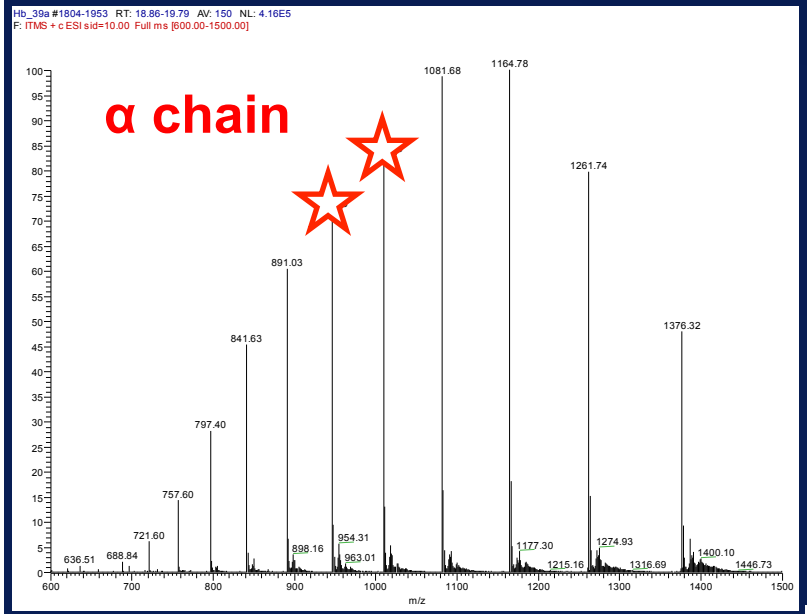
Since MS measure the mass-to-charge (*m*/*z*) ratio, the ESI mass spectrum for a large molecule typically contains multiple signals corresponding to the different charge states.

The target molecules are ionized into multiple charge states producing a waveform spectrum that can be de-convoluted into parent peaks.



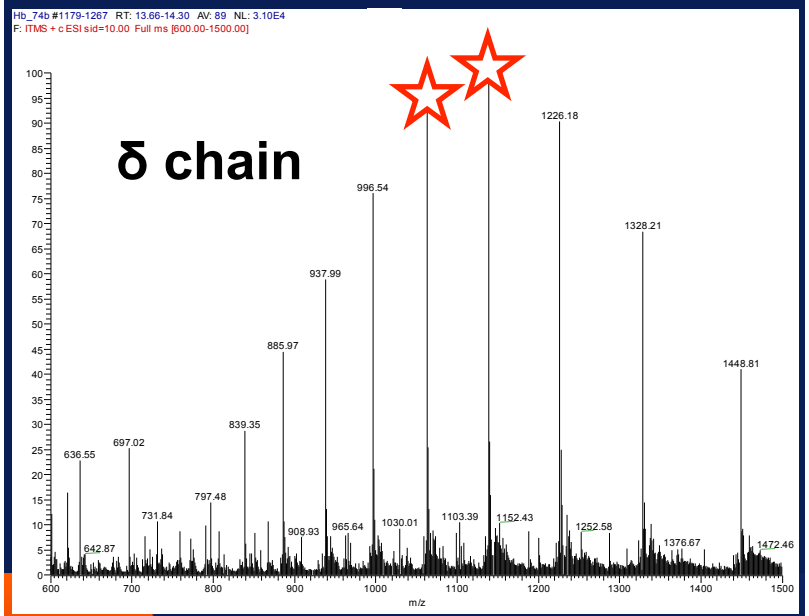


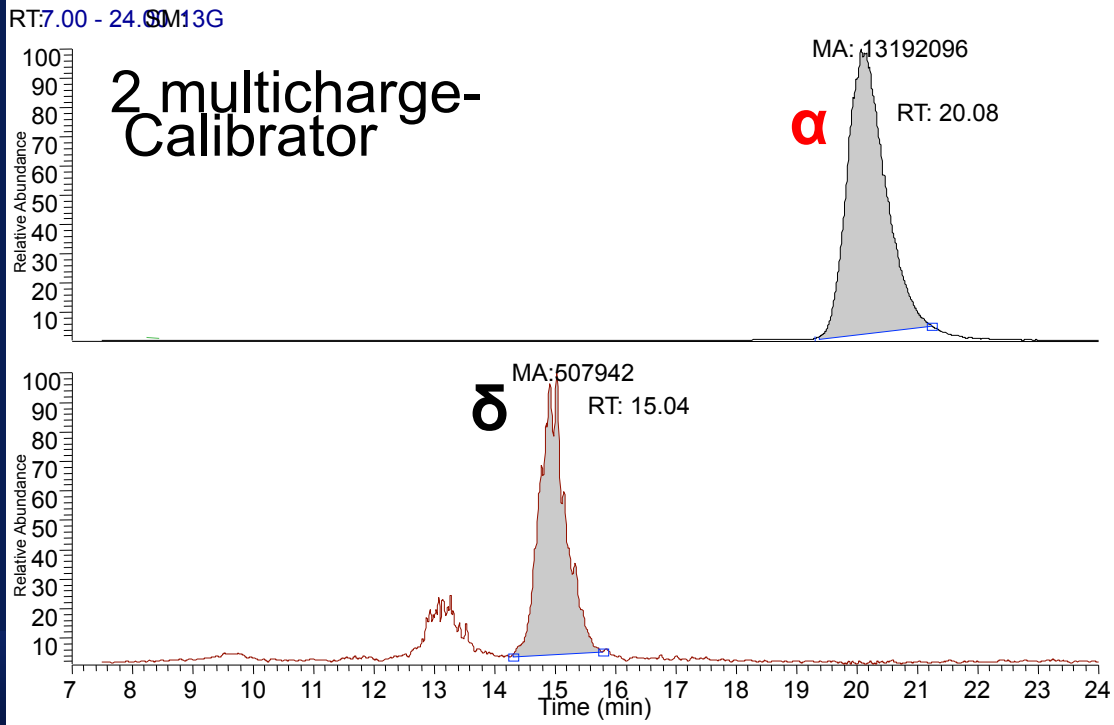
2 MULTICHARGED ION DETECTED



gamma chain m/z	alpha chain m/z
1062	946
1138	1009

HbA_2 = 3.9
Theoretical value = 4.3

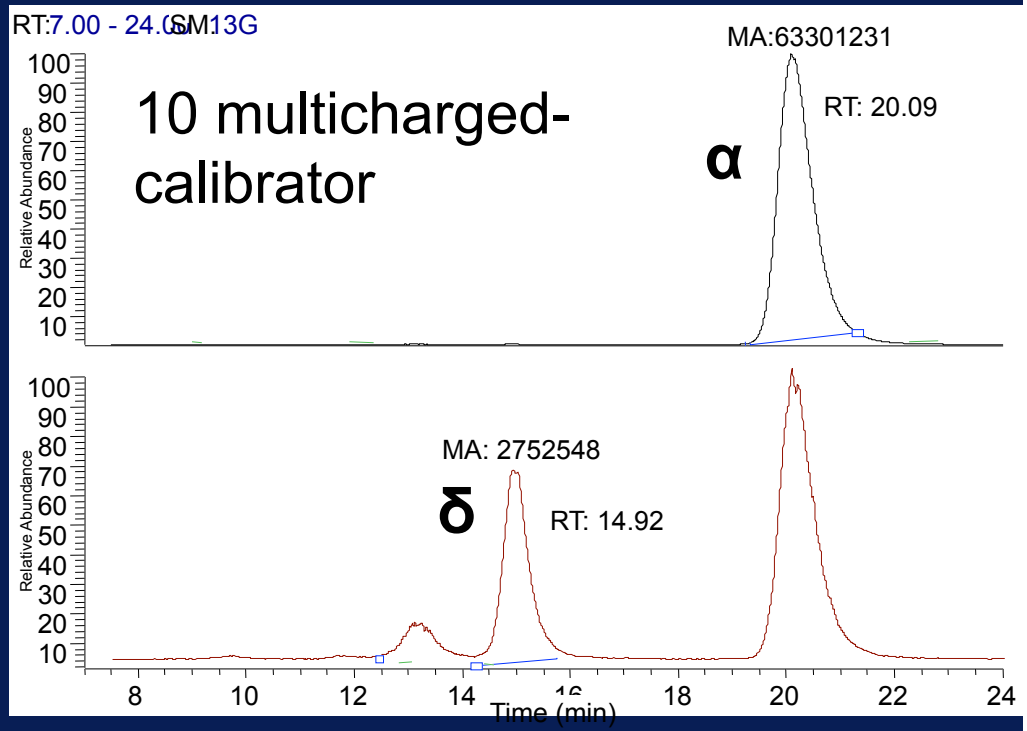




10 MULTICHARGED IONS HAS BEEN DETECTED

gamma chain m/z	alpha chain m/z
797	757
839	797
885	841
937	890
996	946
1062	1009
1138	1081
1225	1164
1327	1262
1448	1375

HbA_2 = 4.3%
Theoretical value = 4,3%



II STEP:
VALIDATION OF THE METHOD

Samples

Human frozen hemolysates treated to remove most of the salt adducts with proteins (no plasma, no leukocytes, no platelets).

First batch: samples 1-16

4 calibrators (HbA2 concentration determined by Tosoh G8 WHO calibrated; 2.5, 3.4, 5.6 and 6.2%)

12 samples to be analyzed in triplicate

Second batch: sample 17-38

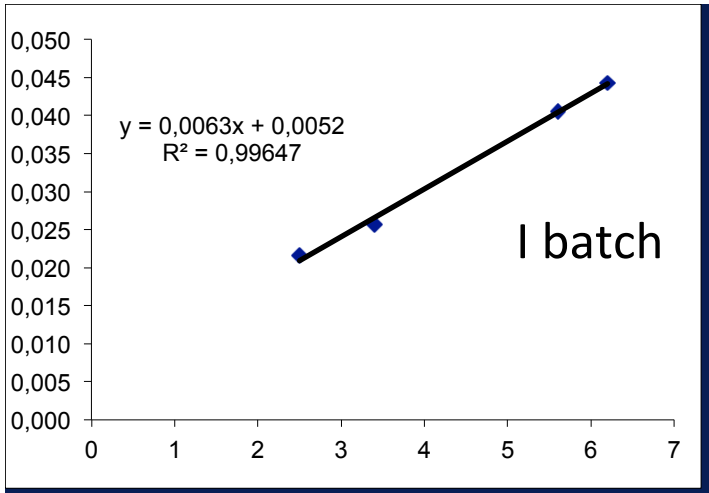
4 calibrators (HbA2 concentration determined by Tosoh G8 WHO calibrated ; 2.5, 3.6, 4.9 and 6.3%)

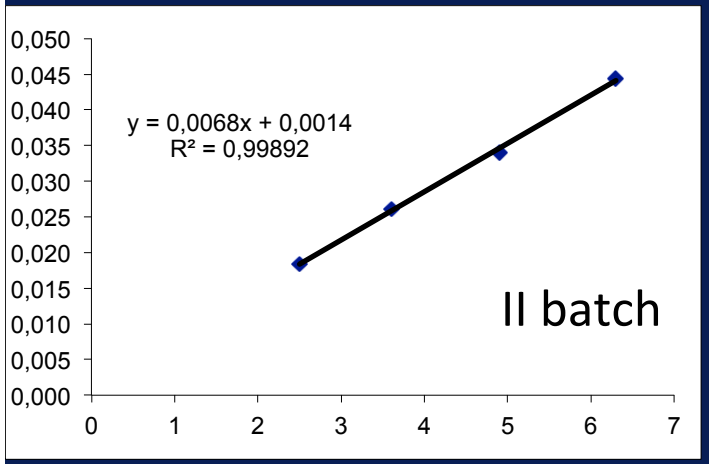
18 samples to be analyzed in triplicate

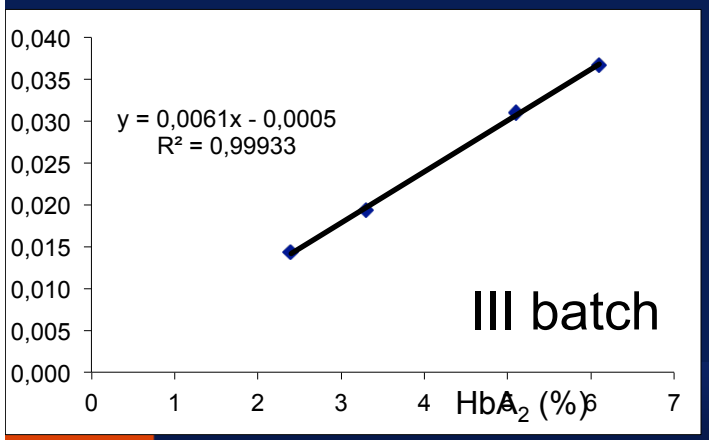
Third batch: samples 39-78

4 calibrators (HbA2 concentration determined by Tosoh G8 WHO calibrated ; 2.4, 3.3, 5.1 and 6.1%)

35 samples to be analyzed in triplicate



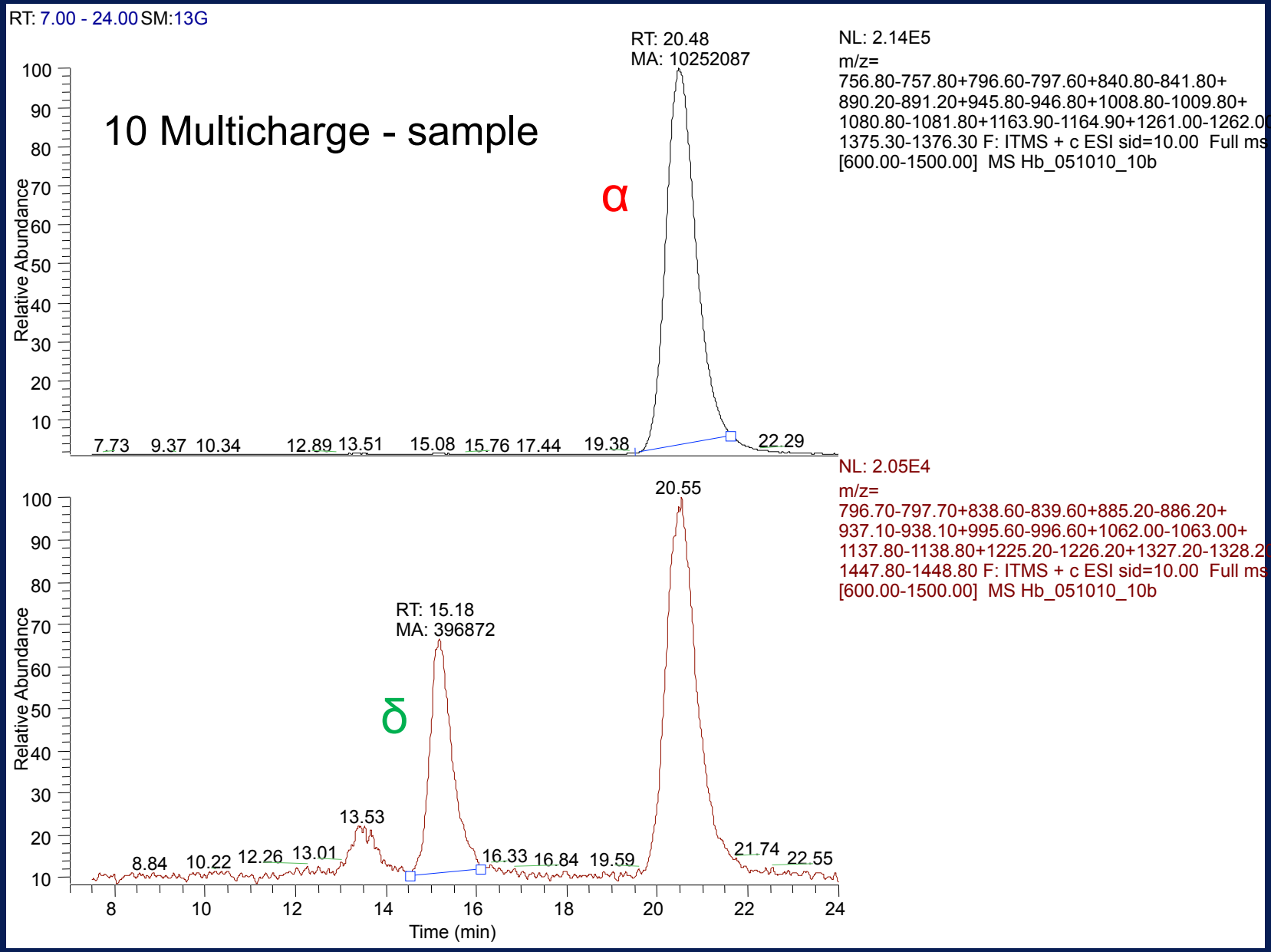




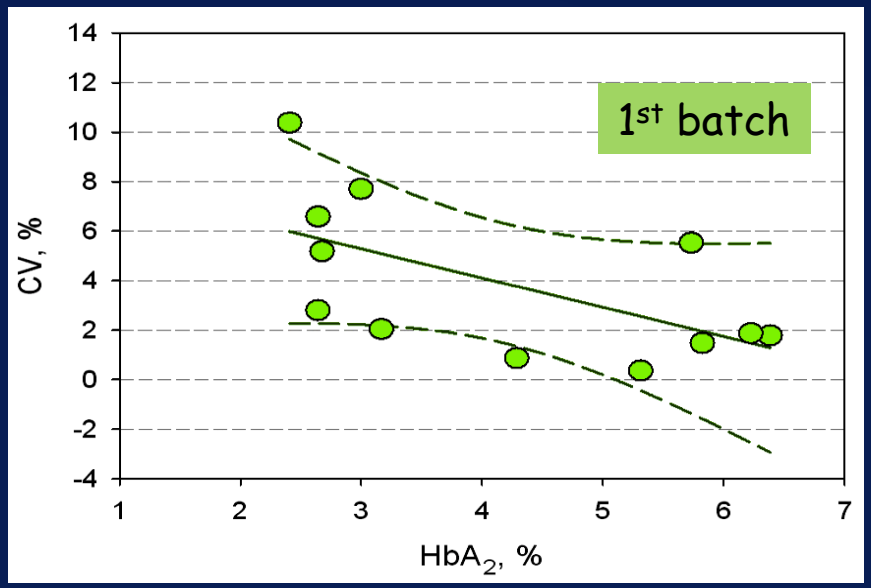
HbA$_2$ (%)

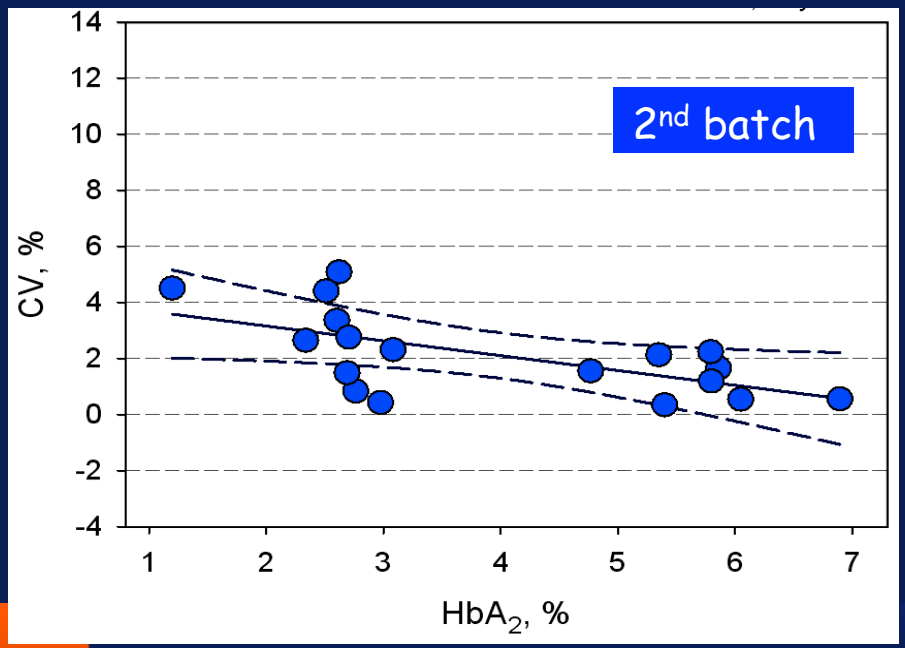
The calibration curve parameters

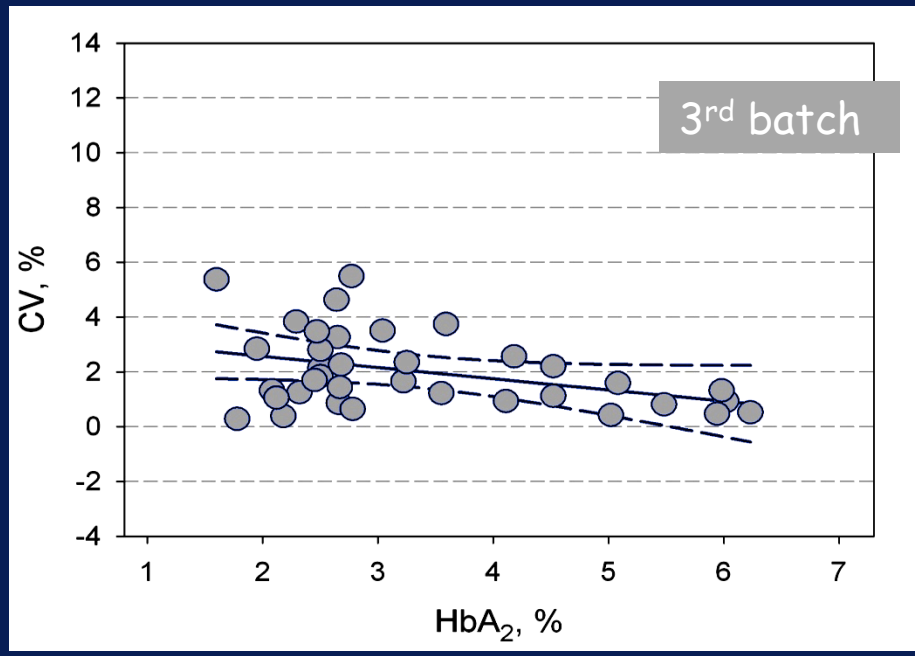
Batch	r^2	slope ($x10^3$)
1	0,997	6,5
2	0,999	6,3
3	0,998	6,1
mean	0,998	6,300
SD	0,001	0,200
CV%	0,10	3,17


RT: 7.00 - 24.00 SM:13G
10 Multicharge - sample
RT: 20.48
MA: 10252087
α
NL: 2.14E5
m/z=
756.80-757.80+796.60-797.60+840.80-841.80+
890.20-891.20+945.80-946.80+1008.80-1009.80+
1080.80-1081.80+1163.90-1164.90+1261.00-1262.00+
1375.30-1376.30 F: ITMS + c ESI sid=10.00 Full ms
[600.00-1500.00] MS Hb_051010_10b
Relative Abundance
7.73 9.37 10.34 12.89 13.51 15.08 15.76 17.44 19.38 22.29
20.55
NL: 2.05E4
m/z=
796.70-797.70+838.60-839.60+885.20-886.20+
937.10-938.10+995.60-996.60+1062.00-1063.00+
1137.80-1138.80+1225.20-1226.20+1327.20-1328.20+
1447.80-1448.80 F: ITMS + c ESI sid=10.00 Full ms
[600.00-1500.00] MS Hb_051010_10b
RT: 15.18
MA: 396872
δ
13.53
8.84 10.22 12.26 13.01 16.33 16.84 19.59 21.74 22.55
Time (min)

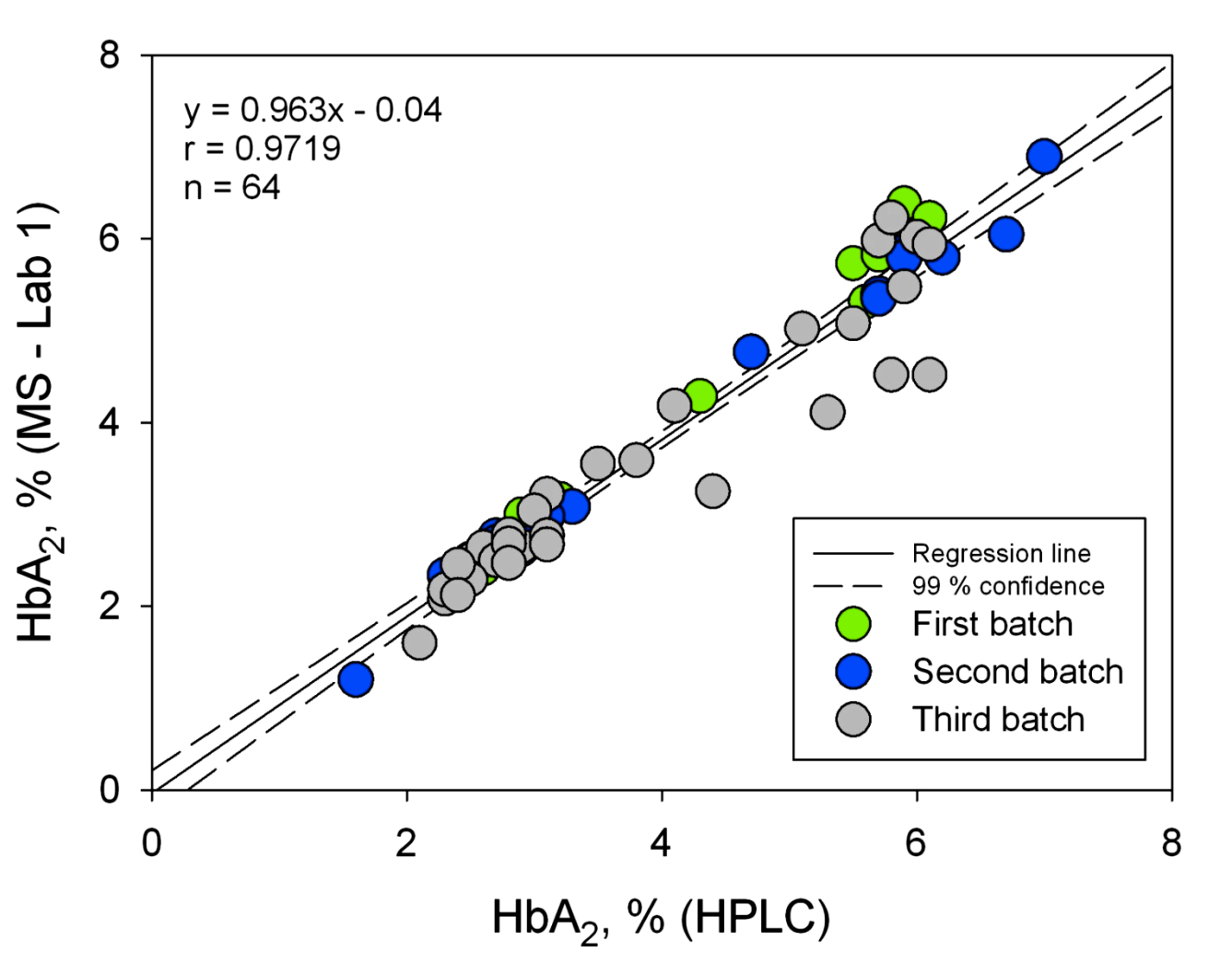
MS precision profile





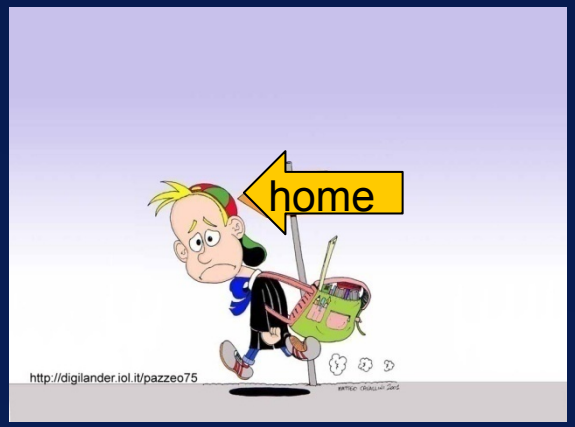


Relationship between HbA_2 measured by the MS and HPLC techniques



III STEP:
INTERLABORATORY COMPARISON

TAKE-HOME MESSAGE



The possibility to perform the quantitative determination of HbA_2 without protein digestion, has been explored.

ESI-MS of the intact Hb chains appears to be a viable method for determining the δ-chain and hence detecting β-thalassemia trait in blood samples.

acknowledgment

Renata Paleari

Federico Abbiati

Andrea Mosca

Flavio Giavarini

all the HbA_2 working group