HbA_{1c} Measurement by IDMS – Current Situation and Future Development

2017 JCTLM Member's and Stakeholder's Meeting
Accurate Results for Patient Care Workshop 2017

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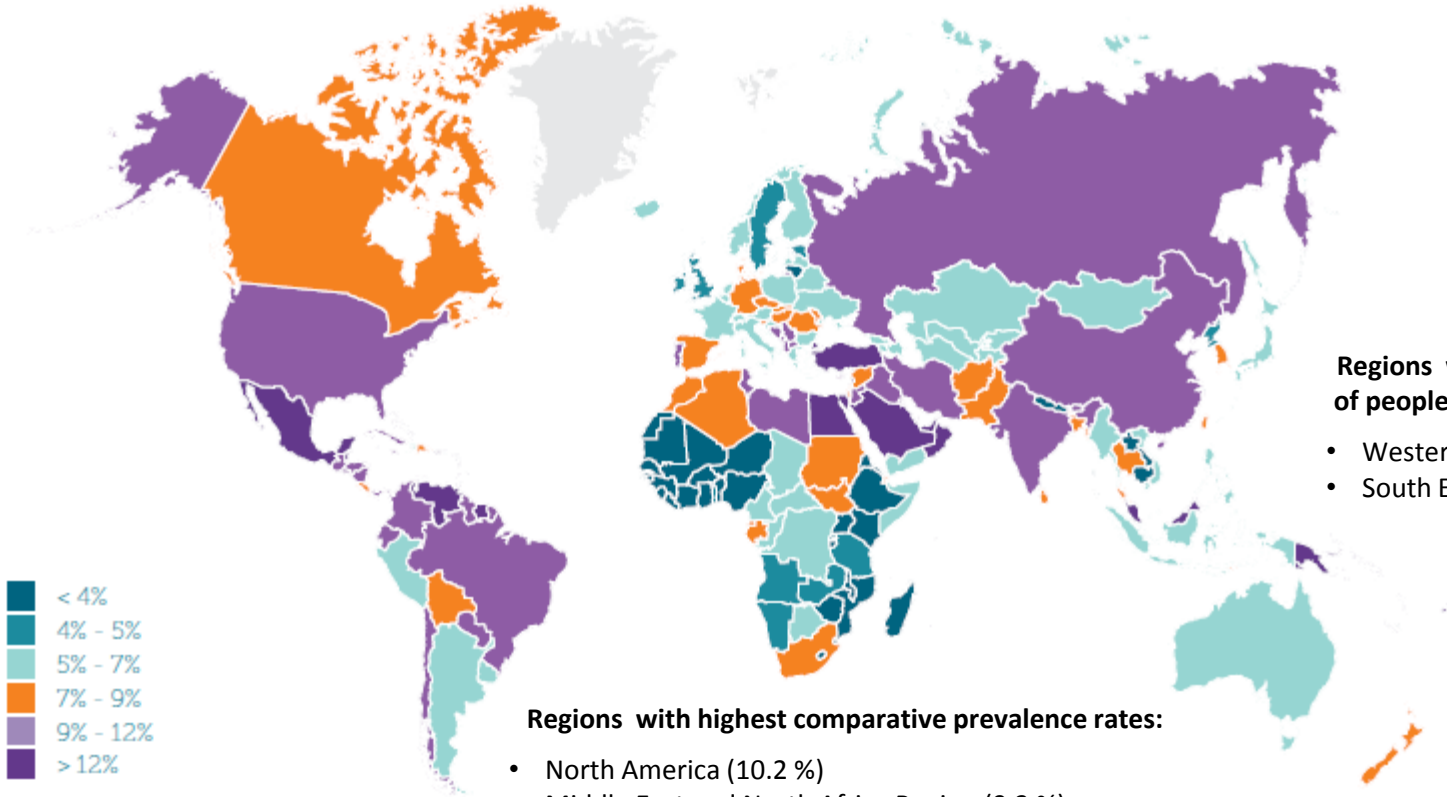
Outline

- Importance of haemoglobin A_{1c} (HbA_{1c}) measurement and its standardisation.
- Principle and procedure of IDMS method for HbA_{1c} measurement.
- Current situation of IDMS method.
- Possible key points to ensure the accuracy of IDMS method.
- Planned CCQM comparisons on HbA_{1c} measurement

Diabetic Conditions Around the World

Map 3.1 Estimated age-adjusted prevalence of diabetes in adults (20-79), 2015



Regions with highest number of people living with diabetes:

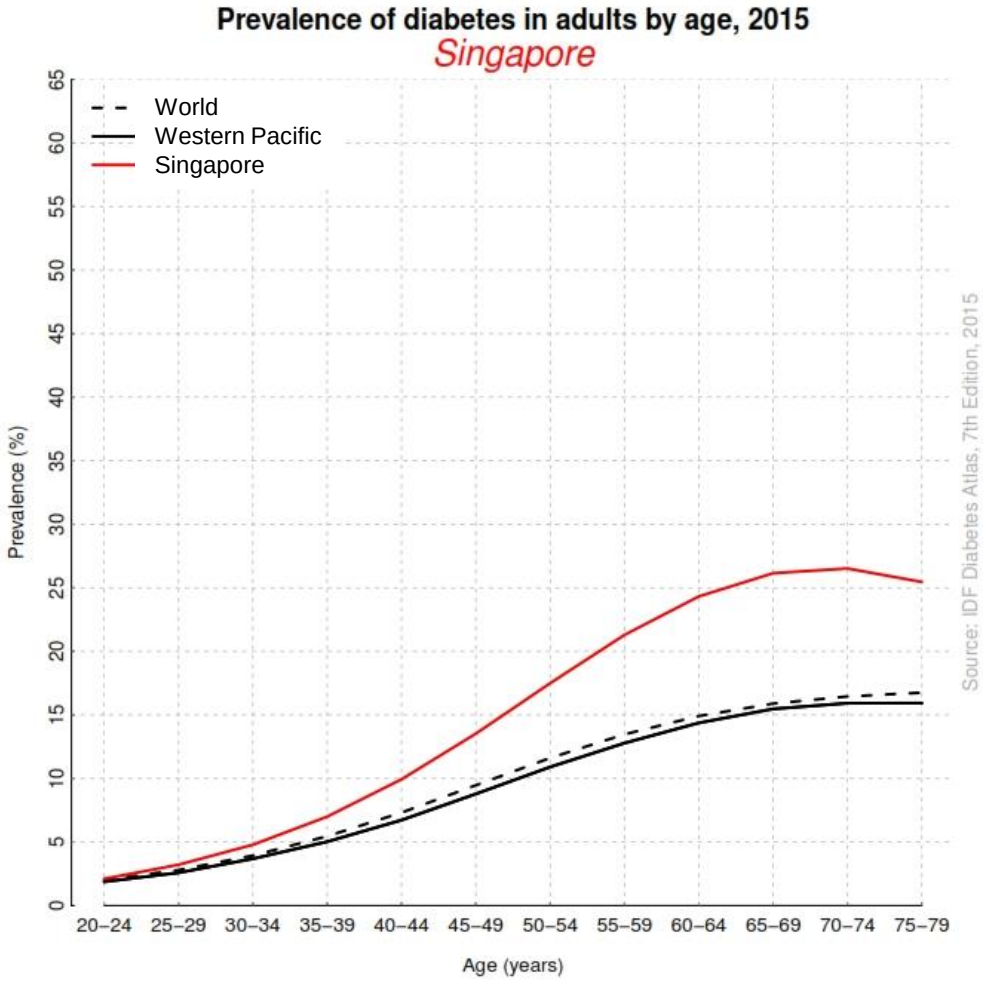
- Western Pacific (77 million)
- South East Asia (59 million)

Regions with highest comparative prevalence rates:

- North America (10.2 %)
- Middle East and North Africa Region (9.3 %)

International Diabetes Federation. IDF Diabetes, 7 ed. Brussels, Belgium: International Diabetes Federation, 2015. http://www.diabetesatlas.org

Diabetic Condition in Singapore



- Singapore has a high prevalence of diabetes.
- Aging Population.
- Some complications resulting from diabetes include cardiovascular disease, blindness, kidney failure, and lower limb amputation.[2]

[1] International Diabetes Federation. IDF Diabetes, 7 ed. Brussels, Belgium: International Diabetes Federation, 2015. http://www.diabetesatlas.org
[2] IDF. Complications of Diabetes. http://www.idf.org/complications-diabetes

Importance of HbA_{1c} Measurement

- HbA_{1c} is an important biomarker for the diagnosis of diabetes mellitus.

  WHO recommendation: a HbA_{1c} level of 6.5% as the cut-off point for diagnosing diabetes.

- HbA_{1c} is an effective biomarker for monitoring the long term blood glucose level in diabetic patients to ensure proper treatment.

  In Singapore, one in nine (11.3%) residents aged 18 to 69 has been diagnosed with diabetes mellitus. HbA_{1c} measurement is used to monitor glycemic control to ensure proper treatment and management of the disease.

  - HbA_{1c} < 7%: optimal glycemic control
    Treatment: mainly nutrition therapy and exercise
  - HbA_{1c} 7 – 9%: "sub-optimal" moderate glycemic control
    Treatment: mainly metformin therapy
  - HbA_{1c} > 9%: "poor" glycemic control
    Treatment: metformin therapy, alternatively insulin therapy

Standardisation of HbA_{1c} Measurement

- Different reference systems with insufficient consistency with one another:
  - US [National Glycohemoglobin Standardisation Program (NGSP)]
  - Japan [Japanese Diabetes Society (JDS)/Japanese Society of Clinical Chemistry (JSCC)]
  - Sweden
- IFCC reference method – the accuracy-based reference method for standardisation of HbA_{1c} measurement.
  - Purified HbA_0 and HbA_{1c} as the calibration standards
  - Purity of calibration standards determined by ion exchange chromatography
- Significant biases were found between IFCC and other reference systems.

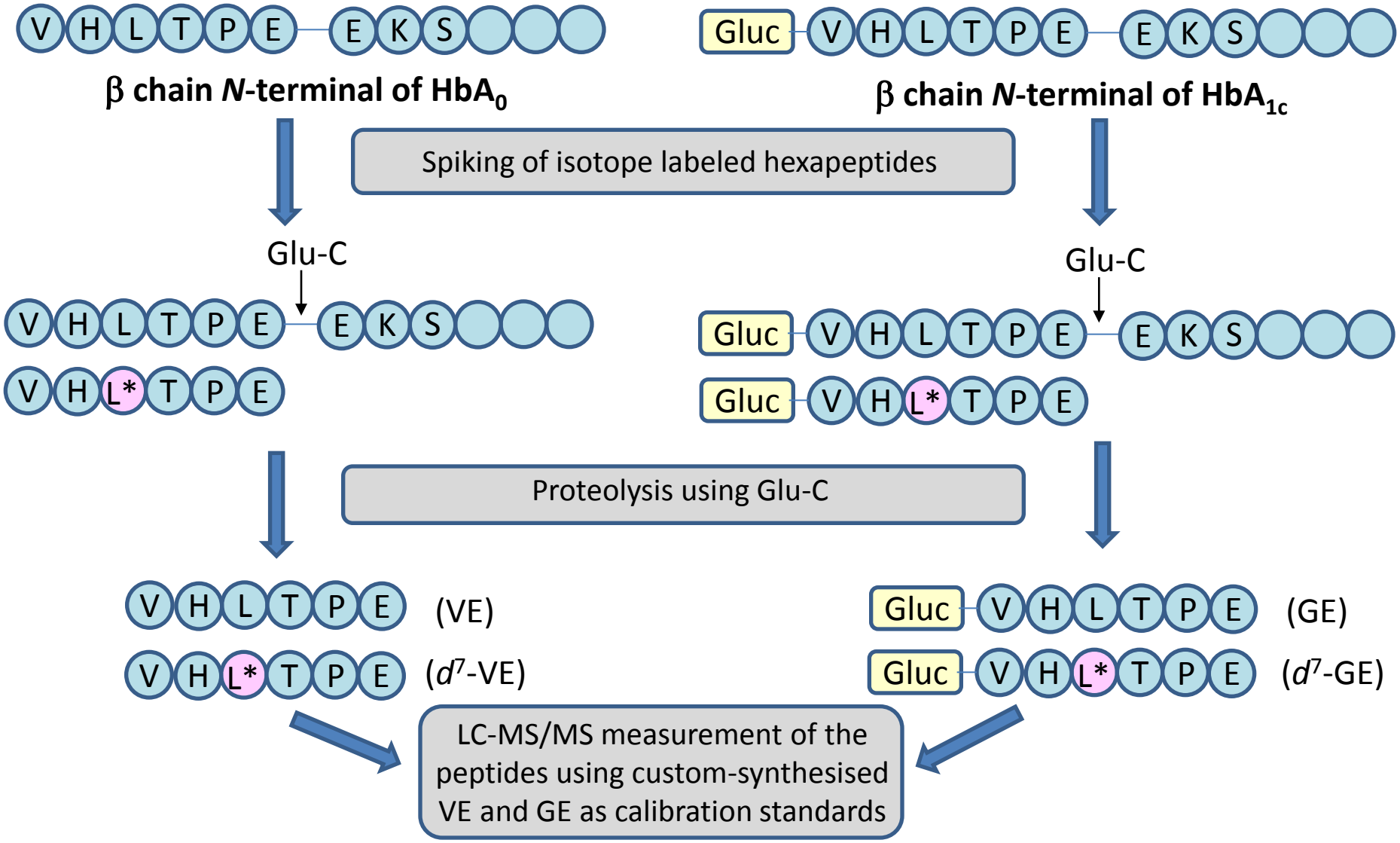
  Master equations are used for conversion, for example:

  NGSP (%)= 0.09148 × IFCC (mmol/mol) + 2.152
- It would be desirable to have an alternative accuracy-based reference method as an independent support for the IFCC reference method.

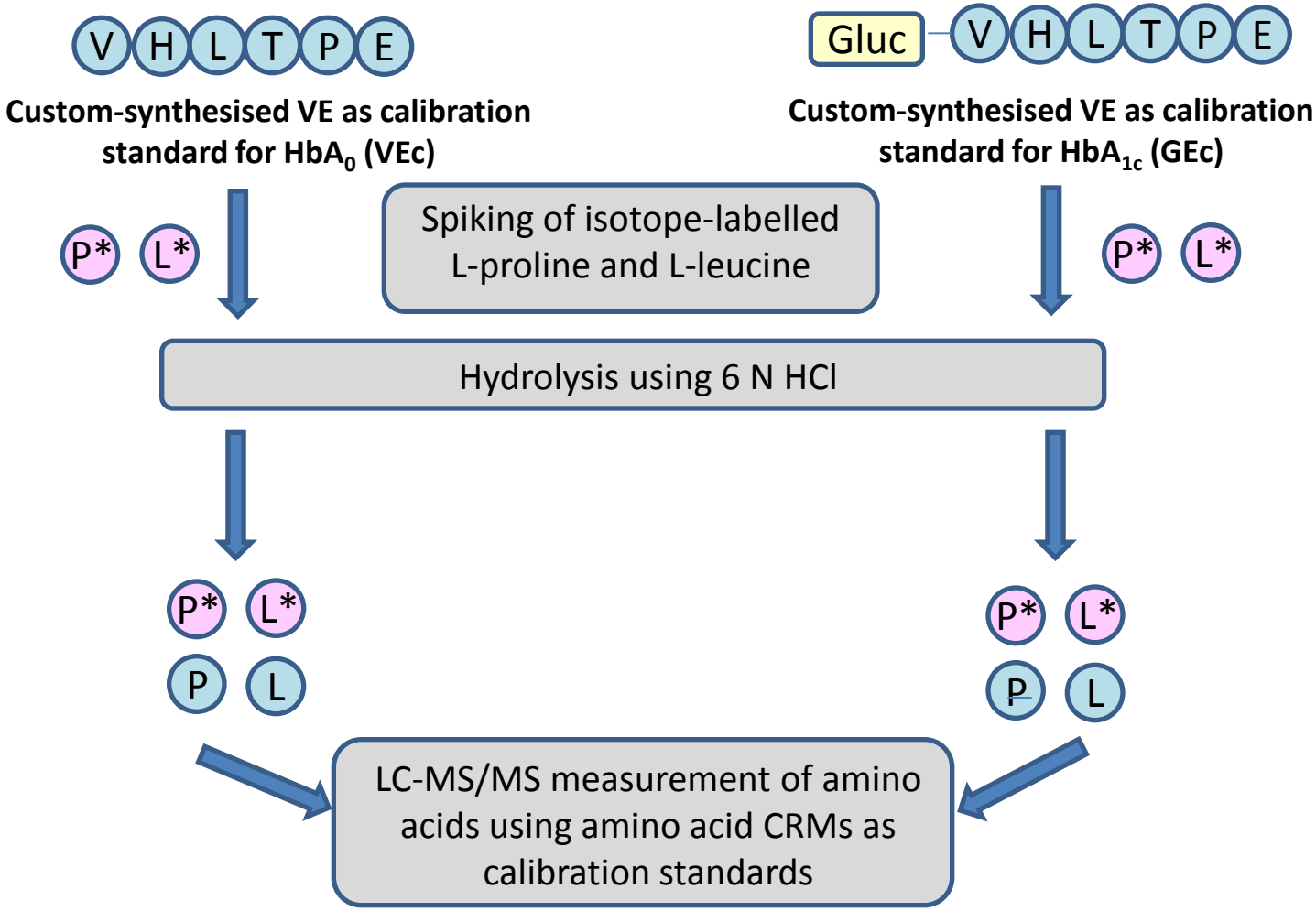
IDMS Method for HbA_{1c} Measurement *vs* IFCC Reference Method

- Similarity
  - Based on proteolysis of HbA_0 and HbA_{1c}, using endoproteinase Glu-C.
- Calibration Standards
  - **IDMS Method:** Hexapeptides as calibration standards (purity determined by another step of IDMS using amino acid CRMs as the calibration standards)
  - **IFCC Reference Method:** Purified HbA_0 and HbA_{1c} as calibration standards.
- Quantification
  - **IDMS Method:** Separate quantification of HbA_0 and HbA_{1c} by IDMS method, using two signature hexapeptides as the calibration standards. HbA_{1c} Level = $HbA_{1c}/(HbA_{1c}+ HbA_0)$
  - **IFCC Reference Method:** External calibration with six levels of HbA_{1c} (0% to 15%). Calibration solutions are prepared by mixing purified HbA_0 and HbA_{1c} solutions.

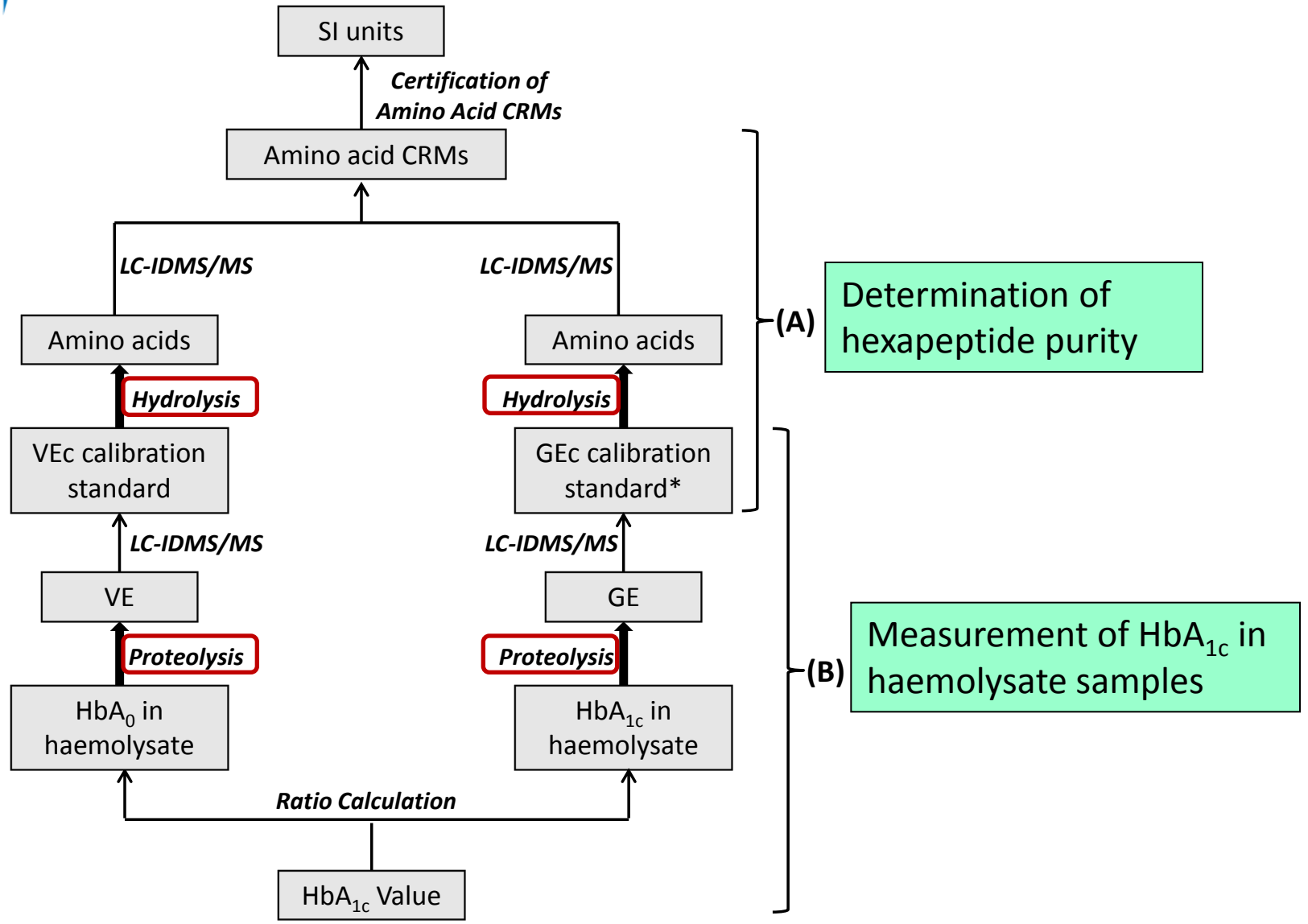
IDMS Procedure for HbA1c Measurement



IDMS Procedure for Determination of the Purity of Hexapeptides as Calibration Standards (VEc and GEc)



Traceability of IDMS Method

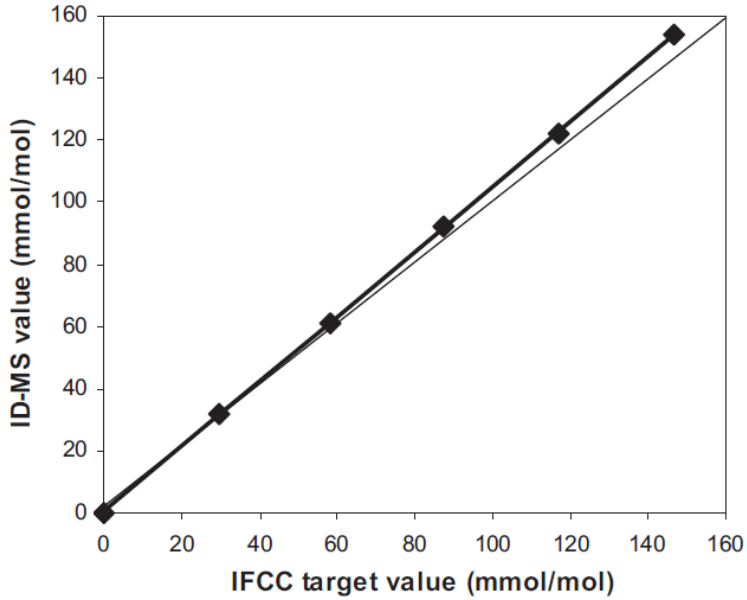


Bias between IDMS Method and IFCC Reference Method?

- Average relative deviation between IDMS method and IFCC reference method: 6.5%

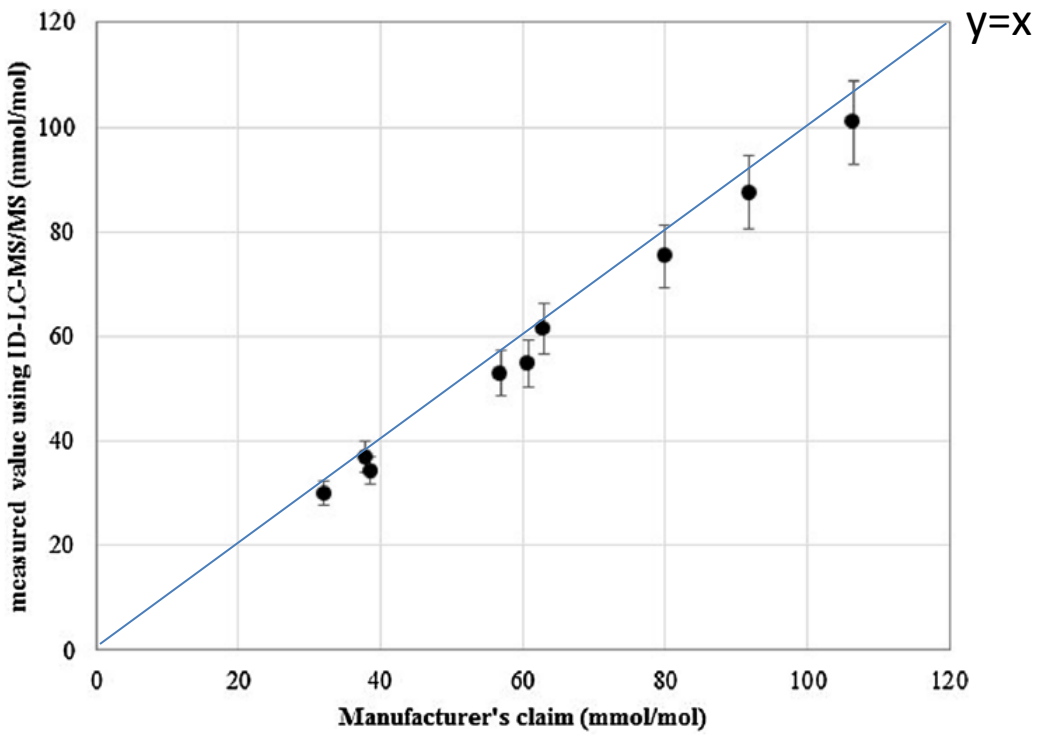
  T. Nakanishi *et al*., *Clin. Chem.*, 2003, 49, 829-831.

- Average relative deviation between IDMS method and IFCC reference method: 3.4%.



P. Kaiser *et al*., *Clin. Chem.*, 2010, 56, 750-754.

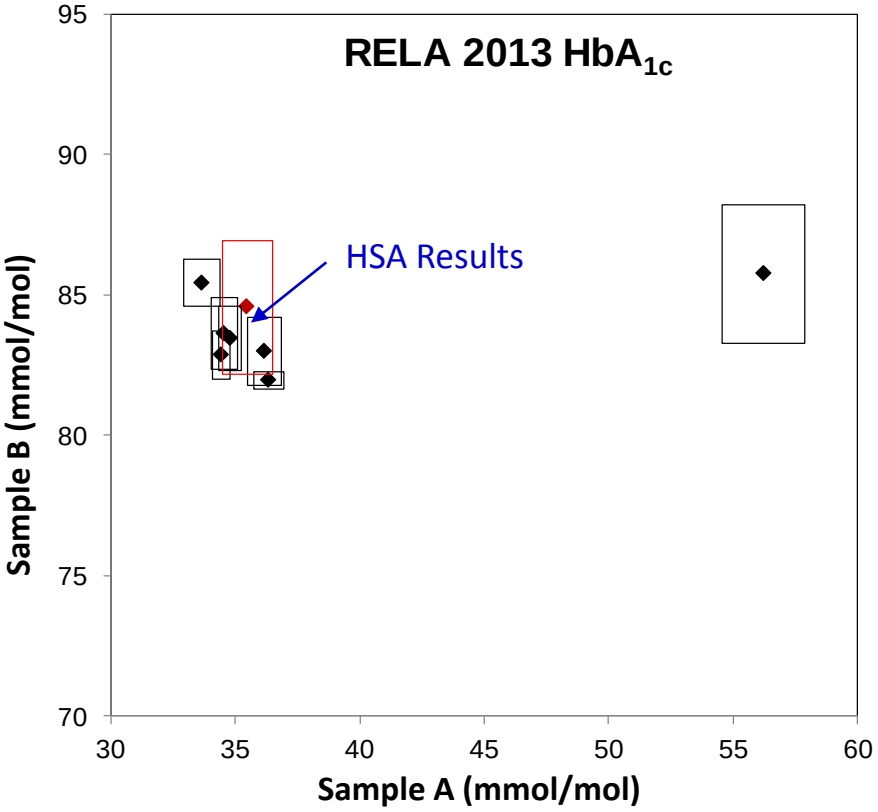
Bias between IDMS Method and IFCC Reference Method?

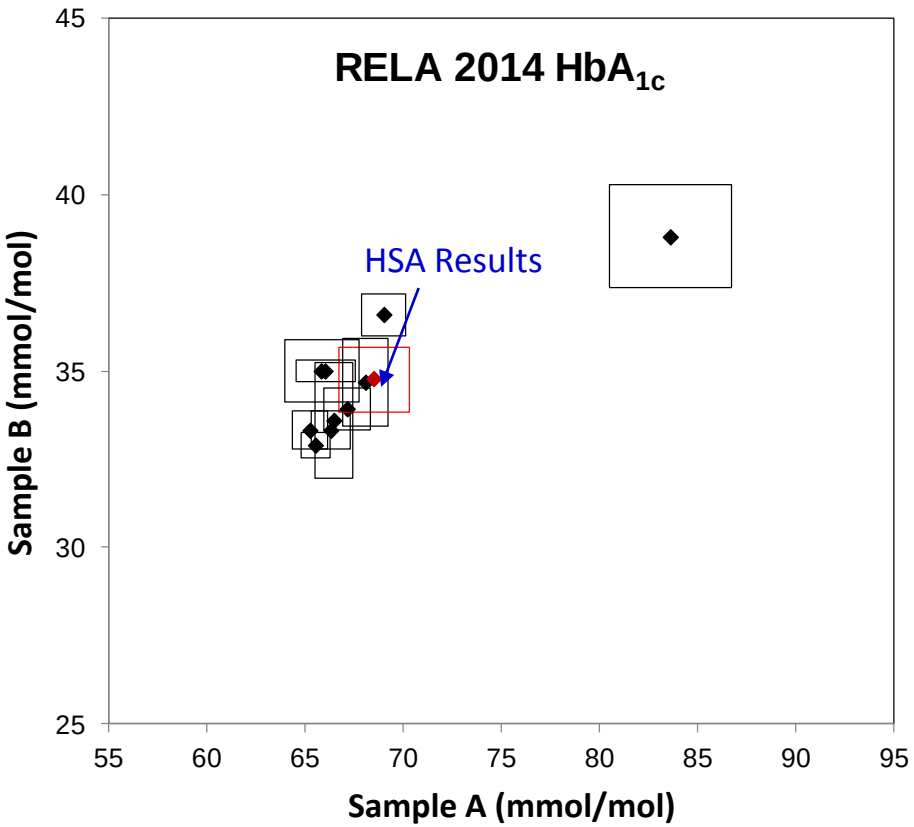


- All IDMS results were found to be lower than ReCCS's certified values with a deviation of about 3% (estimated from this graph)
- Reported MU (5.6 – 6.0%) can cross y=x
- Negative bias?

T. T. H. Tran et al., *J. Chromatogr. A*, 2017, 1513, 183-193.

RELA Comparisons for HbA_{1c}





- With the exception of HSA, all other laboratories used IFCC reference method.
- Relative expanded uncertainties of IDMS method: 2.6 – 2.8% (IFCC) or 1.6 – 2.2% (NGSP).
- Inter-laboratory CV in RELA 2013 and 2014: 1.6 – 3.2% (IFCC) or 1.2 – 1.9% (NGSP).
- Desirable CVs of HbA_{1c} measurement (NGSP): 2% (intra-laboratory) and 3.5% (Inter-laboratory).
- **IDMS method may be comparable with IFCC reference method.**

H. Liu et al., *Anal. Bioanal. Chem.*, 2015, 407, 7579-7587.

Preliminary Comparison of IDMS Methods (LNE vs HSA)

Table 1 Concentrations of VE and GE peptides in RELA 2014 External Quality Assessment materials using ID-LC/MS: comparison of the results published by Liu et al. (HSA) [3] and by LNE

Category	2014 RELA A		2014 RELA B	
	Mean	SD	Mean	SD
VE concentration (μmol/g)				
HSA	3.171	0.021	3.264	0.026
LNE	2.983	0.112	3.040	0.091
Bias (LNE vs HSA)	−5.9%		−6.9%	
GE concentration (μmol/g)				
HSA	0.233	0.002	0.118	0.001
LNE	0.222	0.005	0.109	0.003
Bias (LNE vs HSA)	−4.8%		−7.5%	
HbA_{1c} (mmol/mol)				
HSA	68.50	1.80	34.75	0.93
LNE	69.38	2.17	34.48	1.13
Bias (LNE vs HSA)	1.3%		−0.8%	

- Comparable HbA_{1c} results achieved by HSA and LNE, but significant deviations observed for VE and GE results (about 6%).
- VE and GE peptide calibrators were from different sources and the purities were independently assessed by HSA and LNE.
- Smaller variation may be expected if the same calibrators are used.

N. Clouet-Foraison et al., *Anal. Bioanal. Chem.*, 2017, 407, 5789-5790.

Variations in IFCC Reference Method

Table 1 Variations between the minimum and maximum values in the 2011 to 2015 RELA

Category	2015 RELA		2014 RELA		2013 RELA		2012 RELA		2011 RELA	
	Sample A	Sample B	Sample A	Sample B	Sample A	Sample B	Sample A	Sample B	Sample A	Sample B
Maximum result (mmol/mol)	36	62.9	69	36.6	36.3	85.46	52.8	69.69	86.7	45.03
Minimum result (mmol/mol)	32.13	58.83	65.23	32.9	33.62	82	49.47	66.27	83.95	41.68
Variation between minimum and maximum results	−10.8%	−6.5%	−5.5%	−10.1%	−7.4%	−4.0%	−6.3%	−4.9%	−3.2%	−7.4%
Average variation	−6.6%									

- Average variation of the results from Approved IFCC Network Laboratories for HbA_{1c} using same calibrators can be as large as 6.6% (the largest variation: 10.8%)
- All IFCC Network Laboratories that employed the IFCC reference procedure for HbA_{1c} measurement were using the same batch of calibrators.

H. Liu et al., *Anal. Bioanal. Chem.*, 2017, 409, 5791-5793.

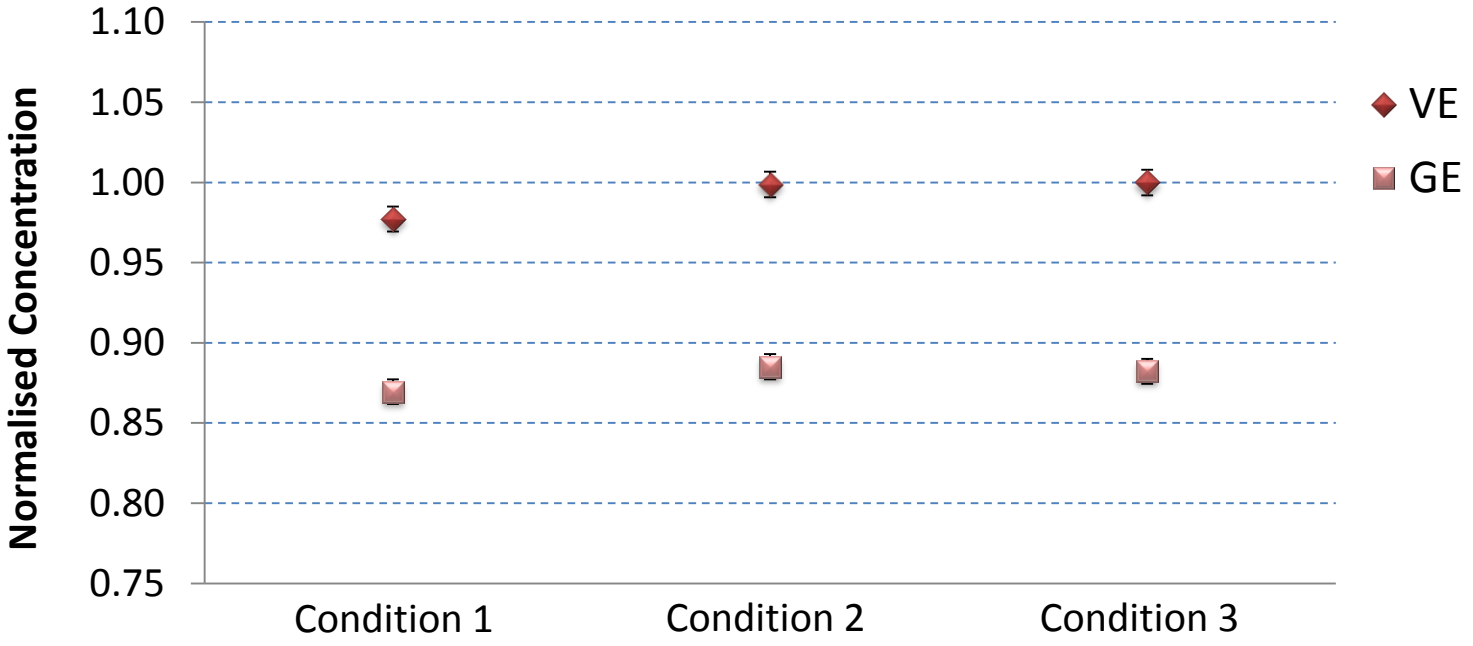
Bias between IDMS Method and IFCC Reference Method?

Two Questions:

- **Is there systematic bias between IDMS method and IFCC reference method due to different calibration standards (peptides vs protein)?**

- **Is comparability between these two methods achievable by optimising the procedure of IDMS method?**

Possible Key Factor: Condition for Peptide Hydrolysis



Condition	1	2	3
6N HCl	✓	×	×
6N HCl with 1% phenol additive	×	✓	✓
Temperature (°C)	110	120	120
Time (h)	69	24	70

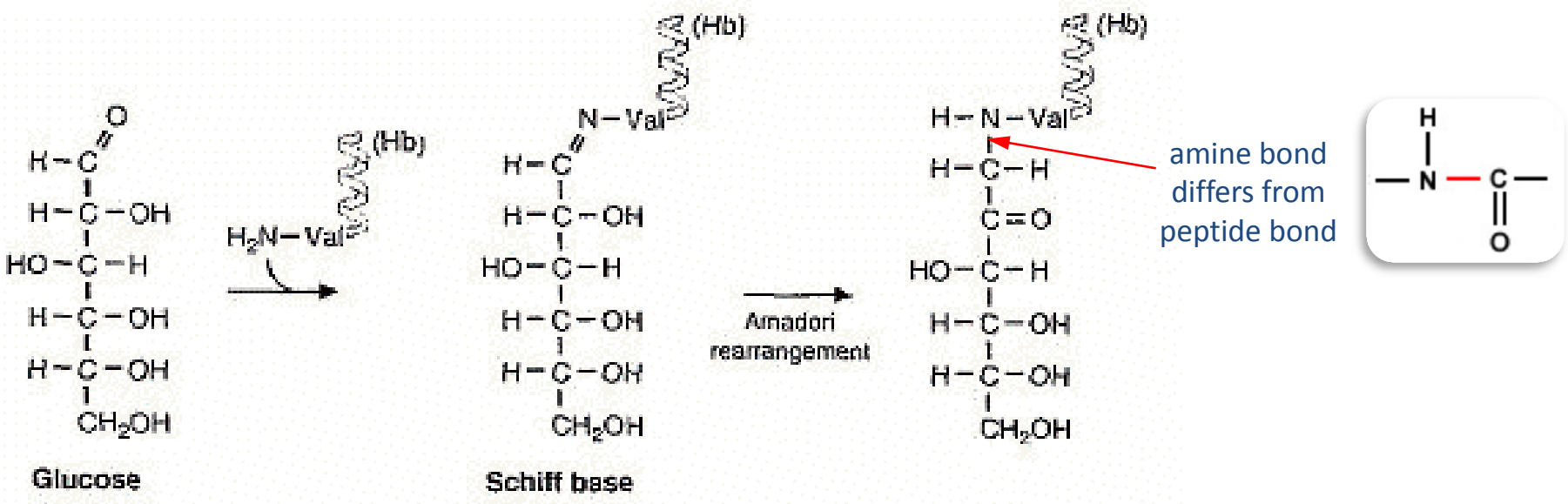
H. Liu *et al.*, *Anal. Bioanal. Chem.*, 2015, 407, 7579-7587.

Possible Key Factor: Condition for Peptide Hydrolysis

	Hydrolysis Conditions
T. Nakanishi *et al*., Osaka Medical College	Not reported
P. Kaiser *et al*., INSTAND e.V.	6 M HCl, 120 °C, 65 h
J. Bi *et al*., NIM *	6 M HCl, 110 °C, 48 h
H. Liu *et al*., HSA	6 M HCl with 1% phenol, 120 °C, 24 h
N. Clouet-Foraison *et al*., LNE	6 M HCl with 1% phenol, 120 °C, 24 h
T. T. H. Tran *et al*., KRISS	8 M HCl, 120 °C, 48 h

* J. Bi et al., Anal. Bioanal. Chem., 2012, 403, 549-554.

Possible Key Factor: Selection of Amino Acid for Quantification



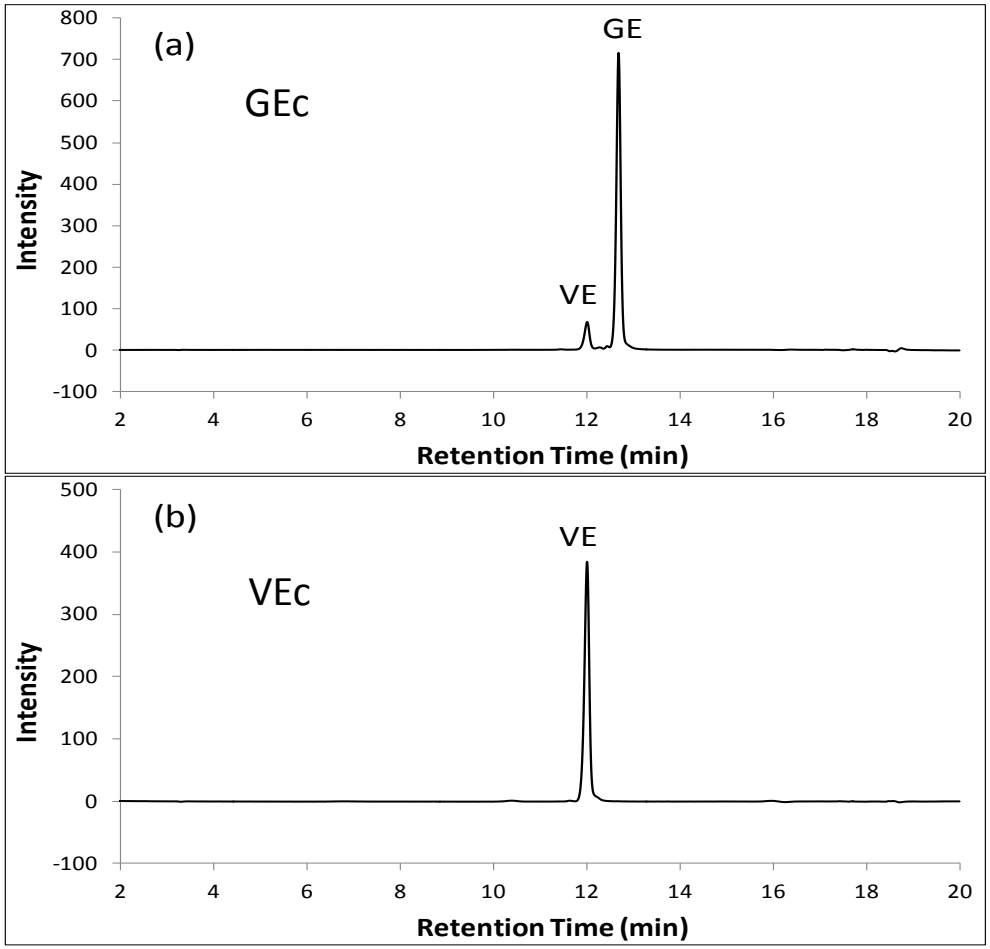
http://www.medbio.info/Horn/Time%205/new_diabetes_march_08.htm

- Amino acids with “Inert” side chain: valine, proline, leucine.
- For GE, valine gave much lower results since the C-N amine bond between glucose and valine differs from a regular peptide bond. Hence, the cleavage of valine during hydrolysis could be affected.
- Proline and leucine were chosen for quantification.

Possible Key Factor: Selection of Amino Acid for Quantification

	AA for Quantification
T. Nakanishi *et al*., Osaka Medical College	Not reported
P. Kaiser *et al*., INSTAND e.V.	Leu, Pro, Thr
J. Bi *et al*., NIM	Leu, Val
H. Liu *et al*., HSA	Leu, Pro
N. Clouet-Foraison *et al*., LNE	Leu, Pro
T. T. H. Tran *et al*.; KRISS	Leu, Pro

Possible Key Factor:
Identification and Quantification of Peptide Impurities



- VE was found in GEc as a major impurity.
- Another step of IDMS measurement was performed to quantify the amount of VE in Gec using VEc as calibration standard.
- The purity value of GEc from IDMS measurement for amino acids was corrected accordingly.

- The purity of VEc was found to be satisfactory.
- The purity value of VEc from IDMS measurement for amino acids was used without correction.

H. Liu et al., *Anal. Bioanal. Chem.*, 2015, 407, 7579-7587.

➢ **Identification of each peptide impurity using Orbitrap or QTOF is necessary if more impurities are found in HPLC.**

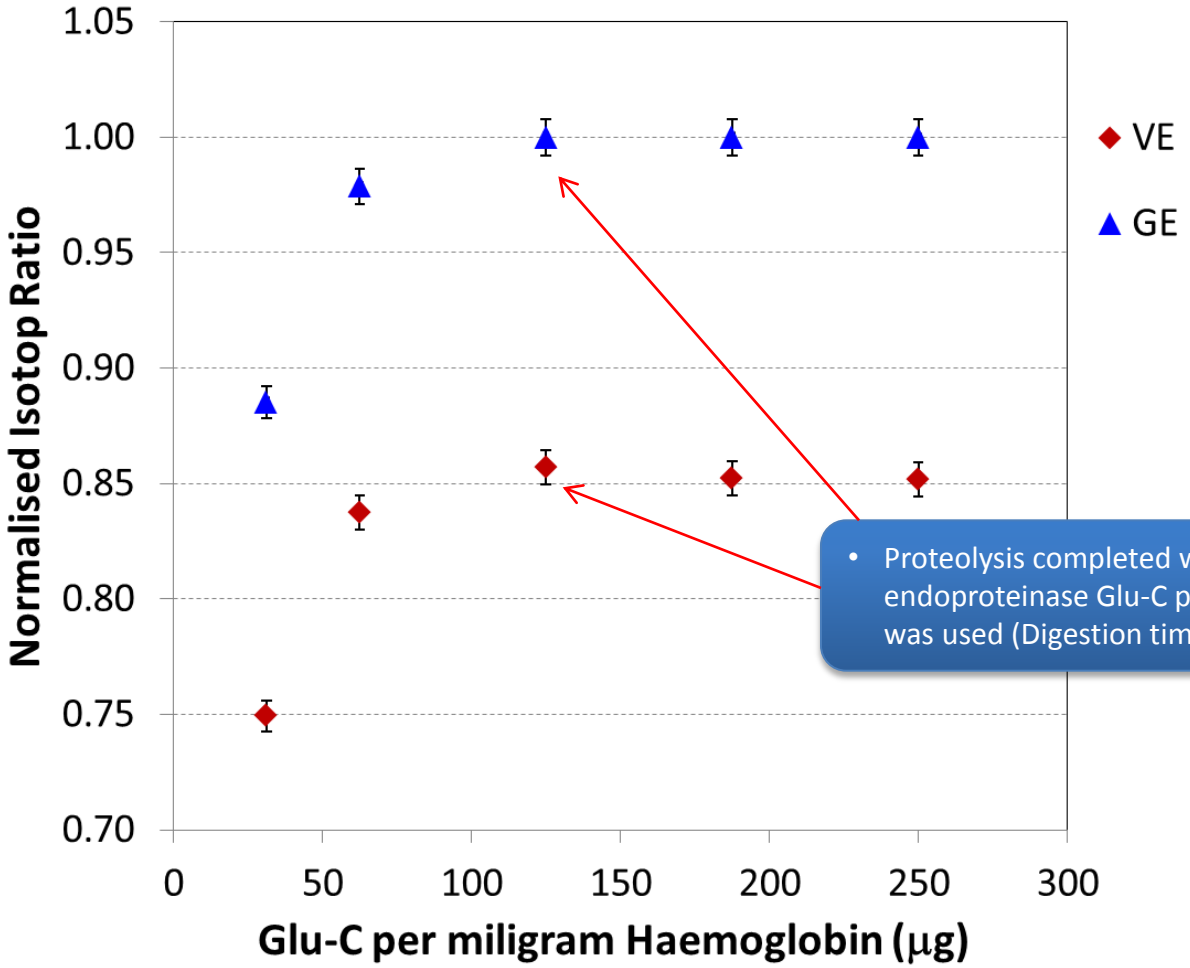
Possible Key Factor: Identification and Quantification of Peptide Impurities

	Impurity Identified	Method of Quantification
T. Nakanishi *et al.*, Osaka Medical College	Not reported	Not Reported
P. Kaiser *et al.*, INSTAND e.V.	Not reported	PICAA
J. Bi *et al.*, NIM	VE as impurity in GE	PICAA
H. Liu *et al.*, HSA	VE as impurity in GE	PICAA
N. Clouet-Foraison *et al.*, LNE	VE as impurity in GE	PICAA
T. T. H. Tran *et al.*; KRISS	VE as impurity in GE	PICAA

- How much do other unidentified impurities affect the results?
- Will mass balance approach be more accurate and reduce the MU?

PICAA: Determination of total peptides and subtraction of impurities using amino acid analysis

Possible Key Factor: Is Complete Proteolysis Necessary?



- Proteolysis completed when 125 μg of endoproteinase Glu-C per mg of haemoglobin was used (Digestion time 24 h).

H. Liu et al., *Anal. Bioanal. Chem.*, 2015, 407, 7579-7587.

Optimisation of the amount of endoproteinase Glu-C.
The error bar of each point was estimated using the pooled CV of VE or GE results in haemolysate samples.

Possible Key Factor: Is Complete Proteolysis Necessary?

	Glu-C Amount (µg per mg Hb)
T. Nakanishi *et al*., Osaka Medical College	50
P. Kaiser *et al*., INSTAND e.V.	10 (same as IFCC reference method)
J. Bi *et al*., NIM	~ 6.7 (assume 15 g/dL Hb in sample)
H. Liu *et al*., HSA	125
N. Clouet-Foraison *et al*., LNE	125
T. T. H. Tran *et al*.; KRISS	~ 13.3 (assume 15g/dL Hb in sample)

Possible Key Factor: Is Complete Proteolysis Necessary?

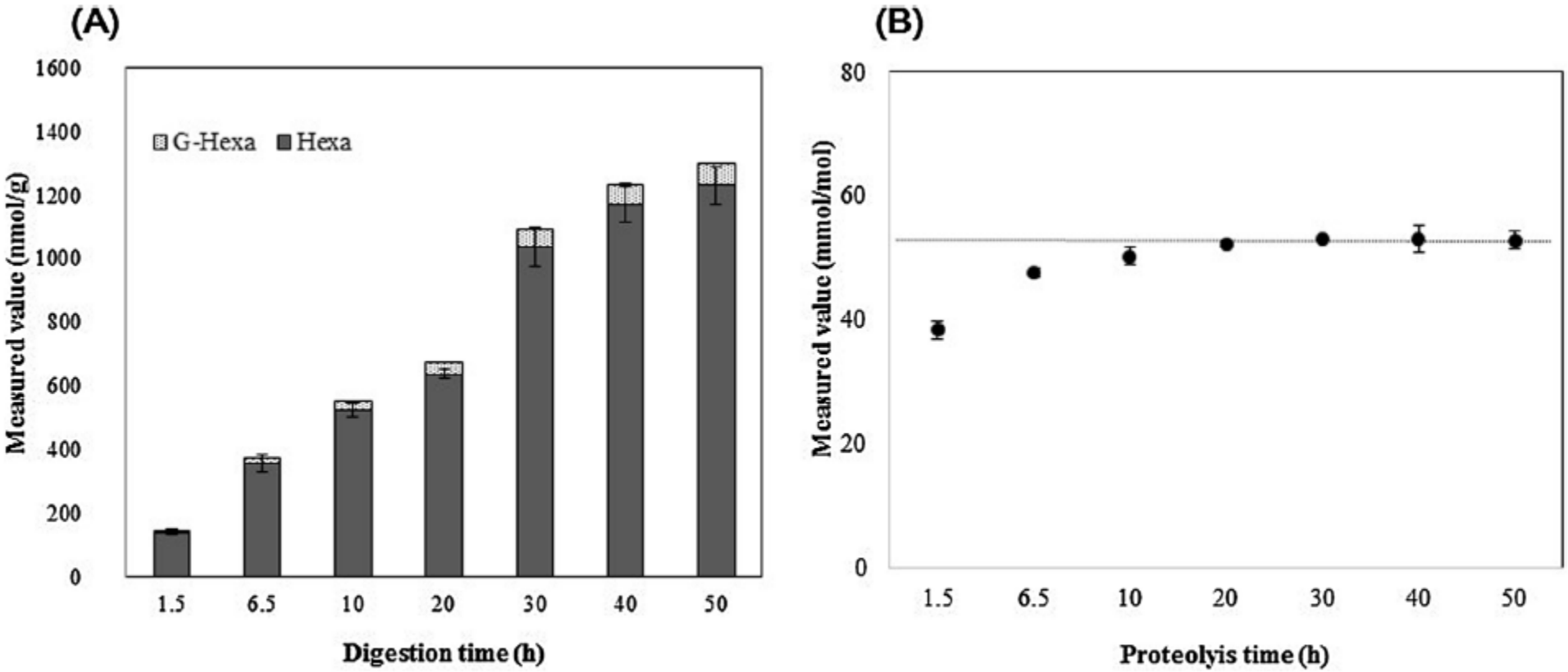


Fig. 6. Optimization of enzyme digestion time. (A) Absolute measured concentration of HbA_{1c}, (B) ratio of HbA_{1c}-to- HbA_0 with different digestion times.

~ 13.3 µg Glu-C per mg Hb

T. T. H. Tran et al., *J. Chromatogr. A*, 2017, 1513, 183-193.

- Proteolysis was incomplete at 40 h (maybe also 50 h) digestion time.
- HbA_{1c}/HbA_0 ratio was stabilised at 20 h digestion time onwards.

Planned CCQM PAWG Comparisons

- Comparisons jointly coordinated by BIPM, NIM and HSA.

2018: CCQM-K115.c Key comparison on peptide purity of GE.

CCQM-K115.2018 Key Comparison on peptide purity of VE.

- •Materials have been prepared by HSA, homogeneity and stability studies are being conducted by BIPM.
- •Institutes that expressed interest:
  LNE, KRISS, PTB, NRC, NMISA, INMETRO, NMIJ, VNIIM
- •Coordinating laboratories to use both PICAA and mass balance methods
- •Participating laboratories to use PICAA method.

- Comparisons jointly coordinated by LNE, HSA, KRISS, and NIM.

2019/2020: Key comparison or pilot study on determination of HbA_{1c} using IDMS method.

- •RELA samples as the study materials.
- •GE and VE materials in CCQM-K115.c and CCQM-K115.2018 as the calibration materials.

Application of IDMS for HbA_{1c} Measurement

HSA EQA Programme

- HbA_{1c} was first included in the 2013 EQA Programme.
- The main objective of the programme is to provide metrologically traceable assigned values to evaluate the results of the participating clinical laboratories.
- Since then, the number of participating laboratories on HbA_{1c} measurement has increased more than twofold (from 15 labs in 2013 to 36 labs in 2017).
- All target HbA_{1c}values are independently determined by HSA using IDMS method

Certification of CRM

- The materials in 2017 HSA EQA Programme have been developed as Certified Reference Materials.

  HRM-3003B HbA_{1c} in Frozen Human Blood (two concentration levels).
- HbA_{1c} CRMs from NIM and KRISS were also certified using IDMS method.
- CRMs from other institutes?

Summary

- The IDMS method for HbA_{1c} may be comparable with IFCC reference method when possible key factors are taken into consideration.
- The IDMS method can be regarded as an alternative accuracy-based reference method for HbA_{1c} measurement, which provides an independent support for the IFCC reference method.
- The IDMS method has been used to provide the assigned/certified values for the HSA EQA Programmes and CRMs for HbA_{1c}.
- CCQM comparisons have been planned to assess the purity of the peptide calibrators and IDMS results of HbA_{1c} from different NMIs/DIs, and to further evaluate the comparability between IDMS method and IFCC reference method.

Acknowledgement

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- Dr Lee Tong Kooi, Division Director, Chemical Metrology Division
- Dr Teo Tang Lin, Laboratory Director, Chemical Metrology Laboratory

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- Dr Vincent Delatour, LNE
- Prof Hongmei Li and Dr Liqing Wu, NIM
- Dr Sang-Ryoul Park and Dr Ji-Seon Jeong, KRISS

Thank you