ID-MS Based Reference Measurement Method for Small Analytes: Vitamin D, Creatinine, Glucose, Cholesterol and Amino acids

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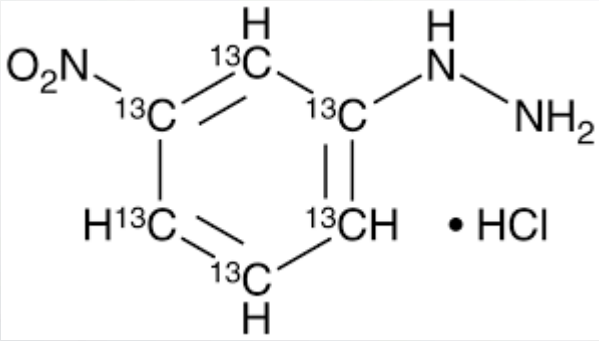
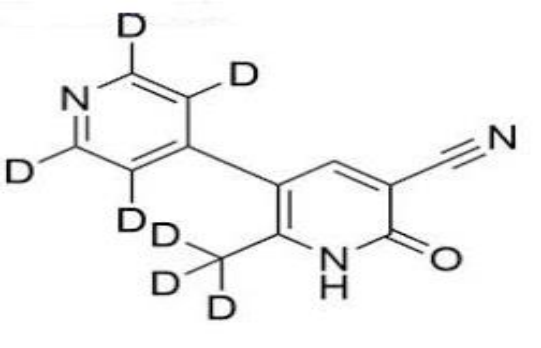
Isotope Dilution/Mass Spectrometry

- Isotope dilution mass spectrometry (IDMS) has become common with the increased use of Mass Spectrometry
- We need to use Internal Standard (IS) because Mass Spectrometry response changes
- We use labeled standards like ^{13}C or deuterium

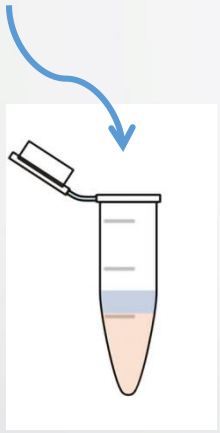




Isotope Dilution/Mass Spectrometry

- A known amount of an isotopically-enriched version of compound is added to the matrix containing the compound of interest
- Mixing the isotopic standard with the sample dilutes the isotopic enrichment of the standard and this forms the basis for the ID method
- Unlike traditional analytical methods which rely on signal intensity, isotope dilution employs signal ratios

Internal Standard $^{13}C_6$-phenylalanine)



Sample

The ratio of the quantity of unlabeled to labeled compound can be measured, and the concentration then can be determined

Concentration of Phe present in matrix(plasma) is determined in part by the ratio of the ion intensity of the Phe to $^{13}C_6$-Phe

Isotope Dilution/Mass Spectrometry

- It is regarded as primary method
- Primary measurement methods, which are traceable to International System of Units like IDMS, provide a viable alternative to estimate true value
- A Primary Method is a method having the highest metrological quality and a complete uncertainty statement can be written down in terms of SI units
- IDMS compensates for errors at all stages of the analysis, from sample preparation to instrument measurement

Isotope Dilution/Mass Spectrometry

- Response factor is calculated and used to determine the mass fraction of the analyte in the sample

$$RF = \frac{A_x x C_{IS}}{A_{IS} x C_x}$$

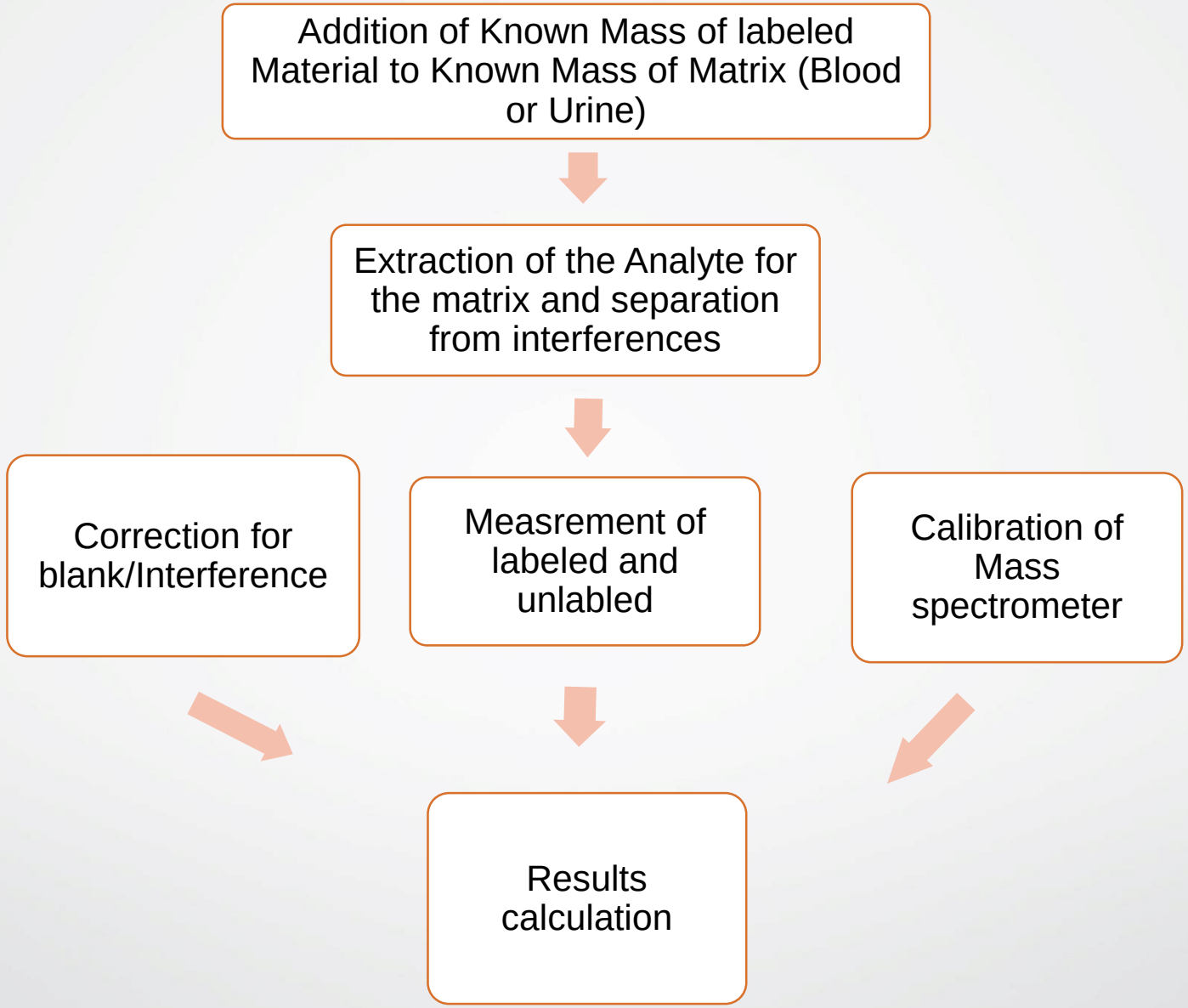
RF Response factor
C_x Conc of native cpd
A_x Peak area of native
A_{IS} Peak area of labeled
C_{IS} Conc of Labeled cpd

Reference Methods for Markers in Blood/Urine

Cholesterol

Glucose

Creatinine



Triglycerides

Urea

Vitamins

Vitamin D Metabolites in Human Serum

CCQM-K132/P169

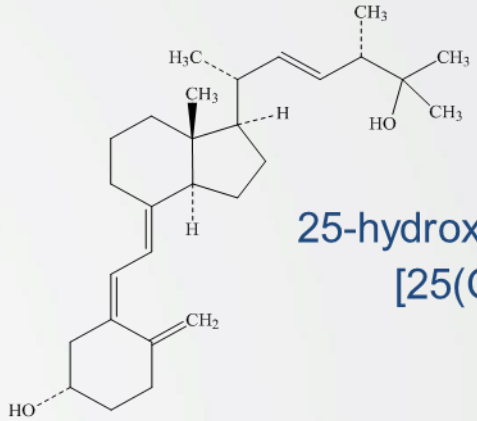
Low Polarity Analytes in a Biological Matrix:
Vitamin D Metabolites in Human Serum

Serum Pool I: High level of $25(OH)D_3$

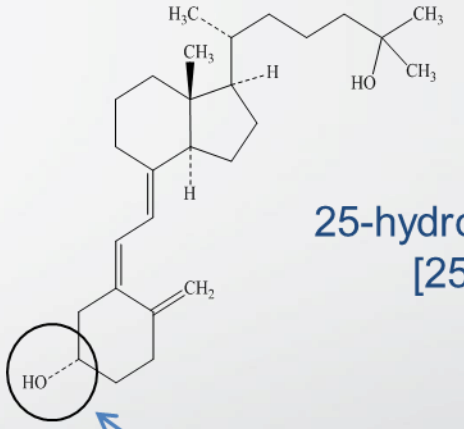
- $25(OH)D_3$ = 30 ng/g – 50 ng/g
- $25(OH)D_2$ = about 50x lower than $25(OH)D_3$
- 3-epi-$25(OH)D_3$ = typically <10% of $25(OH)D_3$ level

Serum Pool II: Normal level of $25(OH)D_3$ with higher than normal level of $25(OH)D_2$

- $25(OH)D_3$ = 15 ng/g – 30 ng/g
- $25(OH)D_2$ = 1 ng/g – 10 ng/g
- 3-epi-$25(OH)D_3$ = typically <10% of $25(OH)D_3$ level



25-hydroxyvitamin D_2
[$25(OH)D_2$]



25-hydroxyvitamin D_3
[$25(OH)D_3$]

3-epimer position

Vitamin D Metabolites in Human Serum

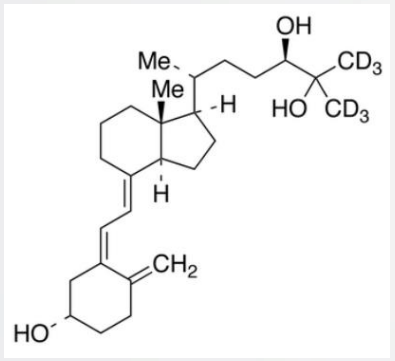
Zivak Tandem Gold LC-MS/MS	
Technique	APCI
Column	Reprosil Fluosil PFP 150 x 2 mm id x 3µm particle size
Mobile phase	78% MEOH : 22% Water + 0.1% Formic Acid
Flow	0.3 mL/ min
Mode	Isocratic

MS Setting	
Dry gas temp	300°C
Vaporizer gas temp	350°C
Drying gas pressure	20psi
Nebulizing gas pressure	55psi
Vaporizer gas pressure	2psi
Detector	600V
CID gas pressure	2.4mTorr

Analyte	Parent (*m/z*)	Daughter (*m/z*)
25-Hydroxy Vitamin D_2	395.3	269.3
25-Hydroxy Vitamin D_3	383.3	257.3
3-*epi*-25-Hydroxy Vitamin D_3	383.3	257.3
IS(D6-25-Hydroxy Vitamin D_3)	389.3	263.3

Internal Standard	Calibrant	Calibration Type
d_6-25(OH)D_3	UME CRM 1308 and Nist SRM 2972a	Single point

Vitamin D Metabolites in Human Serum



0.4 g of the serum sample was weighed into the eppendorf tube

↓

0.6 g of 50% Ammonium Sulfate solution was added

↓

0.3 g D6-25-Hydroxy Vitamin D_3 solution was added

↓

The mixture was vortexed and centrifuged at 14000 rpm for 15 min

↓

200 µL of the upper layer was added into a vial and 200 µL of distilled water was added

↓

The vial was vortexed and 50 µL of the mixture was introduced to the LC-MS/MS

Calibrant	Calibration Type
UME CRM 1308 and Nist SRM 2972a	Single point

TÜBİTAK
ULUSAL METROLOJİ ENSTİTÜSÜ

TÜBİTAK UME

Certificate of the Reference Material

Page 1 / 4

Name of the Material : 25-hydroxy vitamin D3 and 25-hydroxy vitamin D3 in lyophilized serum

Material Code : UME CRM 1308

Issue Date : 09.01.2015

Revision Date : 16.09.2019 (Revision history can be found on the last page)

Validity Period of the Certificate : 3 years from the sales date

Certified Values :

Measurand	Certified Value [1]	Uncertainty [2]	Certified Value [1,3]	Uncertainty [2,3]
25-hydroxy vitamin D3 concentration in lyophilized serum	50.0 ng/g	2.9 ng/g	51.0 ng/mL	3.0 ng/mL
25-hydroxy vitamin D3 concentration in lyophilized serum	48.6 ng/g	2.6 ng/g	49.6 ng/mL	2.7 ng/mL

[1] Certified values are the mean of 6 measurement results obtained from two units of the CRM on two different days by ID-LC-MS technique. The certified values and the uncertainties are traceable to the International System of Units (SI).
[2] The expanded uncertainty of certified value includes characterization, homogeneity, stability components and is stated as the standard uncertainty of measurement multiplied by the coverage factor $k = 2$, which for a normal distribution corresponds to a coverage probability of approximately 95%. The standard uncertainty of measurement has been determined in accordance with GUM "Guide to the Expression of Uncertainty in Measurement".
[3] Certified values and the uncertainties are calculated from the mass fraction (ng/g) using density of the material (1.0206 g/mL) measured at 22 °C.

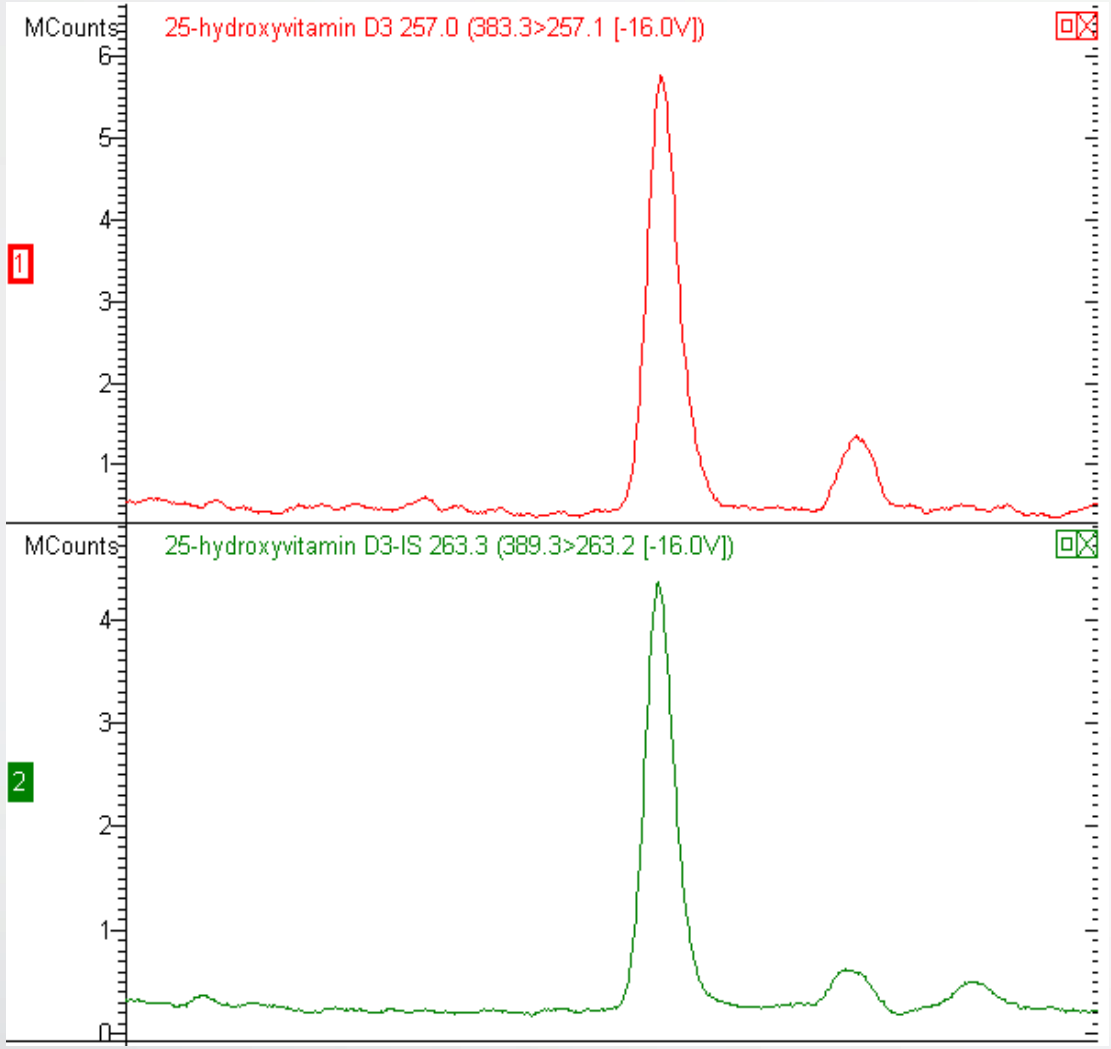
TÜBİTAK UME, as a reference material producer, has been accredited by TÜRKAK according to TS EN ISO 17034 with the accreditation number AB-0001-RM.

Sales Date

Dr. Mustafa ÇETİNTAŞ
Director

The following pages are an integral part of the certificate. The use of current certificate is customers' responsibility.
Most recent certificate can be downloaded from www.ume.tubitak.gov.tr.

Vitamin D Metabolites in Human Serum

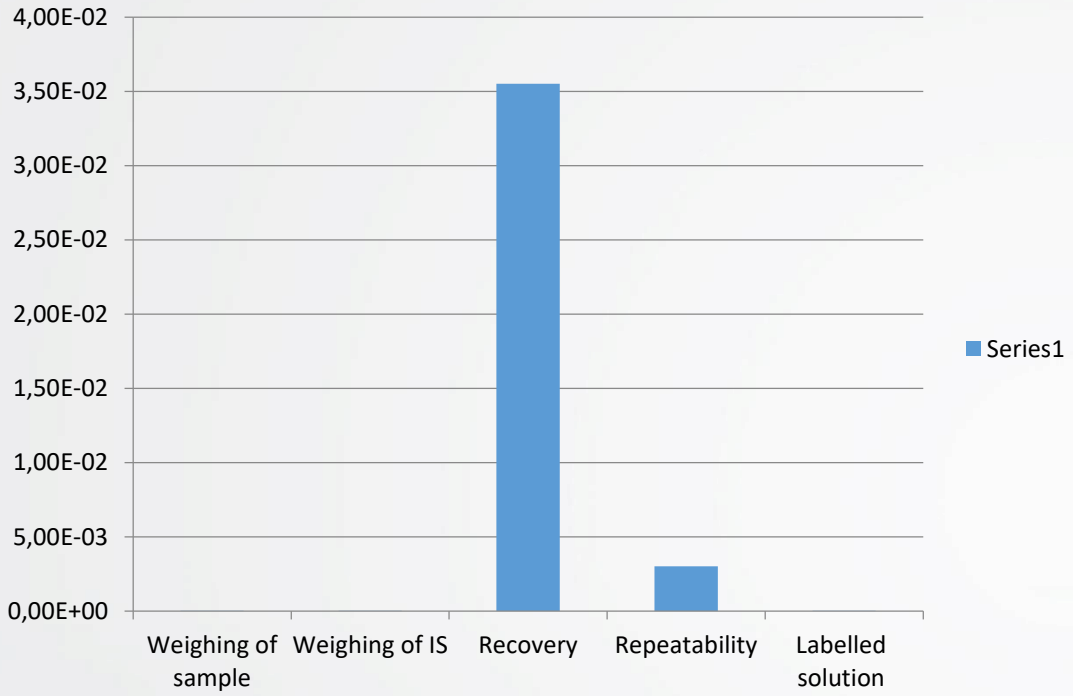


Serum Pool I		
Measurand	**Mass Fraction (ng/g)**	**Expanded Uncertainty (ng/g)**
25(OH)D2	0.50	0.03
25(OH)D3	37.82	2.71
3-epi-25(OH)D3	2.02	0.13

Serum Pool II		
Measurand	**Mass Fraction (ng/g)**	**Expanded Uncertainty (ng/g)**
25(OH)D2	6.05	0.36
25(OH)D3	25.84	1.84
3-epi-25(OH)D3	1.48	0.09

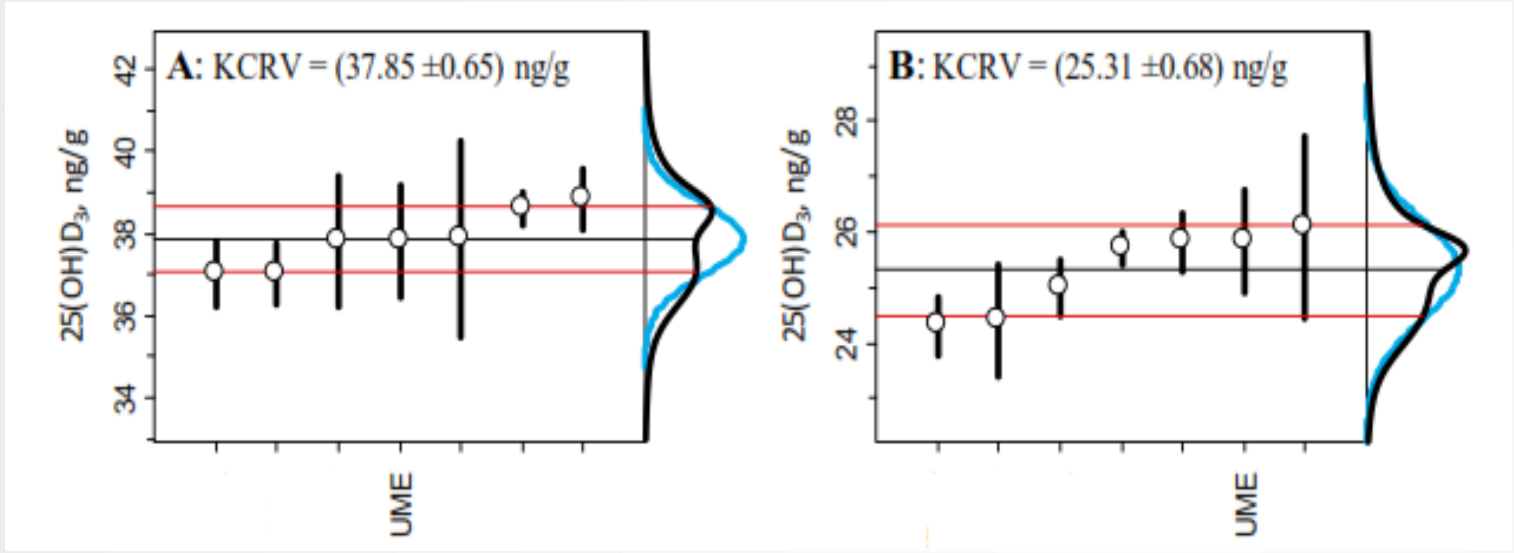
Vitamin D Metabolites in Human Serum

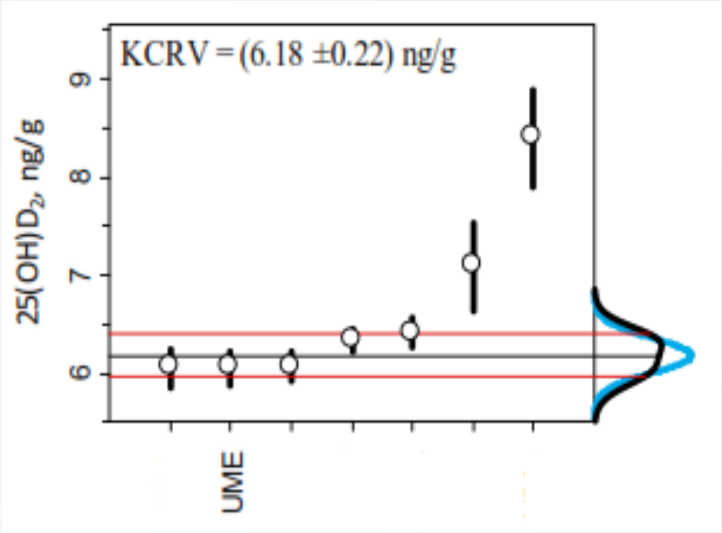
Uncertainty Contributions

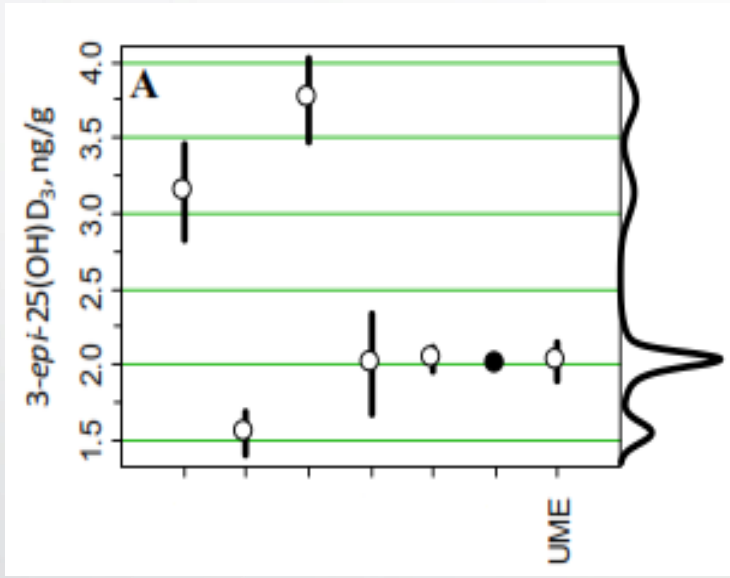


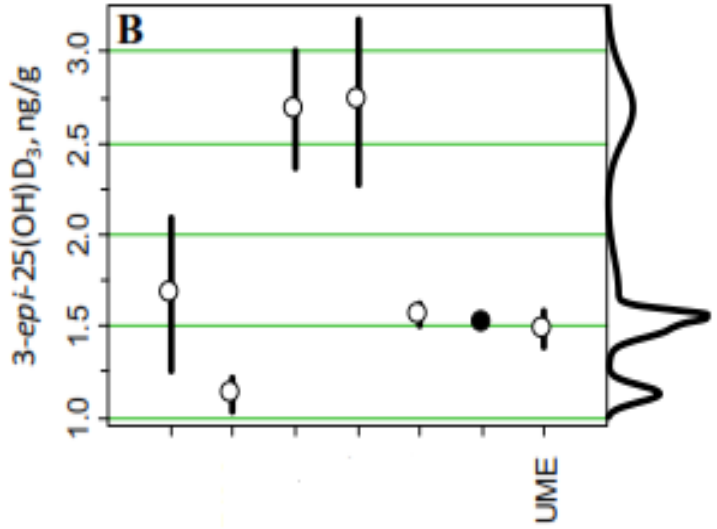
Uncertainty budget of White cap D3			
	Value	u(x)	u(x)/x
Weighing of sample (mg)	406	8.16E-04	2.01E-06
Weighing of IS (mg)	305.64	1.39E-03	4.56E-06
Recovery	1	3.55E-02	3.55E-02
Repeatability	100	4.72E-01	4.72E-03
Labelled stock solution (ng/g)	50	1.32E-04	2.63E-06
			3.58E-02
Result (ng/g) 37.82			
Combined uncertainty	1.35		
Expanded uncertainty	2.71		
% Relative uncertainty	7.16		
% Relative standard uncertainty	3.58		

Vitamin D Metabolites in Human Serum







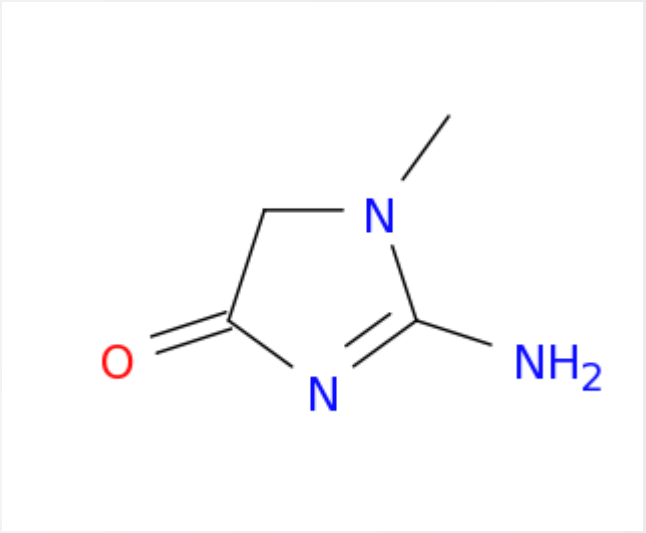


Determination of Creatinine in Human Serum

CCQM-K12.2

Determination of Creatinine in Human Serum

Creatinine (molar mass 113 g/mol) is a polar analyte (pK_{ow} = 1.76) that is present in human serum at relatively low concentrations (1 µg/g to 30 µg/g).



The study material was candidate SRM 1951c Lipids in Frozen Human Serum (Level 2). Participants were provided with three vials of serum for determination of each analyte. Each vial contained 1 mL of human serum.

Determination of Creatinine in Human Serum

Zivak Tandem Gold LC-MS/MS	
Technique	ESI
Column	Reprosil-Por RP 18-NE 3um 75 x 4mm
Mobile phase	75% methanol and 25% of 10 mmol/L ammonium acetate containing 0.4% formic acid (v/v)
Flow	0.5 mL/min
Mode	Isocratic

Analyte	Parent (*m/z*)	Daughter (*m/z*)	Collision Energy (V)	Capillary
Creatinine	114	44	15	100
Creatinine d3	117	47	15	100

Internal Standard	Calibrant	Calibration Type
Creatinine d3	Nist SRM 967a	Single point

Determination of Creatinine in Human Serum

Extraction Procedure

50 µL of serum sample was weighed into 2 mL plastic centrifuge tube

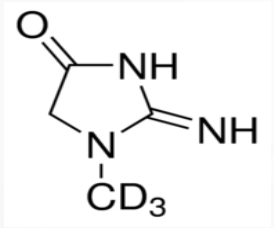
10 µL of the internal standard Creatinine D3 solution (1mg/mL) was added and vortexed

1.2 mL of cold Methanol was added to precipitate the protein

The mixture was Vortexed

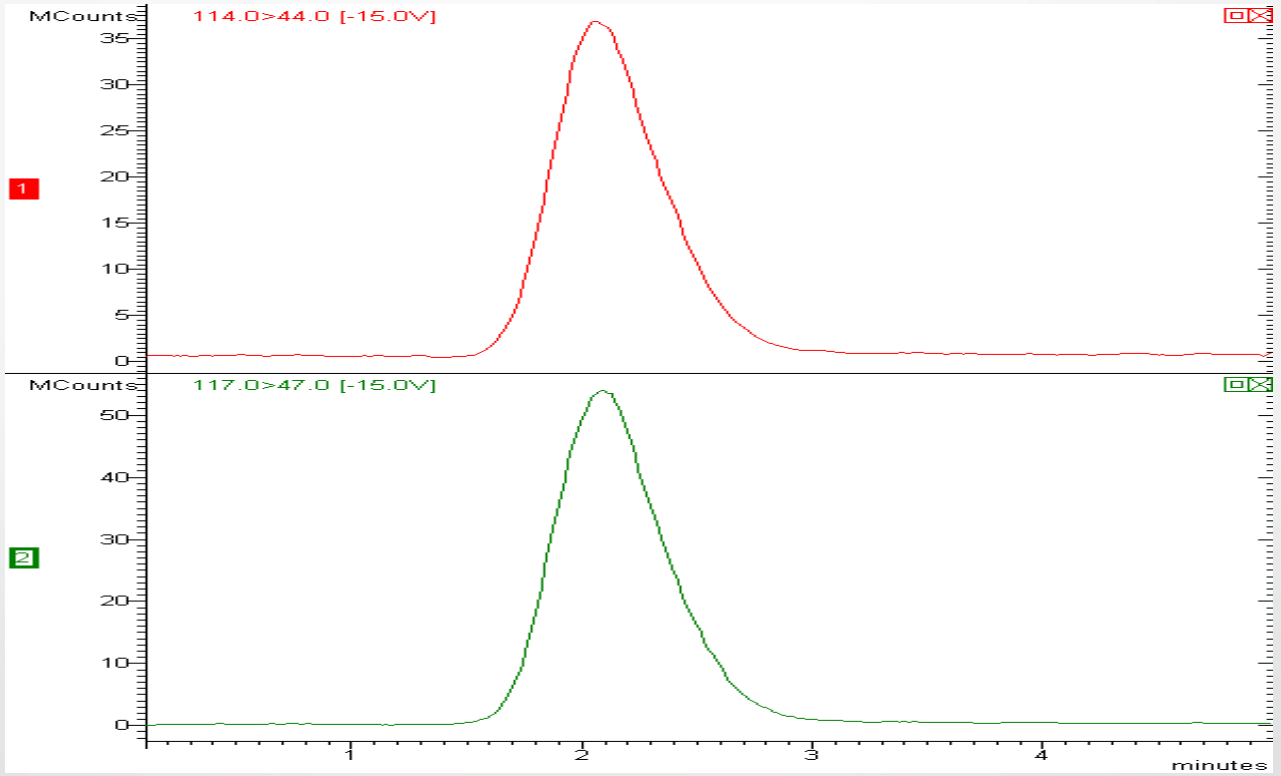
Sample was shaked for 10 min and centrifuged at 11.000×g for 5 min

Supernate was filtered through 0.22 µm syringe filter and analyzed by LC-MS/MS

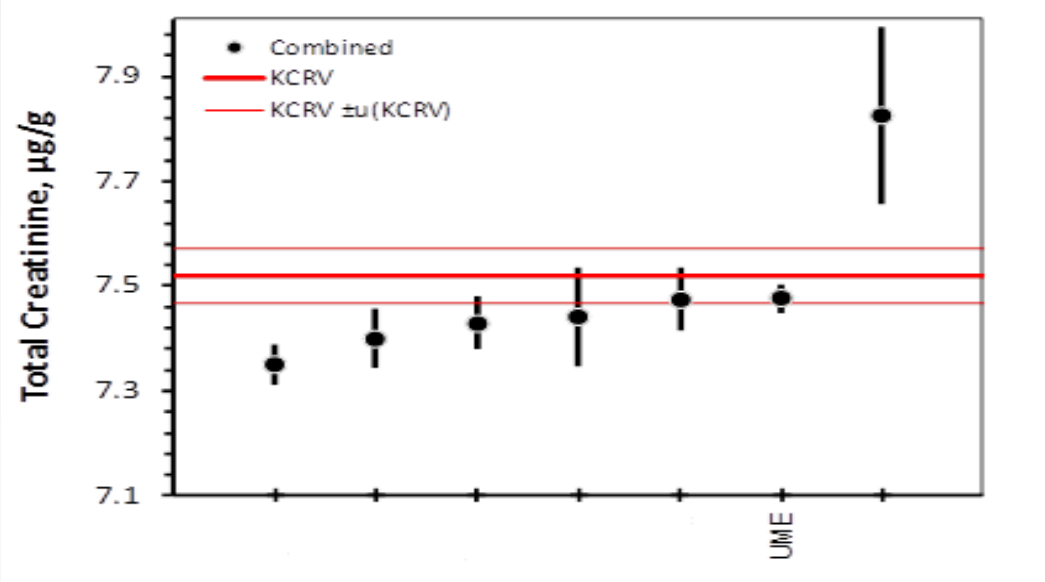


Determination of Creatinine in Human Serum

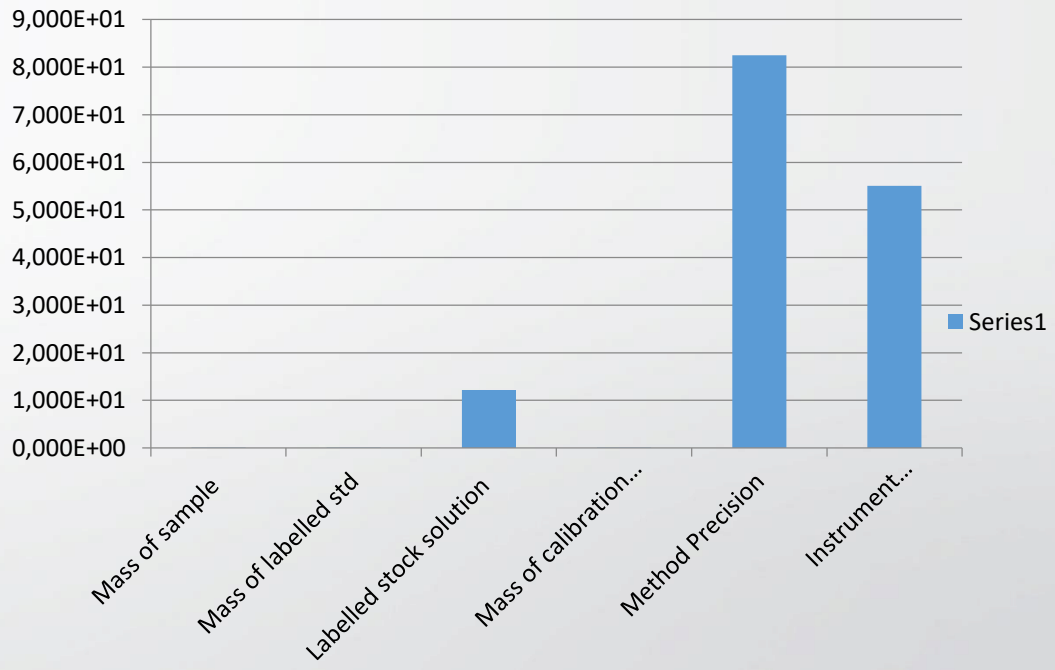
Sample results	Mass Fraction (µg/g)	Combined Standard Uncertainty (µg/g)	Coverage Factor (k)	Expanded Uncertainty (µg/g)
Sample 1	7.47E+00	3.73E-02	2	7.45E-02
Sample 2	7.48E+00	3.87E-02	2	7.74E-02



Determination of Creatinine in Human Serum



Parameter	*Value(X)*	u(x)	u(x)/X
Mass of sample (mg)	5.01E+01	1.72E-06	3.43E-08
Mass of labelled std	*9.25E+00*	5.92E-08	6.40E-09
Labelled stock solution (mg/kg)	*5.00E+01*	3.09E-02	6.19E-04
Mass of calibration standard level 1 (mg)	*4.89E+01*	1.67E-06	3.41E-08
Method Precision	*1.00E+02*	4.19E-01	4.19E-03
Instrument repeatability	1.00E+02	2.63E-01	2.63E-03
Relative Combined Uncertainty			4.99E-03
Result (µg/g)	7.47E+00		
Combined Standard Measurement Uncertainty		3.73E-02	
Expanded Uncertainty (k=2)		7.45E-02	
Relative Uncertainty		9.97E-01	



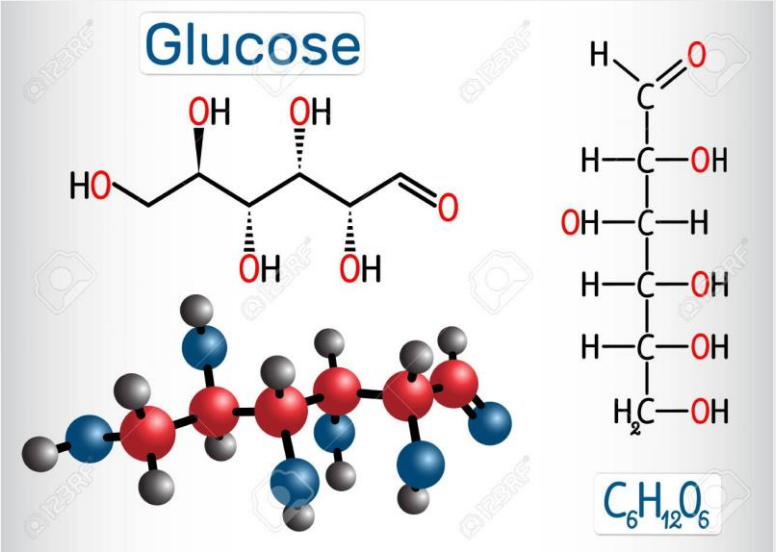
Determination of Glucose in Human Serum

CCQM-K11.2

Determination of Glucose in Human Serum

Glucose (molar mass 180 g/mol) is a polar analyte (pK_{ow} = 2.82), highly water-soluble (909 g/L) analyte that is present in human serum at relatively high concentrations (0.5 mg/g to 1.5 mg/g).

Participants were requested to analyze two vials of material for each analyte.



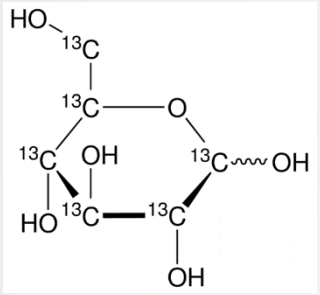
Determination of Glucose in Human Serum

Zivak Tandem Gold LC-MS/MS	
Technique	ESI-ve
Column	Luna 5µ NH2 100 A◦ 250 X 2 mm
Mobile phase	A:5 mmol ammonium formate in 0.05% formic acid, B: acetonitrile (A:20:B 80 by vol)
Flow	0.2 mL/min
Mode	Isocratic

Analyte	Parent (*m/z*)	Daughter (*m/z*)	Collision Energy (V)	Capillary
Glucose	224.7	88.7	12	100
13C6 D-Glucose	230.7	91.7	12	100

Internal Standard	Calibrant	Calibration Type
13C6 D-Glucose	SRM 965 b Glucose in frozen Human	3 point calibration

Determination of Glucose in Human Serum



Extraction Procedure

300 µL of serum sample were weighed into 2 mL plastic centrifuge tube

100 µL of the internal standard 13C6 D-Glucose solution (1mg/mL) was added

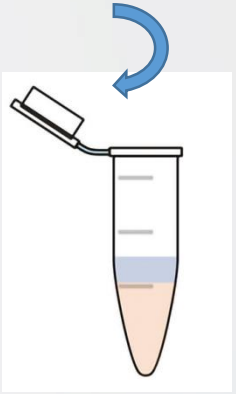
The mixture was vortexed for 1 min and left to stand for 2 h

1.2 mL of acetonitrile was added for protein precipitation

The mixture was vortexed for 1 min and centrifuged

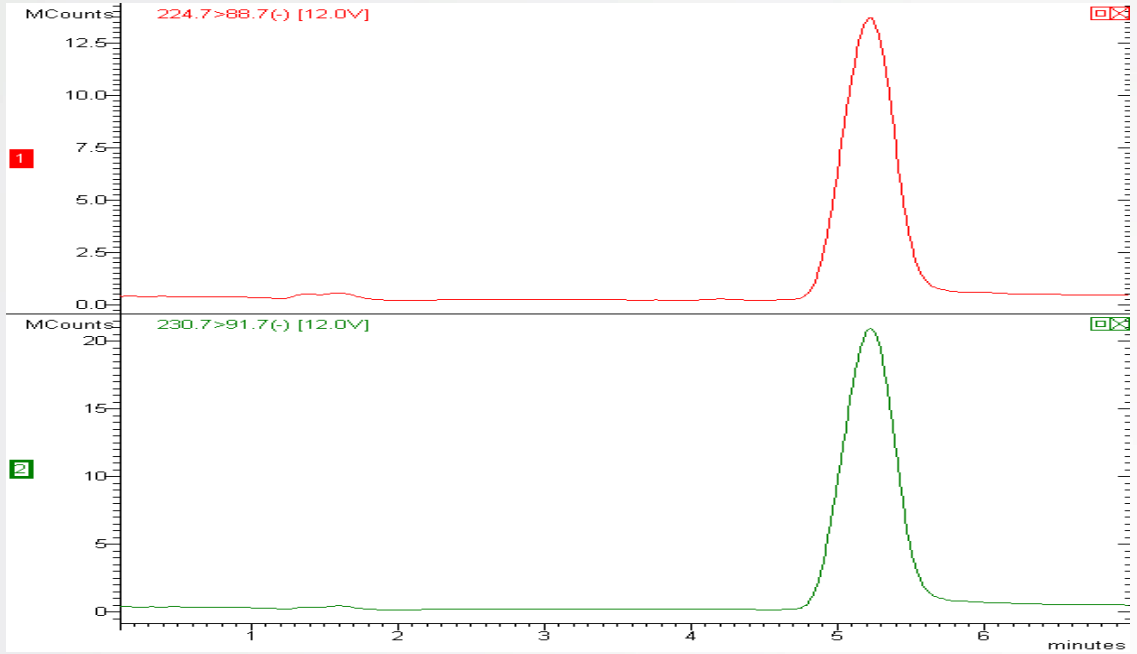
The supernate was filtered through 0.22 µm syringe filter and transferred to vial and analyzed by LC-MS/MS

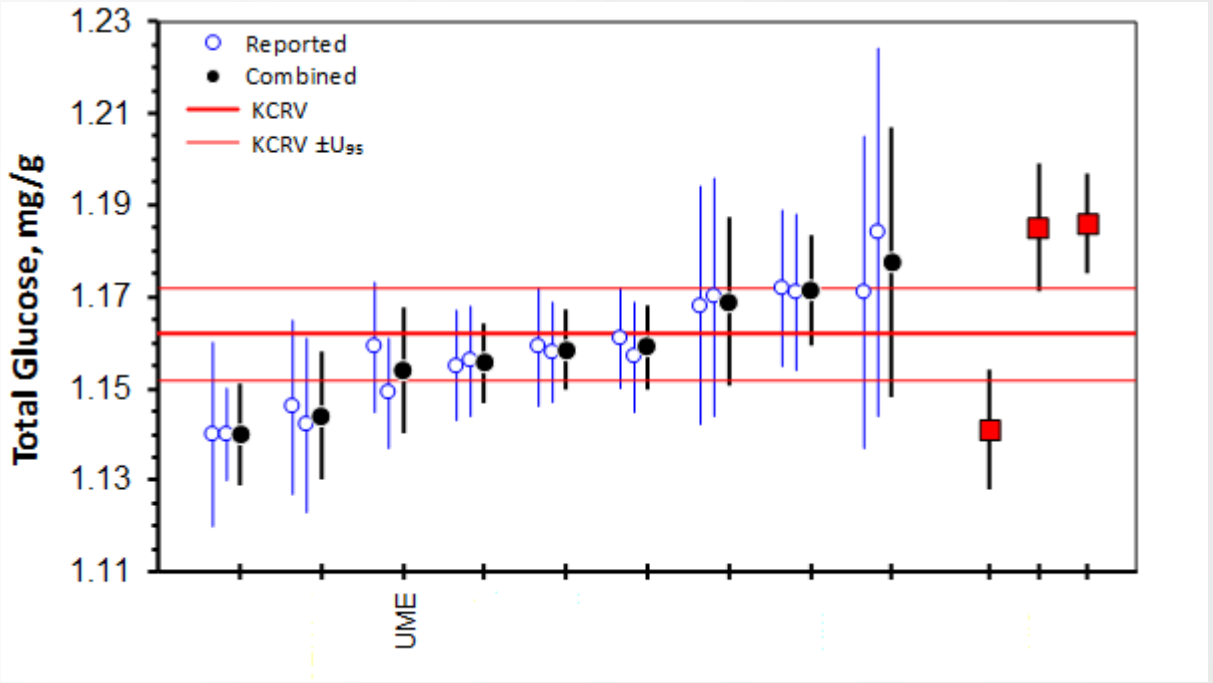
100 µL 3C6 D-Glucose



300 µL serum sample

Determination of Glucose in Human Serum



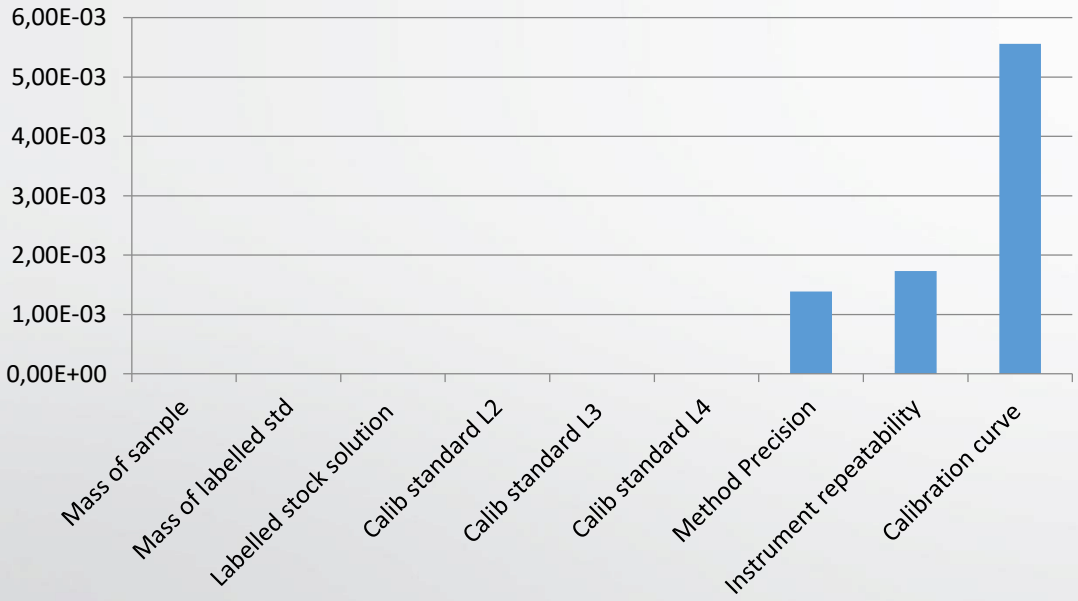


Sample results	Mass Fraction (mg/g)	Combined Standard Uncertainty (mg/g)	Coverage Factor (k)	Expanded Uncertainty (mg/g)
Sample 1	1.16E+00	6.94E-03	2	1.39E-02
Sample 2	1.15E+00	6.15E-03	2	1.23E-02

Determination of Glucose in Human Serum

Uncertainty Parameters

Mass of sample
Mass of labelled std
Labelled stock solution
Uncertainty of calibration standard level 2
Uncertainty of calibration standard level 3
Uncertainty of calibration standard level 4
Method Precision
Instrument repeatability
Calibration curve



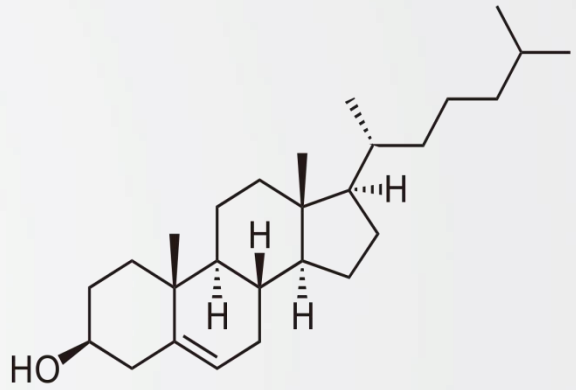
Parameter	***Value(X)***	**u(x)**	**u(x)/X**
Mass of sample (mg)	3.02E+02	6.22E-05	2.06E-07
Mass of labelled std(mg)	*9.79E+01*	6.55E-06	6.69E-08
Labelled stock solution (mg/kg)	*1.00E+03*	9.72E-06	9.72E-09
Uncertainty of calibration standard level 2 (mg)	*3.01E+02*	6.19E-05	2.06E-07
Uncertainty of calibration standard level 3 (mg)	3.03E+02	6.27E-05	2.07E-07
Uncertainty of calibration standard level 4 (mg)	3.03E+02	6.26E-05	2.07E-07
Method Precision	*1.00E+02*	1.39E-01	1.39E-03
Instrument repeatability	1.00E+02	1.73E-01	1.73E-03
Calibration curve	1.16E+00	6.44E-03	5.56E-03
Relative Combined Uncertainty			5.99E-03
Result (mg/g)	1.16E+00		
Combined Standard Measurement Uncertainty		6.94E-03	
Expanded Uncertainty (k=2)		1.39E-02	
Relative Uncertainty		1.20E+00	

Determination of Total Cholesterol in Human Serum

CCQM-K6.2

Determination of Cholesterol in Human Serum

Cholesterol(molar mass 365g/mol) is a low polarity analyte that is present in human serum at relatively high concentration(1 mg/g to 3 mg/g). Cholesterol is predominantly esterified with fatty acids in the blood



The study material for CCQM-K6.2 was candidate SRM 1951c Lipids in Frozen Human Serum (Level 2), which was prepared as a replacement for SRM 1951b Lipids in Frozen Human Serum.

Participants were provided with three vials of serum for the determination of cholesterol. Each vial contained 1 mL of human serum.

Determination of Total Cholesterol in Human Serum

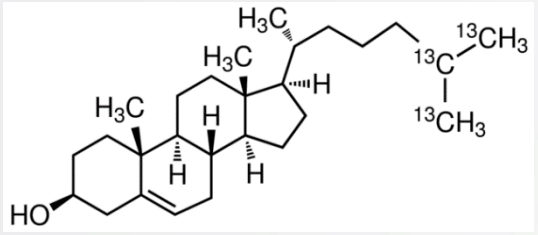
Zivak Tandem Gold LC-MS/MS	
Technique	APCI
Column	Kinetex 2.6u C18 100A 100 mm 2.10 mm
Mobile phase	Isocratic mode ;acetonitrile and methanol (80:20 by vol)
Flow	0.25 mL/min
Mode	Isocratic

Analyte	Parent (*m/z*)	Daughter (*m/z*)	Collision Energy (V)	Capillary
Cholesterol	369	161	12	20
[25,26,27-13C3] Cholesterol	372	161	12	20

Internal Standard	Calibrant	Calibration Type
25,26,27-13C3 Cholesterol	Nist SRM 968e	2 point calibration

968e Fat Soluble Vitamins, Carotenoids, and Cholesterol in Human Serum -Two level concentration were used for calibration

Determination of Total Cholesterol in Human Serum



Extraction Procedure

250 µL of CCQM 6.2 serum sample was placed in test tube

100 µL of the internal standard [25,26,27-13C3] Cholesterol solution (4mg/mL) was added

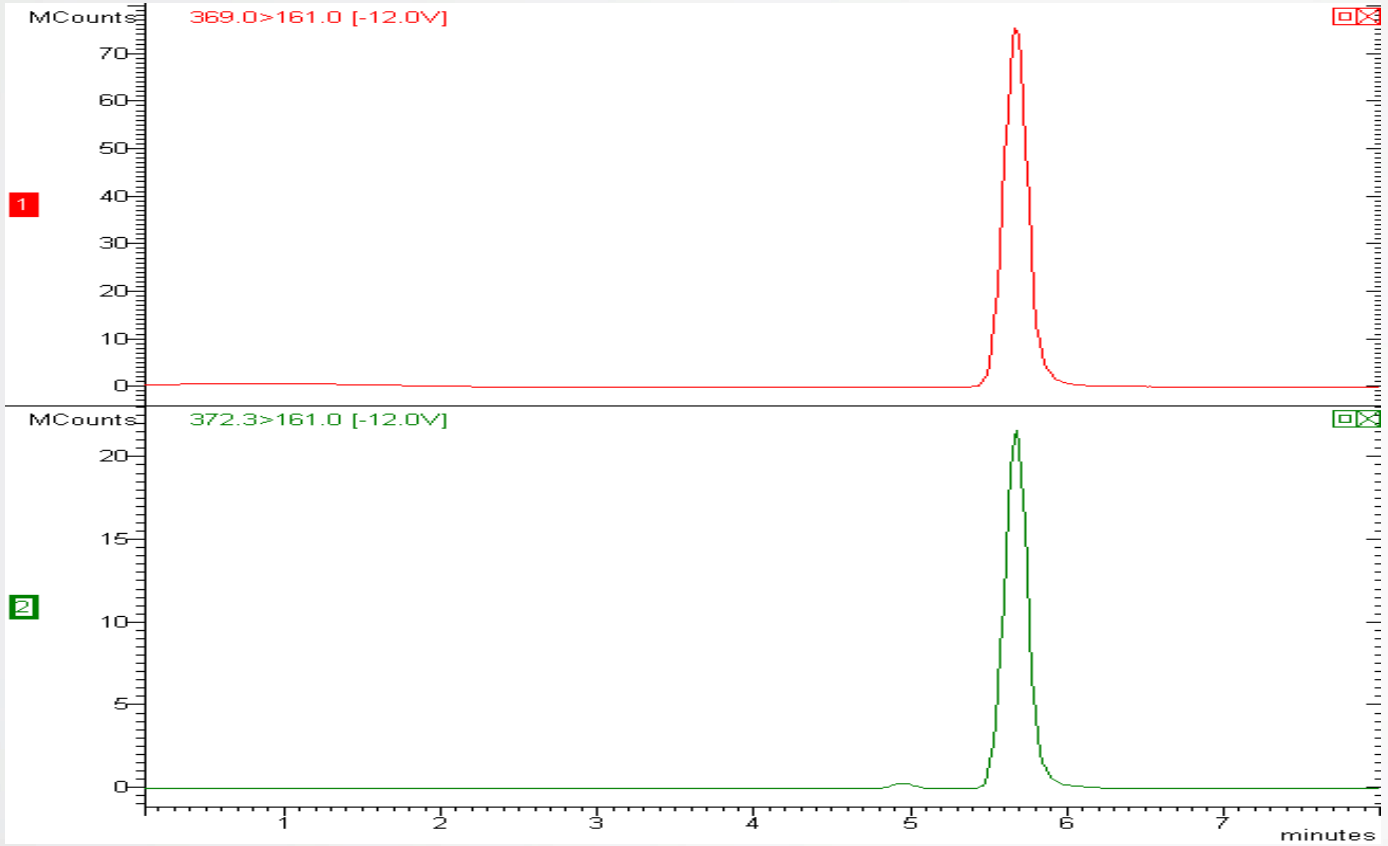
150 µL of an aqueous potassium hydroxide solution (8.9 mol/L) and 1 ml of ethanol were added

The mixture was vortexed for 10 s and heated at 50 °C for 4 h

After hydrolysis, 1 mL of deionized water and 2 mL of cyclohexane were added

After continuous shaking for 5 min the cyclohexane phase was filtered through 0.2µm membrane filter, transferred to vial and analyzed by LC-MS/MS

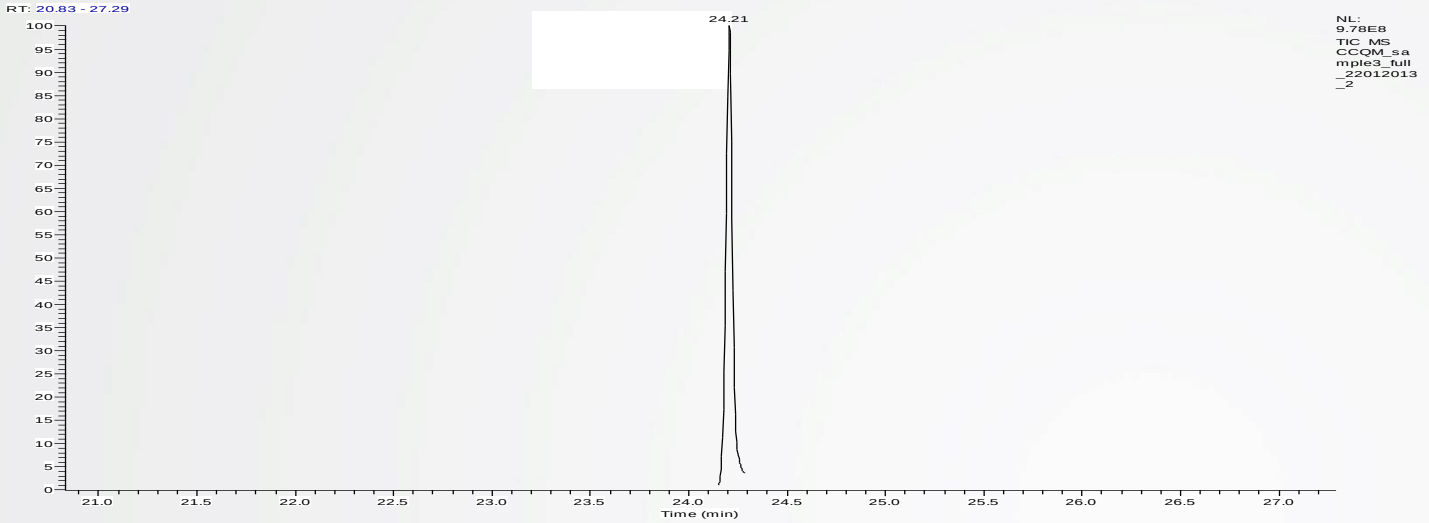
Determination of Total Cholesterol in Human Serum

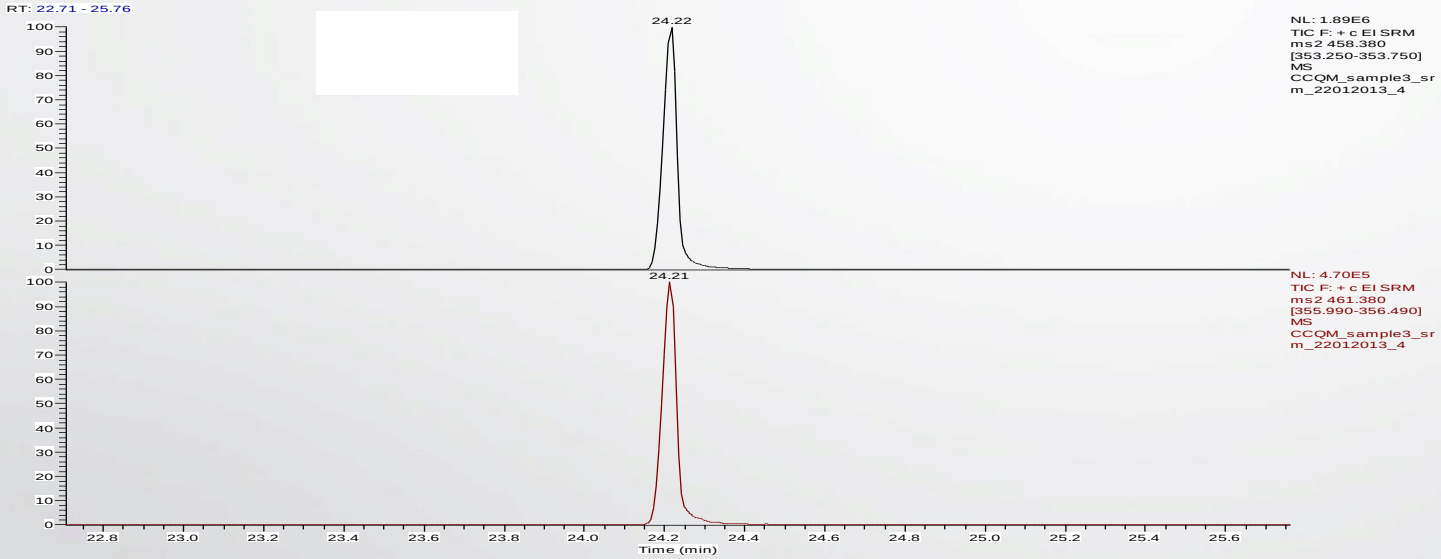


Sample results	Mass Fraction (mg/g)	Combined Standard Uncertainty (mg/g)	Coverage Factor (k)	Expanded Uncertainty (mg/g)
Sample 1	2.27E+00	3.09E-02	2	6.19E-02
Sample 2	2.31E+00	3.30E-02	2	6.60E-02

Determination of Total Cholesterol in Human Serum

GC IDMS method were used for conformation





GC Column : DB-1, 30 m x 0.25 mm x 0,10 µm

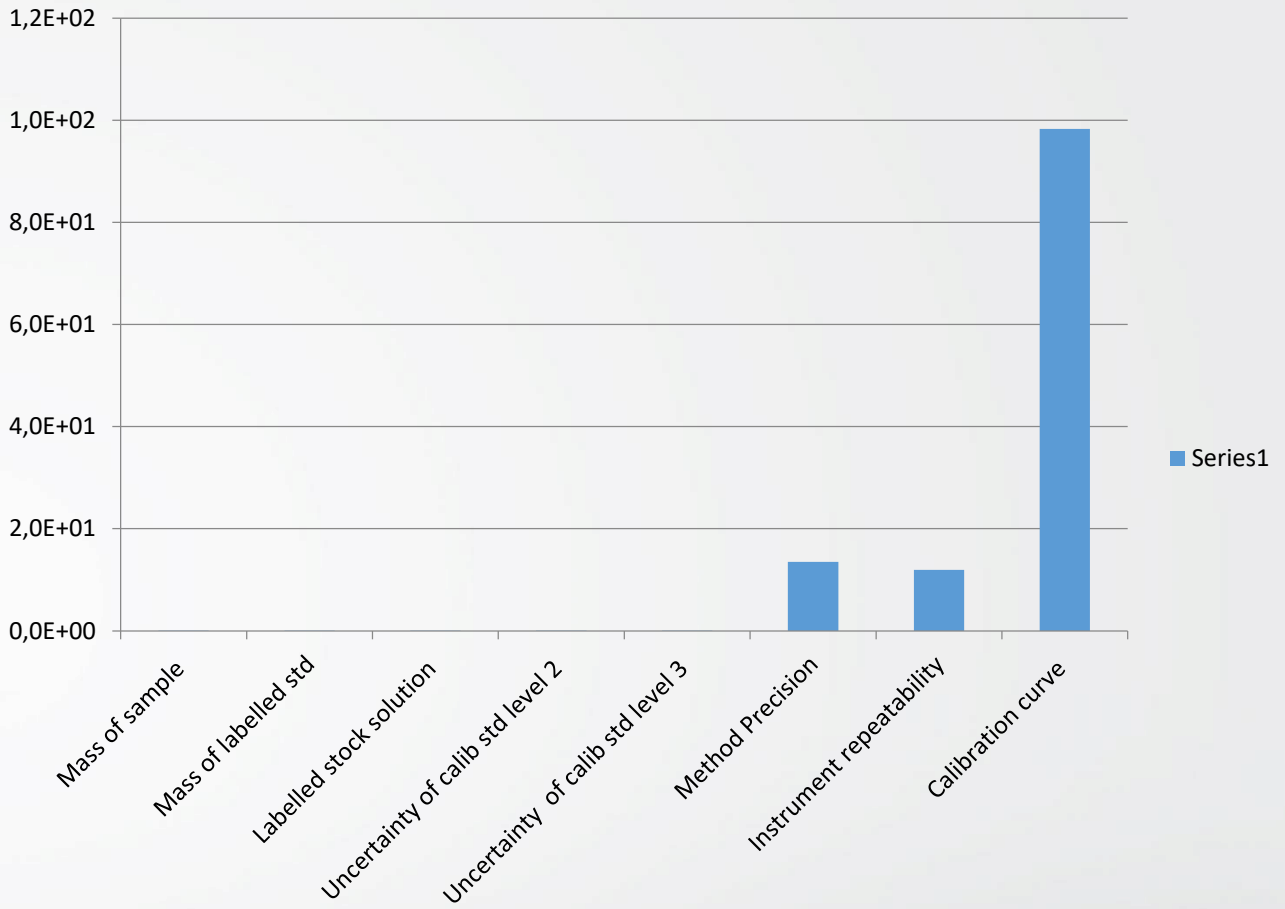
	Rate (^{0}C/min)	Temp (^{0}C)	Hold Time
İnitial		60	1.00
Ramp 1	10	300	5.00

Determination of Total Cholesterol in Human Serum

Uncertainty Parameters Used

Mass of sample
Mass of labelled std
Labelled stock solution
Uncertainty of calibration standard level 2
Uncertainty of calibration standard level 3
Method Precision
Instrument repeatability
Calibration curve

Parameter	*Value(X)*	u(x)	u(x)/X
Mass of sample (mg)	2.05E+02	2.86E-05	1.40E-07
Mass of labelled std(mg)	7.57E+01	3.91E-06	5.17E-08
Labelled stock solution (mg/kg)	4.00E+03	8.30E-03	2.08E-06
Uncertainty of calibration standard level 2 (mg)	2.07E+02	2.93E-05	1.41E-07
Uncertainty of calibration standard level 3 (mg)	2.06E+02	2.91E-05	1.41E-07
Method Precision	1.00E+02	1.89E-01	1.89E-03
Instrument repeatability	1.00E+02	1.77E-01	1.77E-03
Calibration curve	2.27E+00	3.04E-02	1.34E-02
Relative Combined Uncertainty			1.37E-02
Result (mg/g)	2.27E+00		
Combined Standard Measurement Uncertainty		3.09E-02	
Expanded Uncertainty (k=2)		6.18E-02	
Relative Uncertainty		2.73E+00	



Amino Acids in acidic solution

CCQM-K78.a

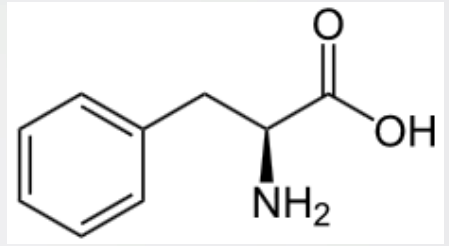
Amino acids in acidic solution

Study Material

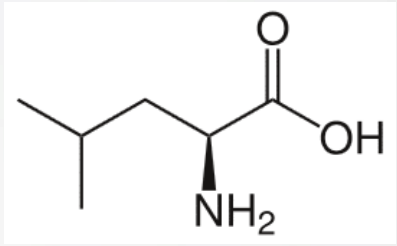
Standard solution in 0.01 N HCl containing,

- phenylalanine (Phe)
- leucine (Leu)
- isoleucine (Ile)
- proline (Pro)

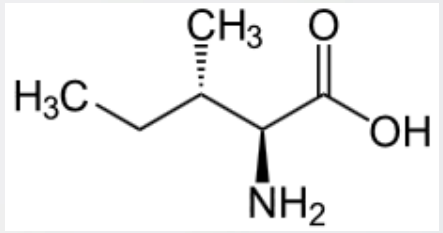
- 0.1 gram sample was diluted with 0.01 N HCl (aq) gravimetrically
- The analytes were derivatized with propyl chloroformate



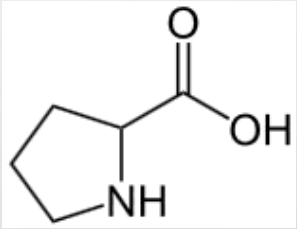
Phenylalanine (Phe)



Leucine (Leu)



Isoleucine (Ile)



Proline (Pro)

Amino Acids in acidic solution

Thermo Scientific Q Exactive, Orbitrap LC/MS

Column: Phenomenex EZ:fast 4u AAA-MS(250 x 2.0 mm i.d.)

AS; Injection volume : 2 µL

Column temperature : 40 °C

Flow rate (mL/min) : 0.250

Mobile phase : A: MeOH: H2O (0.01 M ammonium formate) (1:1)
B: MeOH (0.01 M ammonium formate)

Run length (min) : 22.0

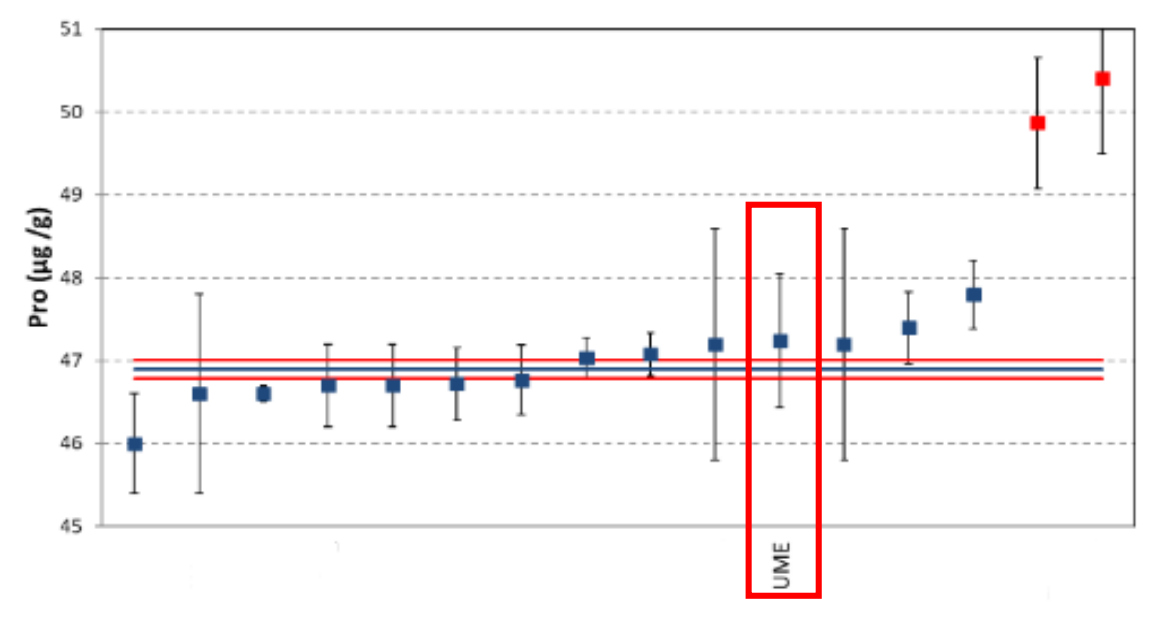
Amino acids	Parent Ions	Daughter Ions
Phenylalanine, derivatized	294	168
Phenylalanine, IS, derivatized	299	198
Leucine, derivatized	260	184
Leucine, IS, derivatized	261	184
Isoleucine, derivatized	260	186
Isoleucine, IS derivatized	261	183
Proline, derivatized	244	152
Proline, IS derivatized	245	152

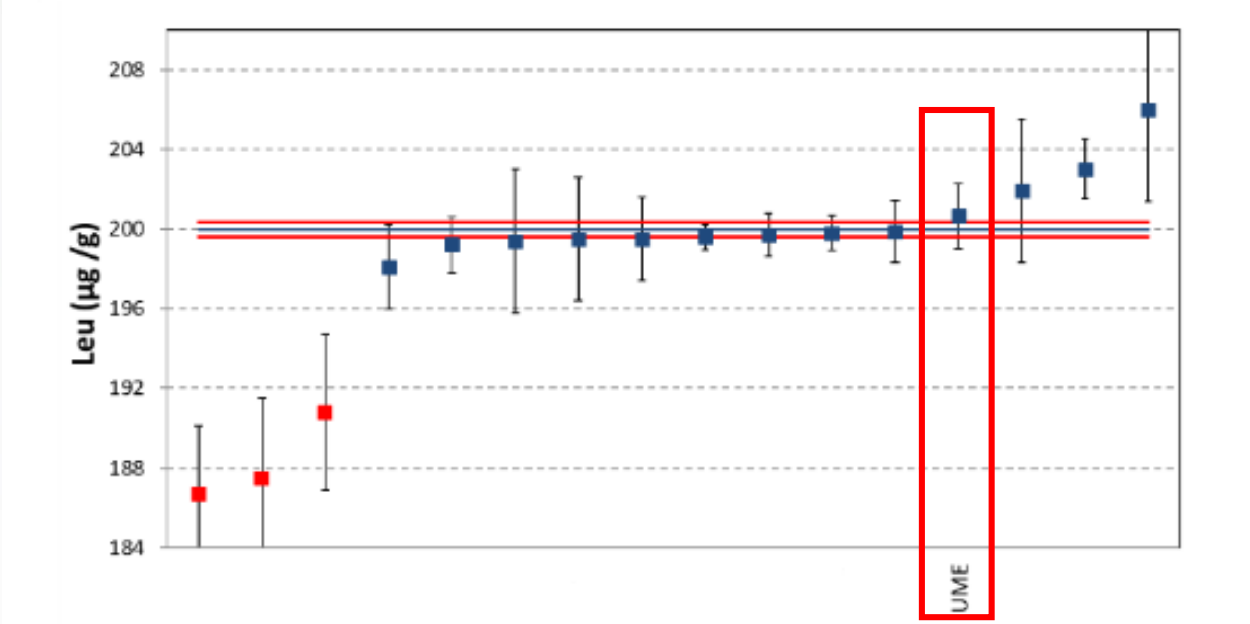
Retention time (min)	Flow (mL/min)	A %	B %
0.00	1.00	65	35
0.00	1.00	65	35
12.00	1.00	45	55
12.01	1.00	20	80
15.00	1.00	20	80
15.01	1.00	00	100
17.00	1.00	00	100
17.01	1.00	65	35
22.00	1.00	65	35

Amino Acids in acidic solution

Internal standards used
L-phenylalanine (Ring-D5, 98%) Cambridge Isotope Laboratories
L-leucine (1-13C, 99%) Cambridge Isotope Laboratories
L-isoleucine (15N, 98%) Cambridge Isotope Laboratories
L-proline (15N, 98%) Cambridge Isotope Laboratories

Amino Acids in acidic solution





Measurand	Mass Fraction (Average, reported value for CCQM K78a)(µg/g)	Mass Fraction (LC-MS)(µg/g)	Mass Fraction (qNMR)(µg/g)	Combined Standard Uncertainty (µg/g)	Coverage Factor (k)	Expanded Uncertainty (µg/g)
Phenylalanine (Phe)	482.59	482.94	482.24	3.60	2	7.20
Leucine (Leu)	200.64	197.49	203.78	1.62	2	3.24
Isoleucine (Ile)	218.98	219.98	217.97	3.50	2	7.00
Proline (Pro)	47.24	47.94	47.10	0.81	2	1.62

Uncertainty budget of Leu (LC-MS)				
		Value	u(x)	u(x)/x
Weighing of sample (mg)		25	1,47E-04	5,88E-06
Weighing of IS (mg)		25	1,57E-05	6,28E-07
Standard stock solution (mg/g)		2500	5,02E+00	2,01E-03
Internal stock solution (mg/g)		2500	7,65E+00	3,06E-03
Intermediate precision		100	1,58E-01	1,58E-03
Recovery		100	5,44E-03	5,44E-05
Repeatability		100	1,95E-01	1,95E-03
Calibration graph		1,8	1,22E-02	6,75E-03
				8,08E-03
Result (mg/g)	197,49			
Combined uncertainty		1,60		
Expanded uncertainty		3,19		
% Relative uncertainty		1,62		
% Relative standard uncertainty		0,81		

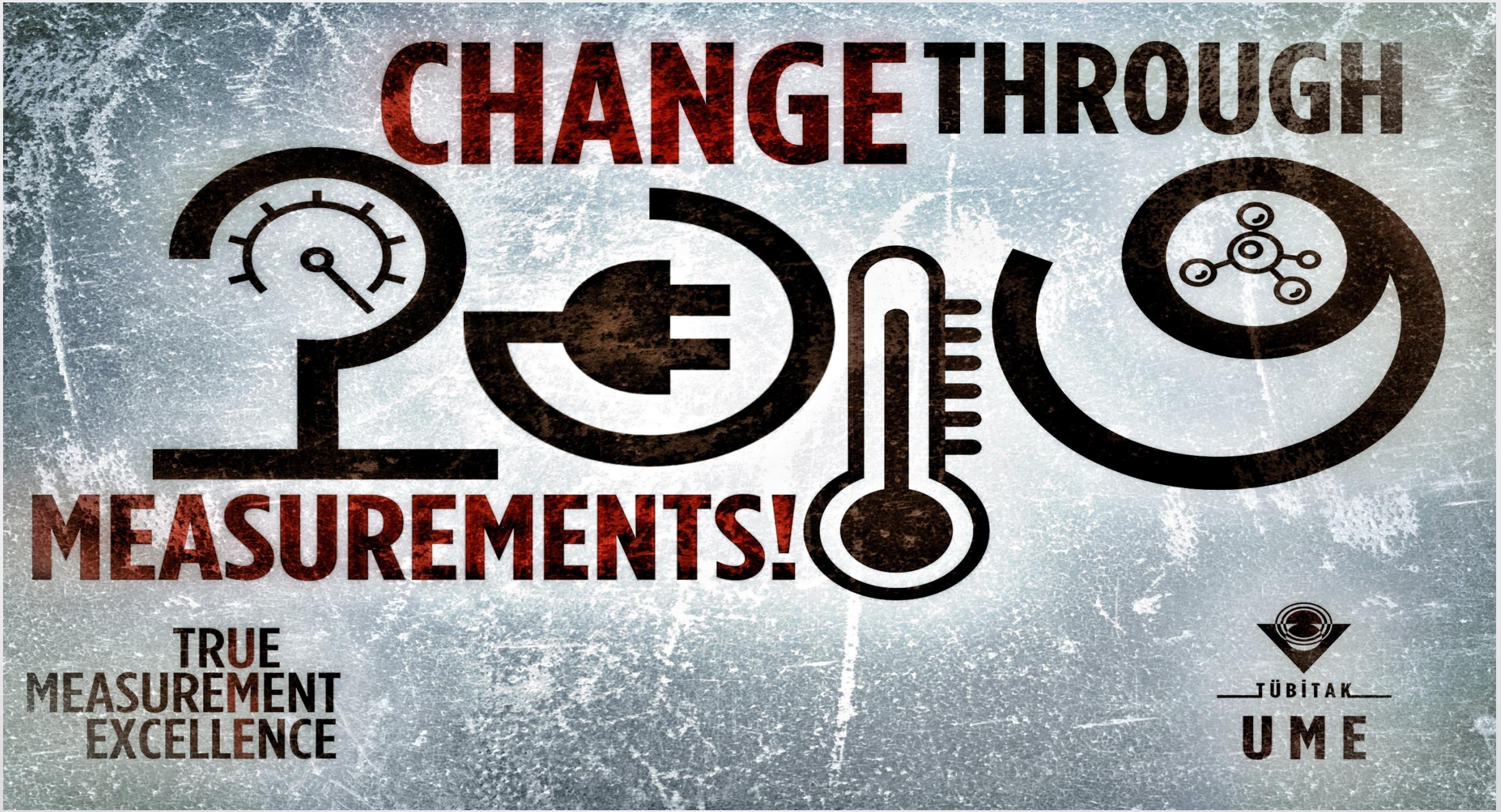
Isotope Dilution/Mass Spectrometry

IDMS is superior to other analytical procedures concerning

- High Trueness together with small uncertainty
- Matrix independent
- Losses of analyte do not affect the accuracy of the result
- SI traceability
- IDMS enhances the accuracy of measurements, especially in difficult sample matrix

Primary Method

Highest Metrological Quality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