

**Measurand characteristics,
Reference measurement procedures and
Reference materials
for HbA1c**

**Kor Miedema, chair
IFCC WG on HbA1c Standardization**

JCTLM presentation, 15-12-2004, Paris

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HbA1c/Glycohemoglobin

- * independent parameter of metabolic control
- * risk parameter for development of complications
- * treatment goal in disease management
- * independent cardiovascular risk parameter
in non-diabetics

AN IMPORTANT ANALYTE >>>STANDARDISE!!!!

Fast haemoglobin's and HbA1c

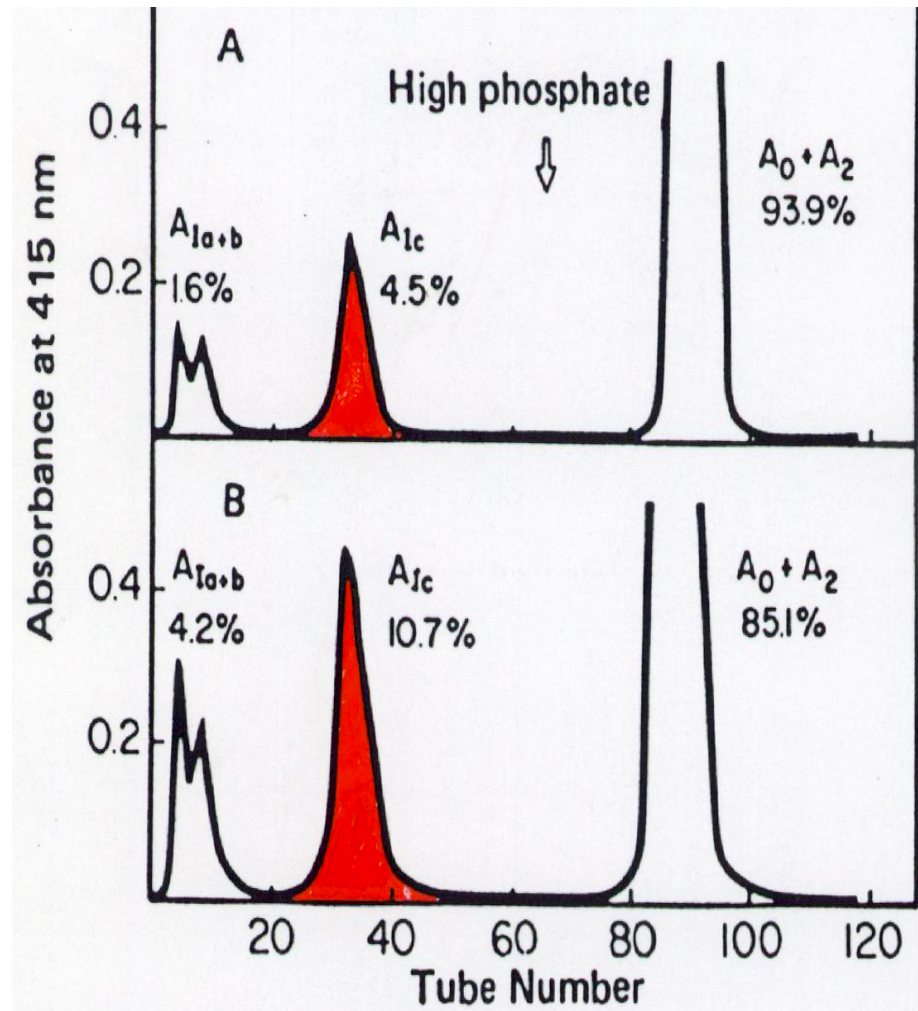
1968: Rahbar detects 'diabetic' haemoglobin's

1971: Trivelli describes routine method

1978: HbA1c = glycosylated/glycated Hb

In HbA1c is glucose attached to the N-terminal valine of the β -chain of HbA0.

Schematic ion exchange chromatogram HbA1c



Assay principles for HbA1c - 1

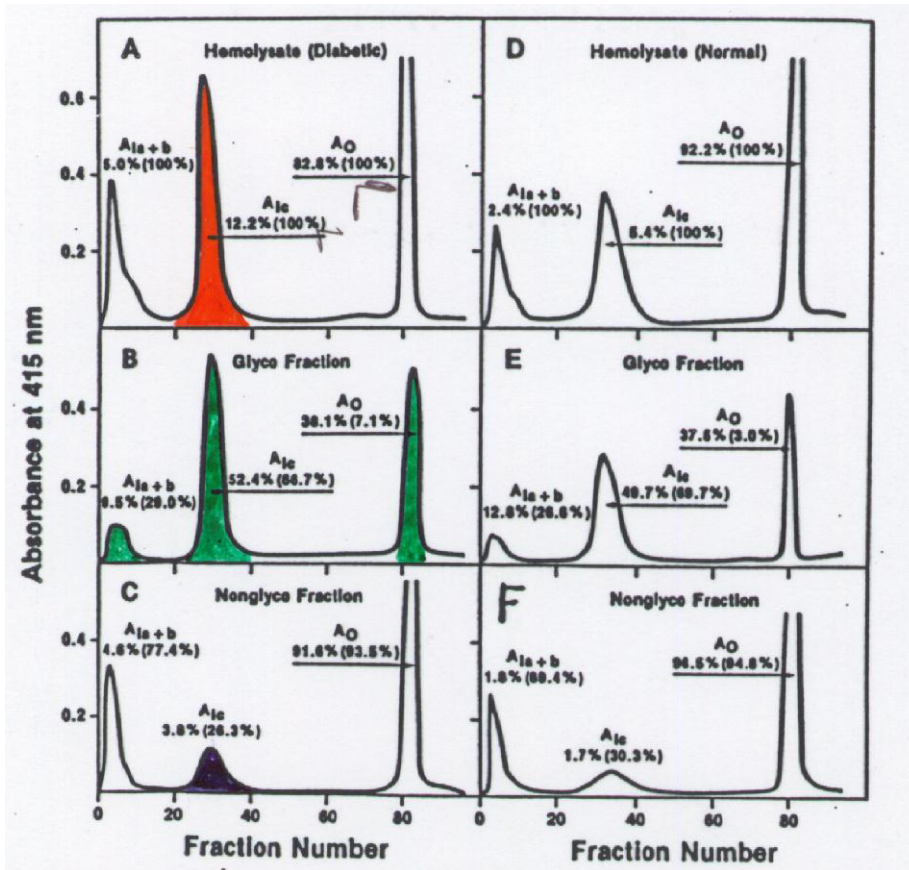
- * ion-exchange chromatography based on very small differences in iso-electric point**
- * affinity chromatography based on covalent binding of cis-diols of glucose in GHb to a boronate matrix**
- * immune assays based on the antigenic properties of β -N-Val glycation**

Assay principles for HbA1c - 2

Definition of the analyte:

- 1) cation exchange chromatography measures β -chain glycation of the N-terminal Valine**
- 2) affinity chromatography measures 'total' glycation (α and β chain, Val and ϵ -Lys)**
- 3) immune assays measures β -chain glycation of the N-terminal Val**

Ion-exchange vs affinity chromatography



- Glycated fraction of affinity elutes in HbA1a+1b, HbA1c and HbA0
- part of non-glycated fraction of affinity elutes with HbA1c
- HbA1c by IEC is NOT specific!

About HbA1c assays

- * ion-exchange chromatography is not specific, contains up to 40 % non-A1c material. (It's a chromatographic illusion!)**
- * affinity chromatography measures in principle all (\uparrow and β -val and \leftarrow -lys) glycated haemoglobin**
- * immune assays differ in specificity of the monoclonal and/or polyclonal antibodies to the different antigenic sites (4, 6 or 8 aa of β -N-Hb)**

Standardisation of HbA1c results

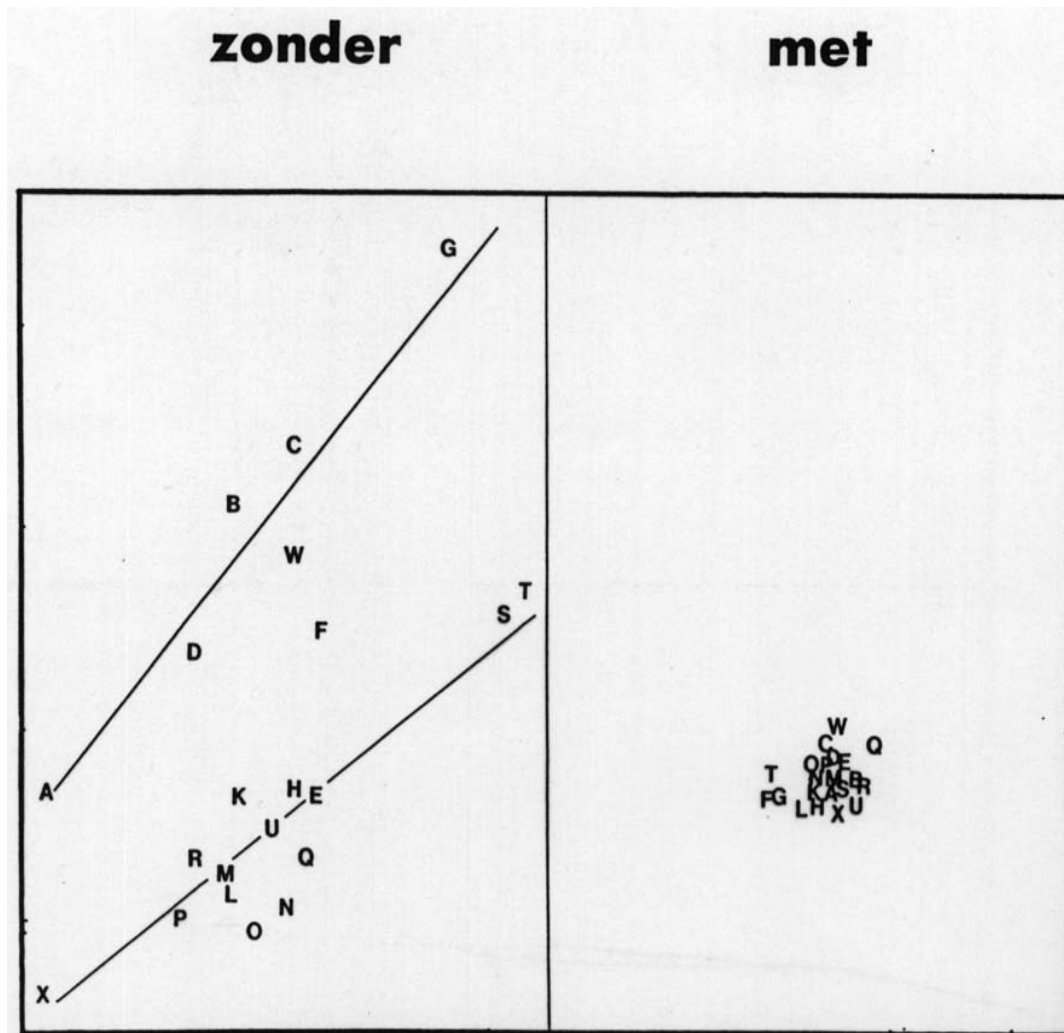
Three approaches:

1 - harmonisation-by-calibration

2- harmonisation by method comparison

3- standardisation by a reference system

Harmonisation-by-calibration



Harmonisation by method comparison

Based on a careful but conscious chosen method to act as Designated Comparison Method when:

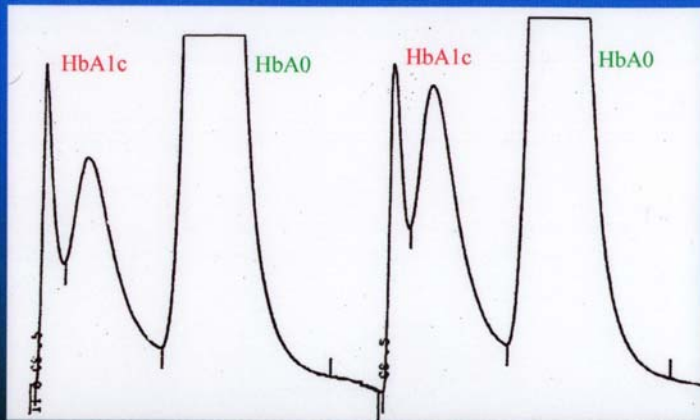
- * the analyte is unstable and not available in purified form**
- * the analyte is heterogeneous**
- * the analyte is measured by a variety of methods based on different properties of the analyte**

Harmonisation schemes based on **DCM**

- **(NGSP) National Glycohemoglobin Standardisation Program, based on BioRex 70 method used in DCCT**
- **Swedish system, based on a very specific Mono-S ion exchange chromatography**
- **Japanese system, based on consensus between the two leading IEC companies, now the value obtained with KO500**

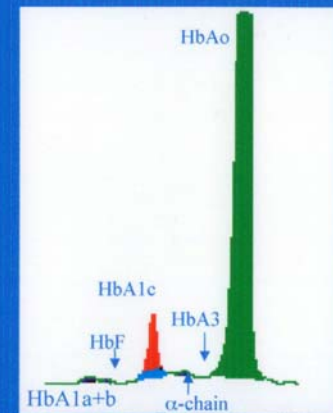
NGSP vs Mono-S ion exchange

BioRex 70 Ion Exchange Chromatography



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

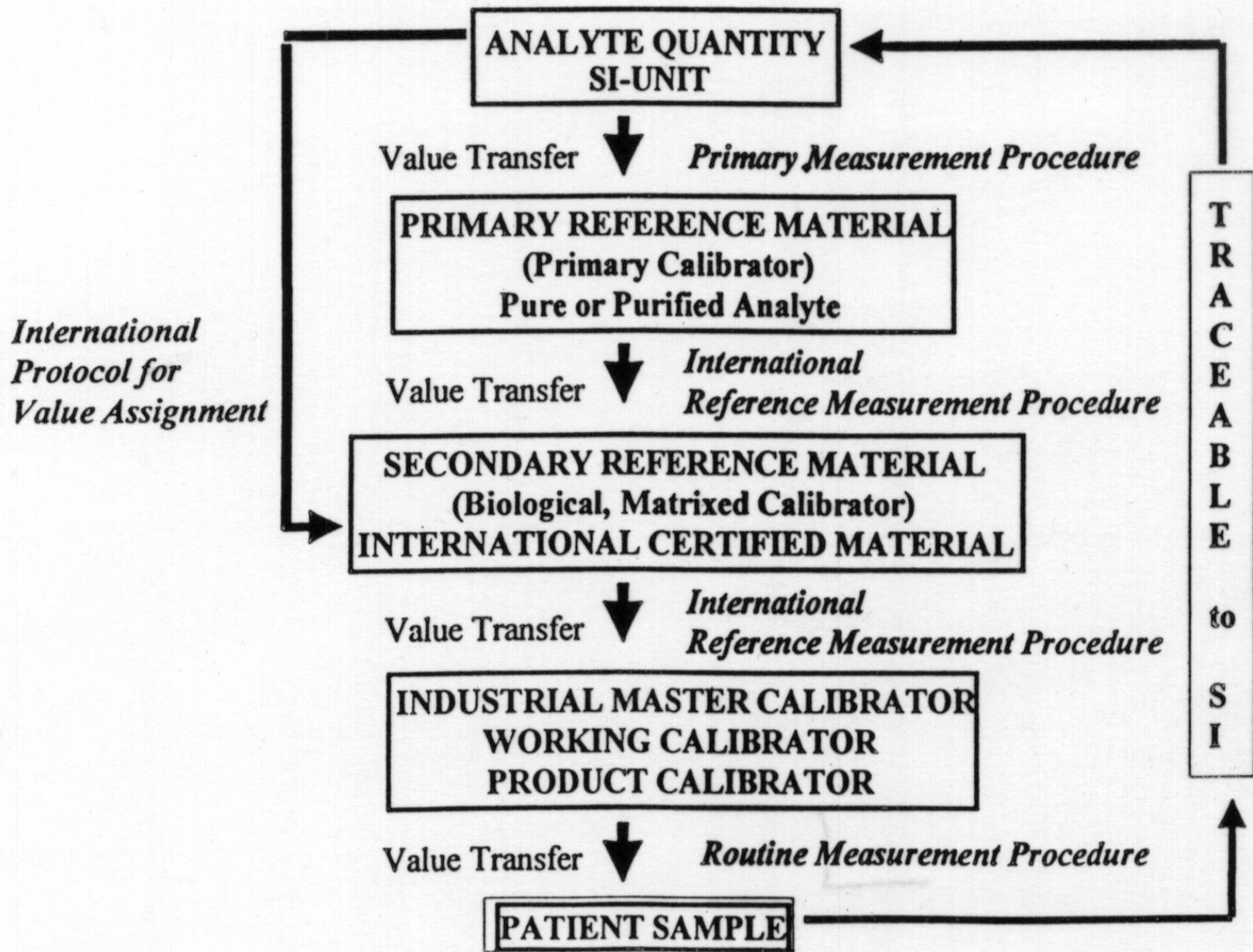


Clin Chem 1986;35:1867-72

The choice of a reference method

- several assay principles used today**
- every method has its own drawback**

**None of the existing methods can be used as
an universal reference method, a new
reference method had to be developed**



Reference System for HbA1c

- * Definition of the analyte**
- * Preparation of pure HbA0 and HbA1c**
- * Development of reference method**
- * Installation of a Reference Lab Network**
- * Preparation of secondary ref. material**

Glycation sites in Glycohemoglobin

- In GHb, glycation occurs at
 - β -N Val 60%
 - α -N-Val 6%
 - β -(ϵ -Lys) 18%
 - α -(ϵ -Lys) 16%
- Glycation can occur at 44 different sites in the HbA0 molecule
- Glycation ratio's are always the same, despite the ultimate level.

HbA1c reference system

**HbA1c is defined as β -N-Valine glycated Hb
(β -N-(1-deoxy)-fructosyl-haemoglobin).**

**Reference methods are developed based on
peptide mapping of Hb after proteolytic cleavage
of haemoglobin by endoprotease Glu-C.**

There is no alternative!!

The premiss is:

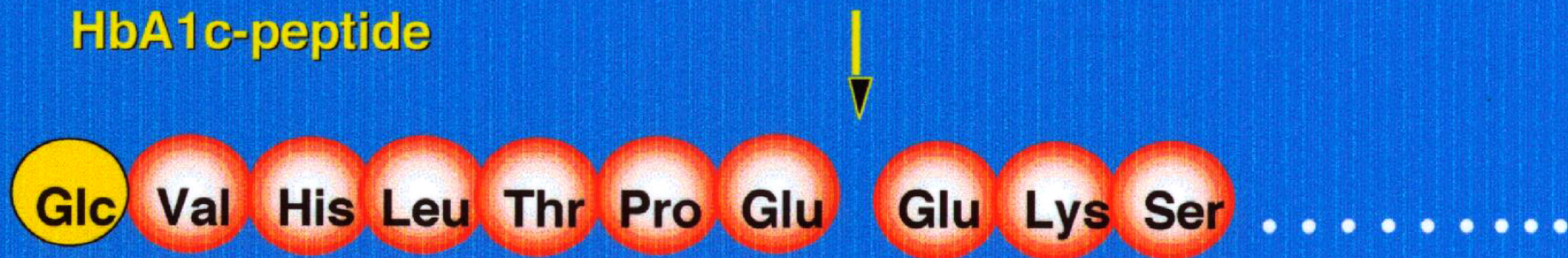
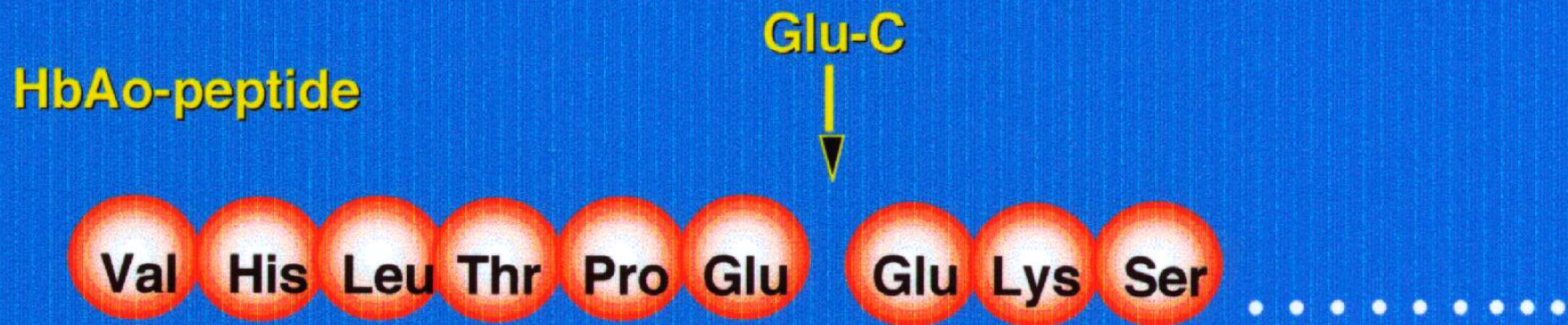
ratio of glycated to non-glycated hexapeptide

equals

the ratio of β -glycated HbA0 to total HbA0

The Analytical Challenge

Proteolytic cleavage of β -chain (146 amino acids)



blood



erythrocytes



hemolysate



enzymatic cleavage



**quantify specific
peptides**



Method A

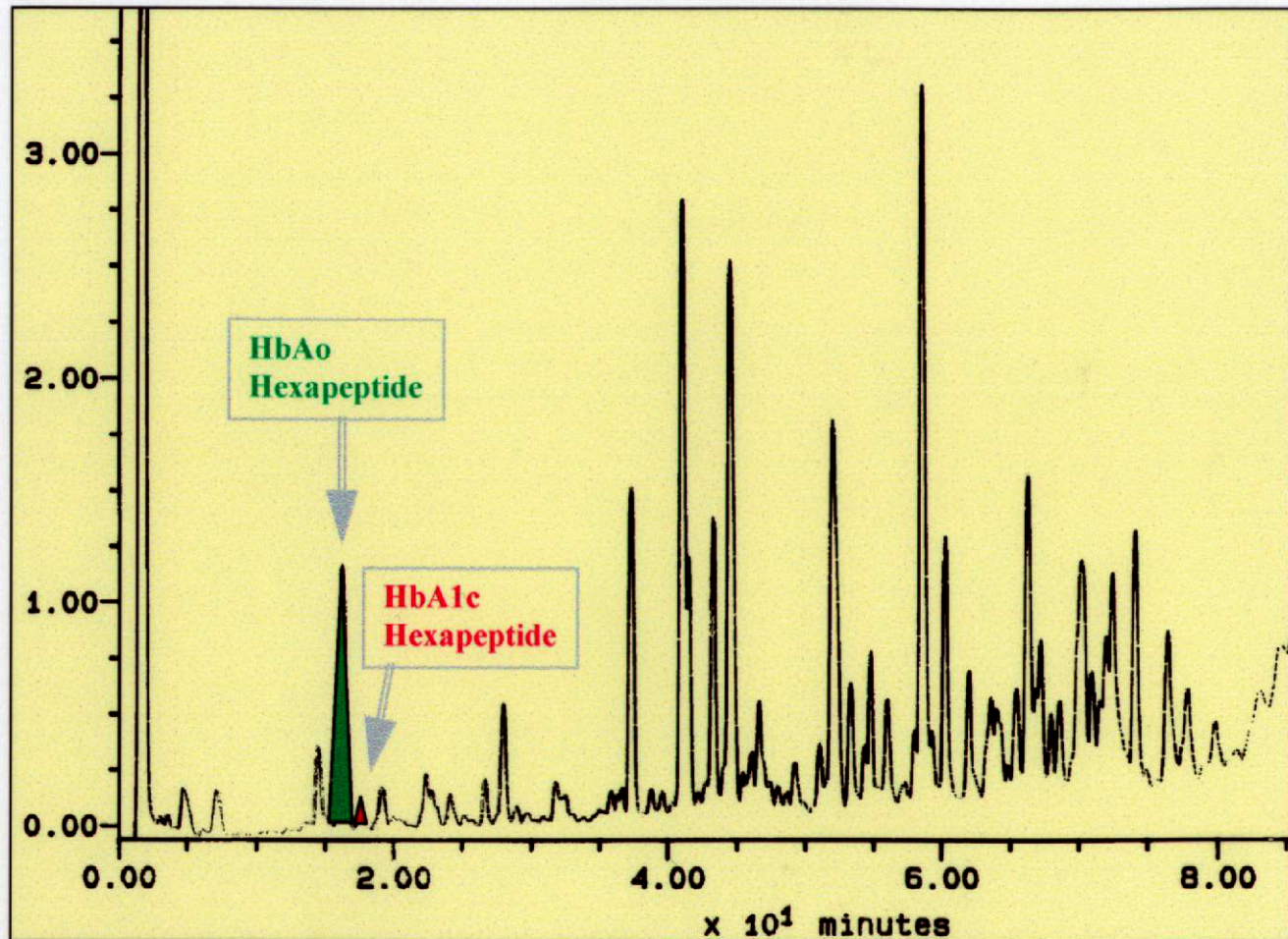
**HPLC - Mass
Spectrometry**



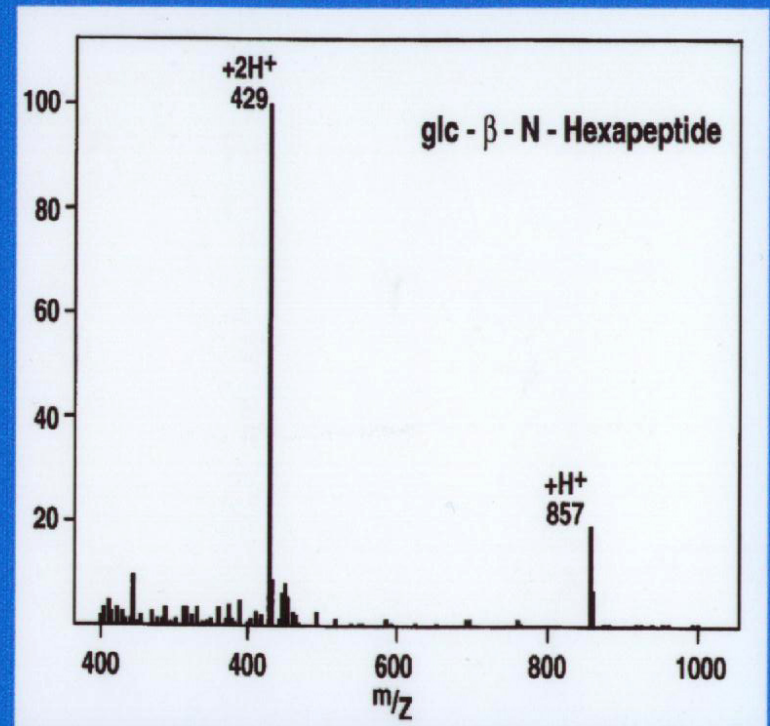
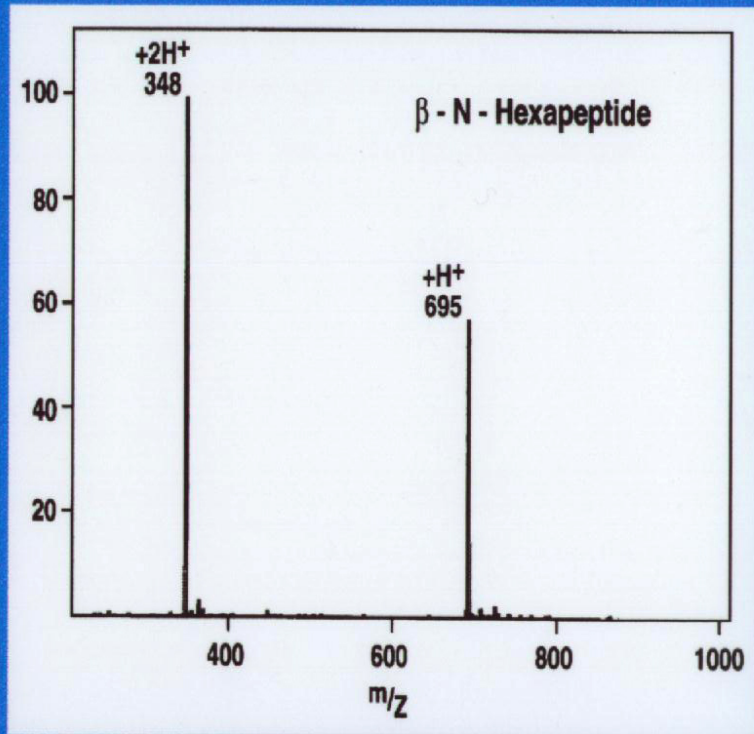
Method B

**HPLC - Capillary
Electrophoresis**

Photometric detection of peptides



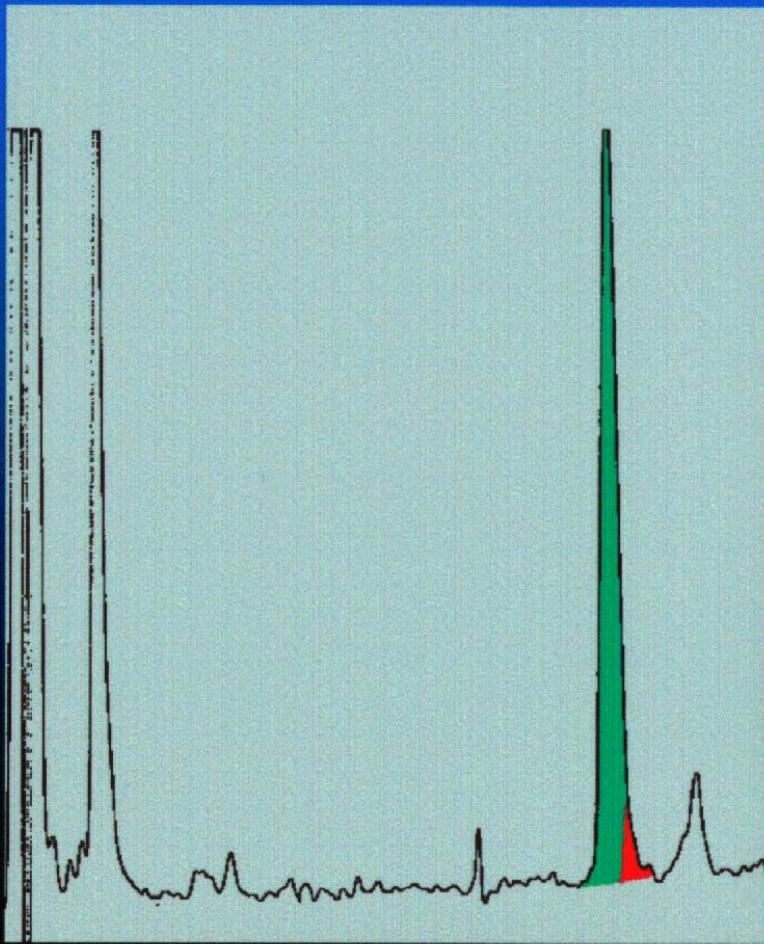
ESI-MS spectra of hexapeptides



Uwe Kobold, Boehringer Mannheim GmbH, Tutzing, Germany.

Two-dimensional separation of N-terminal hexapeptides of hemoglobin

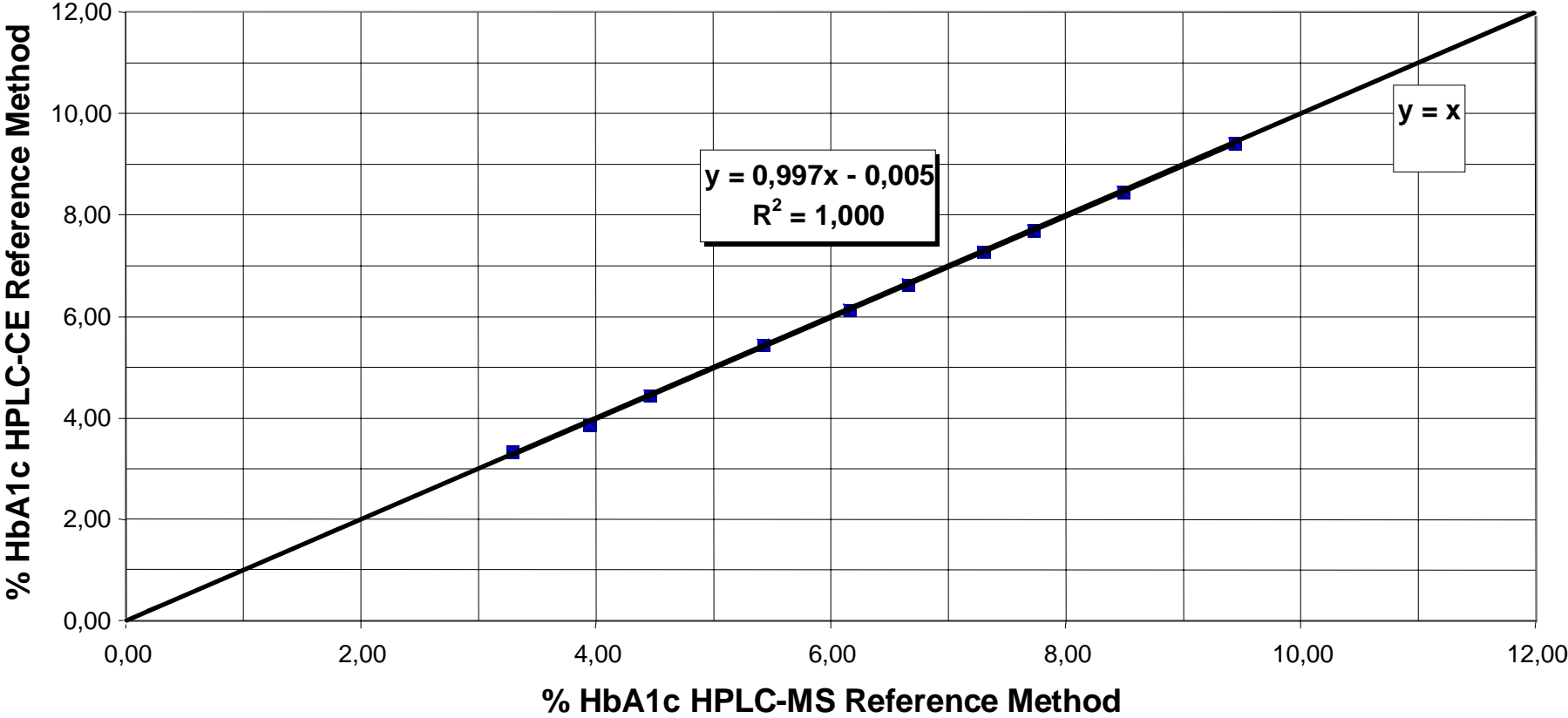
C 18 Chromatography



Capillary Electrophoresis



Comparison HPLC-MS versus HPLC-CE Reference Method (HPLC-MS 4 reference labs,HPLC-CE 6 reference labs)



The reference methods are calibrated with sets of calibrators, each year a new lot, from the Primary Reference Materials:

> 99.5% pure HbA1c

> 97% pure HbA0

Integrity and purity checked by several and different procedures

The HbA1c Reference System

**With the analyte defined, and
the method and materials developed**

the next step is in the network

- method validation**
- international method comparison studies**
- value assignment to calibrators**
- implementation**

IFCC Network of HbA1c Reference Labs

The main task of the IFCC Network is the reliable assignment of HbA1c target values to reference materials, reference panels of blood samples and control materials which are necessary for the implementation and maintenance of the system

IFCC Network of HbA1c Reference Labs

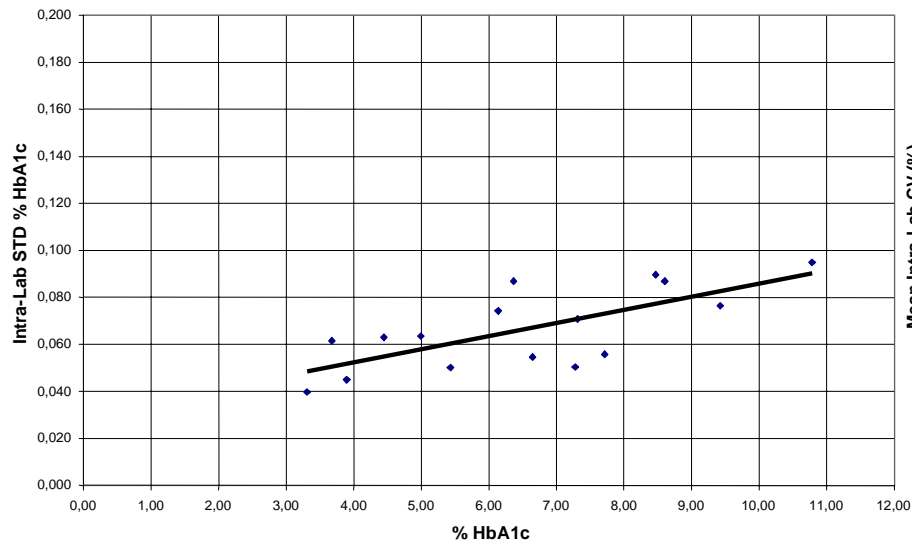
State of affairs:

- 13 intercomparison studies performed**
- results of HPLC/CE and ESI-MS identical**
- stability of the system proven**
- value assignment with very low uncertainty**
- controls included in every study**

Analytical performance of network labs

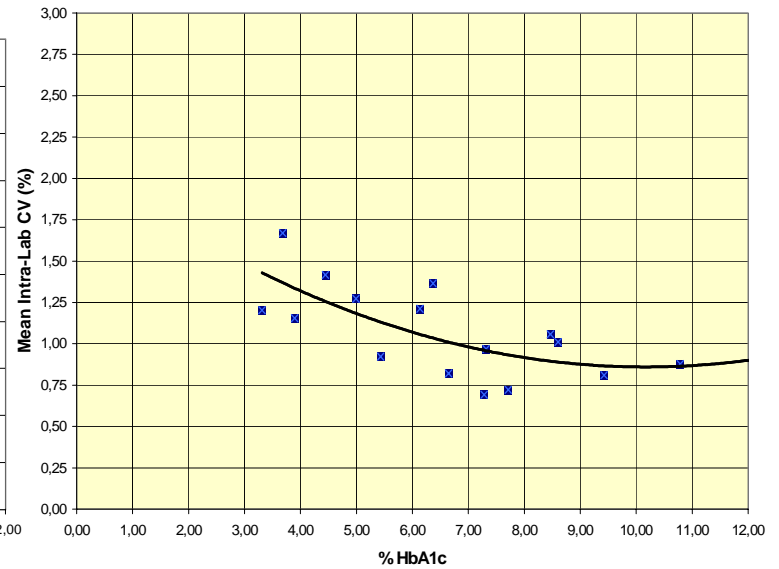
Precision Profile Reference Method -

Intra-Lab Standard Deviation (Florence I and II, 10 Reference Laboratories)



Precision Profile Reference Methods

Intra-Lab CV (Results Florence I and II studies, 10 reference laboratories)



Method Comparison Studies

a: 8 method comparison studies were performed with the existing DCM schemes in USA (NGSP), Sweden and Japan.

B: 3 method comparison studies were performed with all the major manufacturers.

About anchoring DCM's

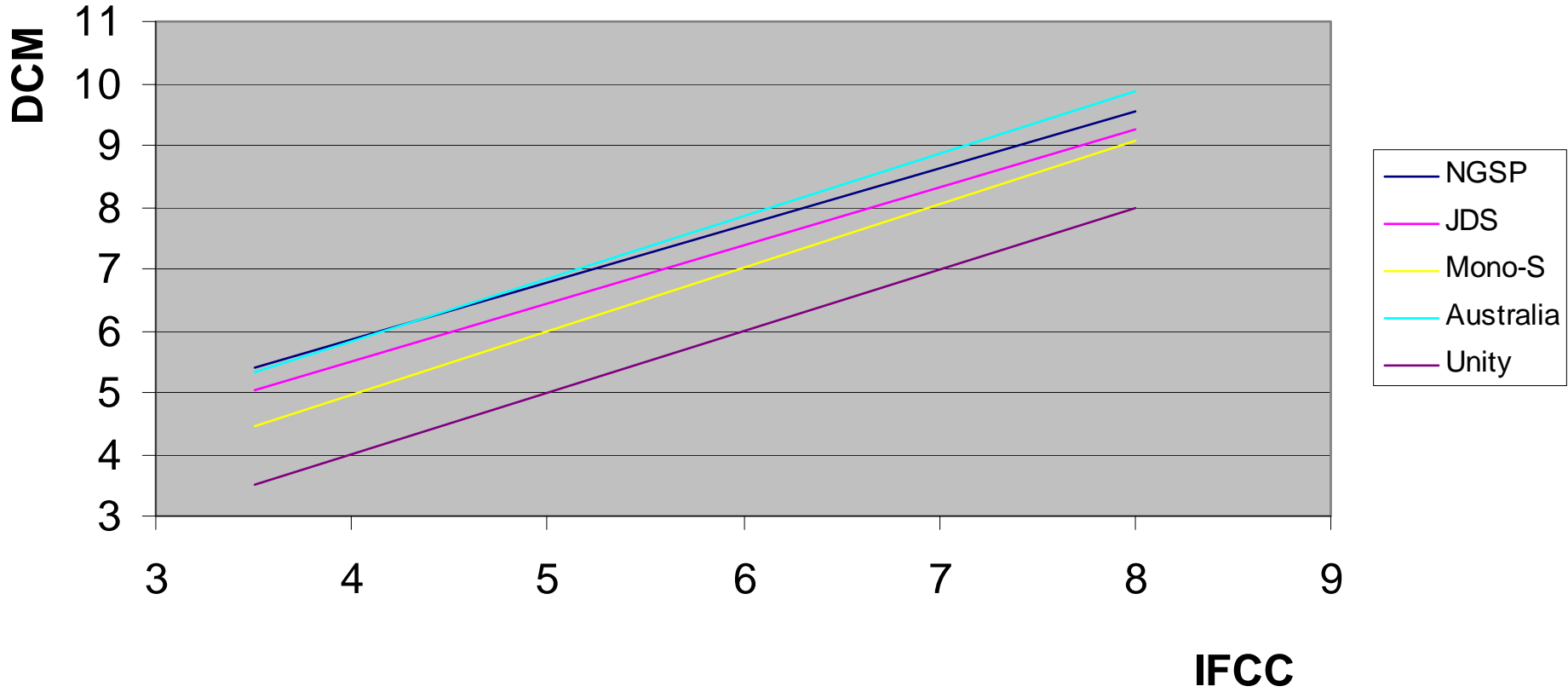
Needed:

- multiple method comparison**
- participation of the whole network on both sides**
- statistical validation of the master equation**

Ends with very precise value assignment to

IFCC SRM and DCM materials.

Master equation (Marrakech)



Correlation between IFCC and DCM's

$$y \text{ HbA1c/NGSP} = 0.915 \text{ HbA1c/IFCC} + 2.15$$

$$y \text{ HbA1c/JDS} = 0.927 \text{ HbA1c/IFCC} + 1.27$$

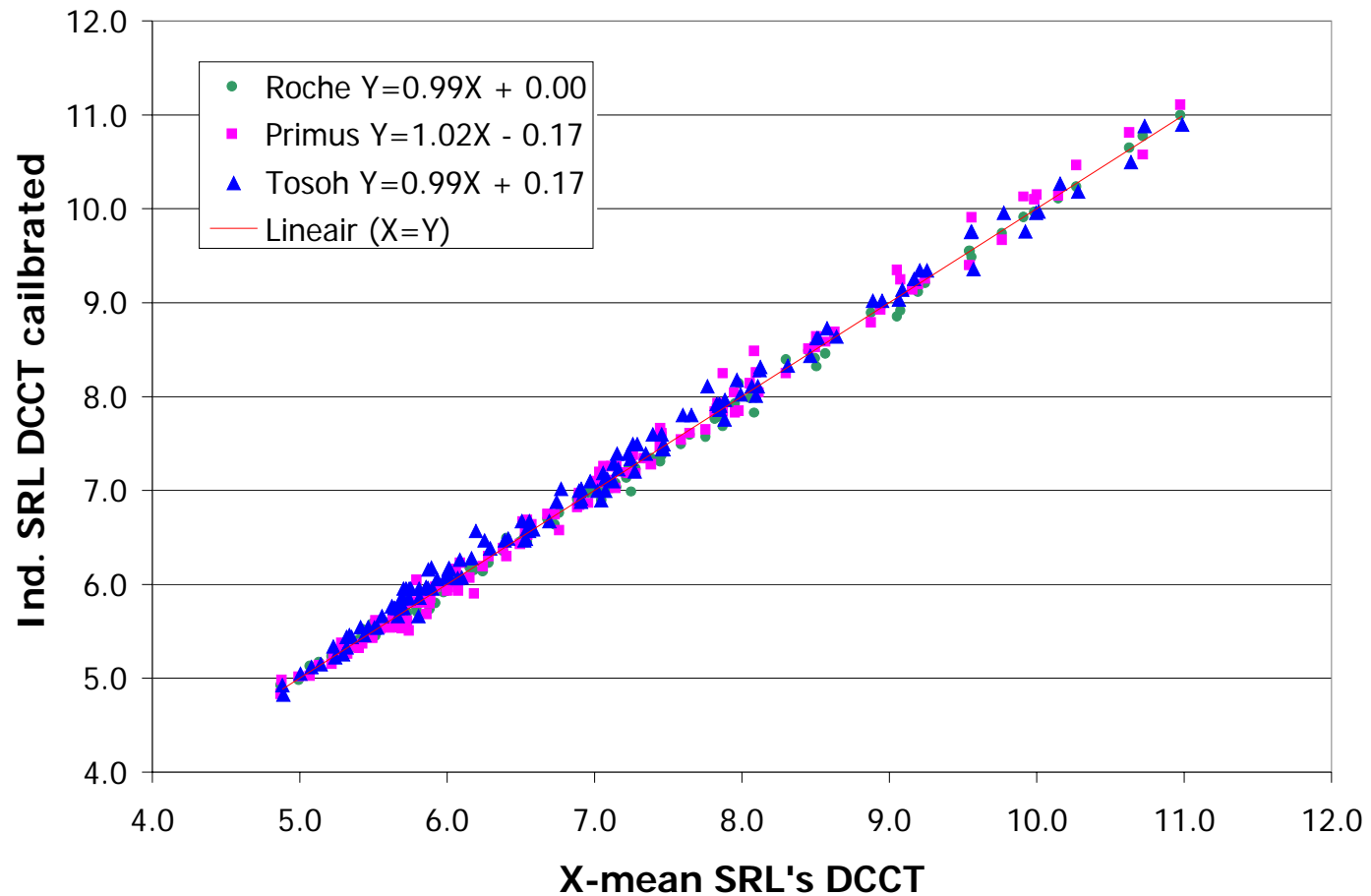
$$y \text{ HbA1c/MonoS} = 0.989 \text{ HbA1c/IFCC} + 0.88$$

Converting % HbA1c to mmol/l glucose:

$$\text{NGSP: Glu} = 1.98 \times \text{HbA1c/NGSP} - 4.29$$

$$\text{IFCC : Glu} = 1.84 \times \text{HbA1c/IFCC} - 0.01$$

X-mean SRL's DCCT vs individual SRL DCCT calibrated



Anchoring Manufacturers Methods

- blood panels with assigned values**
- different certified reference material according to assay design and principle**
- manufacturers should use their own in-house calibration principle**
- correlation with DCM's will be provided**
- manufacturers asks certification of traceability**

Nature of the SRMs

6-8 pools of at least 10 single donations each

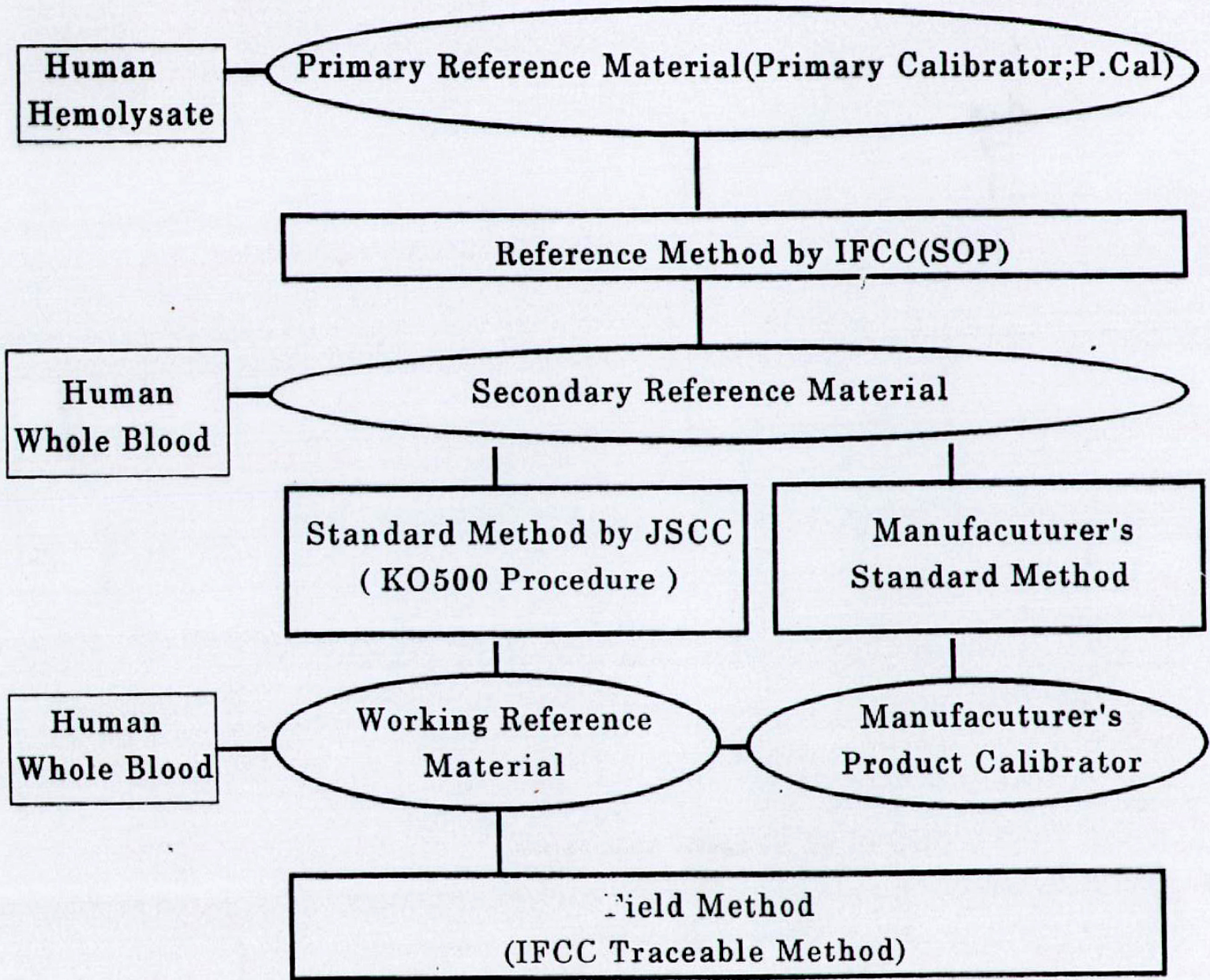
no interferences

no Hb-variants, no Hb-derivatives

normal Hb levels

intended range 3-12 (IFCC)%

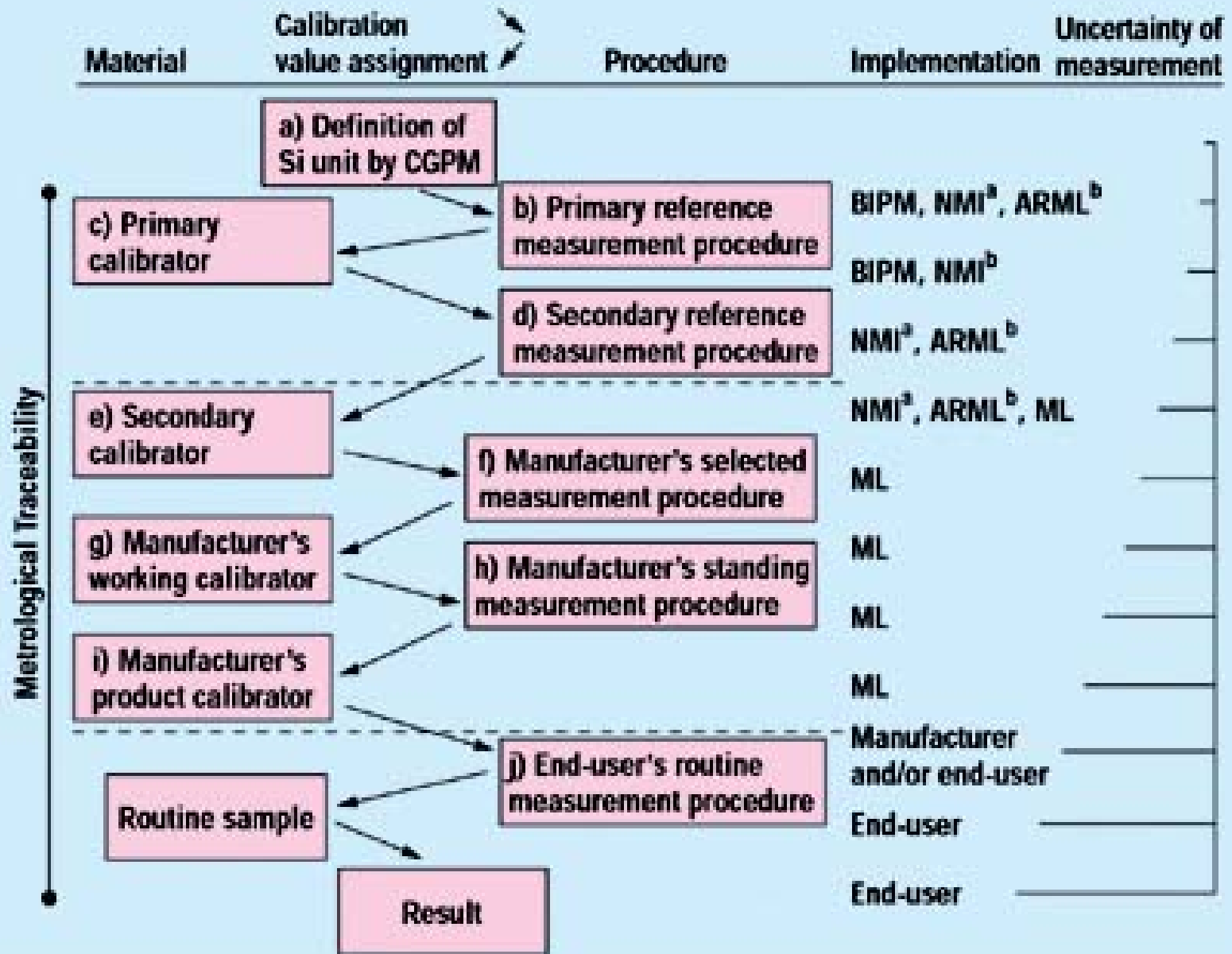
Measurement System of HbA1c in Japan Society of Clinical Chemistry (July, 199



IVD-MD Directory of the EC

“diagnostic manufacturers must guarantee the traceability of their routine tests to reference methods and materials of higher metrological order”.

(ISO TCT 212 demands documentation about how to trace back assigned values)



a. National metrology institute.

b. Accredited measurement reference laboratory. Such a laboratory may be an independent or a manufacturer's laboratory.

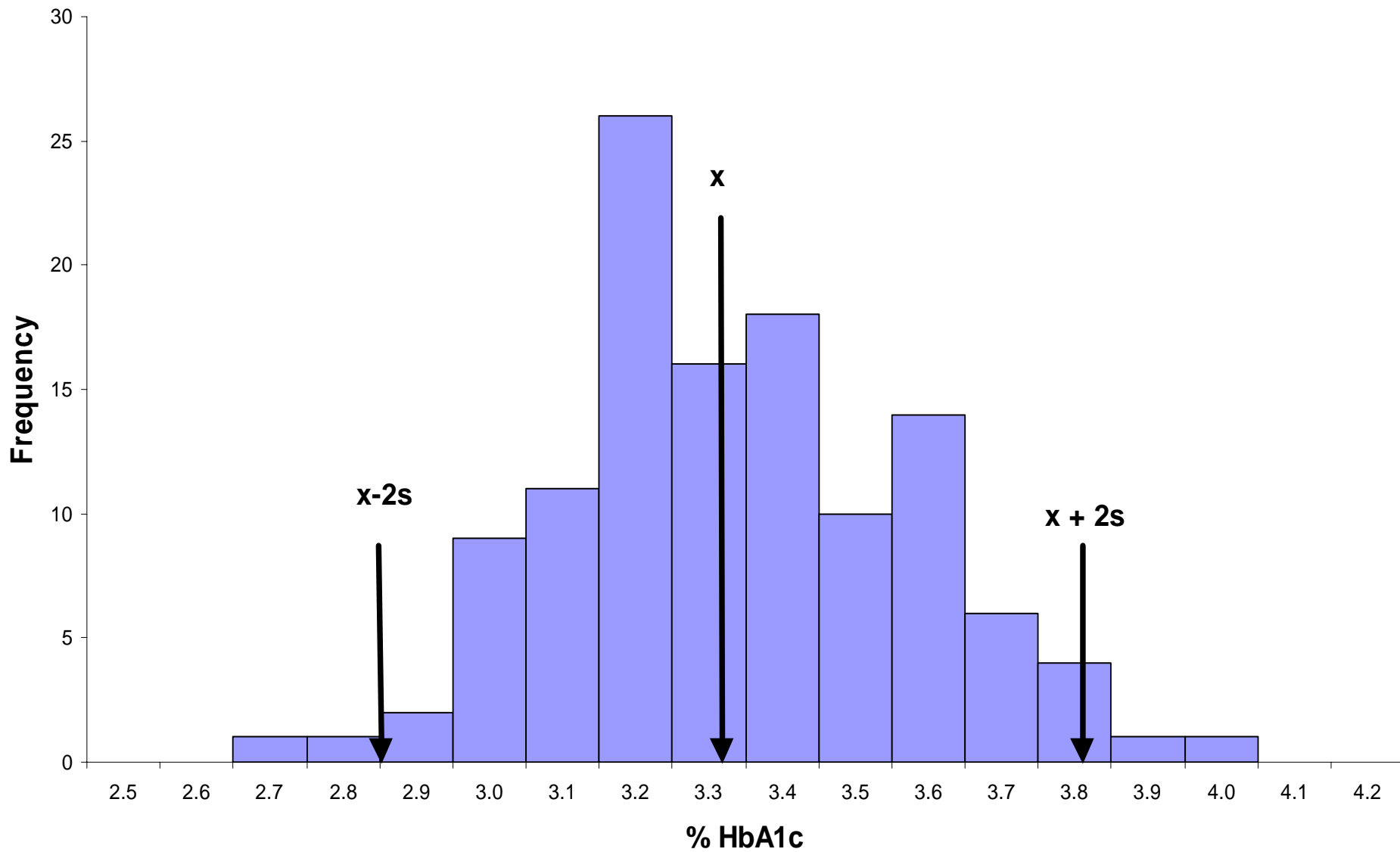


Figure 6. *Results of the Reference Range Study HbA1c*
($n = 120$; 7 reference laboratories, $x =$ mean value, $s =$ standard deviation)

Translation of good clinical diabetes practice in

IFCC HbA1c terms:

non-diabetics **3 - 4 % HbA1c¹**

target for therapy **5 % HbA1c²**

change of therapy **6 % HbA1c²**

1) reference range determined by network

2) diabetic targets recalculated from DCCT

About the name:

| | |
|-----------------------------------|--------------|
| - Glycated Hemoglobin | HbA1c |
| - Glycated Hemoglobin | GHb |
| - β Glycated Hemoglobin | β -GHb |
| - Glycation Index | GI |
| - Diabetes Risk Number | DRN |
| - β -1-N-Deoxy Fructosyl Hb | β -DFH |

**The IFCC WG prefers to keep on the name
HbA1c**

We are measuring

Specific β -N terminal glycation of hemoglobin.

Chemical name 1-N- β -deoxyfructosyl hemoglobin

Name it: HbA1c(IFCC)

Unit: mmolHbA1c/mol tot Hb

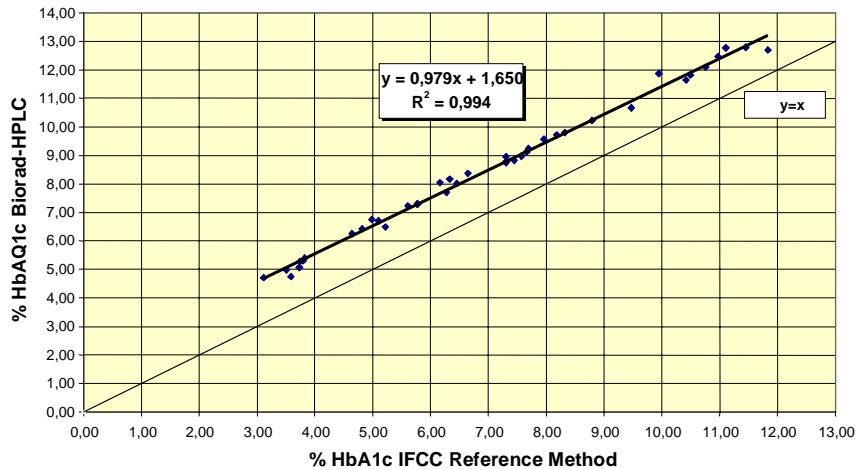
IS:

30 – 100 in practical life.

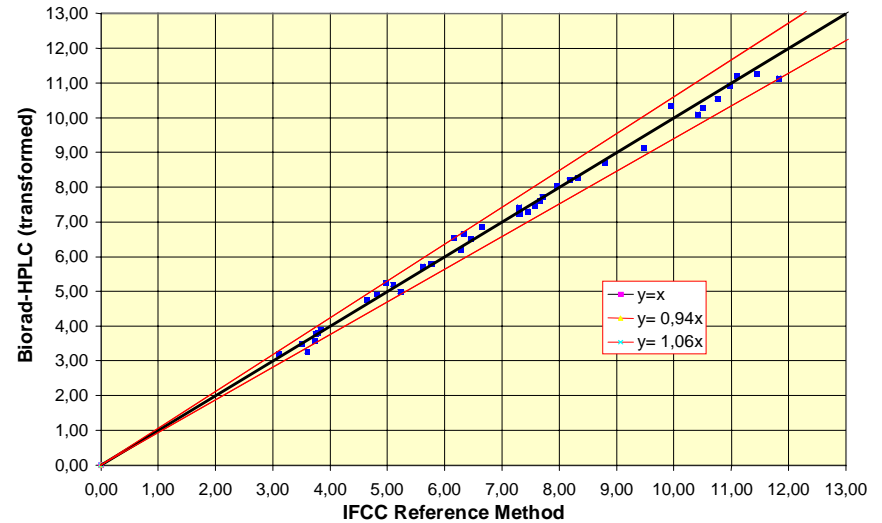
Standardisation of Manufacturers

Methods - 1

a) Method Comparison Biorad-HPLC versus IFCC Reference Method

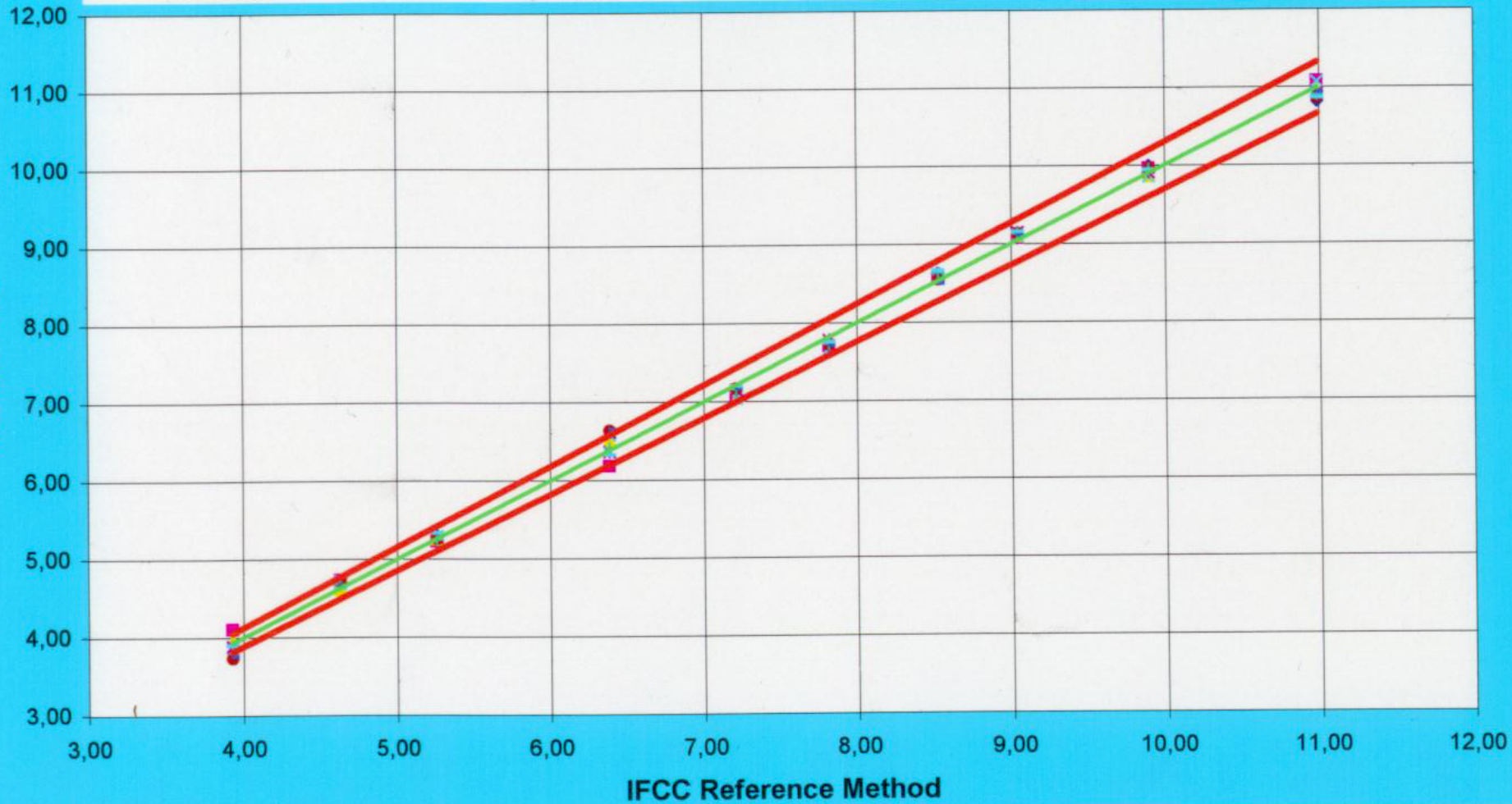


b) Comparison Biorad-HPLC (transformed) versus IFCC Reference Method



Method Comparison IFCC Reference Method Versus Standardized Designated Comparison Methods

- ◆ Diamat
- ✱ CE
- K0500
- Immunoassays
- Menarini
- $y = 1,03x$
- ▲ Primus
- + Tosoh
- $y = 0,97x$
- × Mono S
- Kyoto-Daiichi
- $y = x$



Implementation of the Reference System

- 1. Blood panels (certified reference materials) with assigned values are available for manufacturers for internal calibration**
- 2. IFCC Reference Method will be introduced as anchor for DCM-systems**
- 3. Introducing the IFCC system = IFCC numbers**

The IFCC Reference System for HbA1c

- clear definition of the analyte**
- primary reference material prepared**
- a new reference method developed**
- an international network established**
- suitability of calibrating routine procedures investigated**
- relationship to the old trueness established in order to maintain clinical experience**

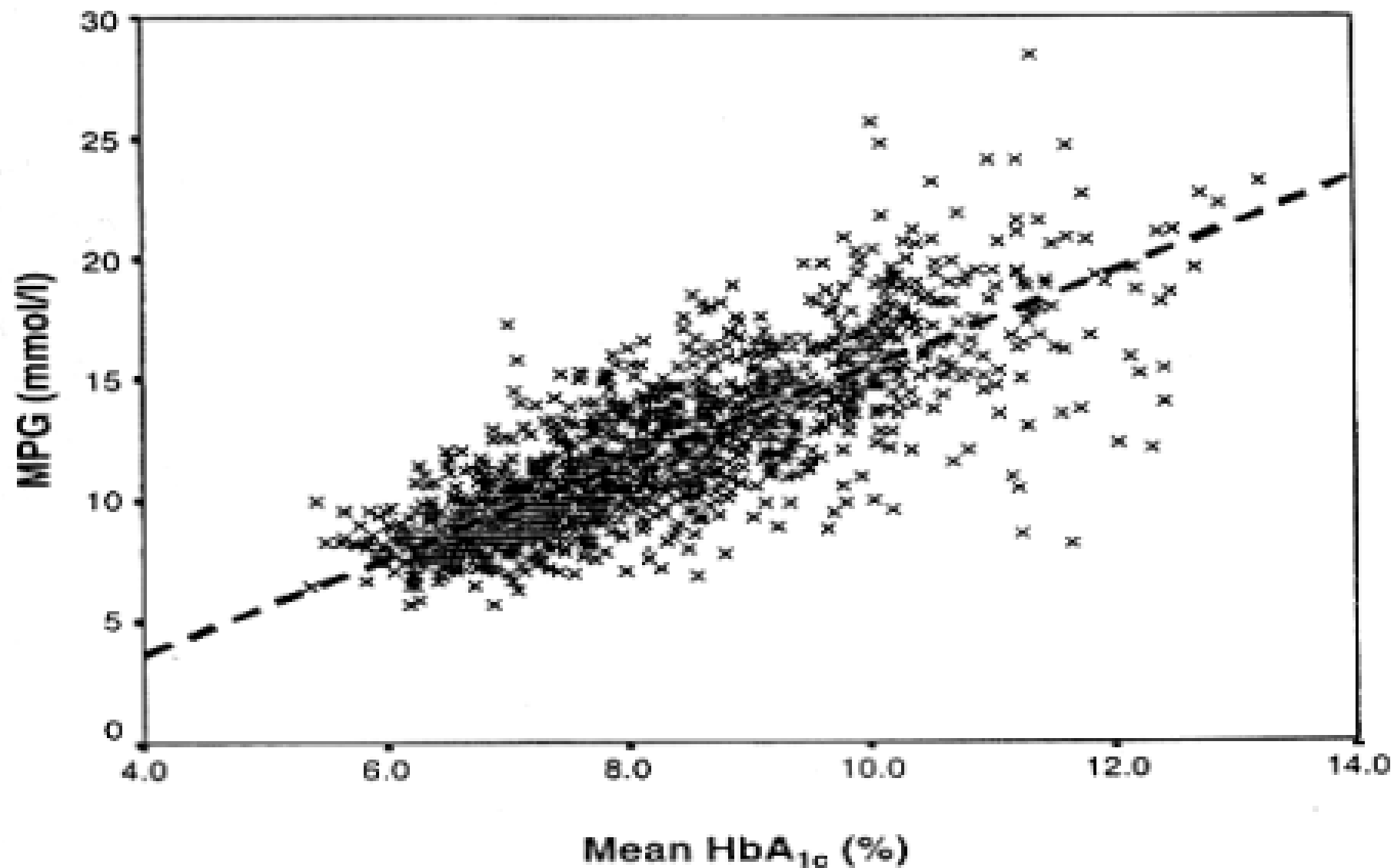


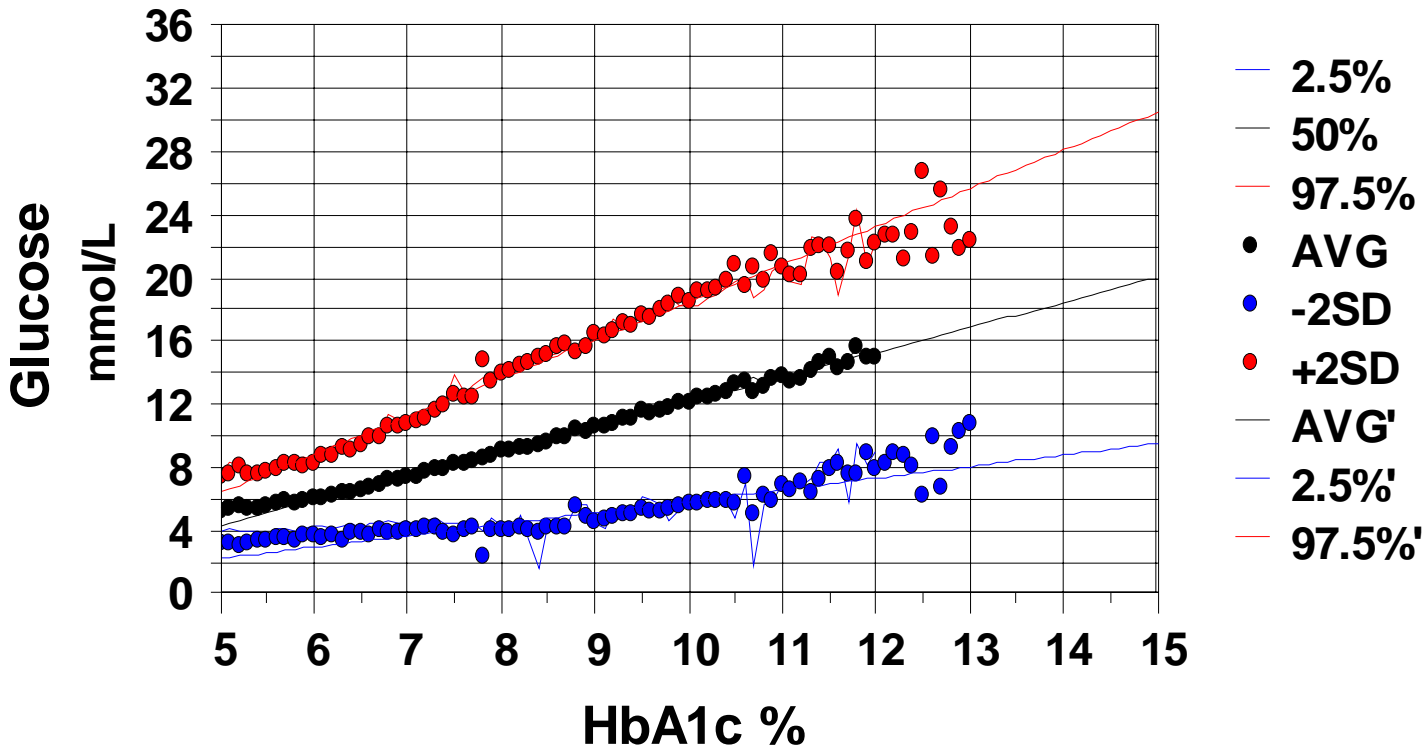
Figure 1—MPG versus HbA_{1c}; $n = 1,439$; $r = 0.82$; $PG \text{ (mmol/l)} = (1.98 \cdot HbA_{1c}) - 4.29$. The dashed line indicates the regression line.

HbA1c & Glucose

HbA1c vs Fasting Glucose

n=36,264

$$\text{AVG} = 1.57\text{HbA1c} - 3.54 \quad \text{STD} = 0.42\text{HbA1c} - 1.05$$



Risk of Sustained Retinopathy and Mean of HbA1c / IFCC in Type 1 Diabetes (DCCT study)

