

CIRME



UNIVERSITÀ DEGLI STUDI
DI MILANO

Centro Interdipartimentale per
la Riferibilità Metrologica in
Medicina di Laboratorio
(CIRME)

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Defining HbA1c: the indispensable decision to approach measurement standardization

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IFCC WG for HbA1c standardization

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Sweden

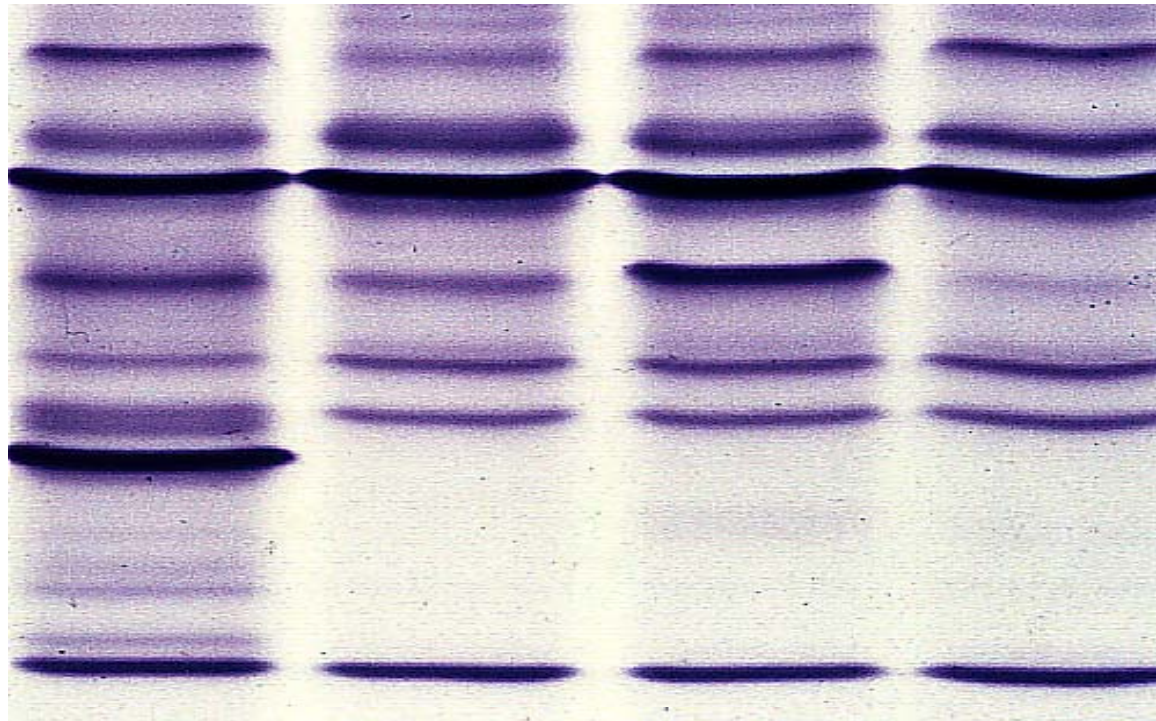
6 November, 2007

Overview

- Biochemical background
- Purification of primary calibrators
- IFCC reference method
- Validation of the reference method

Electrofocusing of hemoglobins

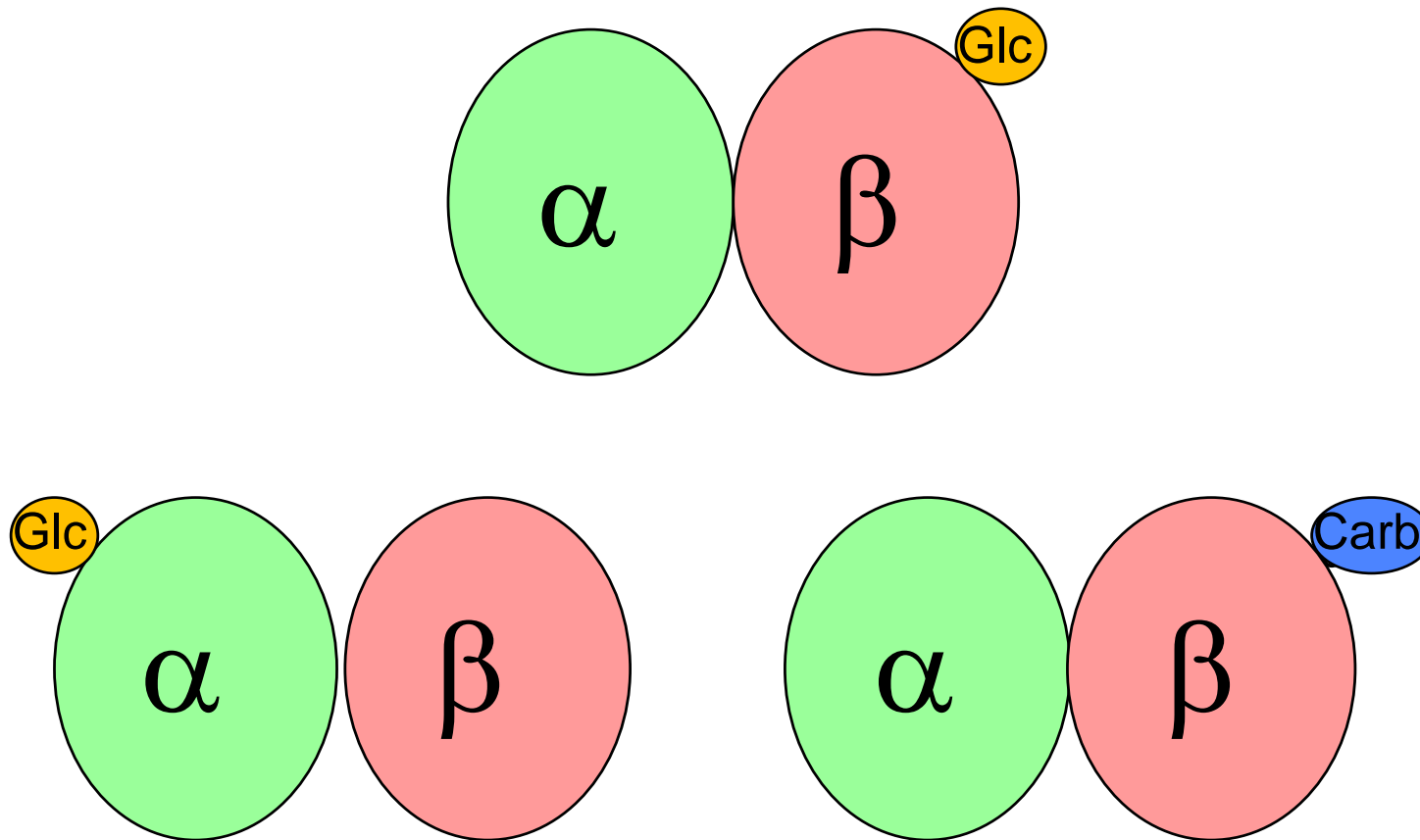
HbS



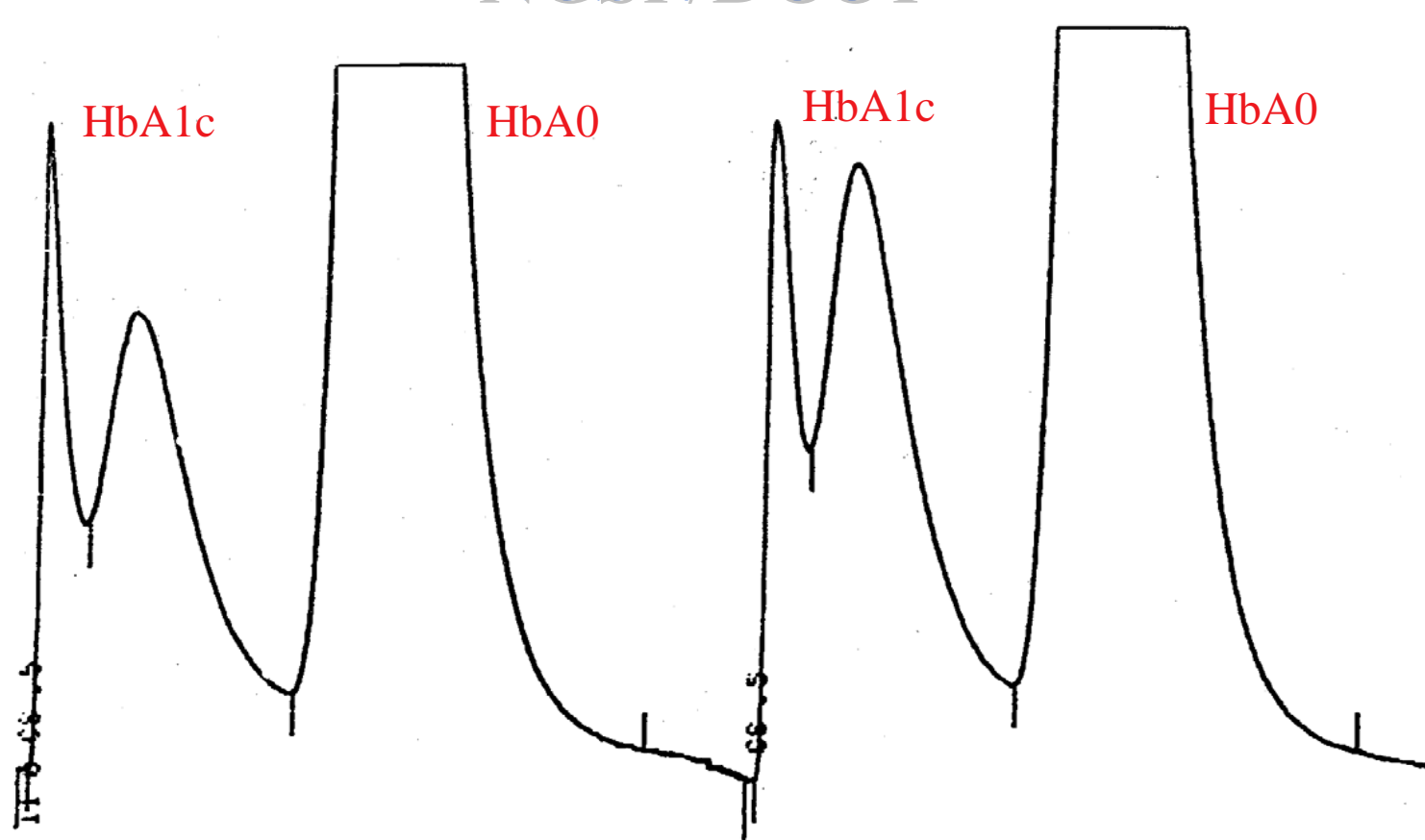
HbA3
HbA1c
HbA0
HbF
Intmed
metHb

HbA2

Contents of the HbA1c peak at ion exchange chromatography

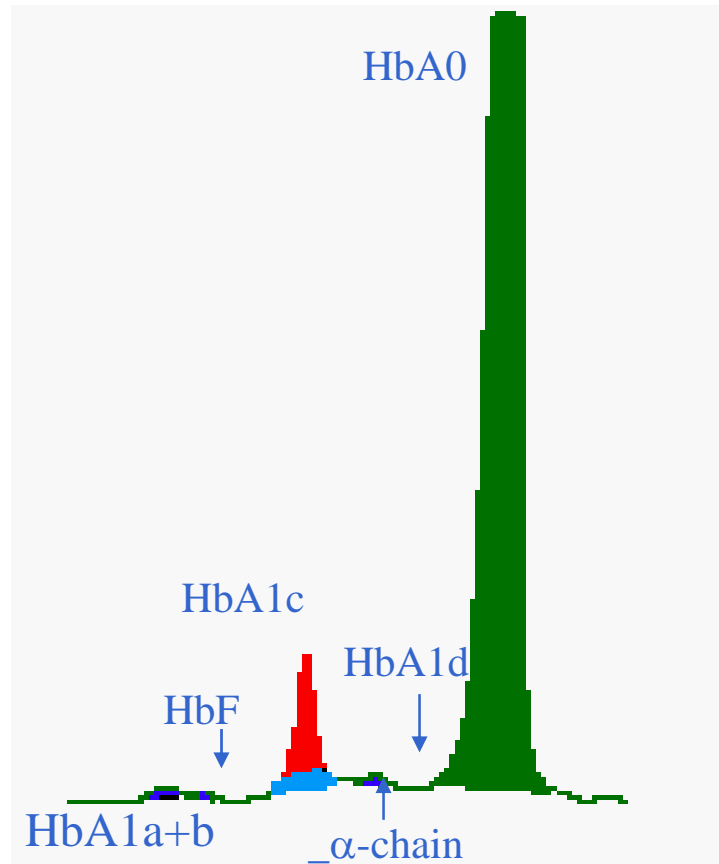


BioRex 70 Ion Exchange Chromatography NGSP/DCCT



Goldstein et al in Clark WL, Larner J, Pohl SL eds
Methods of Diabetes Research Vol 2; 1986:475-504

Mono S Ion Exchange Chromatography of HbA1c



Jeppsson JO et al Clin Chem 1986;35:1867-72

IFCC WG-HbA1c 1995-

- Definition of analyte
- Development of primary calibrators
- Establishment of a validated reference method
- Developing a network of reference laboratories worldwide

Glycation sites of Hemoglobin

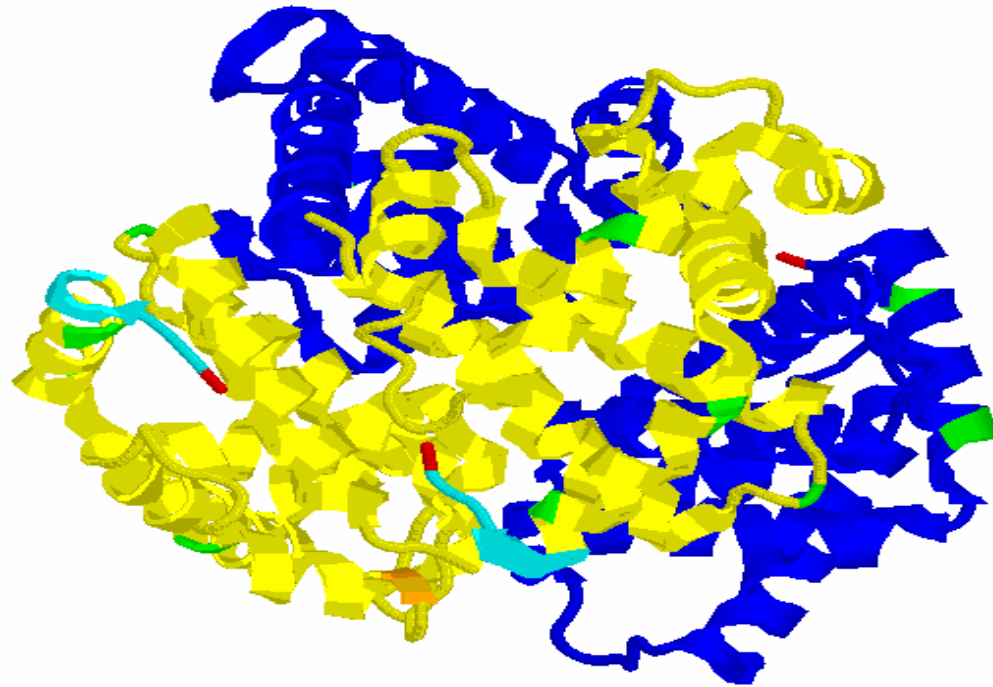
β -Chains: Yellow

α -Chains: Blue

N-terminal

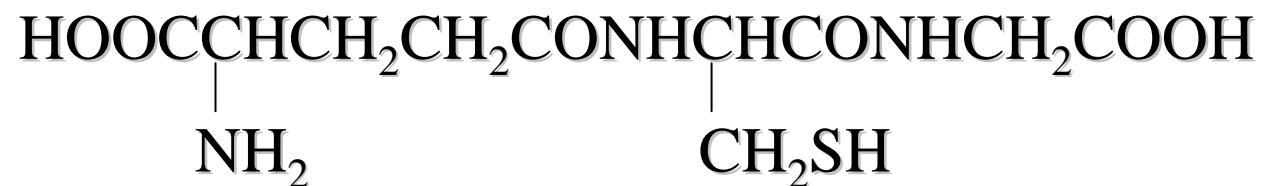
Valine: **red**

Lysine **green**

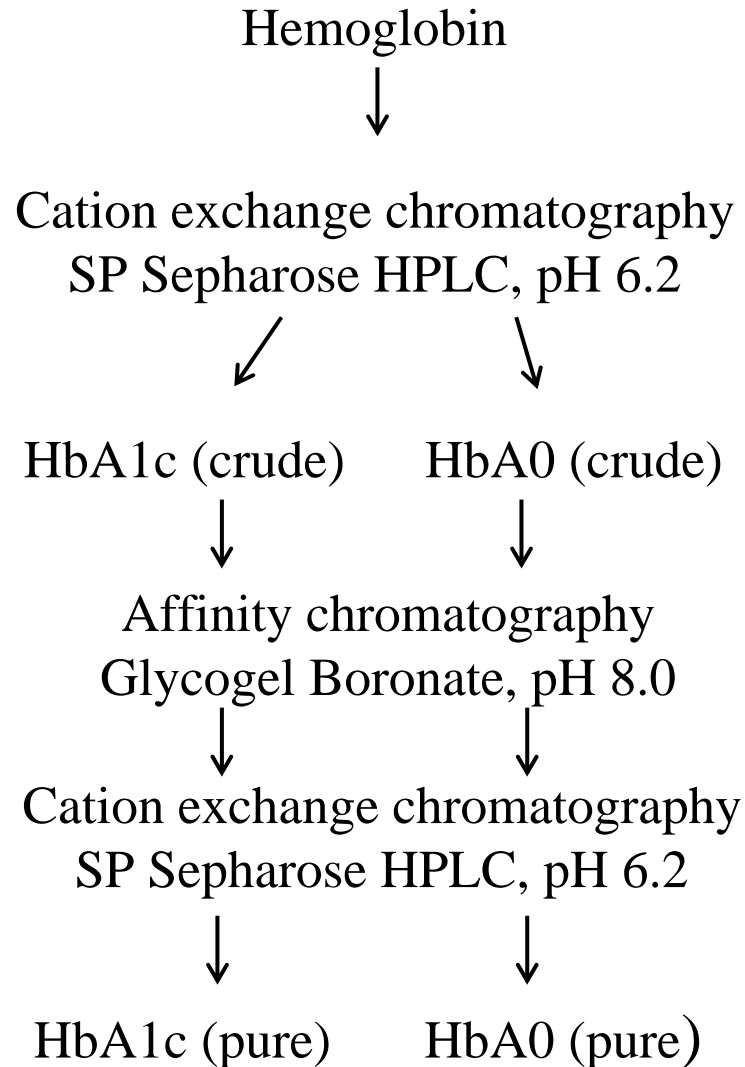


Glutathione

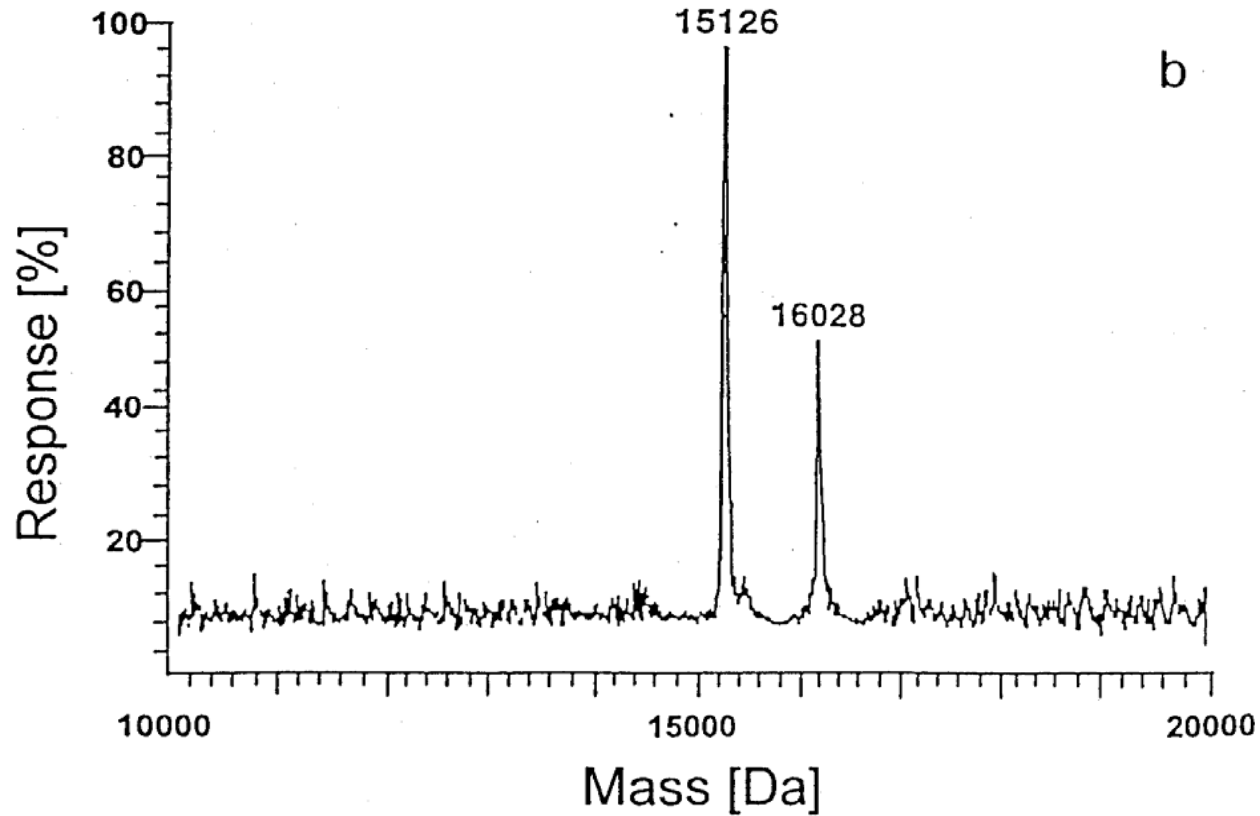
HbA₃ or HbA_{1d}



Preparation of calibrators



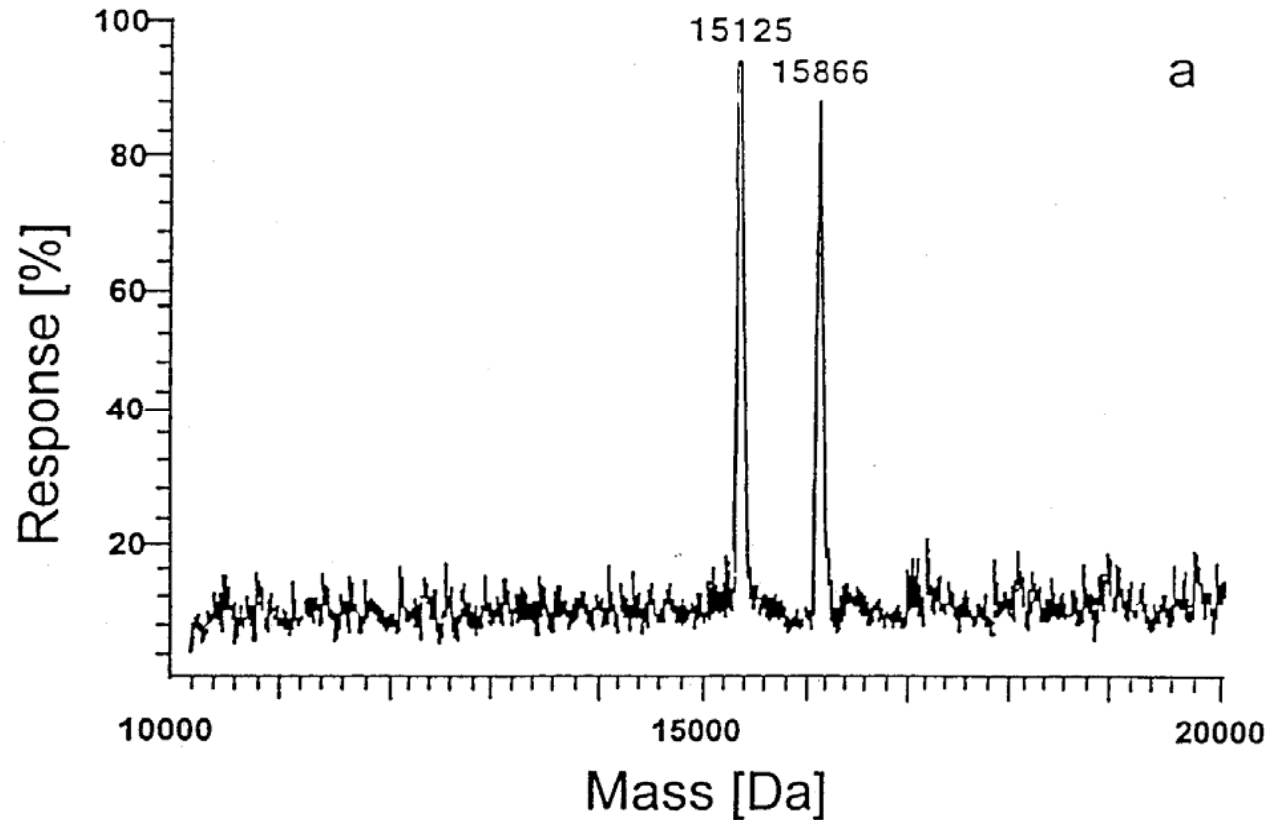
ESI/MS of HbA1c



15126 Da
Non-glycated
 α -chain

16028 Da
Mono-glycated
 β -chain

ESI/MS of HbA0



15125 Da
Non-glycated
 α -chain

15866 Da
Non-glycated
 β -chain

Standard addition experiment with HPLC-ESI/MS

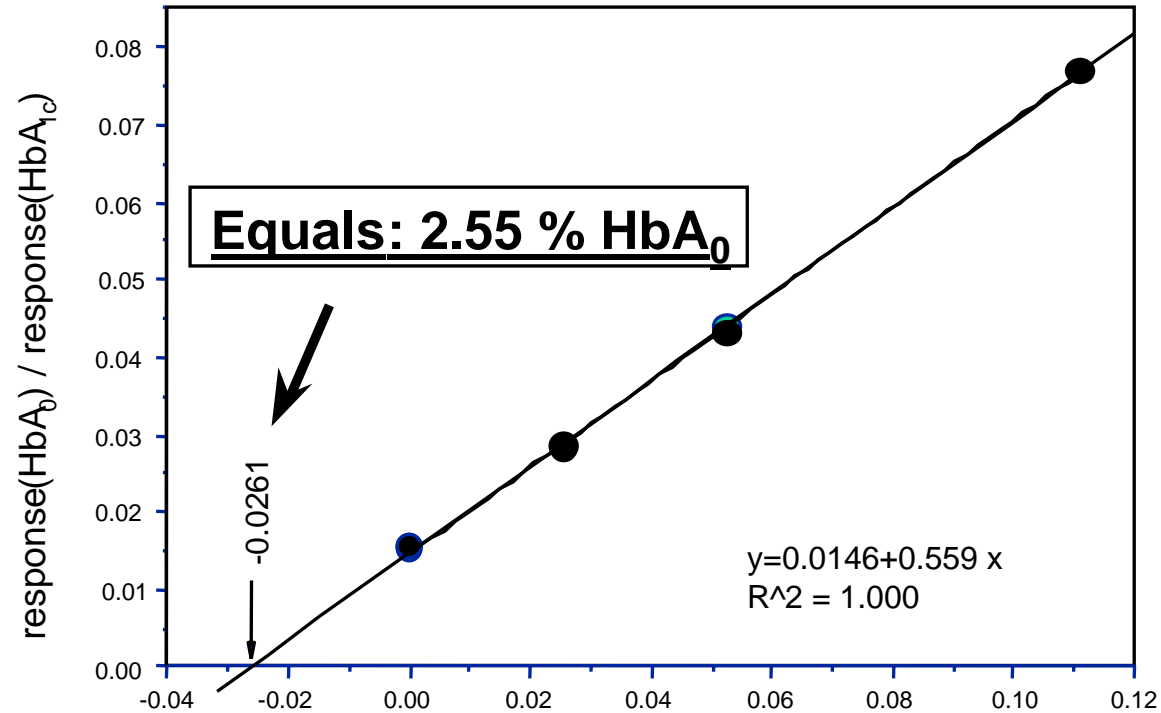


Fig. 13 Ratio: [HbA₀] / [HbA_{1c}]

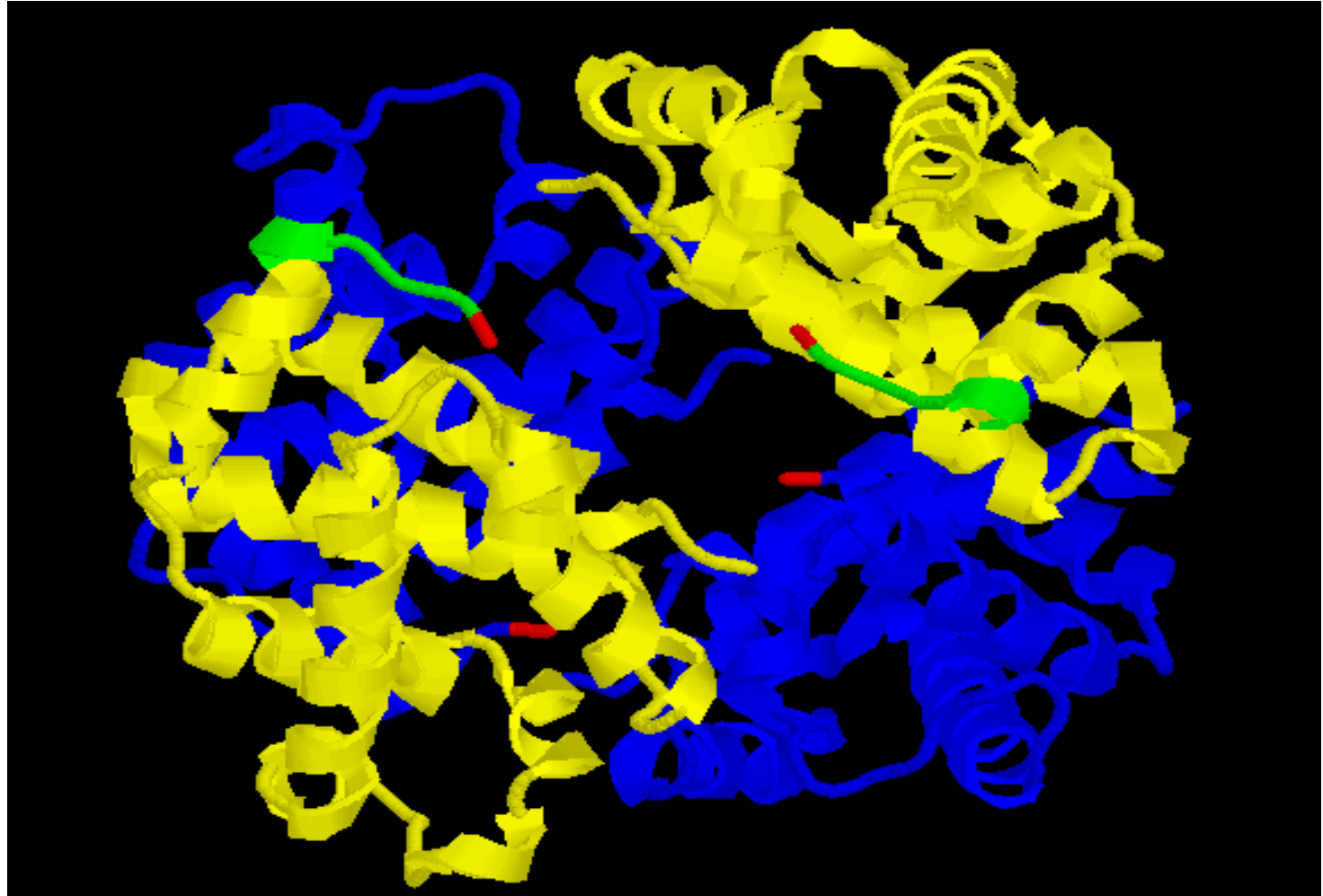
HbA1c is the stable adduct of glucose to the N-terminal amino acid, valine of the β -chain of hemoglobin

β -Chains: Yellow

α -Chains: Blue

N-terminal Valine:
Red

Hexapeptide: Green



Proteolytic Cleavage of β -chain by Endoproteinase Glu-C

VHLTPE

E

KSAVTALWGK VNVDE

VGGE

ALGRLLVVYPWTQRFFE

SFGDLSTPDA VMGNPKVKAHGK KVLGAFS

DGLAHL DNLKGTFATLSE

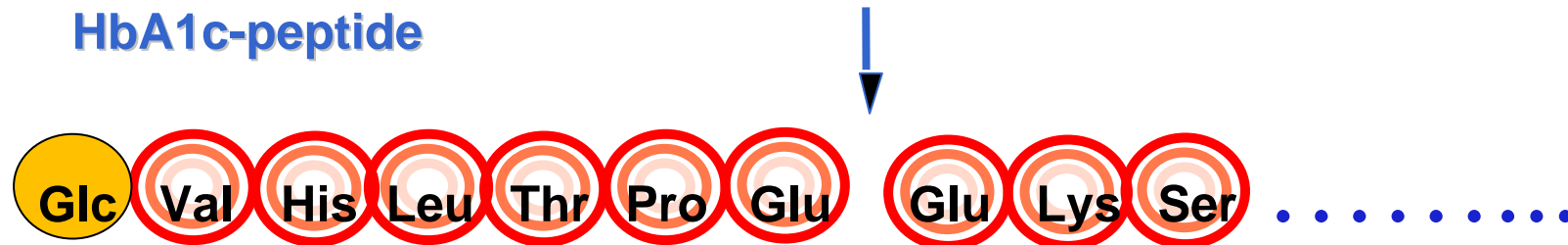
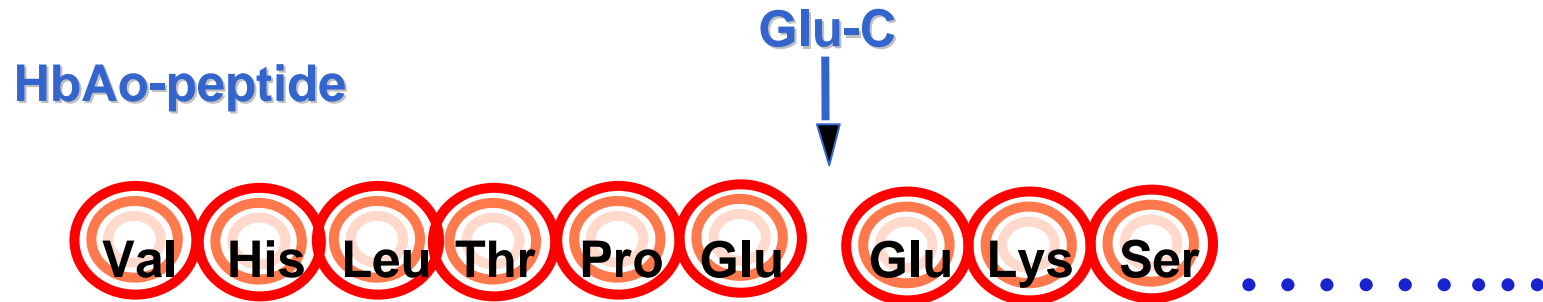
LHCDKLHVDPE

NFRLLGNVLVCVLAHHFGKE

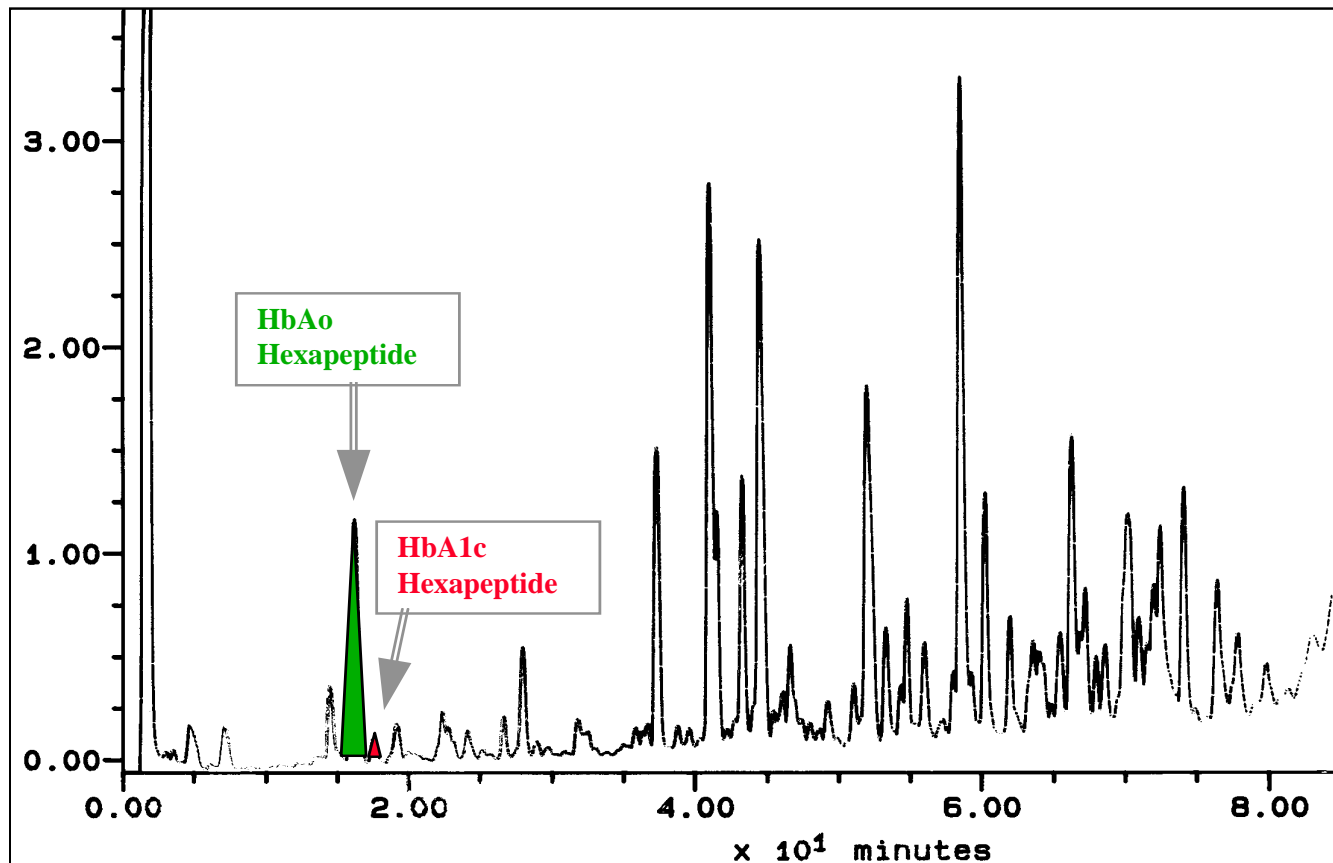
FTPPVQAAYQKVVAGVANALAHKYH

The Analytical Challenge

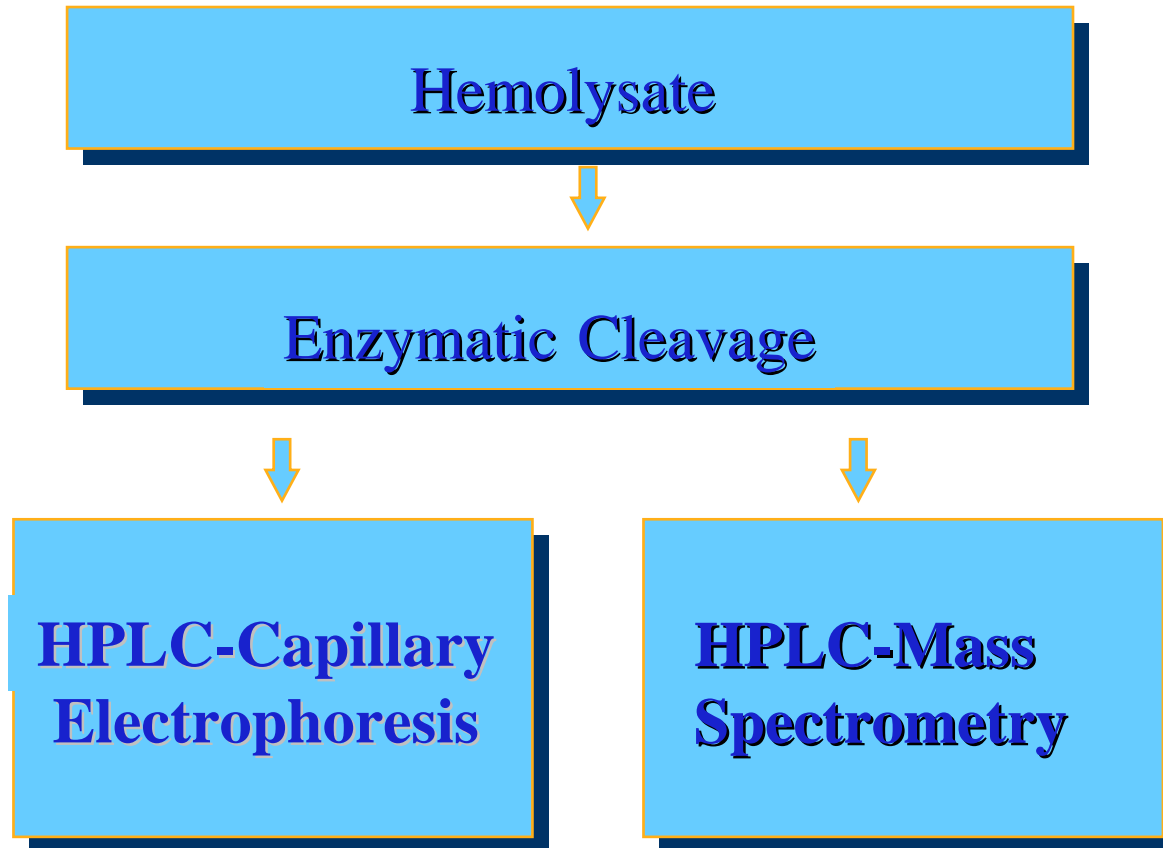
Proteolytic cleavage of β -chain (146 amino acids)



Photometric detection of peptides

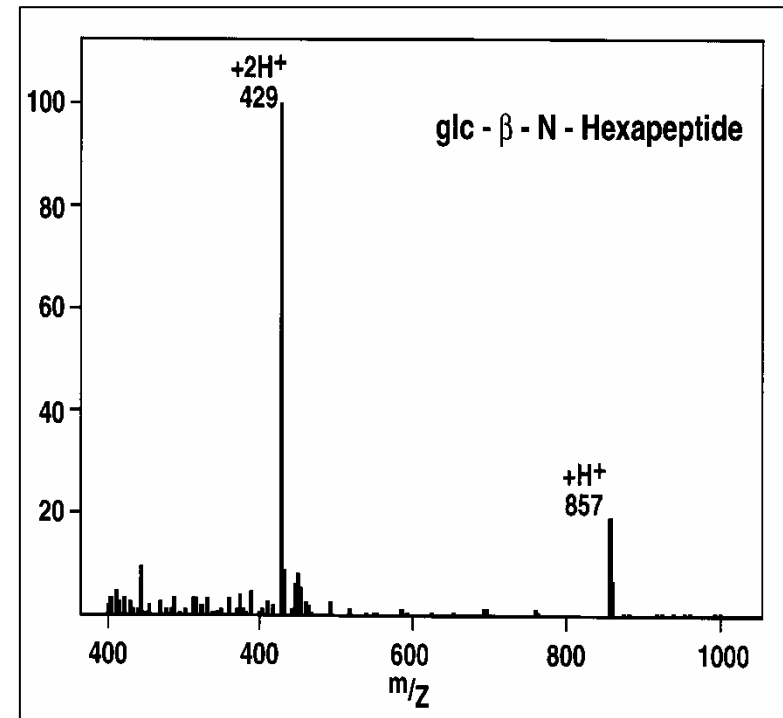
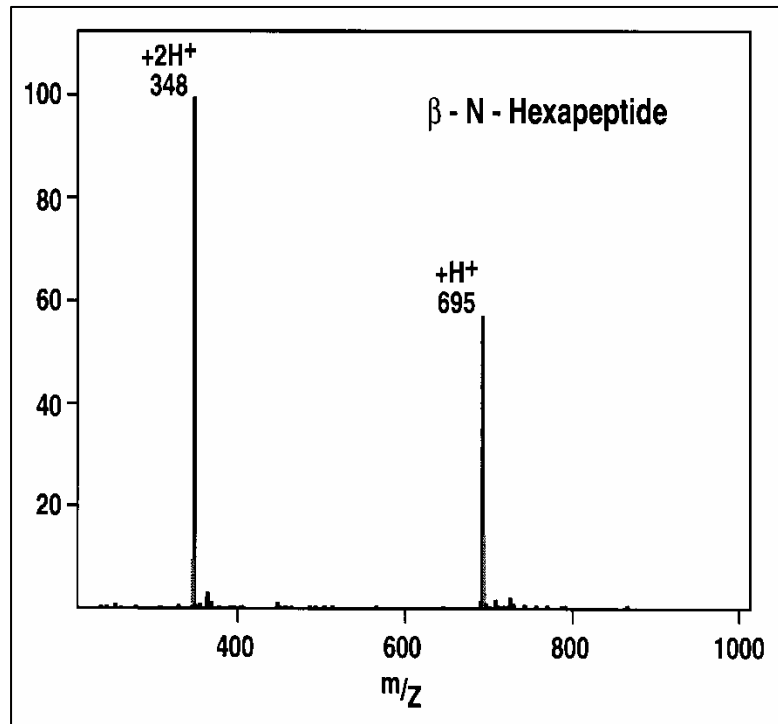


Flow Chart for Reference Methods



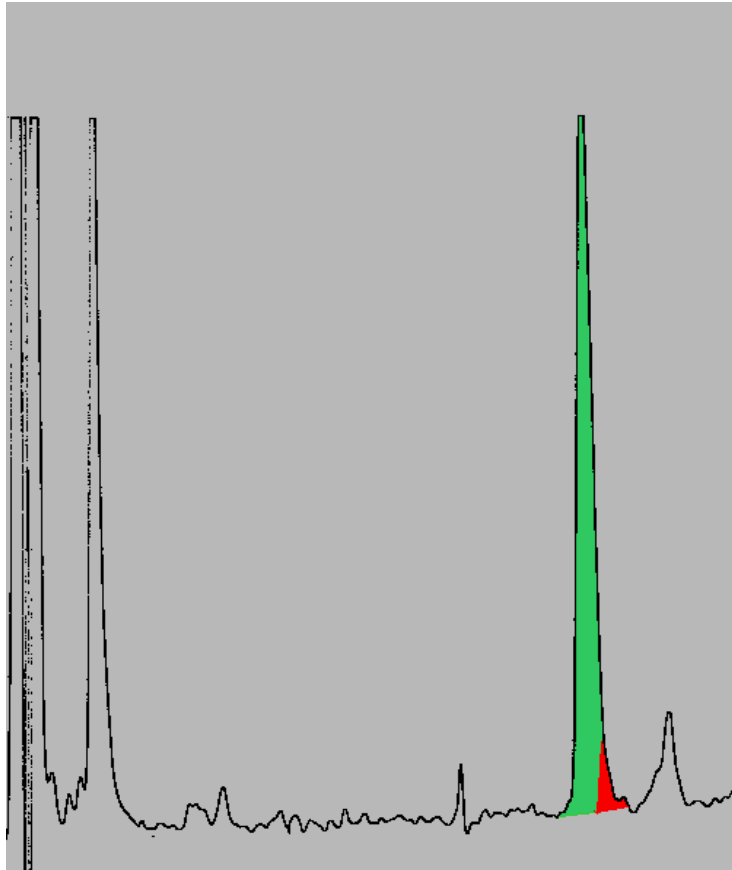
Approved by IFCC 2001

ESI mass spectra of nonglycated and glycated β N-terminal hexapeptides

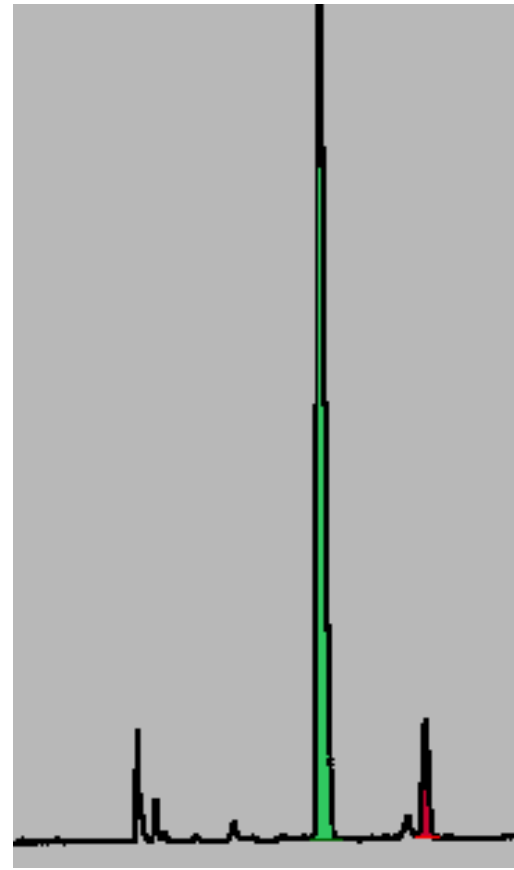


Two-dimensional separation of N-terminal hexapeptides of hemoglobin

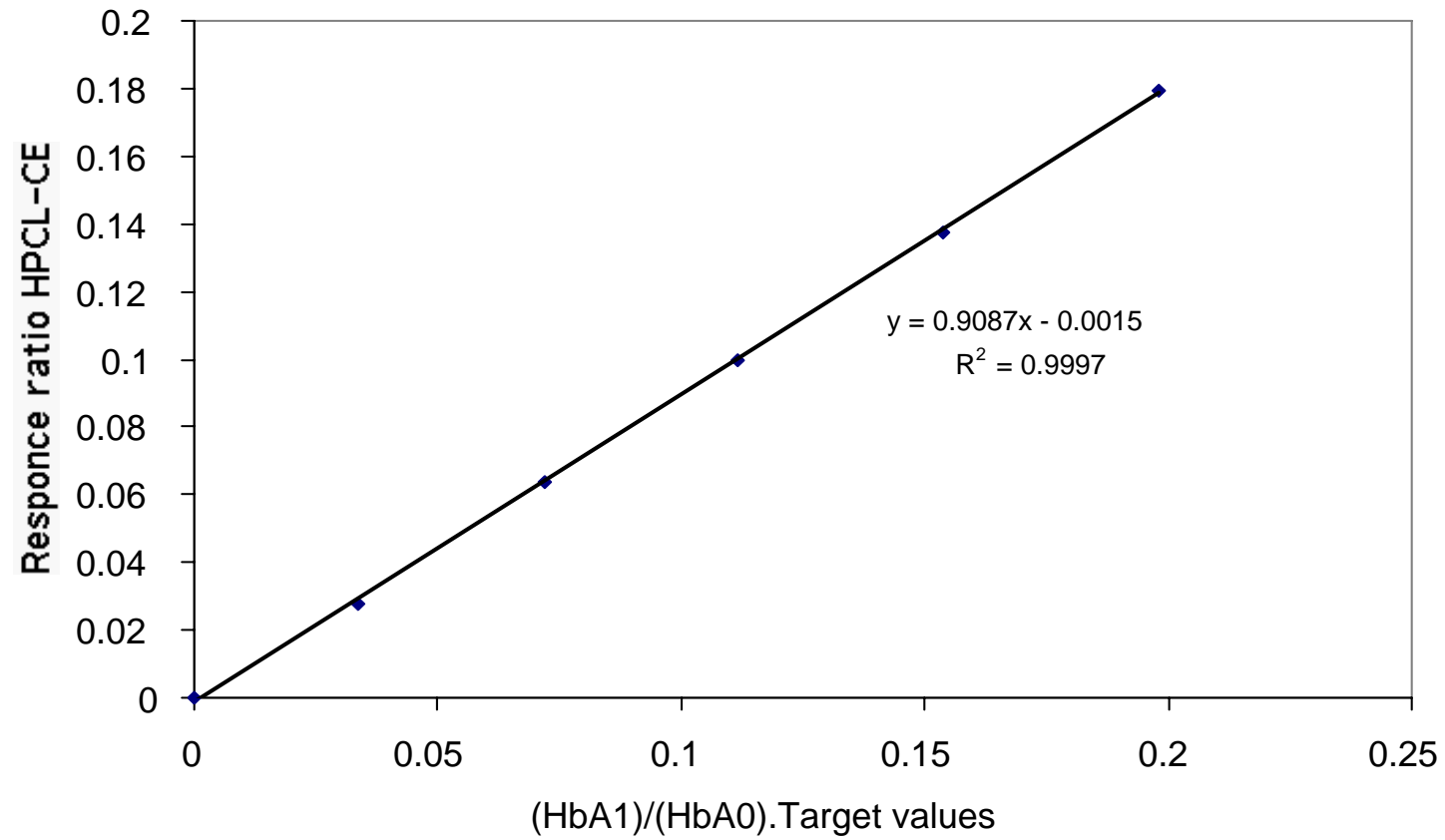
C 18 Chromatography



Capillary Electrophoresis



Calibration function for HPLC-CE



Possible Interferences

- The hexapeptide represent an unique sequence
- Hb S and C
- Acetylated and carbamylated hemoglobin
- Potassium cyanide
- Sodium azid- excluded

Hemoglobinopathies in the N-terminal part

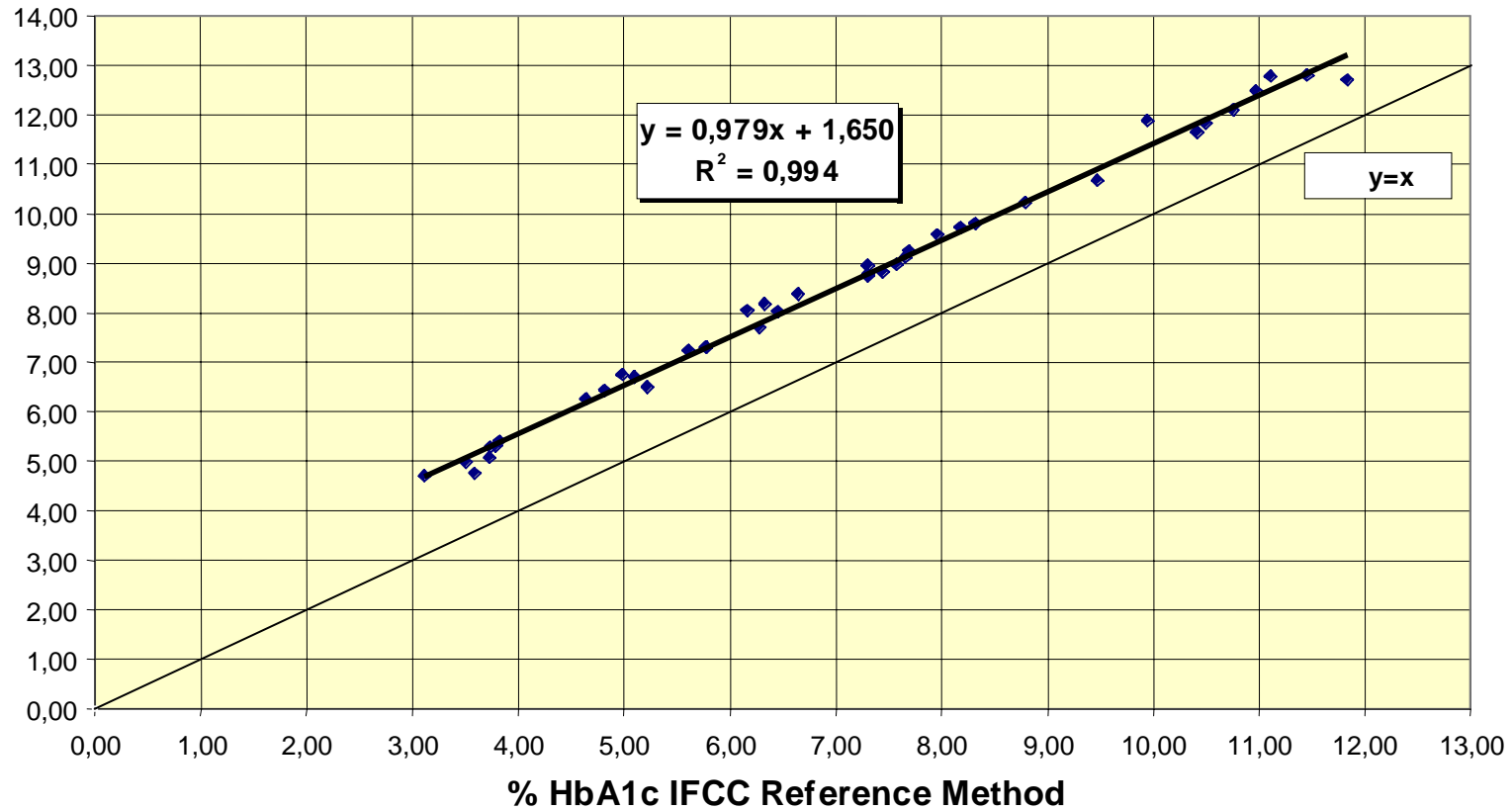
- Hb S Glu → Val
- Hb C Glu → Lys

- Hb Raleigh Val → Ac-Ala
- Hb Okayama His → Gln
- Hb Fukuoka His → Tyr
- Hb Tyne Pro → Ser

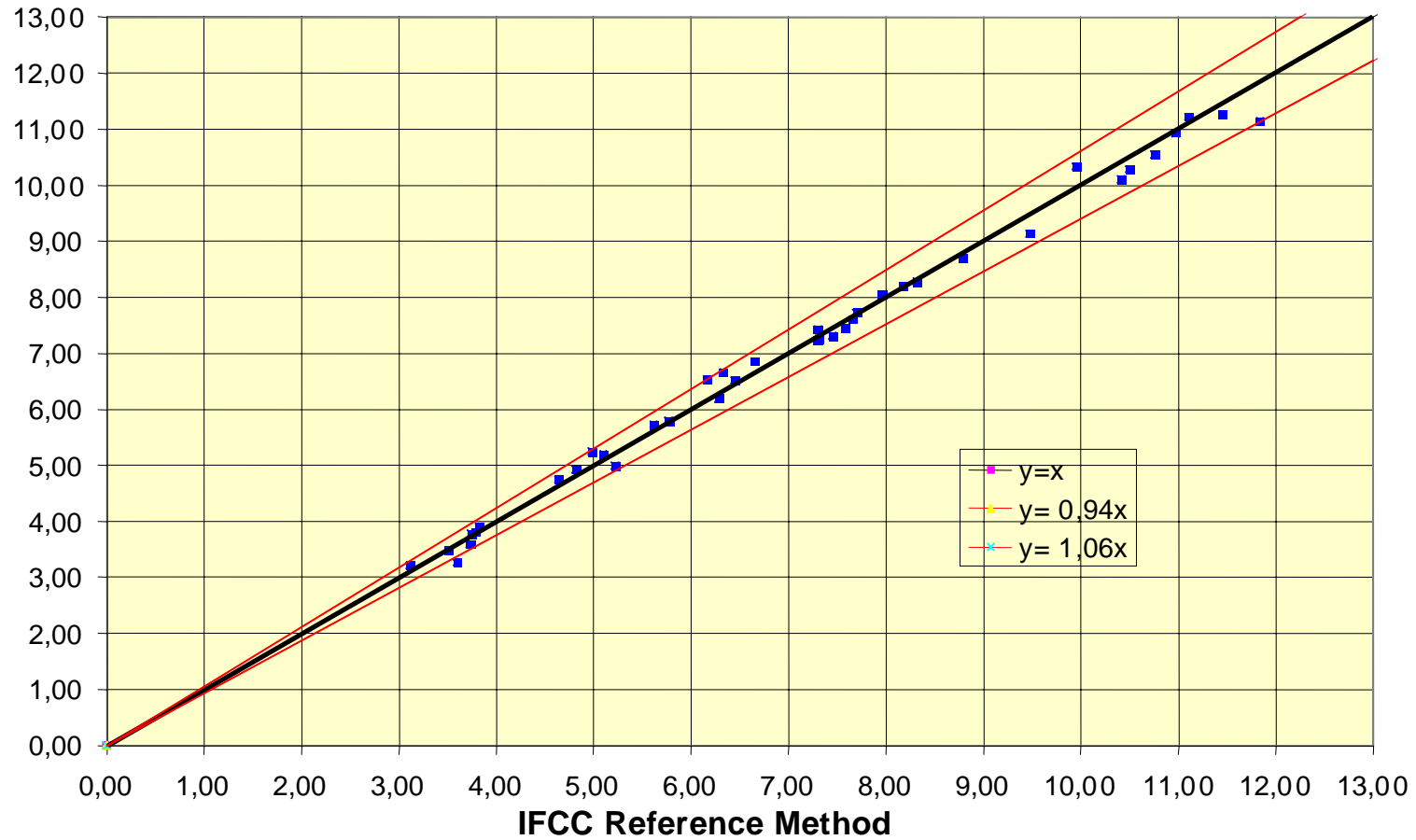
Possible Interferences

- The hexapeptide represent an unique sequence
- Hb S and C
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Comparison between a HPLC and IFCC reference method



Comparison between converted HPLC and IFCC reference method

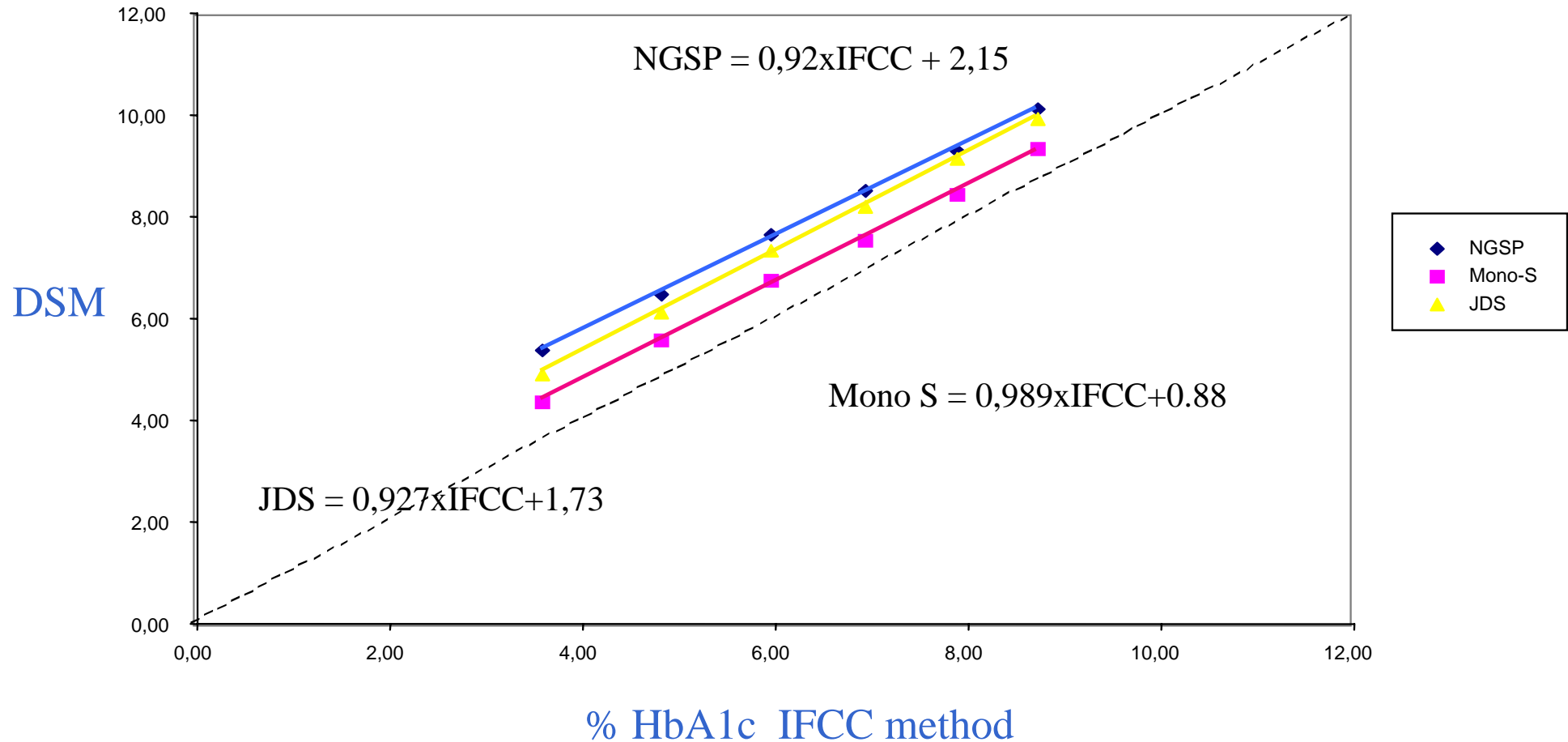


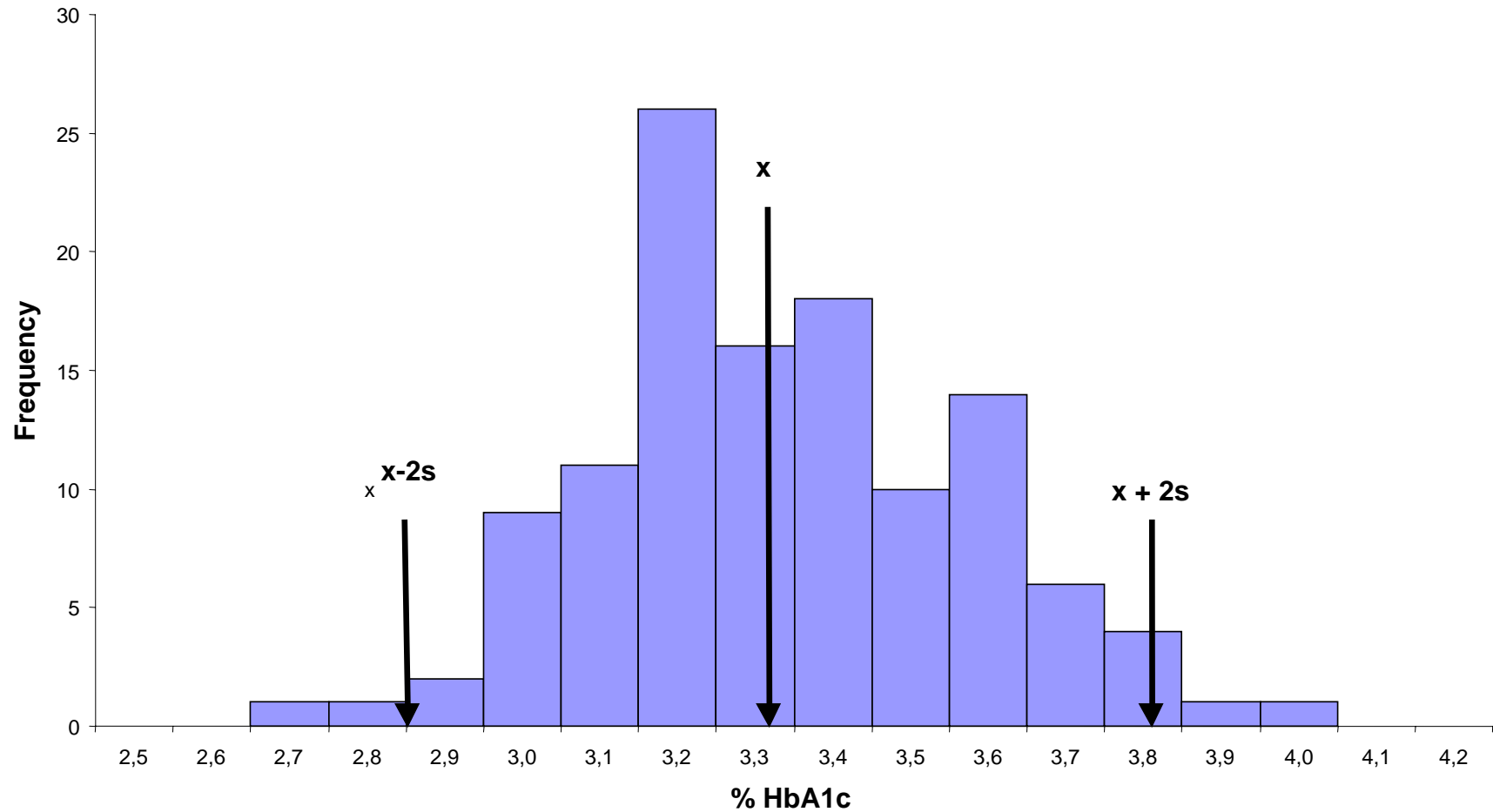
Harmonization of HbA1c

Designated Comparison Methods (DCM)

- National Glycohemoglobin Standardization Program(NGSP) for DCCT in US
- Japan (JDS/JSCC), a set of national calibrators and HPLC/KO500
- Mono S (EQUALIS) ion exchange chromatography in Sweden

Comparison between the IFCC reference method and three designated comparison methods





IFCC: Tentative reference range: 2.8-3.8%
 (Non-diabetics-OGTT: ADA/WHO, n=120)

Summary I

- We defined the analyte HbA1c as the glucose adduct to the N-terminal amino acid, valine of the β -chain of hemoglobin
- Primary calibrators, pure HbA1c (monoglycated β -chain and non-glycated α -chain) and pure HbA0 (nonglycated β -chain and nonglycated α -chain) have been purified
- A reference method has been developed as follows:

Hemoglobin is cleaved into peptides by the proteolytic enzyme endoproteinase Glu-C

The resulting glycated and nonglycated N-terminal hexapeptides of the β -chain are separated from the crude peptide mixture by reversed-phase HPLC

Summary II

- The hexapeptides are quantified by mass spectrometry or capillary electrophoresis at ultraviolet detection
HbA1c is determined as the ratio of the glycated and nonglycated β -N-terminal hexapeptides of hemoglobin
- A strong correlation was obtained between IFCC-RM and all DCM methods
- The specific IFCC reference method gives HbA1c lower values than the DCM methods
- Reference range: 2.8 - 3.8% or 28-38 mmol/mol