NIST Special Publication 1209

Summary of NIST/SIM Chemical Metrology Working Group Training Opportunity: Isotope Dilution-Mass Spectrometry Clinical Measurement Course

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Susan S. Tai
Chemical Sciences Division
Material Measurement Laboratory

Antonio Possolo
Statistical Engineering Division
Information Technology Laboratory

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December 2016

U.S. Department of Commerce
Penny Pritzker, Secretary

National Institute of Standards and Technology
Willie May, Under Secretary of Commerce for Standards and Technology and Director

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1. Introduction

1.1 Course Overview

A week-long training opportunity entitled *Isotope Dilution/Mass Spectrometry (ID/MS) Clinical Measurement Course* was held at the National Institute of Standards and Technology hosted by the Chemical Sciences Division (CSD) on July 18-22, 2016. The training opportunity was offered as a part of the FY16 NIST International and Academic Affairs Office (IAAO)-SIM Engagement Opportunity. Participants from six NMIs from the Sistema Interamericano de Metrologia (SIM), the Regional Metrology Organization (RMO) for the Americas were invited to participate in the course which focused on the application of ID/MS methods for classical clinical biomarker (creatinine, cholesterol, and glucose) measurements. The SIM participants included representatives from the Instituto Nacional de Tecnología Industrial (INTI) – Argentina, Instituto Nacional de Metrologia, Qualidade e Tecnologia (INMETRO) – Brazil, Instituto Nacional de Metrología de Colombia (INM (CO)) – Colombia, Centro Nacional de Metrología (CENAM) – Mexico, Instituto Nacional de Calidad (INACAL) – Peru, and Laboratorio Tecnológico del Uruguay (LATU) – Uruguay.

During the 2015 Chemical Metrology Working Group (CMWG) of SIM meeting, several NMIs requested to receive chemical metrology training to assist in the development of their measurement services for clinical measurements. Katrice Lippa (NIST representative to the CMWG of SIM) and Valnei Cunha (Chair, CMWG of SIM) responded by proposing a one-week course to include a series of in-depth classroom lectures, hands-on and videotaped laboratory training modules, and in-class data analysis.

Coordinated by Jeanita Pritchett and Katrice Lippa, CSD organized a team of experts to provide details for the critical steps in sample preparation, instrumental analysis, and data processing related to clinical measurements. The team included Mary Bedner, Jeanice Brown Thomas, Carolyn Burdette, Johanna Camara, David Duewer, Brian Lang, Mike Nelson, Lane Sander, Lorna Sniegoski, Susan Tai, and Antonio Possolo from the Statistical Engineering Division (SED). Additionally, Mary Satterfield provided an overview of NIST and research efforts within the Material Measurements Laboratory (MML). Furthermore, the participants received biosafety training similar to that offered to NIST staff from Wing (William) Wong to learn how to safely handle biological samples.

1.2 Pre-course survey results

The participants were asked to self-assess their current knowledge and expertise through responses to a pre-course survey. The survey consisted of six subject areas: general knowledge, sample preparation, quantitation, purity, instrumentation, and measurement uncertainty. These results were used to design the most efficient format for the course to ensure that areas of need and interest were addressed throughout the course. The results from the survey are found in Appendix 1.

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1.3 Course Agenda

2016 SIM Clinical Measurement Course Agenda
July 18-22, 2016
National Institute of Standards and Technology
Chemical Sciences Division
227/A105

July 18, 2016 (Monday)

Time	Topic	Instructor(s)	Section
8:00 am	Arrive at NIST; IAAO Briefing; Refreshments	Andrew Conn	
9:15 am	Welcome; Opening Remarks	Katrice A. Lippa	1
9:30 am	Overview of MML Introduction of Attendees	Mary Satterfield	
10:00 am	NIST Clinical Program Overview*; Introduction of Instructors;	Jeanita S. Pritchett; All instructors	2
11:00 am	Group Photo (In Front of Building 101)		
11:30 am	Biosafety Training (224/B309)	Wing Wong	15
12:30 pm	Lunch (NIST Cafeteria; On Your Own)		
1:30 pm	Lab Tour: 227		
2:00 pm	General Traceability and Chemical Metrology*	David L. Duewer	3
2:30 pm	Hazard Reviews*		
3:00	Break		
3:30	Internal Standards for ID/MS and Isotope Dilution in Practice*	Carolyn Q. Burdette, Jeanita S. Pritchett	4

**indicates that session may be videotaped*

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2016 SIM Clinical Measurement Course Agenda
July 18-22, 2016
National Institute of Standards and Technology
Chemical Sciences Division
227/A105

July 19, 2016 (Tuesday)

Time	Topic	Instructor(s)	Section
8:00 am	Arrive at NIST; Breakfast (NIST Cafeteria; On Your Own)		
9:00 am	Chemical Purity*	Mary Bedner and Michael A. Nelson	5
10:30 am	Density Determination (Video); Lab Tour	Brian E. Lang, Jeanita S. Pritchett, Lane C. Sander, and Lorna T. Sniegoski	
11:00 am	Break		
11:30 am	Quantitative Water Determination*	Brian E. Lang	6
12:30 pm	Lunch (NIST Cafeteria; on your own)		
1:30 pm	Calibration Approaches and Data Evaluation (video)	Mary Bedner, Michael A. Nelson, and Lane C. Sander	
3:00 pm	Break		
3:30 pm	Good laboratory Practices for Weighing (Video; Hands-On) (227/B143)	Jeanita S. Pritchett, Lane C. Sander, and Lorna T. Sniegoski	

**indicates that session may be videotaped*

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2016 SIM Clinical Measurement Course Agenda
July 18-22, 2016
National Institute of Standards and Technology
Chemical Sciences Division
227/A105

July 20, 2016 (Wednesday)

Time	Topic	Instructor(s)	Section
8:00 am	Arrive at NIST; Breakfast (NIST Cafeteria; On Your Own)		
9:00 am	Cholesterol and Glucose Overview*	Jeanita S. Pritchett and Lorna T. Sniegoski	7
9:30 am	Lab: Sample Preparation for Cholesterol (Hands-On) (227/B143 and 227/B141)	Jeanita S. Pritchett and Lorna T. Sniegoski	
11:00 am	Break		
11:30 am	Sample derivatization for GC; Separation Challenges in GC*	Jeanita S. Pritchett and Lorna T. Sniegoski	8
12:30 pm	Lunch (NIST Cafeteria; On Your Own)		
1:30 pm	Lab: GC-MS Operation and Sample Analysis (Hands-On) (227/A126)	Jeanita S. Pritchett and Lorna T. Sniegoski	
3:00 pm	Break		
3:30 pm	Data Analysis (Cholesterol)	Jeanita S. Pritchett and Lorna T. Sniegoski	7

**indicates that session may be videotaped*

2016 SIM Clinical Measurement Course Agenda
July 18-22, 2016
National Institute of Standards and Technology
Chemical Sciences Division
227/A105

July 21, 2016 (Thursday)

Time	Topic	Instructor(s)	Section
8:00 am	Arrive at NIST; Breakfast (NIST Cafeteria; On Your Own)		
9:00 am	Creatinine Overview*	Johanna E. Camara and Jeanita S. Pritchett	9
9:30 am	Lab: Sample Preparation for Creatinine (Hands-On) (227/B143 and 227/A142)	Johanna E. Camara and Jeanita S. Pritchett	
11:00 am	Break		
11:30 am	Separation Challenges in LC*	Carolyn Q. Burdette and Lane C. Sander	10
12:30 pm	Lunch (NIST Cafeteria; On Your Own)		
1:30 pm	Lab: LC-MS(/MS) Operation and Sample Analysis (Hands-On) (227/A145)	Carolyn Q. Burdette, Johanna E. Camara, and Jeanita S. Pritchett	
3:00 pm	Break		
3:30 pm	Data Analysis (Creatinine)	Johanna E. Camara and Jeanita S. Pritchett	9
6:00 pm	Social Dinner: Dogfish Head Alehouse (800 W. Diamond Ave. Gaithersburg, MD 20878)		

**indicates that session may be videotaped*

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2016 SIM Clinical Measurement Course Agenda
July 18-22, 2016
National Institute of Standards and Technology
Chemical Sciences Division
227/A105

July 22, 2016 (Friday)

Time	Topic	Instructor(s)	Section
8:00 am	Arrive at NIST; Breakfast (NIST Cafeteria; On Your Own)		
9:00 am	CCQM Data Review; Uncertainty Evaluation	Antonio Possolo	11
11:00 am	Break		
11:30 am	CCQM Data Review; Uncertainty Evaluation (Continued)	Antonio Possolo	11
12:30 pm	Lunch (NIST Cafeteria; On Your Own)		
1:30 pm	Reference Measurement Procedures and JCTLM*	Jeanita S. Pritchett and Susan S. Tai	12
2:00 pm	Other Biomarkers Overview*	Johanna E. Camara and Jeanice Thomas Brown	13
3:00 pm	Break		
3:30 pm	Challenges of Designing Pooled and Spiked Samples*	Johanna E. Camara and Jeanice Thomas Brown	14
4:30 pm	Wrap-Up	Katrice A. Lippa and Jeanita S. Pritchett	

**indicates that session may be videotaped*

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1.4 Participant Listing

Affiliation	Name
Instituto Nacional de Tecnología Industrial (INTI) – Argentina	Illiana Valeria Lobatto
Instituto Nacional de Metrologia, Qualidade e Tecnologia (INMETRO) – Brazil	Wagner Wollinger
Instituto Nacional de Metrología de Colombia (INM (CO)) – Colombia	Sergio A. González-Mónico
Centro Nacional de Metrología (CENAM) – Mexico	Miryan Balderas Escamilla
Instituto Nacional de Calidad (INACAL) – Peru	Galia Ticona Canaza
Laboratorio Tecnológico del Uruguay (LATU) – Uruguay	Ana Silva

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2. Summary

2.1. Post-course survey discussion

After completion of the course, the participants responded to a post-course survey to evaluate the effectiveness of the course. The same format was used as in the pre-course survey; however, the participants were asked how well they felt each topic was presented throughout the duration of the course. Additionally, the participants had the opportunity to provide feedback about what they enjoyed about the course and make suggestions about additional topics that could be added in the future or serve as independent workshops. The general consensus from the participants was that the subject areas with the greatest needs were sufficiently or extensively covered during the course. The results from the post-course survey are found in Appendix 2.
The workshop was a success as highlighted in the comments from the participants. They thoroughly appreciated the organization of the training course and the comprehensive list of topics that were covered. They also valued the willingness of the instructors to maintain contact via email to provide additional technical support and feedback.

2.2. Post-course resources

NIST provided a series of Standard Reference Materials (SRMs) value assigned for cholesterol, glucose, and creatinine in serum- and/or plasma-based materials to each participant and their home institute to aid with method development and expansion of their current capabilities. Additionally, a series of neat chemical SRMs (cholesterol, glucose, and creatinine) were provided for use in calibration solution preparation. Furthermore, videotapes of select lectures, slides of all oral presentations, and training videos were made available to all participants. The title and description of the training videos provided to the participants are found below.

Title / Technical Procedure Title	Time (min:sec)	Description
Calibration and Use of Analytical Balances	12:46	Demonstrations for several electronic balances (different mass ranges) and one mechanical balance
Preparation and Use of Calibration Solutions	19:10	Gravimetric preparation: include use of aluminum weigh boats and gas tight syringe to weigh solids and liquids
Approaches for Quantitation	38:41	Calibration models, peak integration, baselines, and interferences, reference standards and internal standards, experimental design
Method development for liquid chromatography	30:34	Basic guidelines for developing LC methods
Troubleshooting LC Instrumentation and Methods	29:16	Resolving issues associated with instrumentation and methods

2.3. Follow-up SIM comparison

A SIM inter-laboratory comparison for the measurement of glucose, creatinine, and/or cholesterol in a series of serum-based study materials is being planned for the participating NMIs in 2017. The results of this activity may be considered a SIM regional comparison, and will rely on NIST value assignment for the reference value of the study material. Participants will be asked to provide analyte mass fraction (mg/g) value assignment for a study material. Additionally, they will be asked to provide calibrant information, sample preparation and instrumentation details, control data, repeatability data, and a complete uncertainty budget. The NIST experts have agreed to continue to provide metrological support for the participants to address any concerns that may arise during their method development.

2.4 Future training courses

Due to the success of this training course, CSD intends to offer subsequent training opportunities for NMIs in the SIM region. Potential topics could include food metrology and safety, environmental contaminants, or climate change monitoring. Additional surveying of the CCQM SIM community will aid in identifying critical target areas for upcoming training opportunities.

3. Acknowledgements

The course was funded by a FY16 NIST IAAO-SIM Engagement Opportunity and the SIM Technical Committee. A sincere thanks is extended to Andrew Conn from IAAO for his assistance in organizing the logistics for the course. Also, we would like to thank Mary Satterfield, Chief of Staff from MML, for providing an overview of the research activities within MML. Finally, we would like to thank Wing Wong for providing a hands-on biosafety overview for the participants.

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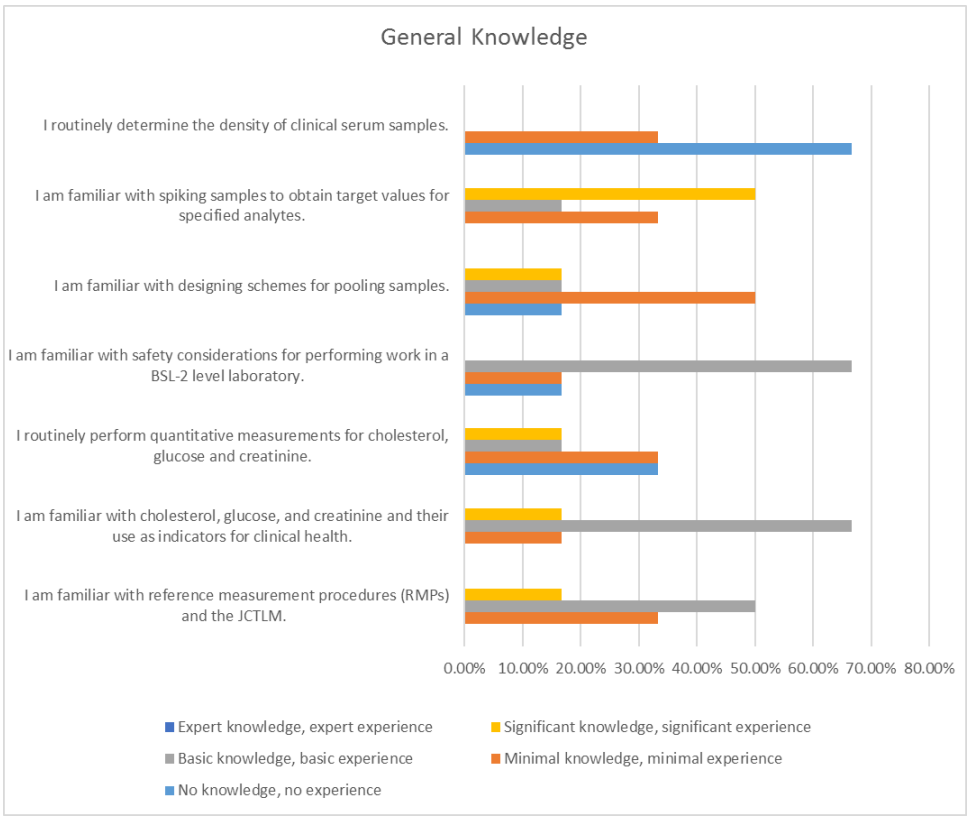
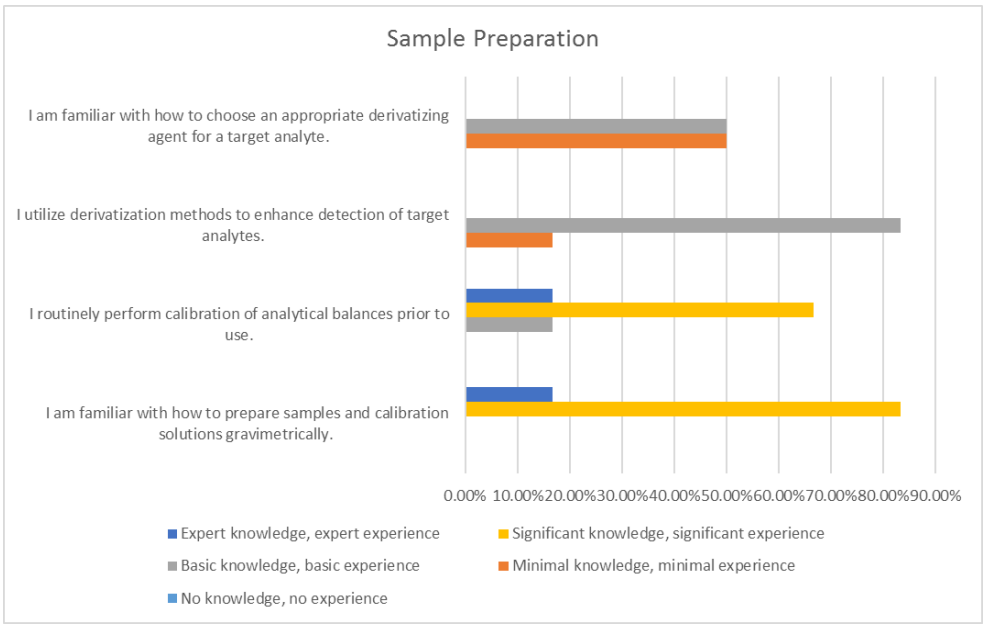
Appendix 1: Pre-course survey results





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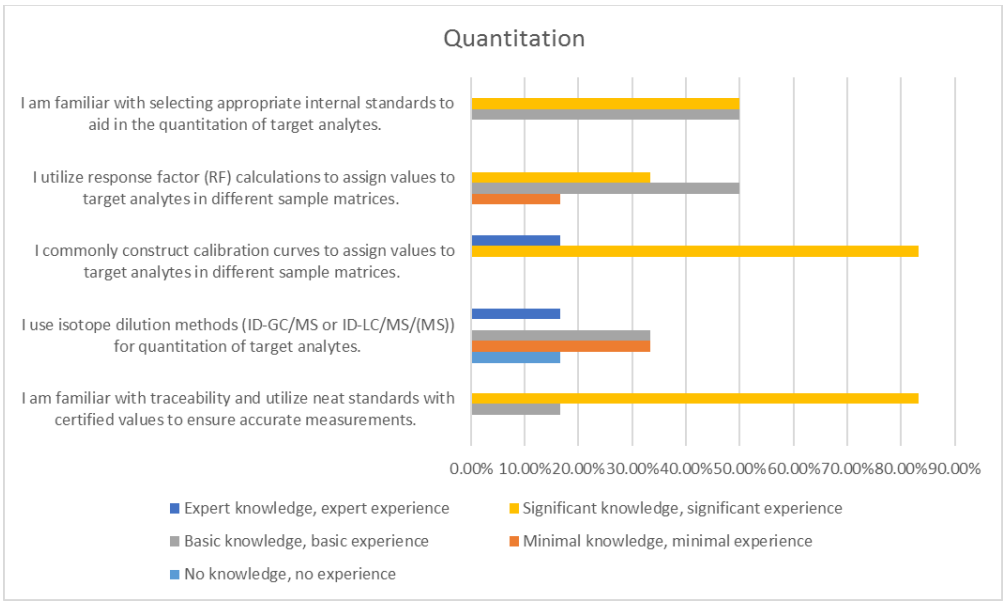

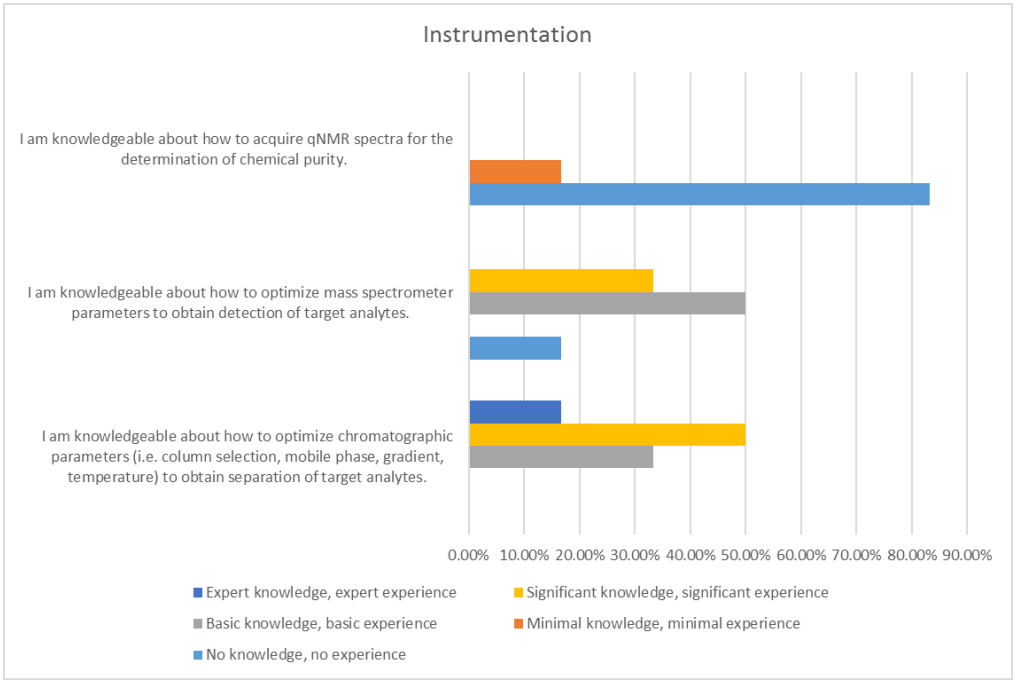

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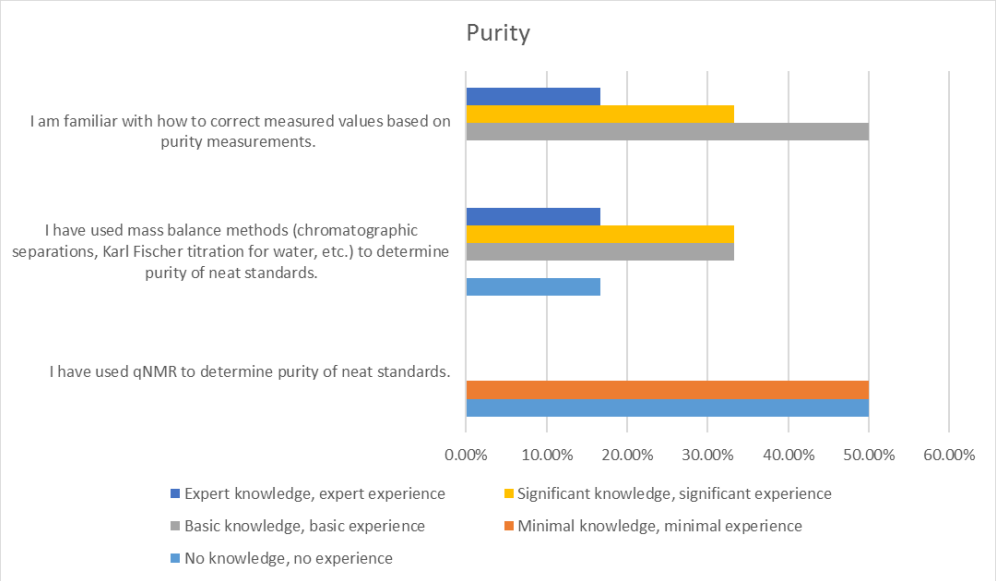

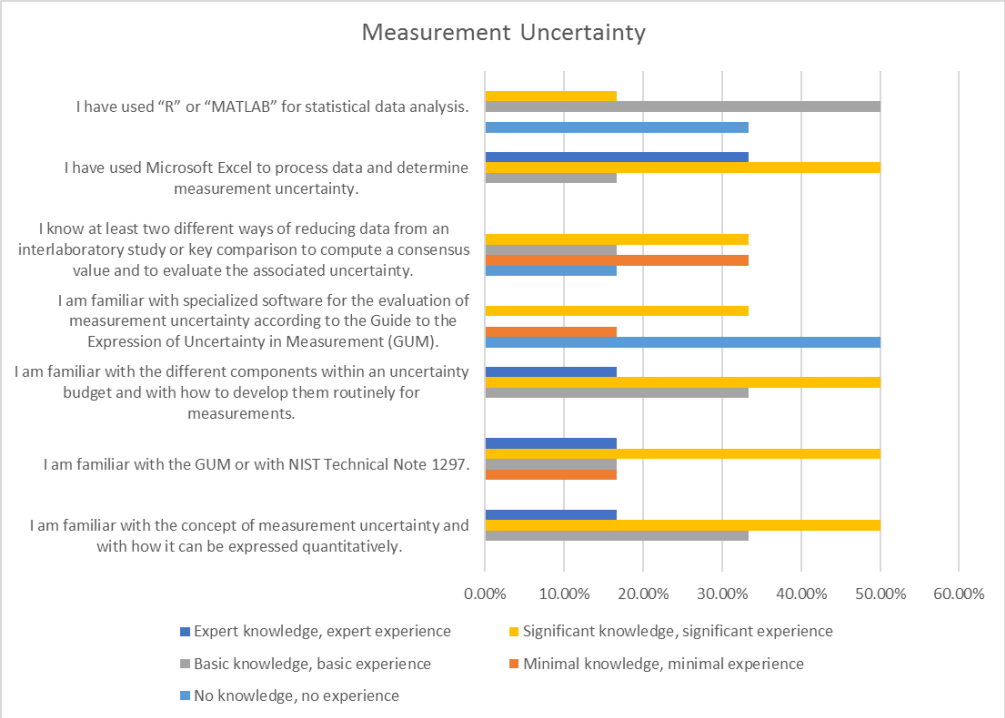

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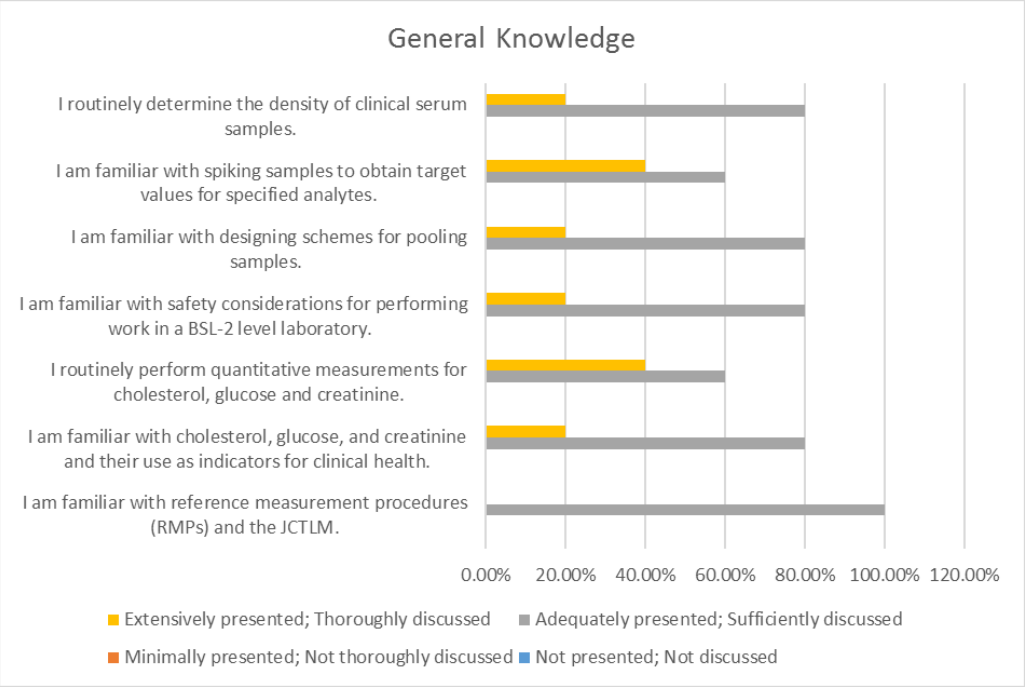
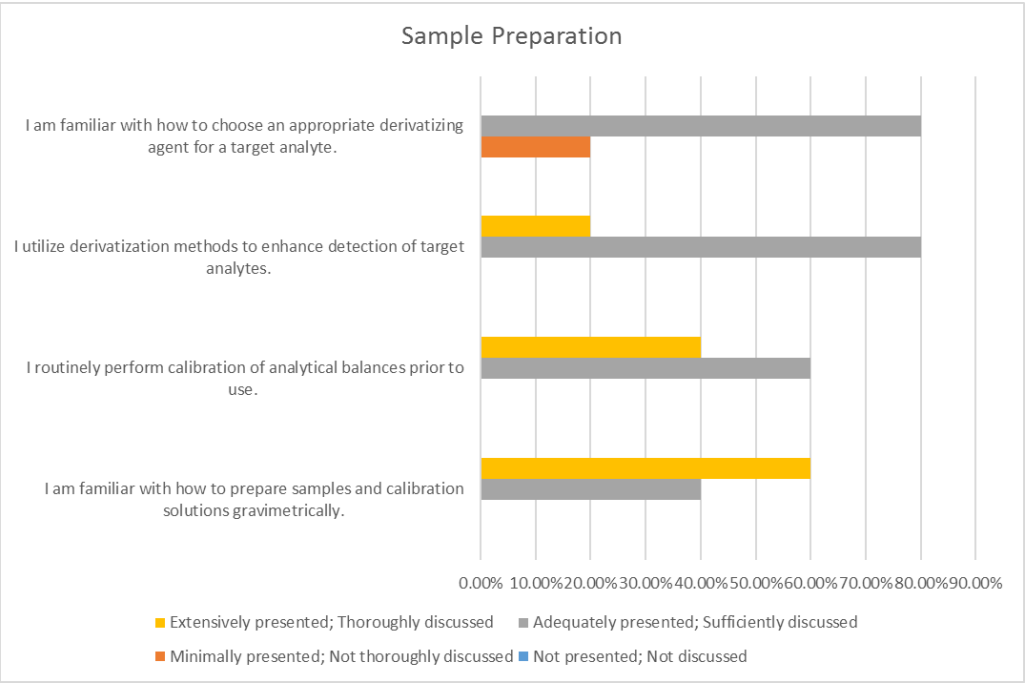
Appendix 2: Post-course survey results





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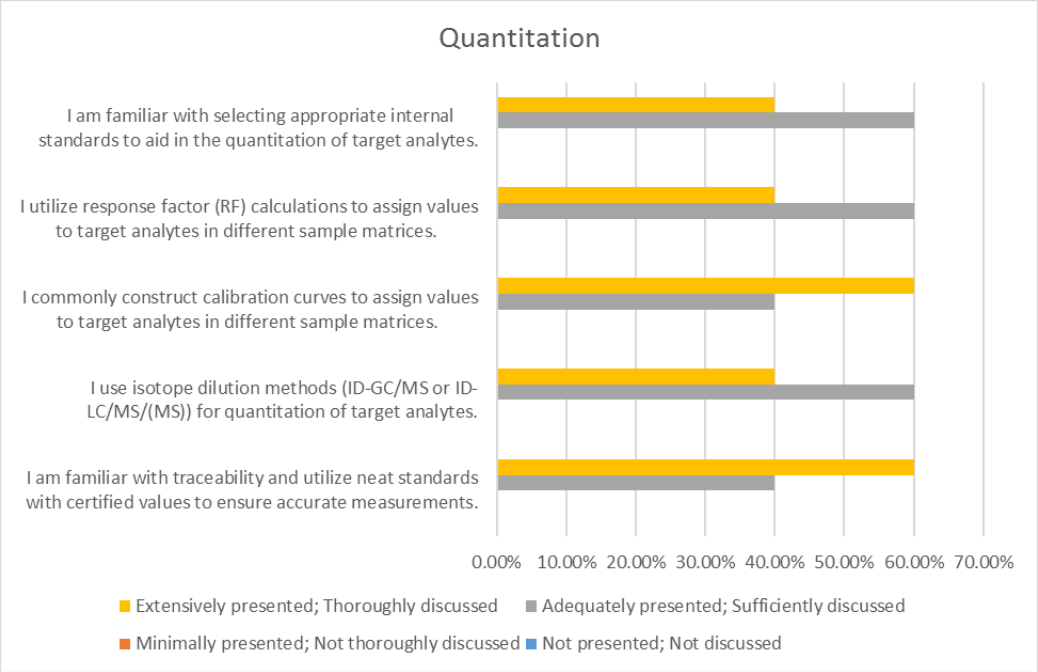

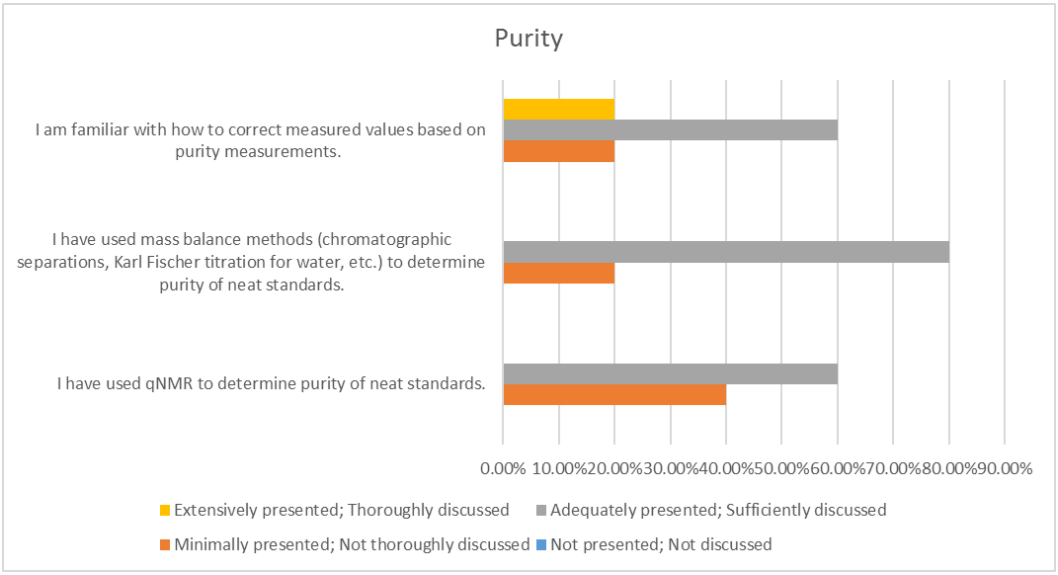

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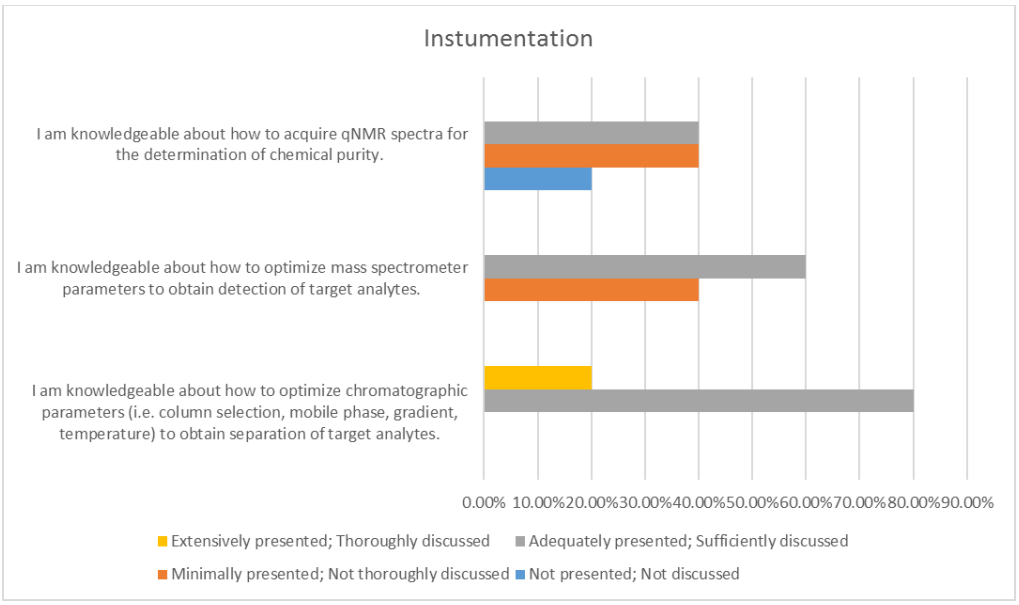

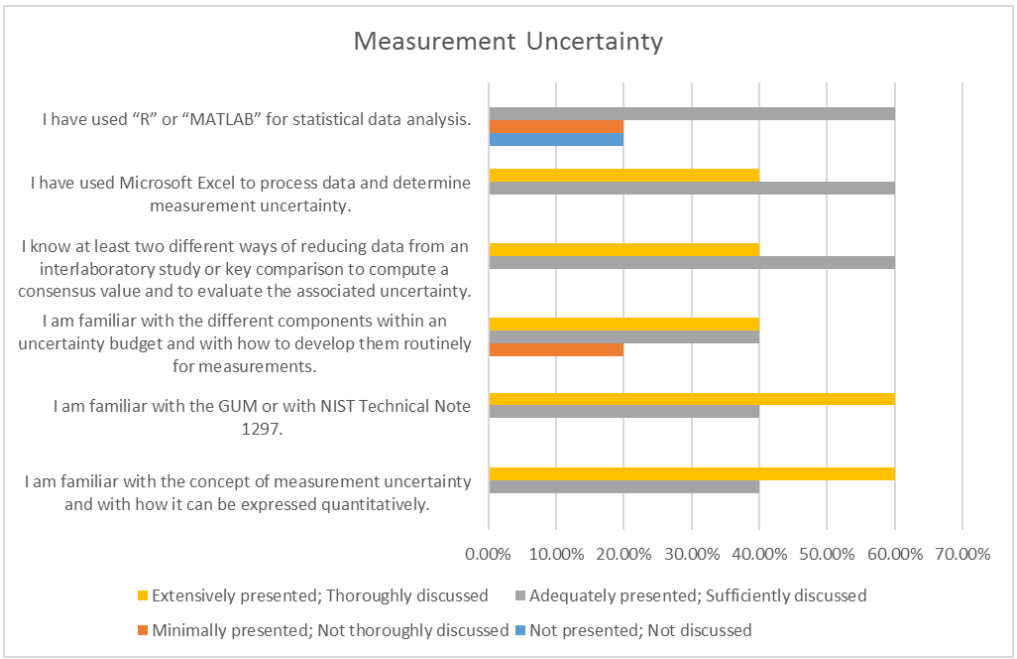

Please describe what you like about the course:

Response 1	*First of all, I want to emphasize the excellent management of the training course. Of course, the patience of all NIST colleagues was appreciated. It was a very pleasant staying.*
Response 2	*The course was very comprehensive, allowed us to have an overview of the considerations to look for in a measurement process. It provided us with valuable information to make improvements in measurement processes for glucose, cholesterol and creatinine that we have currently implemented. We were allowed to have contact with experts from different areas and we leave the door open to keep in touch with them in case of any feedback in the future.*
Response 3	*It covered several really important topics (purity assessment, clinical analysis, Karl Fischer determination, etc).*
Response 4	*The opportunity to learn from different NIST experts and the organization of the course.*

Please describe any additional topics/learning objective that you would've liked covered during the training course:

Response 1	*It can include more details about the preparation of the reference materials, commutability procedure, statistical evaluation of homogeneity and stability.*
Response 2	*I would have liked a little more detail on the side of purity.*
Response 3	*More information on qNMR*
Response 4	*The exposition of each topic was generally right for my level of knowledge on this subject.*
Response 5	*An evaluation of uncertainty of the thorough certification process, including stability and homogeneity.*

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Outside of clinical measurements, please list topics that you or other representatives from your NMI would like covered in a possible future training course:

Response 1	*Determination of heavy metals in food matrix, hydrobiologic products in order to evaluate food safety; Determination of heavy metal to for environmental control (filter air, soil); Determination of heavy metals in minerals; Determination of salts purity, anions, heavy metals by coulometric titration; Determination ethanol purity.*
Response 2	*Determination of purity in organic compounds; Measurement of electrolytes in food and biological samples; Measurement of protein by LC-MS/MS.*
Response 3	*Contaminants in environmental or food samples; Coulometry; Dissolved oxygen (analysis and sensor calibration).*
Response 4	*Purity determinations; Production of reference materials*
Response 5	*Environmental analysis and preparation of matrix CRMs; food safety.*

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Appendix 3: Oral Presentations Delivered at Workshop

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SIM Chemical Metrology Working Group 2016 Training Opportunity

ID/MS Clinical Measurement Course

Katrice A. Lippa

NIST National Institute of Standards and Technology U.S. Department of Commerce

MATERIAL MEASUREMENT LABORATORY

NIST's Role in SIM Chemical Metrology

*In order to most effectively address the unique needs of all 34 countries within SIM, whose capabilities in chemical metrology span a very broad range, we are focusing our SIM Chemical Metrology Working Group activities on **training and capability assessment** rather than participation in MRA-driven Key and Supplemental Comparisons*

This is being accomplished through:

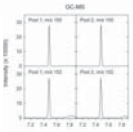
- Training in CMC preparation and review (e.g., SIM CMWG workshop, May 18)
- Hosting guest scientists from SIM NMI/DIs
- **High-impact training courses at NIST** (e.g., ID/MS Clinical Measurement Course, July 2016)

Long-Term Goal: Improved capabilities in chemical metrology across SIM and increased participation in CCQM and CIPM MRA-related activities

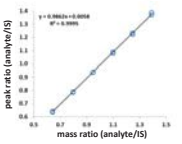
ID/MS Clinical Measurement Course Overview

- Designed to provide SIM NMI/DI laboratory personnel with in-depth classroom and hands-on laboratory experience
- Focus on isotope dilution/mass spectrometry (ID/MS) methods in the application of clinical marker (cholesterol, glucose, and creatinine) measurements
- Include lectures, hands-on sample preparation/lab demonstrations, training videos and hands-on data processing/analysis

Triple Quadrupole MS/MS



Creatinine ID/GC-MS



Preparation and Use of Calibration Solutions Training Video (L. Sander)

Meet the Instructors

Katrice A. Lippa
Scientific Advisor
NIST representative to CMWG

Jeanita S. Pritchett
Research Chemist
Course Coordinator

Mary Bedner
Research Chemist

Carolyn Q. Burdette
Research Chemist

Johanna Camara
Research Chemist

David L. Duewer
Research Chemist

Brian Lang
Research Chemist

Michael A. Nelson
Research Chemist

Antonio Possolo
Chief Statistician

Jeanice Brown Thomas
Research Chemist

Lane C. Sander
Senior Scientific Advisor

Lorna T. Sniegoski
Guest Researcher

Susan Tai
Research Chemist

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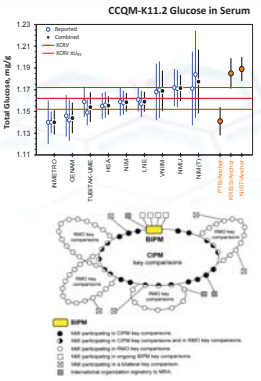
Post-course Resources

- NIST will provide a series of Standard Reference Materials (SRMs) that have been value assigned for cholesterol, glucose, and creatinine in serum- or plasma-based materials.
- A series of pure chemical SRMs (cholesterol, glucose, and creatinine) for use in calibration will also be provided.
- Videotapes of the select lectures may be made available to all participants.

Follow-up SIM Comparison (2017+)

- A SIM interlaboratory comparison for the measurement of glucose, creatinine, and/or cholesterol in a series of serum-based study materials
- Participants will be asked to provide analyte mass fraction (mg/g) value assignment for a study material. Additionally they will be asked to provide the following:
  - Calibrant information
  - Sample preparation and instrumentation details
  - Control data
  - Repeatability data
  - Complete uncertainty budget
- These results may be considered a SIM regional comparison (key or pilot), and will rely on NIST value assignment for the reference value of the study material



Welcome SIM Participants!

- Illiana Valeria Lobatto, Instituto Nacional de Tecnología Industrial (INTI) Argentina
- Wagner Wollinger, Instituto Nacional de Metrologia, Qualidade e Tecnologia (INMETRO) – Brazil
- Sergio A. González-Mónico, Instituto Nacional de Metrología de Colombia (INM (CO)) – Colombia
- Miryan Balderas Escamilla, Centro Nacional de Metrología (CENAM) – Mexico
- Galia Ticona Canaza, Instituto Nacional de Calidad (INACAL) – Peru
- Ana Silva, Laboratorio Tecnológico del Uruguay (LATU) – Uruguay

Clinical Certified Reference Materials and Measurement Services at NIST

2016 SIM Clinical Measurement Course

NIST National Institute of Standards and Technology U.S. Department of Commerce

MATERIAL MEASUREMENT LABORATORY

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Disclaimer

**Certain commercial equipment, instrumentation, or materials are identified to adequately specify the experimental procedure. Such identification does not imply recommendation or endorsement by NIST, nor does it imply that the materials or equipment identified are necessarily the best available for the purpose.*

NIST Biomedical and Health Programs

measurement infrastructure through methods, materials and data

accurate, comparable, reliable measurements

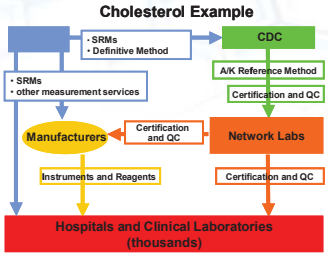
clinically-relevant species
(elements and electrolytes, vitamins, metabolites, contaminants, proteins)

biologically-relevant materials
(human fluids, marine species fluids and tissues)

The accuracy of diagnostic measurements is essential for accurate diagnosis and cost-effective treatment of diseases.

NIST Role in US Reference System for Clinical Measurements

- Maintain existing Definitive Methods and CRMs
- Develop new reference methods and CRMs of "higher metrological order" to meet new needs
- Work with other NMIs to establish equivalence of measurement services
- Ensure that SRMs are commutable with routine clinical assays
- Participate in global reference laboratory network and provide reference laboratory measurement services to *in vitro* diagnostic (IVD) industry



Note that CRMs are not the only option…

Mechanisms/Tools for Dissemination to Customers of a National Metrology Institute's (NMI) Calibration and Measurement Capabilities (CMC) for Chemical Measurements as published in Appendix C of the CIPM MRA:

- Reference Methods / Procedures
- **Certified Reference Materials** / Reference Materials
- Certified Reference Data / Reference Data
- Calibration Services
- Testing Services / **Value-assignment of customer-supplied materials**

CRMs are **ONLY ONE** of these NMI dissemination tools

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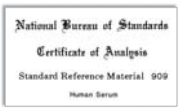
40+ Years of Clinical Diagnostics CRMs at NIST

- ***1960*** Development of pure, crystalline standards for calibration, e.g., urea
- ***1970*** Development of accurate isotope dilution (ID) GC-MS reference measurement procedures (RMPs) for clinical analytes in serum, e.g., cholesterol
- ***1980*** Human serum-based SRMs certified for metabolites and electrolytes, e.g., SRM 909 Human Serum (lyophilized)


National Bureau of Standards
Certificate of Analysis
Standard Reference Material 909
Human Serum
September 15, 1980


1968

40+ Years of Clinical Diagnostics CRMs at NIST

- ***1960*** Development of pure, crystalline standards for calibration, e.g., urea
- ***1970*** Development of accurate isotope dilution (ID) GC-MS reference measurement procedures (RMPs) for clinical analytes in serum, e.g., cholesterol
- ***1980*** Human serum-based SRMs certified for metabolites and electrolytes, e.g., SRM 909 Human Serum (lyophilized)
- ***1990*** New serum-based SRMs are frozen to reduce matrix effects. New focus on reference methods for toxic metals efforts (improve commutability with routine assays)
- ***2000*** Expanded efforts to develop SRMs and RMPs to address *in vitro* diagnostics (IVD) European Union (EU) directive; ID/LC-MS and ID/LC-MS/MS method development, e.g., hormones, creatinine
- ***2010*** SRMs and RMPs developed for serum-based materials assessing nutritional status; contaminants in human fluids (serum, urine, milk)
- ***present***

Roster of NIST's Clinical Diagnostics CRMs

For calibration/traceability

e.g. • **17 pure, crystalline standards:**
SRM 911c Cholesterol
SRM 914a Creatinine
SRM 918b Potassium Chloride (Clinical)

• **2 solutions (ethanol):**
SRM 2972 25-Hydroxyvitamin D2 and D3 Calibration Solutions
SRM 2972a Vitamin D Calibration Solutions (candidate)


SRM 2972 25-Hydroxyvitamin D2 and D3 Calibration Solutions

For method validation, improve accuracy and comparability

e.g. • **20 serum/plasma materials:**
SRM 1951c Lipids in Frozen Human Serum
SRM 1955 Homocysteine and Folate in Frozen Human Serum
SRM 956c Electrolytes in Frozen Human Serum

• **8 urine materials:**
SRM 3668 Mercury, Perchlorate, and Iodide in Frozen Human Urine
SRM 3667 Creatinine in Frozen Human Urine

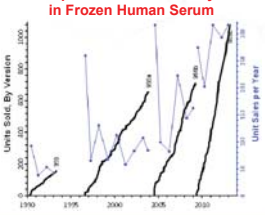

SRM 3950 Vitamin B6 in Frozen Human Serum


SRM 2669 Arsenic Species in Frozen Human Urine

Who purchases these CRMs?

- Other National Metrology Institutes (NMIs)
- Universities
- *In vitro* diagnostics (IVD) manufacturers
- Other US Government Agencies
- Commercial reference material suppliers
- Third-party distributors
- Routine clinical laboratories

Example: SRM 956 Electrolytes in Frozen Human Serum



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New and Updated Clinical Diagnostics CRMs

- **SRM 3669 Arsenic Species in Frozen Urine (elevated levels)** (candidate) – improves accuracy and comparability in arsenic exposure assessments supporting CDC's National Health and Nutrition Examination Survey (NHANES)
- **SRM 909c Human Serum** and **SRM 1950 Metabolites in Human Plasma** to be updated with total transferrin and individual transferrin sialoforms (isoforms), used as biomarkers of iron health status
- **SRM 971 Hormones in Frozen Human Serum** to be updated with several iodide-containing thyroid hormones (e.g., triiodothyronine T3, thyroxineT4) via both LC-MS/MS and LC-ICPMS techniques
- **SRM 2378 Fatty Acids in Human Serum** series of 3 materials from donors with or without supplementation of fish and flaxseed oils

Arsenic keratosis on palms of a patient

Goiter caused by iodine deficiency

Health Monitoring Reference Materials

CRMs for Organic Contaminants in Human Fluids (urine, milk and serum) were developed over ten years in collaboration with CDC's Organic Analytical Toxicology Branch

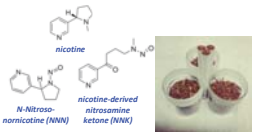
- need for control materials and reference materials to ensure comparability;
- compare the data generated from the annual monitoring program (NHANES) in the US with international data

PCBs, PBDEs, chlorinated pesticides, dibenzo-p-dioxins, dibenzofurans

SRMs 1953 and 1954 Organic Contaminants in Human Milk

Candidate SRM 3222 Cigarette Tobacco Filler currently under development with FDA/Center for Tobacco Products to support the Family Smoking Prevention and Tobacco Control Act

- emulate commercially-available cigarette products subject to routine laboratory testing methods;
- certified values for nicotine, NNK, NNN (nitrosamines specific to tobacco), moisture content and pH



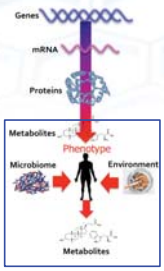
nicotine

N-Nitroso-nornicotine (NNN)

nicotine-derived nitrosamine ketone (NNK)

Technical Contacts: *Lane Sander, Jeanita Pritchett, Jessica Reiner, Bruce Benner*

CRM to Support Metabolomics



Identification, measurement and interpretation of the fingerprint of metabolites or "metabolome" in various cells, biological tissues and fluids that represent the constituents of biochemical cellular processes

The **metabolome** represents the identity and concentration of metabolites at a given point in time

- Profiles are dynamic
- Reflects phenotype
- Range in the 1000s

SRM 1950 Metabolites in Human Plasma was designed to represent a "normal" human plasma and to be used for method validation in metabolomics studies

- Value assignment of over **90 individual constituents** (cholesterol, creatinine et al., hormones, fatty acids, amino acids, vitamins, trace elements and electrolytes)

Technical Contacts: *Dan Bearden, Yamil Simon, Johanna Camara*

Reference Materials to Support Protein Measurements and Proteomics

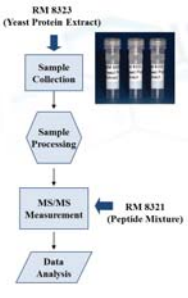
Substantial investment in proteomics research has yet to yield reliable biomarkers

NIST approach:

- Provide standard protocols to provide quality assurance for complex workflow, method- or platform-dependent results
- Develop reference materials to assess various steps of proteomics workflow or verify instrument performance

NIST Materials:

- RM 8323 Yeast Protein Extract is currently available
- RM 8321 Peptide Mixture for Proteomics to be completed FY16
- RM 8313 Digested Yeast Protein Extract is in development



typical workflow for a proteomics experiment

Technical Contacts: *Ashley Beasley-Green, David Bunk*

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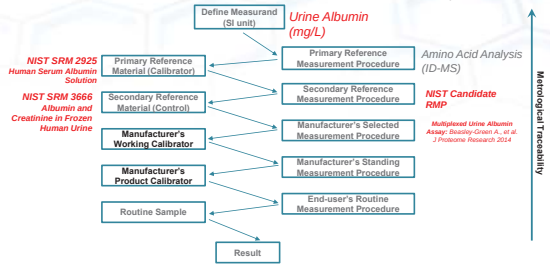
Protein Reference Materials to Support Traceability of Routine Urine Albumin Measurements

Efforts to establish a urine albumin reference measurement system



ISO 17511:2003(E)

Technical Contacts: *Ashley Beasley-Green, David Bunk*

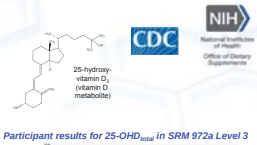
Clinical Quality Assurance Programs (QAPs)

www.nist.gov/mml/csd/qaps.cfm

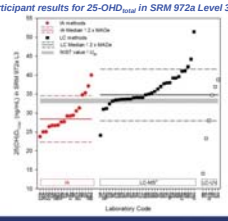
Quality Assurance Programs (QAPs) serve to support measurement comparability for clinical laboratory measurements of nutritional biomarkers in serum and plasma matrices:

- **Micronutrient Measurements QAP** (since 1984)
- **Vitamin D Metabolites QAP** (since 2009)
- **Fatty Acids in Human Serum and Plasma QAP** (since 2012)

Biannual exercises with individual laboratory, exercise consensus and accuracy-based results:



Participant results for 25-OHD$_{total}$ in SRM 972a Level 3



Technical Contacts: *Mary Bedner, Jeanice B. Thomas*

Terms and criteria for NIST CRMs for chemical measurements

- Describes seven modes currently used at NIST for value-assigning SRMs and RMs for **chemical measurements**
- Defines data quality descriptors used at NIST for these SRMs and RMs
  - NIST Certified Value
  - NIST Reference Value
  - NIST Information Value
- Links these modes to these three data quality descriptors


NIST Special Publication 260-136

Definitions of Terms and Modes Used at NIST for Value-Assignment of Reference Materials for Chemical Measurements

http://www.nist.gov/srm/publications.cfm

Modes Used at NIST for Value-Assignment

	Certified Value	Reference Value	Information Value
1. **Certification at NIST Using a Primary Method (Definitive Method or Reference Measurement Procedure (RMP)) with Confirmation by Other Methods**	✓		
2. **Certification at NIST Using Two Independent Critically-Evaluated Methods**	✓	✓	
3. Certification/Value-Assignment Using One Method at NIST and Different Methods by Outside Collaborating Laboratories	✓	✓	
4. Value-Assignment Based On Measurements by Two or More Laboratories Using Different Methods in Collaboration with NIST		✓	✓
5. Value-Assignment Based on a Method-Specific Protocol		✓	✓
6. Value-Assignment Based on NIST Measurements Using a Single Method or Measurements by an Outside Collaborating Laboratory Using a Single Method		✓	✓
7. Value-Assignment Based on Selected Data from Interlaboratory Studies		✓	✓

***NIST Special Publication 260-136**:*
Definition of Terms and Modes used at NIST for Value-Assignment of Reference Materials for Chemical Measurements

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Categories of Assigned Values for NIST CRMs

- **Certified value**: NIST has the **highest confidence** in its accuracy in that sources of bias have been investigated or accounted for by NIST
- **Reference value**: **best estimate** of the true value where sources of bias have not been fully investigated by NIST
- **Information value**: a value that will be **of interest** and use to the SRM/RM user, **but insufficient information** is available to assess the uncertainty associated with the value

Independent Analytical Methods Approach for Certification of Organic Constituents in CRMs

Use of Multiple Analytical Methods

to

exploit differences in

Extraction

Separation and Detection

Isolation and Cleanup

Minimize the Possibility of Undetected Bias in Resulting Certified Mass Fractions

Current NIST Staff involved in Clinical Measurements

Dan Bearden	*metabolomics*
Ashley Beasley-Green	*proteomics, clinical diagnostics SRMs*
Mary Bedner	*organic, clinical QAP*
Bruce Benner	*health monitoring materials*
Jeanice Brown Thomas	*micronutrients QAP*
Carolyn Burdette	*organic, clinical diagnostics SRMs*
John Bowden	*lipidomics*
David Bunk	*protein measurements, clinical diagnostics SRMs*
Johanna Camara	*organic, clinical diagnostics SRMs*
Brittany Catron	*organic, clinical diagnostics SRMs*
Trina Formolo	*protein measurements*
Clay Davis	*inorganic, clinical diagnostics SRMs*
Eric Kilpatrick	*protein measurements*
Lisa Kilpatrick	*proteomics*
Jeanita Pritchett	*clinical standards, health monitoring materials*
Stephen Long	*inorganic, clinical diagnostics SRMs, RMPs*
Mark Lowenthal	*protein measurements*
Jessica Reiner	*health monitoring materials*
Lane Sander	*health monitoring materials*
Tracey Schock	*metabolomics*
John Schiel	*protein measurements*
Yamil Simon	*metabolomics*
Lorna Sniegoski	*organic, clinical diagnostics SRMs*
Susan Tai	*organic, clinical diagnostics SRMs, RMPs*
Lee Yu	*inorganic, clinical diagnostics SRMs*

ID/MS CLINICAL MEASUREMENT COURSE DETAILS

Pre-course survey to all participants to determine level of expertise, main training topics needed

Training for the critical steps in amount of glucose, creatinine, and cholesterol determinations in serum- or plasma-based materials using ID/MS-based methods

Include lectures, hands-on sample preparation/lab demonstrations, training videos and hands-on data processing/analysis

Determination of Liquid Density Training Video (L. Sander)

Preparation and Use of Calibration Solutions Training Video (L. Sander)

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Analytical Techniques for Water and Clinical Research Areas

Mary Bedner

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Focus Area: Water Research

Developing CSD and MML Programs in Water

- Studies of organic disinfection byproducts in water using LC with MS, ECD, and UV detection
- Water profiling with LC-HRMS
- Coordination of water research across MML Divisions and NIST
- Establishing collaborations with IMET
- Stakeholder outreach

Focus Area: Clinical Chemistry

Coordinator for Clinical Quality Assurance Program (ClinQAP) and related activities

- Developed foundational LC-MS NIST methods for vitamin D metabolites
- SRMs: serum, plasma, calibration solutions
- Exploring research opportunities in clinical chemistry including alternative matrices (blood spots, urine) and challenging analytes

Other interests:

Participation in CCQM comparisons for purity of organic substances

Member of MML Metabolomics Interest Group and Precision Medicine Focus Group

Development of community-driven and community-evaluated reference materials

Keywords: water, clinical, quality assurance, separations, mass spectrometry

Vitamin D & Vitamin D Metabolite Determinations in Clinical, Food, and Dietary Supplement Matrices

Carolyn Burdette

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Main Focus: Method development for vitamin and metabolite determinations

Sample Preparation, matrix specific

- Internal Standard choices
- Fat soluble vs water soluble
- Saponification, protein precipitation, etc
- Full extraction without analyte degredation

LC-MS/MS Analysis

- Reversed phase vs normal phase
- ESI vs APCI, MRM transition choices

Develop high throughput RMP for vitamin D metabolites in human serum

Other Research Interests:

Method development for other analytes in similar matrices

- Vitamin K in kelp
- Carotenoids in baby food

Method development for high throughput method flow through analysis of DBS (MML 2014 Angel Investor Award)

Continued support for vitamin D metabolite in human serum measurements

- DEQAS (funded by NIH-ODS)
- Commutability study (headed by Johanna Camara, with VSDP)

SRM Development/Support

- Measurements for certifications and stability

Keywords: vitamins, serum, foods, dietary supplements, LC-MS/MS, SRM development

Vitamin Metabolite Determinations in Clinical and Food Matrices

Johanna Camara

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Main Focus: Method Development for Metabolites in Biological Matrices

- Sample preparation (protein precipitation, liquid:liquid extraction, sample clean-up)
- Validation studies (i.e., recovery)
- LC-MS/MS method development (ESI and APCI)

Method Development for Vitamins in Food Matrices

- Extraction (fortified versus endogenous forms of vitamins)
- LC-MS/MS method development (ESI and APCI)

Other interests:

Support 25(OH)D value assignment of various serum samples utilizing NIST Reference Measurement Procedure

Investigating synthetic serum as a base/diluent for hard-to-achieve low level SRMs (SD project)

Support value assignment/stability of established clinical SRMs (i.e., creatinine in serum and urine)

Design commutability study for NIST vitamin D in serum SRMs

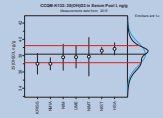
Keywords: clinical, food, vitamins, metabolites, LC-MS/MS, SRMs

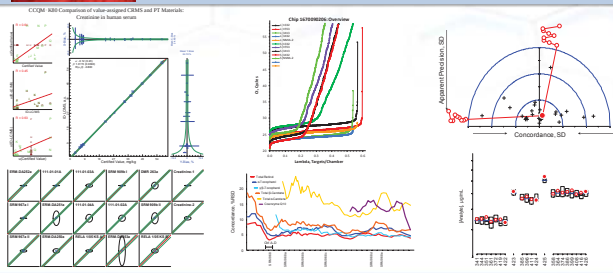
Chemical Data Analysis & Visualization

David L. Duewer

646.00

david.duewer@nist.gov





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Water Analysis and Inorganic Analysis

Brian Lang

646.01

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Focus Area: Water determination

Refining CSD Measurement of Water

- Water by Karl Fischer titration using coluometric and volumetric methods
- Water by thermogravimetric analysis
- Refining uncertainties SRMs by reducing sources of error
- Measurement of water in pure compounds and simple mixtures
- Water in complex matrices

Focus Area: Inorganic Analysis

Measurement of Inorganic materials by Ion Chromatography

- Measurement of ionic materials in pure compounds
- Preparation and validation of NIST Primary Solutions and SRMs

Other interests:

Participation in CCQM comparisons for purity of organic substances

Keywords: water, Karl Fischer, Thermogravimetric Analysis, Ion Chromatography

Metrology for chemical purity and quantitative NMR

Michael A Nelson
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Main Focus: Organic Chemical Purity

- Method development and statistical modeling for traceable ^{1}H-qNMR measurements
- Multi-method approaches for evaluating mass purity
- Novel approaches for data combination and sound evaluation of chemical measurement uncertainty (Bayesian)
- Primary Standard Development
  - Calibration materials for ^{1}H-qNMR, clinical, food, environmental, and elemental analysis measurements
- X-nucleus and 2D-NMR, DSC, LC-UV

Other Areas of Focus:

Spectroscopic assays of organic mixtures

- Method development for traceable 2D-quantitative Time-Zero Heteronuclear Single Quantum Coherence Spectroscopy ($HSQC_0$)
  - structurally-similar species in chemical mixtures (e.g. fatty acids, pharmaceutical and elicit drug compounds, peptides)
- External calibration techniques for ^{1}H-qNMR
- Diffusion-ordered (DOSY) NMR
- NMR methods for forensic applications

Participation in CCQM comparisons for purity of neat organic materials

Keywords: ^{1}H-qNMR, purity, $HSQC_0$, chemical metrology, SRM development, clinical, measurement uncertainty

Applied Statistics and Measurement Uncertainty

— Antonio Possolo —
Statistical Engineering Division
antonio.possolo@nist.gov

Position NIST Fellow, Chief Statistician

Experience Most recent 10 years at NIST, 16 years in industry (General Electric, Boeing), 9 years in academia (Princeton Univ., Univ. of Washington in Seattle, Univ. of Lisboa, Portugal)

Education PhD (Yale Univ., 1983)

Professional Service

- *Associate Member*, Commission on Isotopic Abundances and Atomic Weights (CIAAW, IUPAC)
- *Chair*, Inter-American System of Metrology (SIM) Working Group on Statistics and Uncertainty
- *Member*, Joint Committee for Guides in Metrology, Working Group 1 (GUM)

Selected Publications

- Possolo, A. (2016) Spatial statistics: marks, maps, and shapes. *Quality Engineering* 28(1): 69-90. DOI 10.1080/08982112.2015.1100457
- Possolo, A. (2015) *Simple Guide for Evaluating and Expressing the Uncertainty of NIST Measurement Results*. NIST Technical Note 1900. DOI 10.6028/NIST.TN.1900
- A. R. Montoro Bustos, E. J. Petersen, A. Possolo and M. R. Winchester (2015) Post hoc Interlaboratory Comparison of Single Particle ICP-MS Size Measurements of NIST Gold Nanoparticle Reference Materials. *Analytical Chemistry* 87(17): 8809-8817. DOI 10.1021/acs.analchem.5b01741
- Guenther, F. R. and Possolo, A. (2011) Calibration and uncertainty assessment for certified reference gas mixtures. *Analytical and Bioanalytical Chemistry* 399: 489-500. DOI 10.1007/s00216-010-4379-z

Small molecule determination in Clinical, Forensic, and Environmental Matrices

Jeanita S. Pritchett

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Main Focus: Method development of targeted metabolic LC-MS/MS Assays

LC-MS/MS Optimization

- Ion-pairing agents
- Columns screening (reverse, normal, multimode, HILIC)
- MRM transitions

Assessing Nanotoxicity in Worm Model Systems

- Evaluate change in metabolites present in *C. elegans* and earthworms after oxidative stress and exposure to various nanoparticles (Au, TiO_2, etc…)

Other interest:

SRM Development/Support/Analysis

- GC-MS and LC-MS(/MS) method development and analysis
- Nicotine and tobacco specific nitrosamines in tobacco
- Clinical Diagnostic Markers SRMs
  - Creatinine, Cholesterol, Glucose, Bilirubin, Uric Acid, Folate Vitamers
- Forensic Applications
  - Single use illicit drug material created with inkjet printing
  - Cosmetic treatment effects on hair drug testing

STEM Education

- Embassy Science Fellowship Awardee
- Montgomery College Part-Time Faculty of the Year 2016
- MML Outreach Accolade
- JCTLM Traceability Education Working Group Member

Keywords: nanotoxicity, serum, urine, LC-MS(/MS), GC-MS, method development, SRM development, STEM Education

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Chromatographic Theory and Applications

Lane C. Sander

646.00

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L. C. Sander and S. A. Wise, Anal.Chem. 56:504-510, 1984.

Novel synthetic approaches to stationary phase design

- Polymeric surface modification
- Novel immobilized ligands
- Self-assembled monolayers (SAMs)

Spectroscopic characterization of monolayers

- ^{29}Si NMR: information on bonding chemistry
- SANS: size information
- FTIR: chain conformation
- Raman: chain conformation
- Fluorescence: environment polarity; chain dynamics
- ^{13}C NMR: chain dynamics; chain conformation
- ^{1}H NMR: chain dynamics

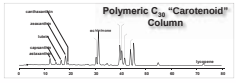
K. A. Lippa, L. C. Sander, and R. D. Mountain, *Anal.Chem. 77:7852-7861, 2005*

Understanding and Controlling Selectivity

- Chromatographic parameters:
  - temperature, phase density, phase length
- Molecular shape recognition
- Shape-constrained isomers
- Planar and nonplanar solutes

Solute Retention Theory

- Stationary phase morphology vs chromatographic performance
- Ordered surfaces vs shape recognition
- Molecular descriptors
- Molecular modeling



L. C. Sander, K. E. Sharpless, N. E. Craft, and S. A. Wise. Anal.Chem. 66:1667-1674, 1994.

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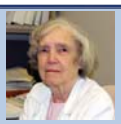

Development of SRMs for Clinical Analytes

Lorna T. Sniegoski

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Focus Area: Definitive methods for clinical analytes in serum and plasma

- Cholesterol
- Glucose
- Urea
- Uric Acid

Focus Area: Determination of cholesterol in food SRMs

Meat homogenate
Egg powder
Whole milk powder
Total diet

Other interests:

Analysis of drugs of abuse in hair, serum, and urine

Keywords: GC-MS, LC-MS, Sample preparation, Solid-phase Extraction Spectrophotometry

NIST MATERIAL MEASUREMENT LABORATORY

Development of Reference Measurement Procedures and Standard Reference Materials for Clinical Analytes

Susan Tai

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Main Focus:

Development of RMPs

Hormones in serum by LC-MS/MS
- Steroid hormones: cortisol, progesterone, testosterone, estradiol
- Thyroid hormones: total T4, total T3

Vitamin D metabolites in serum by LC-MS/MS
- 25-hydroxyvitamin D3, 25-hydroxyvitamin D2, 24R,25-dihydroxyvitamin D3

Antiepilepsy drugs in serum by LC-MS/MS
- Phenobarbital, phenyltoin, lamotrigine, topiramate

Creatinine in serum by LC-MS
Methylmalonic acid in serum by LC-MS/MS

Development of SRMs using RMPs

SRM 971: Cortisol, progesterone and testosterone
SRM 972a, SRM 2973: 25(OH)D3, 25(OH)D2
SRM 900a: Phenobarbital, phenytoin, lamotrigine, topiramate
SRM 967: Creatinine

Other areas:

Support for vitamin D metabolites in serum measurements
- VDSP (funded by NIH-ODS)
- VitDQAP ((funded by NIH-ODS)
- DEQAS (funded by NIH-ODS)

Participation in CCQM comparisons
- 25(OH)D in serum key comparison
- Cortisol and progesterone in serum key comparison (coordinator)
- Norandrosterone in urine (pilot study)
- Lysergide in urine (pilot study)

Participation in international comparisons of T4/T3

Development of SRMs of drugs of abuse
- Urine based (SRM 1507b, SRM 1508a)
- Hair based (SRM 2379, SRM 2380)
- Serum based (SRM 1959)

Keywords: hormones, serum, vitamin D metabolites, LC-MS/MS, RMP, SRM

NIST MATERIAL MEASUREMENT LABORATORY

Vitamin and Micronutrient Analyses in Clinical, Food, and Dietary Supplement Matrices

Jeanice Brown Thomas

646.02

jbthomas@nist.gov

Main Focus: Characterization of NIST clinical- and food-related materials for vitamins and micronutrients

- Area of expertise includes liquid chromatography, sample preparation, spectrophotometry, methods development
- Serves as a coordinator for the NIST Micronutrients Measurement Quality Assurance Program
- Plans research and conducts measurements in support of vitamin analysis in the clinical and food communities for laboratories worldwide

Other Research Interests:

SRM Development/Support
- Conducts measurements for certifications and stability
- Provides continued support to the clinical community for fat- and water-soluble vitamins, carotenoids, and micronutrients in human serum measurements

Keywords: micronutrients, vitamins, serum, foods, dietary supplements, liquid chromatography, quality assurance, SRM development

NIST MATERIAL MEASUREMENT LABORATORY

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Chemical Metrology

2016 SIM Clinical Measurement Course

David Duewer

NIST National Institute of Standards and Technology U.S. Department of Commerce

MATERIAL MEASUREMENT LABORATORY

What is Metrology?

- Metrology is the stuff needed so data can support informed decision making.
  - in a good world, decisions are informed with data
    - which are the results of measurements!
- *Calculus of Confidence*
  - we posit that metrology is the 'formal' system that tells us how well we trust those data

Comparing measurement results

- Informed decisions involve comparing results
  - to other results
    - e.g., to observe a trend
  - to limits
    - e.g., a threshold for action
  - different results in different places or measured at different times…
    - "comparability over space-and-time"

Calculus of Confidence

- The tools of metrology:
  - Traceability
  - Uncertainty
  - Validation
- enable this *calculus of confidence* by which decisions are informed by measurement results with established confidence.

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Tools of the Trade

- Measurement Uncertainty
  - is a non-negative parameter characterizing the dispersion of the quantity values being attributed to a measurand, based on the information used
- Metrological Traceability
  - is a property of a measurement result whereby the result can be related to a reference through a documented unbroken chain of calibrations, each contributing to the measurement uncertainty
- Validation
  - is the provision of objective evidence that a given item fulfils specified requirements where the specified requirements are adequate for an intended use

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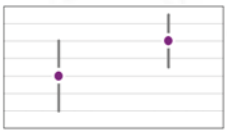
Measurement Uncertainty

- Are these results the same?
- how well do you know the result?
  - essential part of being able to compare!
- are these results good enough?
  - fit-for-purpose

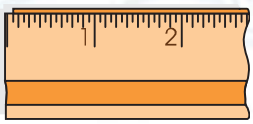
Measurement Uncertainty

- Are these results the same?
- how well do you know the result?
  - essential part of being able to compare!
- are these results good enough?
  - fit-for-purpose



Metrological Traceability

- *Traceability* is how you get units on your result
  - in our simple model, convert from units of your measurement tool to units of the 'standard'
  - the equation adjacent is a familiar "measurement model"
    - it's converts a measured signal to a "calibrated" result



$$C_{Unknown} \quad \frac{C_{Standard}}{S_{Standard}} S_{Unknown}$$

"Measurement Model"

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Comparability of results

- Whole and sole goal of traceability.
  - *raison d'être!*
- results linked to a common reference can be compared
- scope of reference defines scope of comparability
  - Local
    - "Pot of stuff" in your lab
  - Global / temporary
    - WHO standards
  - Global / permanent
    - SI

Traceability in chemistry is…

- Sample dependent
  - identity
    - what am I measuring, anyway?
  - interference
    - do I get the same response for analyte in my calibration material and in it's matrix?
  - morphology
    - is the analysis the same everywhere in my sample?

Traceability in chemistry requires…

- Method validation
  - to establish scope
  - To present a clear measurement model with objective evidence that…
    - the analyte is what's being measured
    - the result is robust to interferences

Method validation

- "checks the model"
  - tests completeness
  - tests assumptions
  - helps establish an uncertainty budget
- identifies relevant parameters to keep under control
- tests scope

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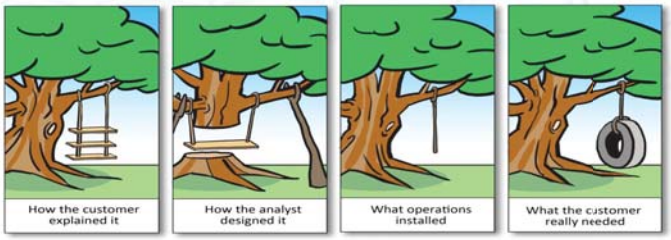
Other Tools

- Fitness for Purpose
- Quality Management Systems
- Data Analysis

Fitness for purpose…



Quality Management Systems

- Catch our blunders.
  - metrology doesn't have any other way to deal with them
  - systematic processes really do work for this
- Provide institutional learning and memory
- Emerging consensus on the role of quality systems in metrology
  - US adoption lags Europe/Asia
- Laboratory Accreditation is fostering consistent implementation

Craft

- Metrology is more akin to a craft than a technology
  - this doesn't mean that 7 year apprenticeships are required!
  - it does mean that two different skilled metrologists might take very different approaches to the same problem
    - *but they should both come to largely equivalent solutions!*
  - matter of style
  - **must be defensible**

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Treasure does not Metrology Make…

- There's a treasure chest of *"Best Practices in Analytical Chemistry and Biochemistry, Data Analysis, and…"* that are in use at NIST
  - this treasure, while precious, doesn't make up *Metrology*
- *Skillful measurements aren't enough – one needs comparability and context to support decision-making*

RESOURCES

JCGM Guides

The *Joint Committee for Guides in Metrology* is a broadly-based coalition of international organizations

- with responsibility to develop, maintain and disseminate guidance documents addressing the general metrological needs of science and technology
- Essential references
  - International Vocabulary of Metrology – Basic and General Concepts and Associated Terms (VIM)
  - Evaluation of measurement data – Guide to the expression of uncertainty in measurement (GUM)
  - http://www.bipm.org/en/publications/guides/
- Other useful resources
  - http://www.bipm.org/
  - including the brochure The International System of Units (SI)

Eurachem Guides

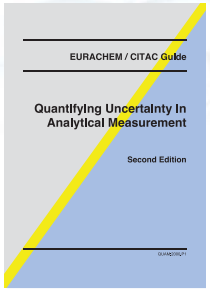
Network of European organizations

working to establish a system for the international traceability of chemical measurements and the promotion of good quality practices.

- Quantifying Uncertainty in Analytical Measurement (QUAM)
  - www.eurachem.org/index.php/publications/guides/quam
- Traceability in Chemical measurement
  - www.eurachem.org/index.php/publications/guides/trc
- The Fitness for Purpose of Analytical Methods
  - www.eurachem.org/index.php/publications/guides/mv
- Guide to Quality in Analytical Chemistry
  - www.eurachem.org/index.php/publications/guides/qa

Other Eurachem Guides and brochures may also be of interest

- www.eurachem.org/index.php/publications



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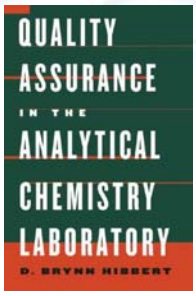
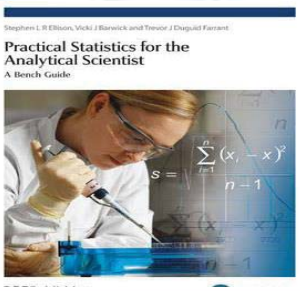
Hibbert – Insightful Overview

- *Quality Assurance for the Analytical Chemistry Laboratory*
  - D. Brynn Hibbert
  - Oxford University Press (2007)
  - ISBN: 978-0-19-516213-4



Ellison – Accessible Statistics

- *Practical Statistics for the Analytical Scientist: A Bench Guide*
  - Steve Ellison & others at LGC Group
  - RSC Publishing (2009)
  - ISBN: 978-0-85404-131-2



Internal Standards for ID/MS and Isotope Dilution in Practice

2016 SIM Clinical Measurement Course

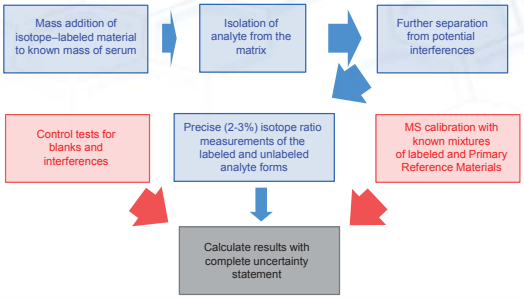
Carolyn Burdette, Johanna Camara, Jeanita S. Pritchett, Lane Sander, Susan Tai

NIST National Institute of Standards and Technology U.S. Department of Commerce

MATERIAL MEASUREMENT LABORATORY

Isotope Dilution/Mass Spectrometry-based Methods:

A General Approach…



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Internal Standard Approach to Quantitation

- One or more compounds are added to both calibrants and unknowns as the internal standard(s)
- Calibration is based on the ratio of responses for analytes and internal standards
- Advantages: losses from transfers, dilutions, etc. are compensated; may compensate changes in instrumental response; less skill is required
- Disadvantages: calculations more complex; internal standards must be identified and used

- Internal standards are added at the earliest opportunity
- Knowledge of volumes is not required
- Quantitative transfers are not required

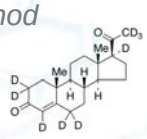
Isotope Dilution

- The internal standard choice is an isotopically labeled form of the analyte of interest
  - At least 2 mass units difference for detection
- Mixed Calibrant: known amount analyte and known amount of isotopically labeled species
- Use signal response ratios to calculate a calibration relationship
  - Response Factor – common in foods/dietary supplements measurements
  - Calibration Curve – common in clinical measurements
- Sample: mixture of a known amount of matrix and known amount of isotopically labeled specie(s)

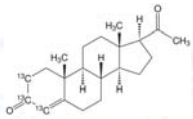
ID/MS as a *Primary Method*

Considerations when implementing isotope dilution:

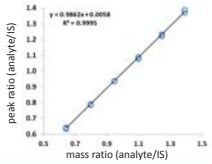
- Natural isotope effects
- Choice of labeled internal standard (d vs ^{13}C)
- Non-equilibration
- Chemical impurities of internal standards
- Instrument calibration errors

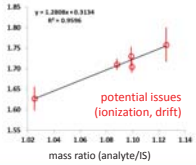


Progesterone-d_9
subject to
intramolecular rearrangement



Progesterone-$^{13}C_3$





Deuterium vs ^{13}C Labeled Isotopes

- Retention time issues
  - Deuterium labeled compound: slightly more polar than unlabeled compound, retention time generally slightly shorter in reversed phase systems
  - ^{13}C labeled compound: retention time co-eluting with unlabeled compound
- H-D exchange / loss of deuterium
  - Deuterium labeled compound: may occur in some cases
  - ^{13}C labeled compound: not an issue
- Provide alternative masses/mass transitions
- Deuterium labeled compounds are usually easier to incorporate, cheaper
- Reactivity can be different (take into consideration)

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Other Types of Stable Isotopes

^{2}H

^{13}C

^{15}N

^{18}O

Isotope	Exact Mass	Abundance
^{1}H	1.007825	0.99985
^{2}H	2.014102	0.00015
^{12}C	12.00000	0.9893
^{13}C	13.003355	0.0107
^{14}N	14.003074	0.9963
^{15}N	15.000109	0.0037
^{16}O	15.994915	0.99762
^{17}O	16.999131	0.00038
^{18}O	17.999159	0.00200

ID/MS

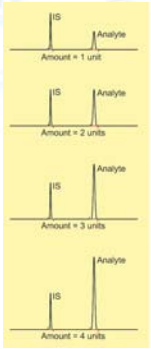
Advantages

- Relative measurements (isotope abundances)
- Ideal internal standard (the same element/compound)
- Correction for signal drift
- Correction for matrix effects
- Correction for volume/sample losses
- Excellent precision and accuracy

Disadvantages

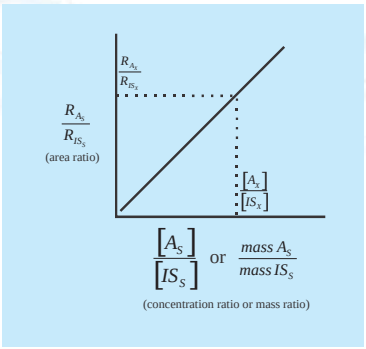
- Price
- Limited availability of isotopically labelled compounds
- Isotopic effects on separation processes (e.g. fully deuterated compounds)
- The measured isotope abundances must be accurate (spectral interferences, mass bias, detector non-linearity, etc.)

Calibrants

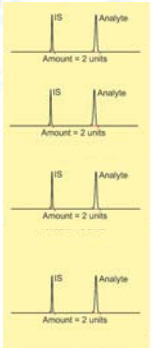


Unknown

Calibration Curve



Calibrants



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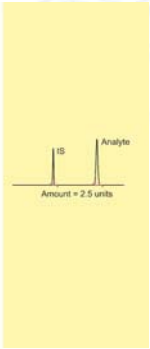

Response Factor

$$RF = \frac{R_x}{R_{IS}} * \frac{[IS]}{[x]}$$

$$[x] = \frac{R_x * [IS]}{R_{IS} * RF}$$

$$mass\ fraction\ (x) = \frac{[x]}{mass\ sample}$$

RF: Response actor
Rx: Response of Analyte
Ris: Response of Internal Standard
[x]: Concentration of Analyte
[is]: Concentration of Internal Standard

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Recommendations

- Relative Response Factor
- Aim for 1:1 ratio or different ratio but keep the same between calibrants and samples
- Average response, slope, etc.
- *Fit for purpose discussion*

Miscellaneous Considerations

Sample handling

- Add the internal standard at the earliest opportunity, i.e., before extraction
- Consider employing mass fraction based quantitation (use fluid masses rather than volumes)
- Devise weighing schemes so “weight by difference” does not involve the difference of two large numbers

Miscellaneous Considerations

Calibration solutions

- Use at least two independently weighed calibrants
- Avoid the use of a *single* “stock solution” – what if an error is made in the original solution?
- Dilutions are OK if analyte masses are too small to be accurately weighted
- Best accuracy results if calibrants closely match unknowns

Miscellaneous Considerations

Sample Introduction (Liquid Chromatography)

- Injection is a volumetric process; quantitation with mass fraction units assumes that the density of calibrants and unknowns are the same (external standard)
- If densities differ, a bias will be introduced through the injection of different masses
- Injection volumes should be as small as practical
  - 10 µL for 4.6 mm i.d. columns
  - 3 µL for 2 mm i.d., columns
- If possible, the sample and calibrant solvent should match the mobile phase composition

Solvent	Density (g/mL)
pentane	0.629
hexane	0.659
cyclohexane	0.779
acetonitrile	0.782
isopropanol	0.786
methanol	0.796
acetone	0.818
toluene	0.867
THF	0.880
water	1.000
methylene chloride	1.336
chloroform	1.500

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Examples

Cholesterol

1. Ellerbe, P.; Meiselman, S.; Sniegoski, L.T.; Welch, M.J.; White, V.E.; *Determination of Serum Cholesterol by a Modification of the Isotope Dilution Mass Spectrometric Definitive Method*; Anal. Chem., Vol. 61, pp. 1718−1723 (1989).

2. Edwards, S.H.; Kimberly, M.M.; Pyatt, S.D.; Stribling, S.L.; Dobbin, K.D.; Myers, G.L.; *Proposed Serum Cholesterol Reference Measurement Procedure by Gas Chromatography–Isotope Dilution Mass Spectrometry*; Clin. Chem., Vol. 57, pp. 614–622 (2011).

Glucose

1. White, V.E.; Welch, M.J.; Sun, T.; Sniegoski, L.T.; Schaffer, R.; Hertz, H.S.; Cohen, A.; *The Accurate Determination of Serum Glucose by Isotope Dilution Mass Spectrometry - Two Methods*; Biomed. Mass Spectrom., Vol. 9, pp. 395–405 (1982).

2. Prendergast, J.L.; Sniegoski, L.T.; Welch, M.J.; Phinney, K.W.; *Modifications to the NIST reference measurement procedure (RMPP) for the determination of serum glucose by isotope dilution gas chromatography/mass spectrometry*; Anal. Bioanal. Chem., Vol 397, pp 1779-1785 (2010).

Creatinine

1. Dodder, N. G.; Tai, S.; Sniegoski, L.T.; Zhang, N. F.; Welch, M.J.; Certification of Creatinine in a Human Serum Reference Material by GC-MS and LC-MS; Clin. Chem., Vol. 53, pp 1694–1699 (2007).

2. Stokes, P.; O'Connor, G.; Development of a Liquid Chromatography-Mass Spectrometry Method for the High-Accuracy Determination of Creatinine in Serum; J. Chromatogr. B., Vol. 794, pp 125–136 (2003).

Cholesterol

- Add isotopically labeled cholesterols to approximately match levels in the sample
  - Cholesterol-d_7 [Cholest-5-en-25,26,26,26,27,27,27-d_7-3-ol(3β)]
  - Cholesterol-$^{14}C_4$
  - Cholesteryl Oleate-$^{14}C_4$
- Hydrolyze and extract the labeled and unlabeled cholesterol
- Convert into trimethyl esters for GC/MS analsysis

- Add isotopically labeled cholesterols to approximately match levels in the sample
  - Cholesterol-$^{13}C_3$ [Cholest-5-en-25,26,27-$^{13}C_3$-3-ol(3β)]
- Hydrolyze and extract the labeled and unlabeled cholesterol
- Convert into trimethylsilyl (TMS) derivatives for GC/MS analsysis

Glucose

- Add isotopically labeled glucose to approximately match levels in the sample
  - Glucose-$^{13}C_6$
- Sodium azide is added and the sample is equilibrated overnight at room temperature
- Deproteinization, concentration, derivatization for GC/MS analysis

Creatinine

- Add isotopically labeled creatinine to approximately match levels in the sample
  - Creatinine-$^{13}C_2$
- Ion-exchange chromatography used to separate creatine from creatinine
- Derivatization for GC/MS analysis

- Add isotopically labeled creatinine to approximately match levels in the sample
  - Creatinine-d_3
- Protein precipitation, concentration, reconstitution, filtration
- Dilution for LC/MS analysis

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Verification Of Accuracy

- Independent Weighings of Primary Reference Compound (neat compounds or calibration solutions)
- Independent Sets for Sample Preparation
- Use of Previous SRMs as Controls (if available) for Validation

Checking for Potential Interferences

- **Blank** – Run injection of only the solvent used to resuspend samples look to see if there is any signal for either the isotopically labeled analyte and the unlabeled analyte
- **Internal Standard** – Look to see if there is any signal for the unlabeled analyte
- **Reference Compound** – Look to see if there is any signal for the isotopically labeled analyte
- **Matrix Blank** – Complete sample preparation without adding the internal standard and look to see if there is any signal for the isotopically labeled analyte
- **Method Blank** – Complete sample preparation without any sample or internal standard and look to see if there is any signal for either the isotopically labeled analyte and the unlabeled analyte

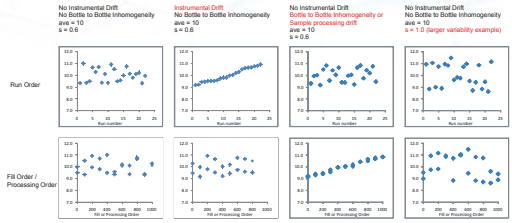
Sample Queue Design: some suggestions

- Prepare an equal number of calibrants and unknown samples (approx)
- Intersperse calibrants and unknowns in the sample queue
- Order samples and calibrants using a random selection scheme
- To permit assessment of within sample and between sample effects, process subsamples from a single bottle for comparison with samples from multiple bottles
- Plot measurements
  - Levels vs run order
  - Levels vs sample processing order (or bottle fill order)

Drift and Inhomogeneity

Plot data as a function of run order and processing order

Randomize samples and calibrants in processing stream



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Use of Controls

- Ideally, a measurement control should offer the same analytical challenges as the sample
  - Matched matrix
  - Matched analyte levels
  - Interferences
  - Bulk properties
  - Commutability
- Analyte levels determined should overlap the certified or reference levels (within measurement uncertainty)
- If suitable SRMs are not available, use other commercial or in-house controls (e.g., spiked blanks)

Concluding Thoughts…

Isotope Dilution Mass Spectrometry has great advantages

- Correction for signal drift
- Correction for matrix effects
- Correction for volume/sample losses
- Excellent precision and accuracy

Careful consideration must be taken into account

- Choice of labeled internal standard
- Equilibration and reactivity of non-labeled vs labeled
- Chemical impurities of internal standards
- Instrument calibration errors

Concluding Thoughts…

The practice of higher order chemical metrology requires a significant commitment by the analyst:

- Knowledge of measurement principles
- Laboratory skills
- Implementation of technology
- Attention to detail

Chemical Purity

2016 SIM Clinical Measurement Course

Michael A. Nelson, Mary Bedner, Michele Schantz, and David Duewer

NIST National Institute of Standards and Technology U.S. Department of Commerce

MATERIAL MEASUREMENT LABORATORY

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Purity……

"Freedom from adulteration or contamination"

"the quality of not being mixed with anything else" or "the quality of or condition of containing some extraneous or foreign admixture, especially of an inferior or baser kind"

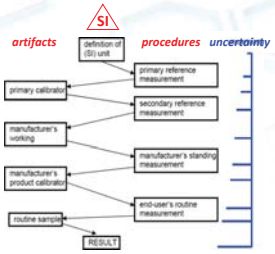
-The Oxford English Dictionary

Overview

- Principles and concepts: The role of purity assessments in clinical metrology
- Analytical techniques and the information each provides
- Interpretation of chemical purity data: Combining distinct measurement inferences to achieve a sound consensus value
- Examples: Evaluation of neat chemical standards

Traceability

- *"… property of a measurement result whereby the result can be related to a reference through a documented unbroken chain of calibrations, each contributing to the measurement uncertainty."* *(International vocabulary of metrology - VIM)*
- Traceability is a property of a measurement result that allows for comparability amongst similarly-calibrated results across time and space
- Neat chemical reference materials have a central role in establishing traceability of chemical measurements



http://www.eurogentest.org/web/info/public/unit1/reference_materials/referenceMaterialsInGeneticTesting.xhtml

Traceability matters

….Traceability of statements of chemical purity to SI units requires complete knowledge of the composition of the material analyzed (NIST1012)

This degree of understanding of chemical composition is an impractical, perhaps unattainable, state of knowledge.

Traceability to SI of neat materials may be *practically* realized through characterization of chemical structure (identification) and amount content.

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Important considerations

- What is the measurand?
  - Purity is a property characterized by the relative amount of an entity of interest within an aggregate body
  - Which quantity will the measurement characterize?
    - Mole Fraction – amount relative to the aggregate of the primary component (PC) and impurity components (IC)
    - Mass Fraction – for organic chemical species, often derived from conversion of amount of substance to mass of substance using a relative molar mass of the PC
    - Unique species, stereoisomers, tautomers, isotopomers, class of compounds?
    - How will the identity be confirmed?

Important considerations (continued)

- What is fit for purpose?
  - Define tolerable limits of uncertainty
    - primary standard or instrument calibration CRM?
    - Matrix measurement calibrator?
    - What is the observed or anticipated uncertainty of the final calibrated result?
    - A sensitivity assessment of the calibration hierarchy may provide insight to the relative weight of the uncertainty component associated with purity adjustments

Measurement methods: Direct determination of the primary component

- Quantitative nuclear magnetic resonance (qNMR) using an internal standard (primary ratio)
- Titrimetry (primary)
  - Measures functional groups through electrochemical or chemical reactivity.
  - Traceable through calibration of titrant
  - Coulometry (H^+); Karl Fischer Titration (H_2O) common for IC determination
- Differential Scanning calorimetry (molar purity)
- Gravimetry
  - Often not viable for accurate organic chemical purity assessments

qNMR – primary ratio direct measurement

Direct ratio measurement of the primary chemical component (PC)

- One measurement technique to determine mass fraction content
- Accuracy may be achieved without detection or complete identification of all impurities
- Limited sample preparation effort, chemical separation, or wet chemistry need
  - Typically no derivatization, reduction, or chromatography is needed for purity assessment of neat materials

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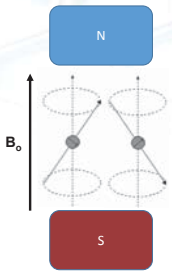
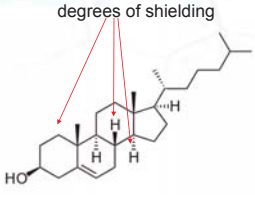
^{1}H NMR – magnetic spin populations



- NMR viable for species with non-zero spins; ^{1}H (near 100 % abundance) provides most sensitive NMR
- Two spin populations ($+\frac{1}{2}$, $-\frac{1}{2}$) precess in magnetic field, one in relative excess
- Precession frequency is influenced by electronic environment: magnetic "shielding" of nuclei

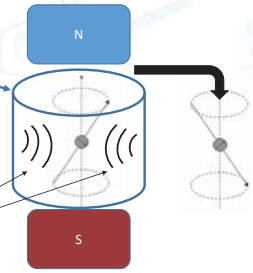
Proton moieties in different chemical environments are subject to different degrees of shielding



^{1}H NMR: Transition energy absorption

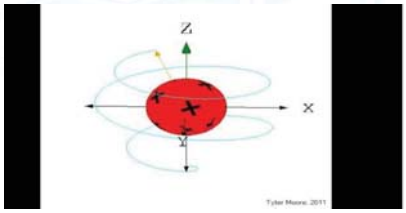
Radio Frequency (RF) Coil

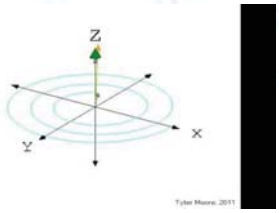
RF pulse with energy equal to transition energy of nucleus (Larmor Frequency) – dependent on external magnetic field and chemical structure environment



- Absorption of transition energy (RF) changes angle of precession
- After absorption has ended, nuclei relax to thermodynamic equilibrium

^{1}H NMR: Dipole precession and T_1 relaxation

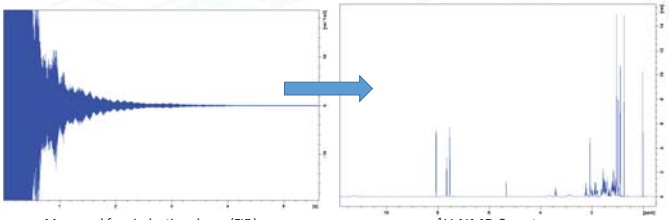




^{1}H NMR: signal processing

The amplitude of each spin component of the FID is directly proportional to the number of corresponding resonant nuclei

Fourier Transformation of FID converts time domain signal to frequency domain ^{1}H Spectrum: allows inference of chemical structure and relative amount of substance for ^{1}H.



Measured free induction decay (FID)

^{1}H NMR Spectrum

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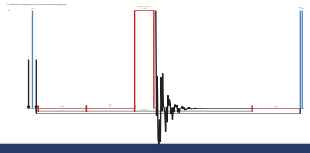
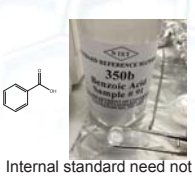
quantitative experiment: ^{1}H-qNMR with internal standard

- ^{1}H experiment w/90° excitation pulses
- Sufficient scan recycle delay (8 x T_1) and acquisition times
- Suitable signal averaging for high S/N
- Rapid analysis after dilution to evaluate labile species



qNMR sample preparation



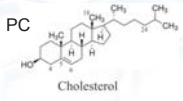
Internal standard need not be compound-specific; Must have known, preferably high (>99 %), purity, contain suitable nuclei with resonances distinct from those of PC, demonstrate stability, and have mutual solubility

Measurement methods: qNMR

$$P_{sample} = \frac{A_{sample}}{A_{std}} \times \frac{N_{std}}{N_{sample}} \times \frac{M_{sample}}{M_{std}} \times \frac{m_{std}}{m_{sample}} \times \frac{b_{std}}{b_{sample}} \times P_{std}$$

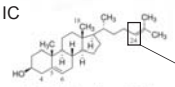
A	Integrated area of signal peak
N	proton multiplicity of moiety associated with signal
m	mass (kg)
b	buoyancy correction factor
M	molecular mass (kg/mol)
P	purity (%, or mass fraction, kg/kg)

PC



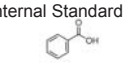
Cholesterol

IC

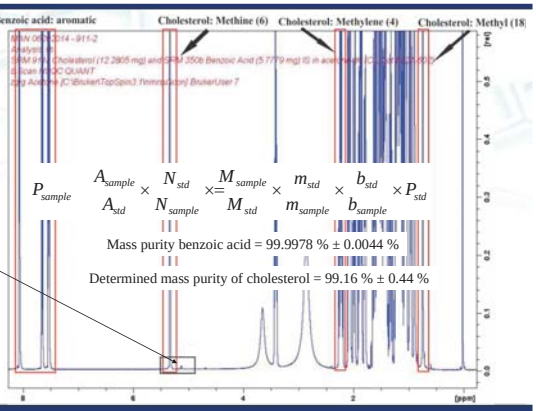


5,24-Cholestadiene-3β-ol

Internal Standard



Benzoic Acid



$$P_{sample} = \frac{A_{sample}}{A_{std}} \times \frac{N_{std}}{N_{sample}} \times \frac{M_{sample}}{M_{std}} \times \frac{m_{std}}{m_{sample}} \times \frac{b_{std}}{b_{sample}} \times P_{std}$$

Mass purity benzoic acid = 99.9978 % ± 0.0044 %

Determined mass purity of cholesterol = 99.16 % ± 0.44 %

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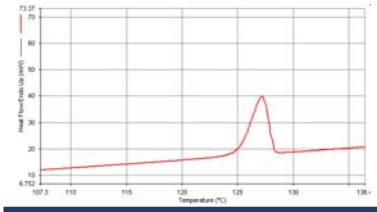
Measurement methods
-Quantification of primary component (DSC)

- Differential Scanning Calorimetry
  - Estimate of molar purity via melting point depression
  - Measures change in enthalpy with respect to temperature



- The heat flow through a sample cell is measured relative to that of a reference cell containing no sample
- Viable samples include high-purity (>98.5 %)crystalline materials with distinct first order phase transitions
- Suitable for 1 mg – 3mg of material

Measurement methods
-Quantification of primary component (DSC)

- DSC may complement Mass Balance approach, but not always viable
- **Requires 98.5 % or greater molar purity**
- IC must be soluble in melt and insoluble in crystalline material
- More advanced data analysis is required if degradation occurs during melting
- Sample must not form conjugates with solvent
- Sample must not sublime

Measurement methods:
-Identification and quantification of impurity components (IC)

Mass balance (MB) approach

$$w_{PC} = 1 - \sum_i \left(\frac{m_{IC}}{m_{CM}}\right)_i$$

m_{IC} = *determined mass of impurity component*
m_{CM} = *mass of composite material*

Considered a primary method for purity assessment

-Requires ***thorough*** chemical investigation and control of multiple measurement techniques; assumes characterization of all mass components; time/labor intensive

Measurement methods:
-Identification and quantification of ICs

Mass balance (MB) approach

$$w_{PC} = 1 - \sum_i \left(\frac{m_{IC}}{m_{CM}}\right)_i$$

m_{IC} = *determined mass of IC*
m_{CM} = *mass of composite material*

Impurity components (IC) may be:

i = 1: identified organic compounds
i = 2: unidentified organic compounds
i = 3: organic solvents
i = 4: water
i = 5: inorganic species and elements (e.g. bases, acids, redox reagents used in the synthesis)
i = 6: macromolecules or particles
i = 7: other stuff we haven't yet thought about

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Mass Balance Approach
-Quantification of organic impurities

- NMR – survey structural moieties of prominent and low-level impurities, may have limited specificity for highly-related isomers (low-level stereoisomers, enantiomers)

- Chromatographic approaches tailored to chemical properties of the PC provide high-resolution separation of structurally related compounds and are sensitive (generally)
  - o GC with MS, ECD, FID
    - ✓ GC-MS libraries (EI) facilitate identification (NIST Mass Spec Database)
    - ✓ FID closest to 'universal' detector – i.e. provides near-equivalent responses
  - o LC with MS, UV/DAD, CAD, NMR (off-line or on-line)
  - o CE with MS, UV/DAD

Massive Toolbox

Mass Balance Approach
-Quantification of organics: chromatography

- o MS spectra provide best structural information, followed by absorbance spectra from DAD
- o Absolute identification requires retention time and spectral matching with known standard
- o Use of orthogonal methods to ensure comprehensiveness of identification/detection (e.g. GC and LC; different column chemistries)
- o Use of compatible detectors in series to maximize information (e.g. UV and MS)

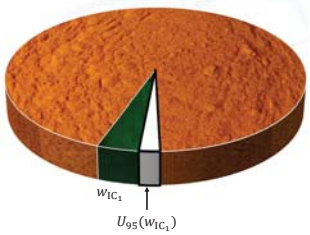
*Using data from **both NMR and separation methods** combined with chemical inference is highly valuable for IC species identification*

Mass Balance Approach
-Quantification approaches for identified organic impurities

$$w_{PC} = 1 - \sum_{i_1:\, identified\ organic\ impurities} \left(\frac{m_{IC}}{m_{CM}}\right)_i$$

Direct determination methods:

- qNMR (approach the same as for the PC)
- calibrated detection for chromatographic techniques using external, experimentally-derived response factors
  - requires reference standards for IC's of reasonably high purity
  - requires concentrations to be in the linear range of the detector



Mass Balance Approach
-Quantification of identified organic impurities using calibrated chromatography (external)

- Gravimetrically prepare solution(s) of impurity component (IC) reference standard (RS) to match concentration in solution of composite material (CM)
- Analyze using chromatographic method
- Determine response factor:

$$\text{Response Factor IC } (RF_{IC}) \quad \frac{Conc_{IC,RS} \times Purity_{IC}}{A_{IC,RS}}$$

- Gravimetrically prepare solution of CM
- Analyze using chromatographic method
- Determine mass fraction of IC in CM (be mindful of units):

$$\text{Mass Fraction IC in CM} \quad \frac{A_{IC,CM} \times RF_{IC}}{Conc_{CM}}$$

$Conc_{IC,RS}$ = concentration of impurity component in the reference standard solution
$purity_{IC}$ = purity of impurity component reference standard
$A_{IC,RS}$ = peak area of the impurity component in the reference standard

$A_{IC,RS}$ = peak area of the impurity component in the composite material
$Conc_{CM}$ = concentration of composite material in solution

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Mass Balance Approach -Estimated quantification for unidentified (or tentatively identified) organic impurities

Relative response % peak area in chromatography:

- ratio of the impurity component peak area (A_{IC}) to the peak area of the primary component (A_{PC})
- assumes (often incorrectly, except for FID) the same response factor as the PC
- requires both A_{IC} and the A_{PC} to be in the linear range of the detector

$$\text{Relative amount IC (mg/g)} \quad \frac{A_{IC}}{A_{PC}} \times 1000$$

Pseudo- 'calibration' using a range of external, experimentally-derived response factors for other structurally-related compounds and the PC:

- uses approach described on previous slide to generate response factors (RFs), but all RFs (including PC) used to estimate impurity mass fraction
- assumes the response factor is in the range of the identified impurities and the PC (better, but still an assumption)

Mass Balance Approach -Estimated quantification for unidentified (or tentatively identified) organic impurities (cont'd)

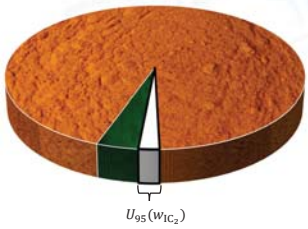
$$w_{PC} = 1 - \sum_i \left(\frac{m_{IC}}{m_{CM}}\right)_i$$

i_2: $unidentified\ organic\ impurities$

Relative response % peak area and pseudo-'calibration' in chromatography:

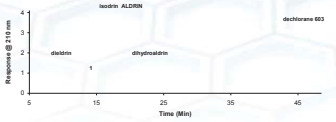
Must use realistic evaluations of amount fraction uncertainties given the assumptions and other factors:

- Structure and/or molecular weights
- Response factors
- Measurement bias (e.g. co-eluting peaks)
- Measurement precision (for chromatographic assessment, may be the smallest component of uncertainty; NOT the *only* component).

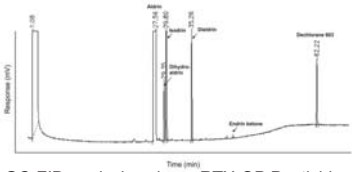


$U_{95}(w_{IC_2})$

Chromatography Tips and Lessons Learned: Aldrin



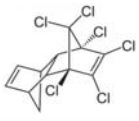
LC-UV_{210} analysis using a SB-C18 column



GC-FID analysis using a RTX-OP Pesticides 2 column

CCQM k55b purity of Aldrin

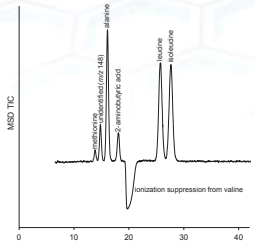
- Structure amenable to both LC and GC analysis – best orthogonality
- Enhanced efficiency (resolution) of capillary GC improves detectability of impurities
- FID closest to a 'universal' detector for chromatography (i.e. provides near-equivalent responses without a need for standards)



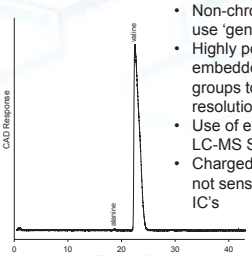
Chromatography Tips and Lessons Learned: L-Valine

CCQM k55.c purity of L-valine

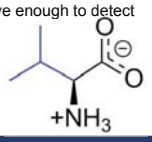
- Non-chromophoric, so unable to use 'general' UV detector
- Highly polar, use column with embedded acidic ion pairing groups to achieve retention and resolution
- Use of external standards with LC-MS SIM for quantitation
- Charged aerosol detection (CAD) not sensitive enough to detect IC's



$LC\text{-}MS_{SIM}$ analysis using a Primesep 100 column



LC-CAD analysis using a Primesep 100 column



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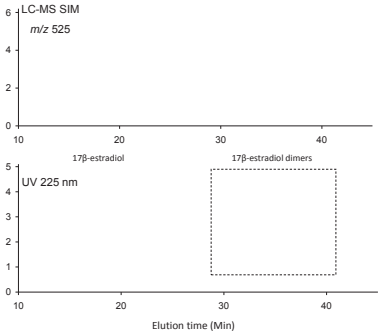
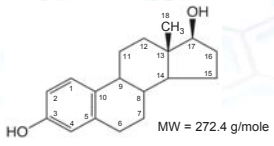
Chromatography Tips and Lessons Learned: Estradiol



CCQM k55.a purity of estradiol

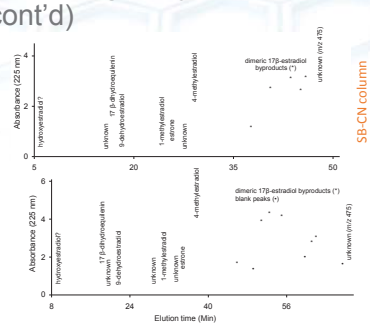


MW = 272.4 g/mole

"... trifluoroacetic acid was added to the mobile phases to suppress formation of dimeric oxidation products ... on the HPLC column. Peaks corresponding to these compounds sometimes appeared during the reversed-phase HPLC analysis of some samples..."

Segmuller BE, Armstron BL, Dunphy R, Oyler AR. Identification of autoxidation and photodegradation products of ethynylestradiol by on-line HPLC-NMR and HPLC-MS. J Pharm Biomed Anal 2000;23:927-937

Chromatography Tips and Lessons Learned: Estradiol (cont'd)



- Dimers are not impurities in the reference standard, but rather are chromatographic artifacts
- Repeating the experiment with an acidic mobile phase suppresses the dimerization reactions
- Must verify that peaks correspond with 'real' impurities:
  - Absent in the blank
  - Peak area increases proportionally to the injected sample amount
  - Cross-check with NMR or other orthogonal methods
- In this case, different column chemistries provide different elution orders for unknowns

Chromatography Tips and Lessons Learned: Estradiol (cont'd)

LC-UV of identified Organic IC's: Calibration with Response Factors from External Standards

Values in mg/g sample

Compound	LC/UV-ph Mean	LC/UV-ph SD	LC/UV-CN Mean	LC/UV-CN SD
?-Hydroxyestradiol	0.12	0.08	0.19	0.04
17β-Dihyroequilenin	0.32	0.02	0.31	0.01
9-Dehydroestradiol	0.17	0.02	0.17	0.02
1-Methylestradiol	0.31	0.03	0.32	0.03
Estrone	1.07	0.02	1.10	0.03
4-Methylestradiol	4.88	0.09	5.23	0.13

ph = phenyl column CN = cyano column

Chromatography Tips and Lessons Learned: Estradiol (cont'd)

LC-UV of unidentified organic IC's: Pseudo- 'calibration' using range of response factors derived from identified impurities and the PC

Values in mg/g estradiol

Evidence	*Assumptions* MW, g/mol	*Assumptions* Rel Abs	Rel Area ph	Rel Area CN	Rel mg/g Least	Rel mg/g Most
~17β-dihydroequilenin, 9-dehydroestradiol	260 - 280	0.1 - 0.8	0.018	*na*	0.002	0.015
~17β-dihydroequilenin	260 - 280	0.1 - 0.8	*na*	0.006	0.001	0.005
~17β-estradiol,1-methylestradiol	260 - 300	0.8 - 1.1	0.011	*na*	0.008	0.013
~1-methylestradiol, estrone	260 - 300	0.8 - 1.1	0.014	*na*	0.011	0.017
~ estrone, 4-methylestradiol	260 - 300	0.8 - 1.1	*na*	0.011	0.008	0.013
Long-retained, m/z of 475, shifted UV	450 - 550	0.1 - 1.5	0.069	0.082	0.114	2.483
				Total:	0.144	2.547

Rel Abs = (molar response factor unknown) / (molar response factor estradiol)
Rel Area = (peak area unknown) / peak area estradiol)
Least = MIN(Rel Area)*MIN(Rel Abs)*MIN(MW)
Most = MAX(Rel Area)*MAX(Rel Abs)*MAX(MW)

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Mass Balance Approach
-Quantification of solvent impurities

$$w_{PC} = 1 - \sum_{i_3:\, solvent\ impurity} \left(\frac{m_{IC}}{m_{CM}}\right)_i$$

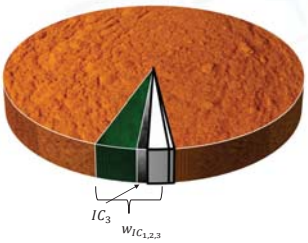
Artifact of synthesis or purification procedures:

GC-Headspace analysis

NMR

TGA

LC (limited)



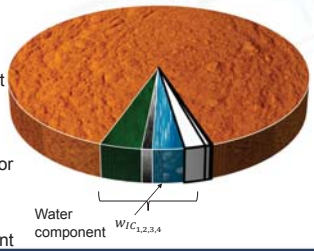
Mass balance approach:
-Quantification of water

$$w_{PC} = 1 - \sum_{i_4:\, H_2O} \left(\frac{m_{IC}}{m_{CM}}\right)_i$$

- Karl Fischer Titration
- Thermogravimetric analysis

For high-purity materials water is often the largest impurity component, particularly for hygroscopic materials

Perform evaluations in controlled conditions and/or characterize potential for water composition changes over time and varying relative humidity-material may accrue water during time between purity assessment and implementation as calibrant

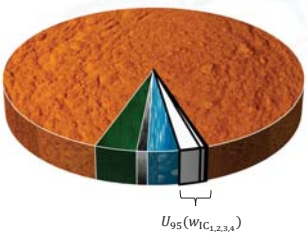


Mass balance approach:
-Quantification of water

$$w_{PC} = 1 - \sum_{i_4:\, H_2O} \left(\frac{m_{IC}}{m_{CM}}\right)_i$$

If performed carefully and precisely, KF titration uncertainties may be small

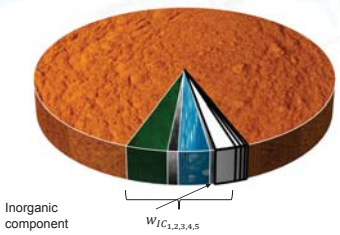
Be sure that material is not accruing water over time of sampling and analysis replication



Mass balance approach:
-Quantification of inorganics

$$w_{PC} = 1 - \sum_{i_5:\, inorganics\ and\ elements} \left(\frac{m_{IC}}{m_{CM}}\right)_i$$

- Inductively Coupled Plasma Mass Spectrometry (ICP-MS)
- Ion Chromatography



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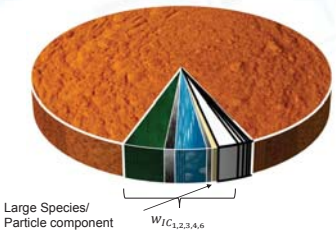
Mass balance approach: -Identification and quantification of macromolecules/particles

$$w_{PC} = 1 - \sum_{i_6:\, macromolecules/particulates} \left(\frac{m_{IC}}{m_{CM}}\right)_i$$

Inferable/measureable contamination:

- polymers
- Insoluble particulate matter
- Other stuff….



Mass balance approach -Identification and quantification of total ICs

$$w_{PC} = 1 - \sum_{i} \left(\frac{m_{IC}}{m_{CM}}\right)_i$$

Derived mass fraction purity via Mass Balance approach

qNMR and Mass Balance Inference: What is fit for purpose?

qNMR allows for traceable, accurate mass fraction determinations with a single, selective measurement technique

Accurate quantification of water, specific stereoisomeric impurities, and inorganics is impractical via NMR

Purity Consensus

Determinations from disparate methods to evaluate the same measurand may be combined

Metrological assessment of purity is a skill which requires experience and expertise. Each material has a unique composition and poses different measurement problems.

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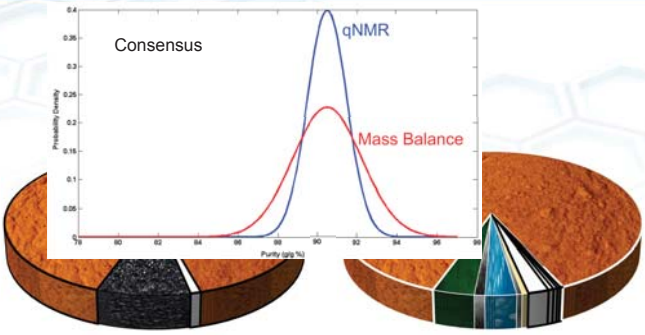
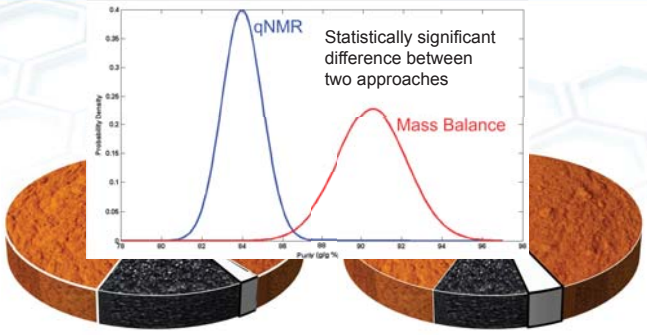




Purity assessment of neat vitamin D metabolite RM's

Primary materials used to develop clinical calibration solutions

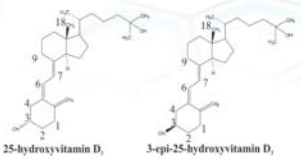
The challenges:

- Isomeric IC's
- Transient species in solution
- Light-sensitive

The approach:

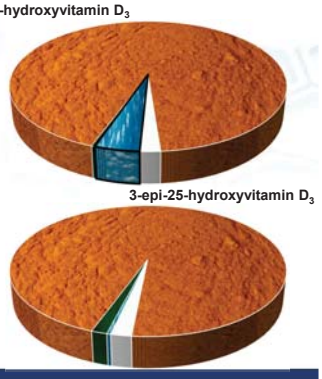
- qNMR direct measurement of PC
- Limited mass balance approach to evaluate ICs

Both are critical, uniquely valuable methods for evaluating neat primary organic materials.



Purity assessment of neat vitamin D metabolite RM's: Mass balance approach

	Organic Ana ysis Methods[1,2]			Volatiles and Water Methods[2]		
	LC-UV ($Column_{\lambda}$ $_{UV}$)	LC-UV (%)	LC-MS-MS (%)	TGA (% g/g)	Karl Fischer Titration (% g/g)	Mass Balance Purity Result (% g/g)[2]
25(OH) D_3	CN_{220}	99.8		4.41 (0.22)	4.44 (0.16)	
		99.6				
	CN_{265}	99.6			4.54 (0.20)	
	$C18_{265}$	99.5				
	$F5_{220}$	99.8				
	$F5_{265}$	99.6				
Mean	99.66 (1.01)			4.46 (0.11)		95.21 (1.02)
3-epi-25(OH)D_3	CN_{220}	98.4		0.4 (0.04)		
	CN_{265}	98.3				
	$F5_{220}$	98.6				
	$F5_{265}$	98.4				
Mean	98.43 (1.01)			0.4 (0.04)		98.03 (1.03)



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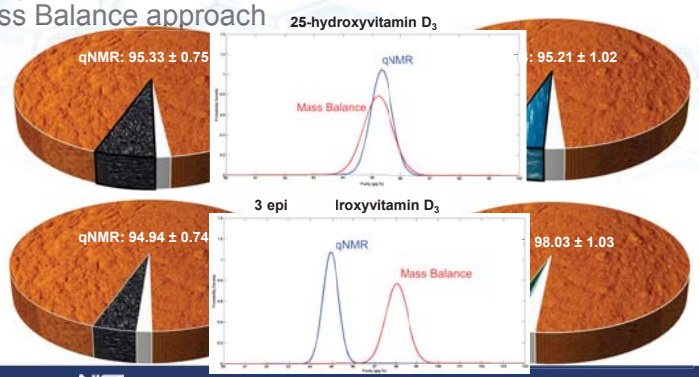
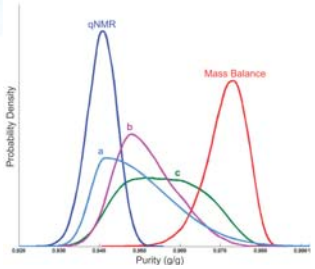
Purity assessment of neat vitamin D metabolite RM's: Mass Balance approach



Combining data and deriving probability distributions

- Use all available inference to make decisions based on chemical knowledge and analyst expertise for the measurement system. *Equal-weighted mean may not demonstrate full state of knowledge.*
  - A) two-piece normal fit
- Bayesian consensus:
  - B) analyst-derived prior distribution (triangle)
  - C) non-informative (weighting values by uncertainty distribution)

Nelson MA, Bedner M, Lang BE, Toman B, Lippa K. Anal. Bioanal. Chem. **2015** 407(28):8557-69



Conclusions

- Each method provides important, distinct information regarding the composition of the neat chemical material
- Biases may be associated with any measurement; they may be discovered using orthogonal methods

Photo obtained from countryliving.com

- The more information, the better….
  Inference can be gleaned from each technique that may confirm other determinations, uncover bias, or lead to impurity component identification
- The accuracy of your clinical measurement is dependent upon the quality of the determined purity of your primary calibrant
  - Decide what measurand and tolerance limits work best for you
  - Give heed to your chemical intuition and evaluate uncertainty intervals that reflect your state of knowledge gleaned from the measurements.
  - Expertise and prior knowledge are statistically valuable!

Quantitative determination of Water

2016 SIM Clinical Measurements Course

Brian Lang

NIST National Institute of Standards and Technology U.S. Department of Commerce

MATERIAL MEASUREMENT LABORATORY

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Outline

- Importance of Water Determination
- Water by Gravimetric Methods
- Karl Fischer Titration Methods
- Water Calibration Standards
- NIST Solutions to Karl Fischer Problems

Importance of Water in Clinical Samples

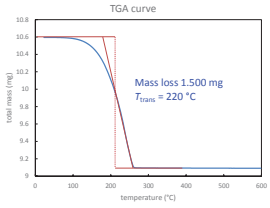
- Water is often on of the major components of clinical samples and neat chemicals used as primary standards
- Water content can be quite variable
  - Will vary due to hydrophobicity of the sample
  - Will also vary from lot to lot
  - In extreme cases will vary due to external factors such as temperature and relative humidity
- Knowledge of water content is important for mass balance calculations and traceability
- Water content is primarily measured either by loss on drying methods or by direct detection

Water Determination by Gravimetric Methods

- Loss on drying (by heating or desiccation)
  - Simplest method for estimation of water and solvents
  - Easily traceable to SI
  - Non selective
  - Water may or may not be completely removed
- Thermogravimetric Analysis (TGA)
  - Mass loss as a function of temperature
  - Useful in determining mass loss temperature
  - Best used in conjunction with a secondary technique such as Karl Fischer or mass spectrometry

Thermogravimetric Analysis

- Thermogravimetric Analysis (TGA)
  - Mass loss as a function of temperature
  - Useful in determining mass loss temperature
    - Mass loss temperature can give indication of the volatile compound
  - Best used in conjunction with a secondary technique such as Karl Fischer or mass spectrometry with organics
  - Accuracy and precision on the order of 0.1 % or better



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Thermogravimetric Analysis

Example: calcium oxalate monohydrate

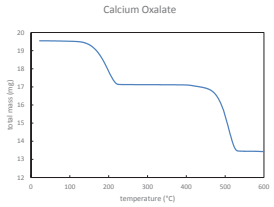
- Useful as a validation method
- Has three mass loss events
- Below ~200 °C, loss of water
  - Ideally 12.3298 % water, but the stoichiometry can vary
  - Water should be measured independently
- ~500 °C, decomposition

  $CaC_2O_4 \rightarrow CaCO_3 + CO$

  Ideally 21.866 % of mass after loss of water
- ~840 °C, decomposition (not shown)

  $CaCO_3 \rightarrow CaO + CO_2$

  Final mass 43.777% of CaC_2O_4 mass

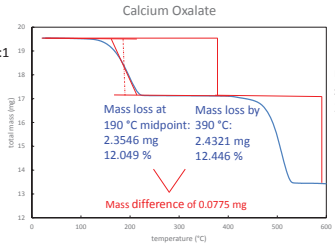


Thermogravimetric Analysis

Example: calcium oxalate monohydrate

Initial mass: 19.5418 mg

mass loss from 1:1 stoichiometry 12.3298 %



mass loss from stoichiometry 21.866 %

Mass loss by 595 °C: 3.7279 mg 21.788 % of mass at 390 °C

Karl Fischer Basics

- Karl Fischer reactions consume stoichiometric amounts of water and iodine with a series of reactions
  - Karl Fischer is selective for water
- I_2 is measured by consumption of I_2 in KF reagent (volumetric method) or by generation of I_2 from I^- present in the solution (coulometric method)
  - I_2 directly correlated with amount of water present in the sample.
- In most cases it is more sensitive to water than gravimetric methods
- I_2 is measured by potentiometry of the reagent in KF cell

Reactions in the Karl Fischer system

$B{\cdot}I_2 + B{\cdot}SO_2 + B + H_2O \rightarrow 2BHI + BSO_3$

$BSO_3 + ROH \rightarrow BH^+ROSO_3^-$

B : a base, usually imidazole 

ROH : alcohol, most often methanol where R = CH_3

Volumetric Karl Fischer Titration

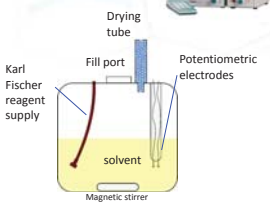
Advantages

- Simple
- Generally one solution
- May be adapted for a wide variety of solvent systems
- Easiest of use with solid samples

Disadvantages

- Needs daily external calibration for best results
- Not as sensitive as coulometry
- Prone to have water absorption in the feed lines when not used for long periods of time

Typical volumetric Karl Fischer apparatus



Volumetric Karl Fischer Cell

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Coulometric Karl Fischer Titration

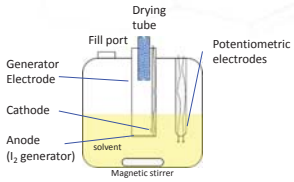
Advantages

- Most sensitive method for water determination
- Some solvent variation is available
- Does not need daily exchange of solvent

Disadvantages

- Atmosphere exchange can be problematic for solid samples
- More limited on solvent selection than the volumetric method

Typical coulometric Karl Fischer apparatus



Coulometric Karl Fischer Cell

Karl Fischer Oven Methods

- Oven heats sample, carrier gas transports water vapor to Karl Fischer cell
- Useful for samples that are not miscible in the solvent
- Helpful for measuring solid samples with the coulometric system
- Two typical variations
  - Sample in tube furnace
  - Samples in headspace vials

Issues

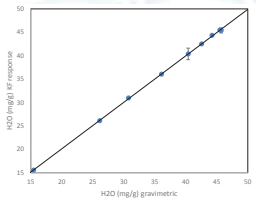
- Need quantitation and validation of water transfer
- Typically takes longer to release water using oven methods than for direct addition
- Heating may also cause breakdown of organic material, forming new water as a byproduct

Tube type Karl Fischer oven

Karl Fischer oven using headspace vials. Above single use furnace, and to the right with an outosampler

Calibration and Validation

- Coulometric system
  - Electronics should be calibrated regularly
  - Use standards to check and adjust calibration
- Volumetric system
  - Calibrations for volumetric solutions should be done daily against a water standard
- Calibrate against a well-characterized standard material
- Record of daily titrations of calibration solutions will show trend and drifts of the system
- Calibration curves using a range of concentrations/ total water content will demonstrate linearity in the titration system
- Use gravimetric addition for standard solutions



Calibration curve for Coulometric Karl Fischer using water in octanol solutions

Calibration Solutions

- Water (Type 1 deionized or better)
  - Water is easily obtainable to high purity (>99.999%)
  - Straight water may cause too large KF response
  - Straight water difficult to measure in small quantities
- SRM 2890 (water saturated octanol)
  - High level water (~47.3 mg/g)
- NMIJ CRM 4222-a (water in mesitylene)
  - For low level water (~0.1 mg/g)
- In-house water in octanol standards
  - Gravimetrically prepared calibration solutions
  - Equilibrium solution of water saturated octanol
- Commercial water standards
  - Validated against SRM 2890

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Karl Fisher Methods: Solid Samples

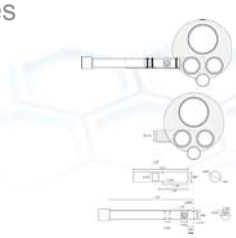
- Solubility of solids
  - Karl Fisher works best if samples completely dissolve
  - Insoluble samples may work well if they can be dispersed either through the solvent or by a homogenizer
  - For some insoluble samples, the oven method will be best
- For solids, volumetric methods may be preferable to coulometry
  - You can mix and match many solvents with the volumetric methods
    - Chloroform
    - Formamide
    - Acetonitrile
    - Pyridine
  - For methanol based Hydranal, Keep methanol around 50%

Karl Fisher Methods: Solid Samples

- Addition of solid samples will require a blank
  - Open fill port for same length of time for all samples
  - Open fill port without addition of sample for same time
- Coulometry with solid samples is problematic since opening the fill port will introduce water
  - For low water samples, water from opening the fill port may offset the benefits of using the coulometic method

Solutions

- Place entire apparatus in dry glove box
  - NMIJ
- Use an air-lock to minimize and keep water addition to sample more consistent
  - NIST



Karl Fisher Methods: Liquid samples

- Handle liquids using syringe
  - Gas tight syringes work best for most cases
  - Use type 2 needle to prevent coring of septum
  - For some insoluble samples, the oven method will be best
- Make sure liquids are miscible
  - Liquids immiscible with the KF solvent will have longer titration times
  - Co-solvents with KF medium may help
  - Immiscible liquids may need to be run in oven

Karl Fisher Methods: Interferences

- Many compounds will interfere with the Karl Fischer reaction
  - Side reactions can happen due to interactions with the analyte or with impurities
  - Generally by the uptake of I_2, biasing the signal high
    - Reducing agents
- Changing the pH of the KF solution may help mitigate side reaction
  - Decreasing pH often works best
    - Salicylic Acid
  - Increasing the pH for highly acidic materials
    - Imidizole
- Use orthogonal methods to confirm the actual water content

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Karl Fisher Methods: Interferences

Case study: Catechins

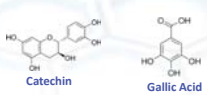
- We were tasked to measure the water content of catochins (flavonoids)
  - Gallic Acid first sample ran
- Initial Karl Fisher measurements gave high water content
- Turned KF solution from yellow to bright **RED**

Cause:

- Gallic acid has an active aromatic ring
  - Electrophilic substitution

Solution:

- Acidified KF reagent with 5 g Salicylic Acid per 50 ml
  - Observed water consistent with TGA



Catechin

Gallic Acid

Epicatechin gallate

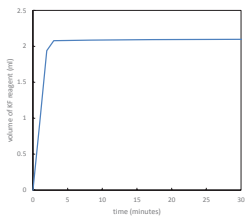
General Karl Fisher Methods

How long should I run the titration?

- Time versus endpoint
- Normal setting on many instruments is to run until the electrochemical endpoint
  - many samples will reach the endpoint within 5 minutes
- Advanced methods will allow the titration to run longer to ensure all of the water reacts

Case study: SRM 3222 (well behaved)

- Ran for 30 minute
- Endpoint reached within 3 min
  - 2.077 ml (drift corrected) **99 %**
- Total signal with endpoint correction
  - 2.086 ml
- Titration was not complete until minute 12



SRM 3222 volumetric titration

NIST Solutions

- Glass Joints on the Karl Fischer cell
  - Use apiezon grease (N or M) to get better seals
  - Do not use silicone vacuum grease
- Cover breakable ampoules with septum
  - Move contents to autosampler vial
  - Use septum directly on vial
- Cover KF apparatus in dry nitrogen blanket
  - Use disposable glove bag that can be closed
  - Continually flow nitrogen gas
- For small samples place in small pan
  - Platinum DSC crucibles work well
- In house water in octanol standards
  - Gravimetrically prepared calibration solutions
  - Equilibrium solution of water saturated octanol

Questions?

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Thank You!

Overview: Cholesterol and Glucose

2016 SIM Clinical Measurement Course

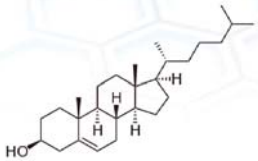
Jeanita S. Pritchett and Lorna T. Sniegoski

NIST National Institute of Standards and Technology U.S. Department of Commerce

MATERIAL MEASUREMENT LABORATORY

Background: *Cholesterol*

- Present at relatively high concentrations in serum
- Low polarity
- Predominantly esterified with fatty acids in blood
- Precursor molecule for several biochemical pathways
- Associated with cardiovascular diseases including heart disease and stroke



Cholesterol

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Sources of Cholesterol Measurement Inaccuracy

Pre-analytical Issues

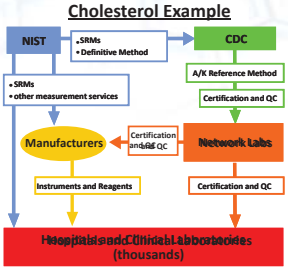
- Intra-individual biological variation (age, sex, body weight)
- Behavioral factors (diet, alcohol use, exercise)
- Pregnancy,
- Trauma
- Surgery
- Acute illness
- Chronic diseases
- Diet
- Acute exercise
- Sample collection
- Sample handling
- Storage/shipment

Analytical Issues

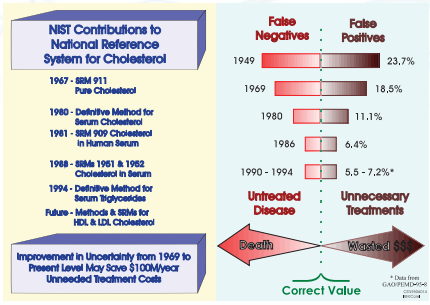
- Method or sequence of chemical reactions
- Reagent
- Measurement instrument
- Approach to calibration

NIST Role in National Reference System for Clinical Measurements

- Maintain existing Definitive Methods and SRMs
- Develop new reference methods and SRMs of "higher metrological order" to meet new needs
- Work with other NMIs to establish equivalence of measurement services
- Participate in global reference laboratory network and provide reference laboratory measurement services to IVD industry



Improved Cholesterol Measurement Accuracy Saves Health Care Costs



NIST Cholesterol Standard Reference Materials (SRMs)

- **Crystalline, Neat Material**
  - SRM 911c: Cholesterol
- **Matrix-based Material**
  - SRM 909c: Cholesterol
  - SRM 968e: Fat-soluble Vitamins, Carotenoids and Cholesterol in Human Serum
  - SRM 1951c: Lipids in Frozen Human Serum
  - SRM 1952a: Cholesterol in Freeze-Dried Human Serum
  - SRM 1950: Metabolites in Frozen Human Plasma

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NIST Cholesterol Reference Measurement Procedure (RMP)

- **Sample Preparation**
  - Weigh serum sample containing 0.2 mg cholesterol, add known mass of cholesterol-$^{13}C_3$ in ethanol, and equilibrate.
  - Add alcoholic KOH and heat at 37 ºC for 3 hrs to saponify esters.
  - Extract with 2 mL hexane, vortex, evaporate 1 mL aliquot from hexane layer under N_2.
  - Derivatize with BSA, and heat at 60 ºC for 30 min.
- **Calibration Standards**
  - Prepare primary standard solution by dissolving known mass of SRM 911c Cholesterol (purity 99.2 ± 0.4%) in known mass of ethanol (warm in hot water bath/ swirl gently).
  - Add constant mass of cholesterol-$^{13}C_3$ in ethanol to series of tubes and add masses of primary standard solution to the tubes such that the unlabeled/labeled cholesterol ratio ranges from 0.7 to 1.2.

NIST Cholesterol Reference Measurement Procedure (RMP)

GC/MS MEASUREMENTS

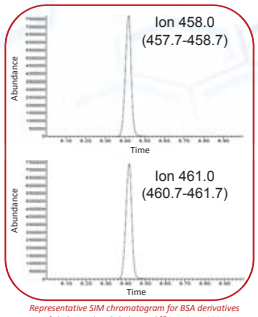
GC Conditions: 30 m DB-5ms (0.25 mm i.d., splitless, Column temperature: 200 ºC, 0.5 min hold time, 20 ºC/min to 300 ºC, 5 min hold time, for a total run time of 10.5 min.

MS Conditions: Quadrupole instruments, electron ionization at 70ev, Selected ion monitoring (SIM) monitoring of m/z 458 and 461(derivatives)

Measurements: Run standards containing known ratios of unlabeled to labeled material along with samples. Analyze standards first, followed by samples, then by the samples and standards in reverse order.

Quantification of Cholesterol in Serum

- The measured ratios for each standard were subjected to linear regression analysis, and the least squares fit was then used to calculate the weight ratios for the samples from the measured intensity ratios.
- Cholesterol concentrations are calculated from the mass of cholesterol-$^{13}C_3$ added and the mass of the serum sample.



Representative SIM chromatogram for BSA derivatives of cholesterol and cholesterol-$^{13}C_3$ in SRM 909c. (ROA 839.2-10-094)

Cholesterol peak area

or SIM

Labeled cholesterol peak area

Measured ratio =cholesterol derivative peak area/ labeled cholesterol derivative peak area

File	Sample	458	461	Meas ratio	Wt ratio
7121601	A	11483101	9366665	1.2260	
7121602	B	6970085	6679305	1.0435	
7121603	C	4921338	5781178	0.8513	
7121604	D	8854639	7793544	1.1362	
7121605	E	8241659	8658520	0.9519	
7121606	F	6153390	8051145	0.7643	
7121607	1	5924205	5444088	1.0882	1.0312
7121608	2	8276540	7700101	1.0749	1.0174
7121609	3	5882439	5310343	1.1077	1.0513
7121610	4	6923793	6210678	1.1148	1.0587
7121611	4	8766741	7896222	1.1102	1.0539
7121612	3	4748232	4262075	1.1141	1.0579
7121613	2	8939006	8321708	1.0742	1.0167
7121614	1	6308155	5800799	1.0875	1.0304
7121615	F	6025873	7873018	0.7654	
7121616	E	5027475	5249192	0.9578	
7121617	D	6437946	5[illegible]		
7121618	C	4709121	5[illegible]		
7121619	B	5160100	4923722	1.0480	
7121620	A	7177768	5799342	1.2377	

Calculated weight ratio from regression equation variables =measured ratio-intercept/ x variable

Regression equation variables

	Coefficients	Standard Error	t Stat	P-value	Lower 95%	Upper 95%	Lower 95.0%	Upper 95.0%
Intercept	0.089735	0.017532	5.118342	0.006894	0.041058	0.138412	0.041058	0.138412
X Variable 1	0.968282	0.0184	52.62432	7.8E-07	0.917196	1.019368	0.917196	1.019368

ANOVA	df	SS	MS	F	Significance F
Regression	1	0.154853	0.154853	2769.319	7.8E-07
Residual	4	0.000224	5.59E-05		
Total	5	0.155077			

Mean	WR	MR1	MR2	Mean
A	1.1812	1.2260	1.2377	1.2318
B	0.9749	1.0435	1.0486	1.0458
C	0.7833	0.8513	0.8516	0.8514
D	1.0911	1.1362	1.1456	1.1409
E	0.8969	0.9519	0.9578	0.9548
F	0.7023	0.7643	0.7654	0.7648

SUMMARY OUTPUT	
Regression Statistics	
Multiple R	0.999279
R Square	0.998558
Adjusted R Square	0.998197
Standard Error	0.007478
Observations	6

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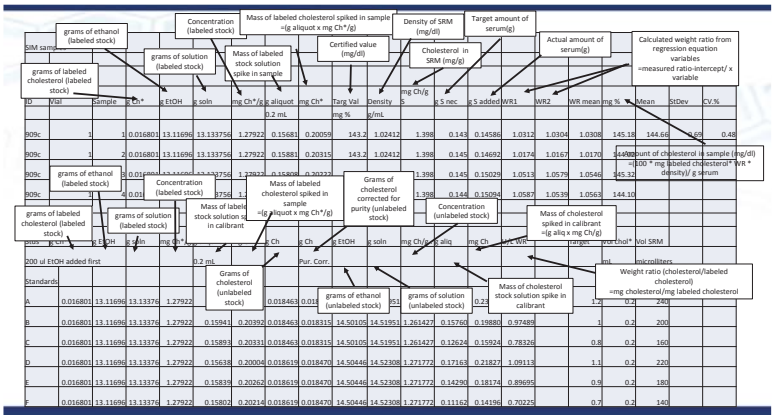
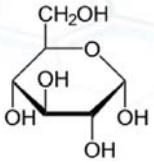


Background: Glucose

- Glucose is a six-carbon monosaccharide
- Serves as the major source of energy for cells in the body
- Its concentration in blood is carefully regulated in healthy individuals through production of insulin that acts to stimulate absorption of glucose by the cells in liver, muscle, and adipose tissue



Glucose

Significance of Glucose in Diabetes

- Diabetes is a metabolic disorder where the body isn't able to regulate levels of glucose in the blood.
- Either insulin production or activity is reduced, leading to elevated blood levels.
- Blood glucose is measured to determine if diabetes is present and if so, to what extent is the glucose of normal ranges.
- The nature and timing of treatments depend upon these measurements, so accuracy in these measurements is important to properly diagnose and treat.

Image source: conpherm.com

Diagnostic Testing for Diabetes

- Routine clinical laboratory measurements of blood glucose generally use enzymatic methods based upon hexokinase, glucose oxidase, or other enzymes that act on glucose.
- Such methods may not be specific to glucose and method-method differences can be large.
- Thus, there is a need for higher order methods to provide an accuracy base to which the routine methods can be compared.
  - Definitive method
  - Adapted Reference Measurement Procedure (ID-GC-MS method)

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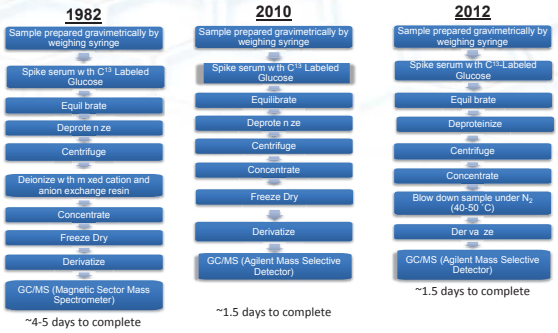
NIST Glucose Standard Reference Materials (SRMs)

- **Crystalline, Neat Material**
  - SRM 917c: D-Glucose (Dextrose)
- **Matrix-based Material**
  - SRM 965b: Glucose in Frozen Human Serum
  - SRM 1950: Metabolites in Frozen Human Plasma
  - SRM 1951c: Lipids in Frozen Human Serum
  - **SRM 909c: Frozen Human Serum (stability issues)**

Modification of Glucose Method



NIST Glucose Modified Reference Measurement Procedure (RMP)

- **Sample Preparation**
  - Weigh serum sample containing glucose, gravimetrically add known mass of glucose-$^{13}C_6$ in distilled water.
  - Add 0.1 mL aliquot of sodium azide solution, swirl, then allow to equilibrate overnight.
  - Deproteinize samples by adding ~2.5 volumes of ice-cold absolute ethanol, mix, then centrifuge (2500 rpm for 15 min).
  - Transfer supernatant, concentrate to dryness at 40 to 50 °C under a stream of nitrogen.
  - Derivatize with butylboronic acid in pyridine (95 °C for 50-60 minutes).
  - Add acetic anhydride, mix, let stand 1-2 hrs, evaporate under stream of nitrogen at 40 to 50 °C.
  - Reconstitute in isooctane containing 1% acetic anhydride (warm in hot water bath).
  - Dilute further with isooctane-acetic anhydride; GC-MS analysis.

NIST Glucose Modified RMP (continued)

- **Calibration Standards**
  - Prepare primary standard solution by dissolving known mass of SRM 917c Glucose (purity 99.6 ± 0.1%) in known mass of distilled water.
  - Add constant mass of glucose-$^{13}C_6$ in distilled water to series of tubes and add masses of primary standard solution to the tubes such that the unlabeled/labeled glucose ratio ranges from 0.7 to 1.2.
  - Calibrants are derivatized in a similar manner as the samples.

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NIST Glucose Modified Reference Measurement Procedure (RMP)

GC/MS MEASUREMENTS

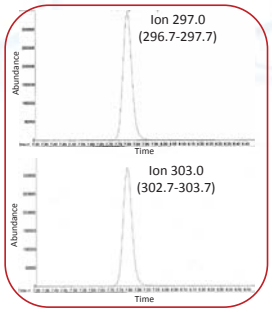
GC Conditions: 30 m DB-5ms (0.25 mm i.d., split injection (20:1) at 200 °C temperature program: 150 °C, one minute hold time, 40°C/min to 200 °C, 10 min hold time.

MS Conditions: Quadrupole instruments, electron ionization at 70ev, MS Quad 150 °C, MS Source 230 °C. Selected ion monitoring (SIM) monitoring of m/z 297 and 303 (derivatives).

Measurements: Run standards containing known ratios of unlabeled to labeled material along with samples. Analyze standards first, followed by samples, then by the samples and standards in reverse order.

Quantification of Glucose in Serum

- The measured ratios for each standard were subjected to linear regression analysis, and the least squares fit was then used to calculate the weight ratios for the samples from the measured intensity ratios.
- Glucose concentrations are calculated from the mass of glucose-$^{13}C_6$ added and the mass of the serum sample.



Representative SIM chromatogram for BBA derivatives of glucose and glucose-$^{13}C_6$ in SRM 1950. (ROA 646.2-15-047)

Derivatization for Gas Chromatography

2016 SIM Clinical Measurement Course

Jeanita S. Pritchett and Lorna T. Sniegoski

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MATERIAL MEASUREMENT LABORATORY

What is derivatization?

- Derivatization is the process of chemically modifying a compound to produce a new compound which has properties that are suitable for analysis using GC.
- Derivatization also reduces analyte adsorption in the GC system and improves detector response, peak separations, and peak symmetry.
- Derivatives are used for the following reasons:
  - To improve resolution and reduce tailing of polar compounds (-OH, -COOH, =NH, -NH_2, -SH, and other functional groups
  - To analyze relatively nonvolatile compounds
  - To improve analytical efficiency and increase detectability
  - To improve stability of compounds

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What does derivatization accomplish?

- Increases volatility:
  - Eliminates the presence of polar groups
  - Derivatization targets O, S, N, and P functional groups (with hydrogens available)
- Increases detectability
- Increases stability

Conditions for choosing a derivatizing agent

- Produce a derivatization reaction that is 95-100% complete
- Will not cause any rearrangements or structural alterations during formation of the derivative
- Does not contribute to loss of the sample during the reaction
- Produce a derivative that will not interact with the analytical column
- Produce a derivative that is stable with respect to time

Main types of derivatizations

- Silylation
  - Readily volatizes the sample
  - Most prevalent method
- Alkylation
  - Used as the first step to further derivatizations or as a method of protection of certain active hydrogens
- Acylation
  - Commonly used to add fluorinated groups (ECD)
- Chiral derivatization

Silylation derivatization

- Replaces active hydrogens with a TMS (trimethylsilyl) group.
- Silylation occurs through nucleophilic attack (SN2). The better the leaving group, the better the siliylation.
- Silylation reagents will react with water and alcohols first. *Care must be taken to ensure that both sample and solvents are dry.*
- *Solvents should be as pure as possible*. This will eliminate excessive peaks. Try using as little solvent as possible as this will prevent a large solvent peak.
- Pyridine is the most commonly used solvent. Although pyridine may produce peak tailing, it is an acid scavenger and will drive the reaction forward

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Silylation derivatization (continued)

- In many cases, the need for a solvent is eliminated with silylating reagents. (If a sample readily dissolves in the reagent, it usually is a sign that the derivatization is complete).
- Many reagents require heating (not in excess of 60 °C for about 10-15 minutes, to prevent breakdown). Hindered products require long term heating.
- The ease of reactivity of the functional group toward silylation follows the order:

- The order of alcohols being:

Advantages and disadvantages of silylation

- **Advantages**
  - Ability to silylate a wide variety of compounds
  - Large number of silylating reagents available
  - Easily prepared
- **Disadvantages**
  - Silylation reagents are moisture sensitive
  - Must use aprotic (no protons available) organic solvents

Common silylating reagents

- BSA (Bistrimethylsilylacetamide)
- BSTFA (Bistrimethylsilyltrifluroacetamide)
- MSTFA (N-methyl-trimethylsilyltrifluroacetamide)
- MTBSTFA (N-methyl-N-t-butyldimethylsilyltrifluoroacetamide)
- HMDS (Hexamethyldisilzane)
- TMCS (trimethylchlorosilane)
- TMSI (Trimethylsilylimidazole)
- TMS-DEA (trimethlysildiethylamine)
- Halo-methylsilyl derivatization reagents (BMDMCS and CMDMCS)

Acylation derivatization

- Acylation reduces the polarity of amino, hydroxyl, and thiol groups and adds halogenated functionalities for ECD.
- In comparison to silylating reagents, the acylating reagents target highly polar, multifunctional compounds, such as carbohydrates and amino acids.
- Acylation converts these compounds with active hydrogens into esters, thioesters, and amides.
- Acylations are normally carried out in pyridine, tetrahydrofuran, or another solvent capable of accepting the acid by-product.
- The presence of a carbonyl group next to the halogenated carbons enhances the ECD.

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Advantages and disadvantages of acylation

- **Advantages**
  - Addition of halogentated carbons increases detectability by ECD
  - Derivatives are hydrolytically stable
  - Increased sensitivity by adding molecular weight
  - Acylation can be used as a first step to activate carboxylic acids prior to esterfications (alkylation)
- **Disadvantages**
  - Acylation derivatives can be difficult to prepare
  - Reactions products (acid by-products) often need to be removed before analysis
  - Acylation reagents are moisture sensitive
  - Reagents are hazardous and odorous

Common acylating reagents

- Fluorinated anhydrides
  - TFAA- trifluoroacetic anhydride
  - PFPA-pentafluropropionic anhydride
  - HFBA-heptafluorobutyric anhydride
- Fluoracylimidazoles
  - TFAI-trifluoroacetylimidazole
  - PFPI-Pentafluoropropanylimidazole
  - HFBI-Heptaflurobutyrylimidazole
- MBTFA-N-Methyl-bis(trifluoroacetamide)
- PFBCl-Pentafluorobenzoyl chloride
- PFPOH- pentafluropropanol

Alkylation derivatization

- Alkylation reduces molecular polarity by replacing active hydrogens with an alkyl group.
- Used to modify compounds with acidic hydrogens, such as carboxylic acids and phenols to produce esters, ethers, alkyl amines and alkyl amides.
- The principle reaction employed for preparation of these derivatives is nucleophilic displacement.
- Alkylation can be used in conjunction with acylation and silylation.

Advantages and disadvantages of alkylation

- **Advantages**
  - Wide range of alkylation reagents available
  - Reaction conditions can vary from strongly acidic to strongly basic
  - Some reactions can be done in aqueous solutions
  - Alkylation derivatives are generally stable
- **Disadvantages**
  - Limited to amines and acidic hydroxyls
  - Reaction conditions are frequently severe
  - Reagents are often toxic

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Common alkylating reagents

- DMF (dialkylacetals)
- TBH (tetrabutylammonium hydroxide)
- BF_3 in methanol or butanol
- PFBBr (pentafluorobenzyl bromide)

GC Chiral Derivation

- These reagents target one specific functional group and produce individual diastereomers of each of the enantiomers.
- GC determination of enantiometric purity is facilitated by using enantio-pure derivatization reagents.
- There are two ways of separating enantiomers by chromatography:
  - Separation on an optically active stationary phase
  - Preparation of diastereomeric derivatives that can be separated on a non-chiral stationary phase

Reagents for optical purity

- TPC (N-trifluoroactyl-L-prolyl chloride)
- MCF ((-) menthylchloroformate)



General problems with gas chromatography

2016 SIM Clinical Measurement Course

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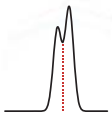
Common issues affecting separation with GC

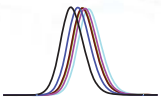
- Poor resolution
- Poor retention time reproducibility
- Fronting Peaks
- Tailing Peaks
- Split Peaks
- Carryover/Ghost Peaks
- High Bleed
- Unstable Baseline
- Response Variation
- No Peaks
- Broad Peaks

Poor resolution



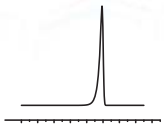
Causes	Solutions
Non-selective stationary phase	• Select appropriate stationary phase and column dimensions.
Poor efficiency	• Optimize carrier gas linear velocity and GC oven temperature program.
Sample overload	• Adjust sample concentration or amount on column.
Incorrect analytical conditions used	• Optimize temperature program, flow rates, and column parameters.

Poor retention time reproducibility



Causes	Solutions
Leaks	• Check for leaks at the injector and press-fit connections. • Replace critical seals.
Analyte adsorption	• Maintain inlet liner and GC column. • Use properly deactivated liners, seals, and columns.
Resolution/integration issues	• Avoid sample overload.
Incorrect column/oven program	• Optimize column temperatures and oven temperature program.
Incorrect or variable carrier gas flow rate/linear velocity	• Optimize the carrier gas glow and linear velocity.
Poor control of oven temperature programming	• Verify GC oven program falls within instrument manufacturer's recommendation.
Incorrect oven equilibration time	• Extend GC oven equilibration.
Manual injection: delay between pushing start and actual injection	• Use autosampler or standardize manual injection procedure.

Fronting Peaks



Causes	Solutions
Incompatible stationary phase	• Select appropriate stationary phase
Column overloading	• Decrease amount injected, dilute sample. • Increase column inner diameter and/or film thickness.

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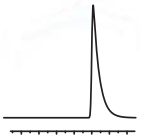
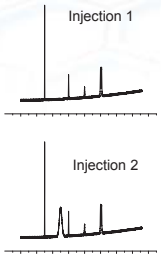
Tailing Peaks



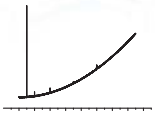
Causes	Solutions
Adsorption due to surface activity or contamination	• Use properly cleaned and deactivated liner, seal, and column. • Trim inlet end of column. • Replace column if damaged.
Adsorption due to chemical composition of compound	• Derivatize compound.
Leak in system	• Check for leaks at all connections, replace critical seals if needed.
Installation issues	• Minimize dead volume. • Verify that the column is cut properly. • Verify correct installation distances.

Carryover/Ghost Peaks



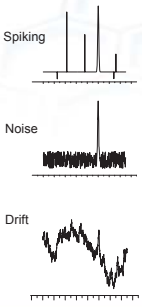
Causes	Solutions
Contaminated syringe or rinse solvent	• Replace rinse solvent. • Rinse or replace syringe.
Backflash (sample volume exceeds liner volume)	• Inject smaller amount. • Use a liner with a larger internal diameter. • Increase head pressure to contain the vapor cloud. • Use slower injection rate. • Increase split flow. • Use liner with packaging. • Use pressure-pulse injection.
Last analysis ended too soon	• Extend analysis time to all components and/or matrix interferences to elute.

High Bleed



Causes	Solutions
Improper column conditioning	• Increase conditioning time and/or temperature.
Contamination	• Trim column and/or heat to maximum temperature to remove contaminants. • Replace carrier gas and/or detector gas filters. • Clean injector and detector.
Leak in system and oxidation of stationary phase	• Check for oxygen leaks across the entire system and replace seals and/or filters. • Replace column.

Unstable Baseline (spiking, noise, drift)



Causes	Solutions
Carrier gas leak or contamination	• Leak check connections and replace seals if needed.
Injector or detector contamination	• Clean system and perform regular maintenance.
Column contamination or stationary phase bleed.	• Condition, trim, and rinse column.
Septum coring/bleeding	• Replace septum. • Inspect inlet liner for septa particles and replace liner if needed.
Loose cable or circuit board connections	• Clean and repair electrical connections
Variable carrier gas or detector gas flows	• Verify flow rates are steady and reproducible; may need to replace or repair flow controller. • Leak check system.
Detector not ready	• Allow enough time for detector temperatures and flows to equilibrate.

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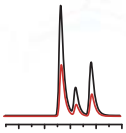
Response Variation

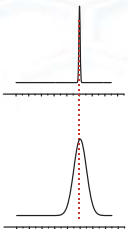


Causes	Solutions
Sample issues	• Verify sample concentration. • Verify sample preparation procedure. • Verify sample decomposition/shelf life.
Syringe problems	• Replace syringe. • Check autosampler operation.
Electronics	• Verify signal settings and adjust if needed. • Repair or replace cables or boards.
Dirty or damaged detector	• Perform detector maintenance or replace parts.
Flow/temperature settings wrong or variable	• Verify steady flow rates and temperatures, then adjust settings and/or replace parts if needed.
Adsorption/reactivity	• Remove contamination and use properly deactivated liner, seal, and column.
Leaks	• Check for leaks at all connections and repair connections as needed.
Change in sample introduction/injection method	• Verify injection technique and change back to original technique. • Check that split ratio is correct. • Verify that the splitless hold time is correct.

No peaks

Causes	Solutions
Injection problems	• Obstructed syringe; clean or replace syringe. • Verify there is sample in the syringe. • Injecting into wrong inlet; reset autosampler. • Verify carrier gas is flowing.
Broken Column	• Replace column
Column installed into wrong injector or detector	• Re-install column.
Detector problems	• Signal not recorded; check detector cables and verify that detector is turned on. • Detector gas turned off or wrong flow rates used; turn detector on and/or adjust flow rates.

Broad Peaks



Causes	Solutions
High dead volume	• Minimize dead volume in the GC system; verify proper column installation, proper connectors, proper liners, etc.
Low flow rates	• Verify injector and detector flow rates and adjust if needed. • Verify make-up gas glow and adjust if needed.
Slow GC oven program	• Increase GC oven programming rate.
Poor analyte/solvent focusing	• Lower GC oven start temperature.
Column film is too thick	• Reduce retention of compounds by decreasing film thickness and length.
Sample carryover	• See Carryover/Ghost Peaks solutions.



Overview: Creatinine

2016 SIM Clinical Measurement Course
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NIST National Institute of Standards and Technology U.S. Department of Commerce

MATERIAL MEASUREMENT LABORATORY

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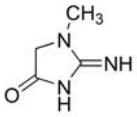
Introduction

- The concentration of creatinine in serum is a diagnostic marker for chronic kidney disease (CKD)
- Early detection of CKD, followed by drug treatments, can prevent or postpone kidney failure
  - Most simple, widespread method of detecting kidney disease is through measurement of blood creatinine concentrations
- Recognizing that more accurate blood creatinine measurements will lead to better diagnosis of early stage kidney disease, the Laboratory Working Group of the National Kidney Disease Education Program (NKDEP) outlined a series of recommendations, including development of a reference material



Creatinine

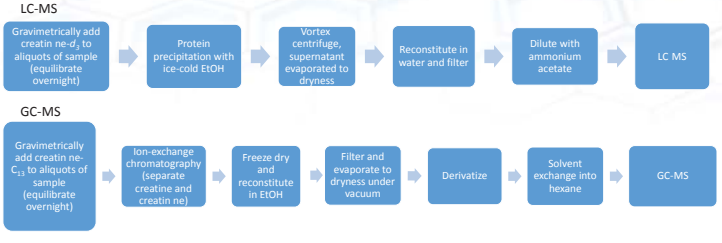
Creatinine Reference Materials at NIST

- Crystalline, neat material
  - SRM 914a Creatinine
- Matrix-based material
  - SRM 967a Creatinine in Frozen Human Serum
  - SRM 3667 Creatinine in Frozen Human Urine
  - SRM 909c Frozen Human Serum
  - SRM 1950 Metabolites in Human Plasma

Example: Certification of Creatinine in Human Serum for SRM 967

- Material for SRM 967
  - Level I: unspiked (endogenous) pooled serum from postmenopausal females
  - Level II: spiked pooled serum from postmenopausal females
- Reference Compound
  - SRM 914a creatinine pure compound
- Internal Standard
  - Creatinine-$^{13}C_2$ for ID/GC-MS
  - Creatinine-d_3 for ID/LC-MS
- Methods
  - ID-GC/MS definitive method coupled with ion exchange chromatography
  - ID-LC/MS higher-order RMP coupled with protein precipitation
- Measurement Protocol
  - Duplicate (or single) aliquots from each vial (2 vials for GC/MS, 3 for LC/MS)
  - Control: SRM 909b (serum-based), Levels I and II

Comparison of Sample Preparation Steps



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Example: Creatinine RMP ID/GC-MS, ID/LC-MS

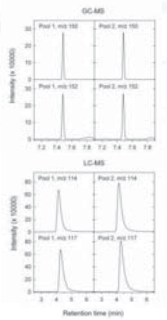

Table 2. Quality control measurements of SRM 909b from the GC-MS and LC-MS sample sets.

Method	Set	Pool 1 (μmol/L)	Pool 2 (μmol/L)
GC-MS	1	55.6	467.0
	2	55.8	468.0
	3	55.4	466.7
Mean		55.6	467.2
SD		0.2	0.7
LC-MS	1	56.1	465.6
	2	55.8	465.9
	3	56.1	467.5
Mean		55.9	466.3
SD		0.4	1.0
Certified SRM value		56.18	467.4
Expanded uncertainty		0.55	5.3
Difference, GC-MS, %		−1.0	−0.04
Difference, LC-MS, %		−0.50	−0.23

Table 3. Calculation of the expanded uncertainty for SRM 967.

Variable	Pool 1, μmol/L	Pool 2, μmol/L
Combined mean, $\bar{c}$	56.53	346.20
Combined variance, σ^2	0.38	1.60
Combined standard uncertainty, u_c	0.91	3.69
Expanded uncertainty, U	1.82	7.37

Dodder et al. Certification of Creatinine in a Human Serum Reference Material by GC-MS and LC-MS, *Clin. Chem. 2007; 53 (9) 1694-1699*

Creatinine RMP

SAMPLE PREPARATION

Weigh serum sample containing creatinine, add known mass of d_3-creatinine stock solution, and equilibrate overnight

Add three volumes relative to total sample volume of ice cold ethanol; vortex; stand for 5 min (precipitate protein)

Centrifuge for 20 min; transfer supernatant; evaporate to dryness

Reconstitute in water; filter, transfer to autosampler vial

CALIBRATION STANDARDS

Prepare primary standard solution by dissolving known mass of SRM 914a Creatinine (purity 99.7 ± 0.3%) in known mass of water

Add constant mass of d_3-creatinine in water to series of tubes and add masses of primary standard solution to the tubes such that the unlabeled/labeled creatinine ratio approximates 1

Creatinine RMP

LC/MS MEASUREMENTS

LC Column: Phenomenex (Torrence, CA); Luna C18 (2); 15 cm x 2.0 mm; 3μm particle size

LC parameters: gradient: isocratic (10 mM aqueous ammonium acetate); 0.200 mL/min; column temperature, 22 °C; injection volume, 5 μL

MS Conditions: Positive-mode electrospray ionization
Single ion monitoring (SIM) monitoring of *m/z* 114 and 117

Quantitation: Relative Response Factor

The relative response factors (RRF) were calculated from calibrant samples according to the equation:

$$RRF = \frac{\text{Area Creatinine}_{\text{Calibrant}} \times \text{Mass Internal Standard}_{\text{Calibrant (μg)}}}{\text{Area Internal Standard}_{\text{Calibrant}} \times \text{Mass Creatinine}_{\text{Calibrant (μg)}}}$$

Mass fractions of creatinine in controls and SRM samples were calculated based on the following equations:

$$\text{Mass Fraction (μg/g)} = \frac{\text{Area Creatinine}_{\text{Sample}} \times \text{Mass Internal Standard}_{\text{Sample (μg)}}}{\text{Area Internal Standard}_{\text{Sample}} \times \text{RRF} \times \text{Mass Sample}_{\text{(g)}}}$$

**Convert to mg/dL using density of the serum*

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SD Project (Current efforts): Development of a Low-Level Creatinine Material Formulated with Synthetic Serum

Johanna Camara

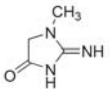
Background

- NIST has been involved with the standardization efforts of the National Kidney Disease Education Program (NKDEP) for many years
- Creatinine is a marker of kidney function
  - Elevated levels indicate that creatinine is not being cleared by the kidneys
- SRM 967a Creatinine in Frozen Human Serum
  - Sells 140 units/year
  - 2 levels of creatinine: adult normal (0.847 mg/dL) and high levels (3.877 mg/dL)
- NIST has Reference Measurement Procedures for creatinine in serum
  - Welch, MJ *et al. Anal. Chem.*, **1986**, *58* (8), pp 1681–1685 (ID-GC-MS)
  - Dodder, NG *et al. Clin. Chem.*, **2007**, 53:9, 1694-1699 (ID-LC-MS)
- NKDEP has voiced concern that the current SRM does not cover the pediatric range (≈0.4 mg/dL)

NIST MATERIAL MEASUREMENT LABORATORY

Current Routine Methods

- Jaffe assay (1886)
  - Serum plus picric acid in alkaline medium changes to orange color measured at 520 nm
  - Cheap, fast, easily automated (≈300 results/hour)
  - Interferences: hemolysis, icteremia, lipemia, ammonium heparin, protein, glucose
- Enzymatic methods
  - Fewer interferences compared to Jaffe (hemoglobin, bilirubin, ascorbic acid)



$$\text{Creatinine} + H_2O \xrightarrow{\text{Creatininase}} \text{Creatine}$$

$$\text{Creatine} + H_2O \xrightarrow{\text{Creatinase}} \text{Sarcosine} + \text{Urea}$$

$$\text{Sarcosine} + H_2O + O_2 \xrightarrow{\text{SOD}} \text{Formaldehyde} + \text{Glycine} + H_2O_2$$

$$2\,H_2O_2 + \text{4-AA} + \text{Toos} \xrightarrow{\text{POD}} 4\,H_2O + \text{Quinone dye } (\lambda \text{ max } 556 \text{ nm})$$

(Diasystems)

NIST MATERIAL MEASUREMENT LABORATORY

Options for Filling the Gap

- Obtaining large volumes of pediatric donor serum is not feasible
- Creatinine remains in charcoal-stripped serum, so it cannot be used to dilute normal serum
- Commercial synthetic serum options offer new choices for dilution and spiking to produce desired levels of clinical analytes in serum

NIST MATERIAL MEASUREMENT LABORATORY

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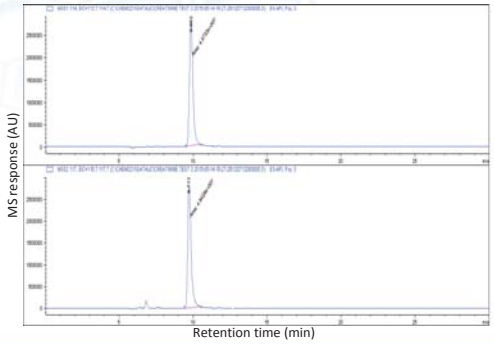
What is Synthetic Serum?

- Mixture of chemicals specifically designed to have physical and chemical characteristics similar to human serum/plasma (pH, density, viscosity, protein, lipid, electrolyte, phospholipids)
  - SeraFlx E
    - Does not contain vitamins, steroids, minerals, hormones, drugs, DNA/RNA, antibodies
    - Designed for analysis of endogenous compounds
    - Contains phospholipid to control for MS ion suppression
  - SeraFlx M
    - Glucose-free
    - Designed for analysis of xenobiotic compounds
  - Pre-Market material from Sigma
    - Unknown content
    - Likely human serum albumin in sodium phosphate buffer

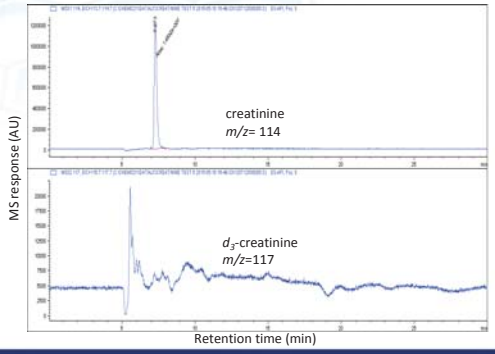
Initial Characterization of Creatinine in Artificial Matrices

- Do these commercially prepared materials contain detectible creatinine and, if so, how much?
- ID-LC-MS analysis
  - SRM 914a Creatinine calibration standard
  - d_3-creatinine internal standard
  - Ice-cold ethanol protein precipitation
  - Centrifugation
  - Dry down under N_2(g)
  - Resuspend in H_2O
  - Filter
  - Analyze by LC-MS

LC-MS of SRM 967a Level 1



LC-MS of SeraFlx-E without Internal Standard



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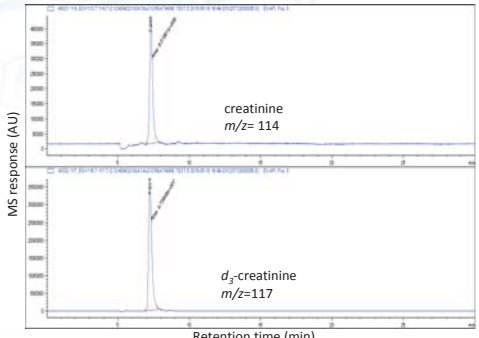
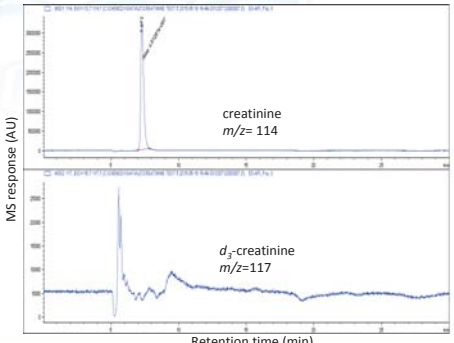
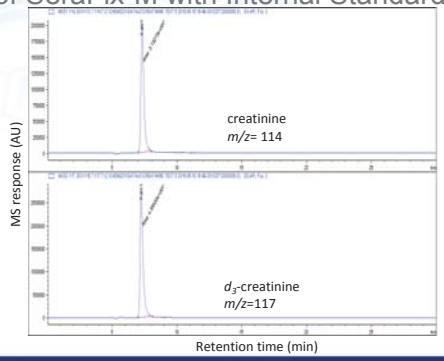
LC-MS of SeraFlx-E with Internal Standard



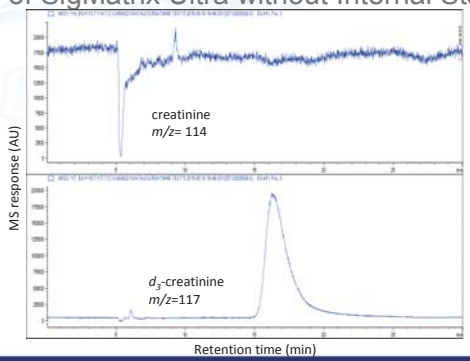
LC-MS of SeraFlx-M without Internal Standard



LC-MS of SeraFlx-M with Internal Standard



LC-MS of SigMatrix Ultra without Internal Standard



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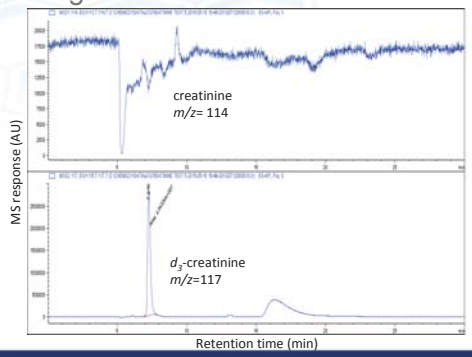
LC-MS of SigMatrix Ultra with Internal Standard



Creatinine Screening Values

Sample	Creatinine (µg/g)
SeraFlx-E	0.50
SeraFlx-M	3.30
SigMatrix Ultra (pre-market)	0.00

Spiked Recovery of Creatinine

- Each material was spiked in batches at several clinically-relevant creatinine levels and equilibrated overnight
- Each batch was split and processed in triplicate by ID-LC-MS
- Recovery was calculated as (measured/expected) x 100 %

Spiked Recovery of Creatinine in Synthetic Serum Matrices

Sample	Target Concentration (µg/g)	Calculated Concentration (µg/g)	Mean Measured Concentration (n=3) (µg/g)	% Recovery
SeraFlx-E 0.5	0.5	0.509	0.509	N/A
SeraFlx-E 4	4	3.969	3.916	99
SeraFlx-E 8	8	9.005	8.419	94
SeraFlx-E 16	16	16.786	16.476	98
SeraFlx-M 3.3	3.3	3.295	3.295	N/A
SeraFlx-M 8	8	4.231	4.454	105
SeraFlx-M 35	35	35.436	36.764	104
SigMatrix Ultra 0	0	0	0	N/A
SigMatrix Ultra 4	4	4.097	4.230	103
SigMatrix Ultra 8	8	8.345	8.681	104
SigMatrix Ultra 32	32	32.788	32.921	100

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Conclusions

- SeraFlx-E, SeraFlx-M, and SigMatrix Ultra remain viable candidate bases or diluents for further study
- SeraFlx-E or SigMatrix Ultra could be spiked with crystalline creatinine to achieve ≈0.4 mg/dL goal level
- SeraFlx-E or SigMatrix Ultra could be used to dilute “normal serum” 50:50 to achieve ≈0.4 mg/dL goal level
- SeraFlx-M may be appropriate “as is” or could be spiked with small amount of crystalline creatinine to achieve ≈0.4 mg/dL goal level

Future Plans

- Requesting additional SD funds for FY2016 to continue project
- Prepare candidate mixtures for routine method/laboratory evaluation
- NKDEP has offered to help facilitate a round robin study of candidate materials
  - Manufacturers and clinical laboratories running routine serum creatinine methods
    - Jaffe assay
    - enzymatic methods
  - Siemens, Beckman, Roche, Abbott, Ortho

- Acknowledgments
  - Dr. Greg Miller (NKDEP)
    - Input into SD proposal
    - Assistance with round robin study
  - Mitzi Rettinger (Cerilliant)
    - Discussion of synthetic serum options
    - Pre-market Sigma material

Certification of Creatinine in Standard Reference Material® 3667 Creatinine in Frozen Human Urine by Liquid Chromatography-Mass Spectrometry

Johanna Camara and Karen W. Phinney

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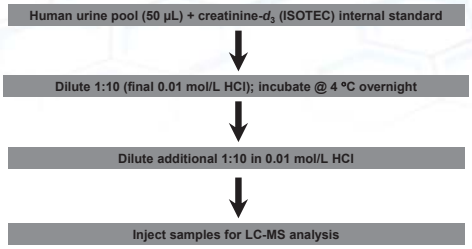
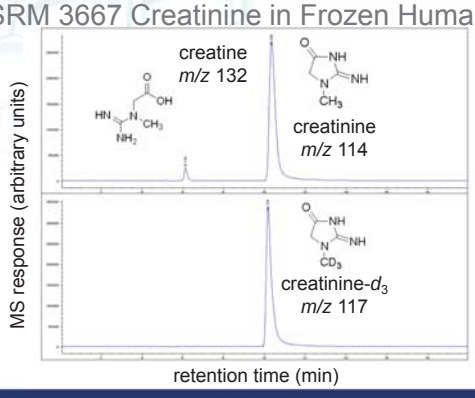
LC-MS of Creatinine in Urine



LC-MS Parameters

- Instrumentation: Agilent 1200 liquid chromatograph coupled with an Agilent 6130 Quadrupole mass spectrometer detector
- Column: Luna C18(2) 25 cm x 4.6 mm, 5 µm particle (Phenomenex)
- Flow rate: 0.5 mL/min at 22.0 ºC
- Mobile phase: 10 mmol/L ammonium acetate in water
- Gradient: Isocratic
- Injection volume: 5 µL
- MS settings: ESI+; capillary, 1.5 kV; gas temperature, 350 ºC; drying gas flow, 12.0 L/min; nebulizer pressure, 50 psig; fragmentor, 90; dwell time, 144 ms
- Single ion monitoring (SIM): creatinine, *m/z* 114 ; creatinine-d_3, *m/z* 117

LC-MS SRM 3667 Creatinine in Frozen Human Urine



Determination of Precision

Day	Mean creatinine (µg/g)	Standard Deviation	% CV
1 (n=36)	613.56	6.51	1.06
2 (n=24)	611.92	1.86	0.30
Overall	612.90	5.21	0.85

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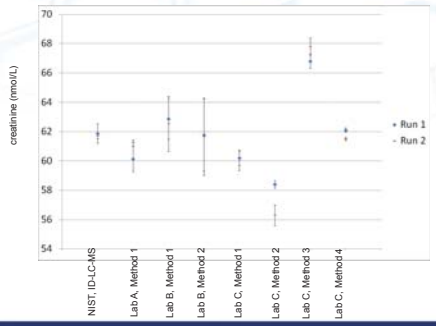
Certified Values for Creatinine in SRM 3667 Creatinine in Frozen Human Urine

Mass fraction (µg/g)	Mass concentration[a] (mg/dL)
613 ± 13	61.8 ± 1.3

[a]Mass concentration was calculated from the mass fraction using the measured urine density, 1.00816 g/mL. The uncertainty in the urine density measurements was incorporated in the value that is reported relative to units of volume.

Interlaboratory Comparison Study of Creatinine in SRM 3667



A Unit of SRM 3667 Creatinine in Frozen Human Urine

Liquid Chromatographic Separation and Mass Spectrometric Challenges

2016 SIM Clinical Measurement Course

Carolyn Burdette, Johanna Camara, Jeanita S. Pritchett, Lane Sander

NIST National Institute of Standards and Technology U.S. Department of Commerce

MATERIAL MEASUREMENT LABORATORY

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Separation by LC vs by MS

- Ideally you would have every analyte physically separated from each other and no coelution with any other compounds. This is not always practical or possible.
- Compounds with the same exact mass and fragmentation (e.g. isotopes) need to be physically separated by LC.
- Compounds with different masses can coelute from the column because the mass spectrometer can detect the different compounds.
- Other compounds found in the matrix can be reduced through sample preparation and chromatographic parameters.

Throughput vs Quality

- Always strive for separation quality and analyte sensitivity first, then focus on reducing analysis time.

Watch and learn

Method Development for Liquid Chromatography

Begin sections… stop by 19:28

Integration

- Chromatographic resolution and/or selective detection is always the best solution!
- *Always* inspect how baselines are set by the data system.
- If appropriate, adjust integration settings and/or manually reintegrate to achieve best estimate of peak area.
- Use peak areas rather than peak heights: peak area does not change with changes in retention.

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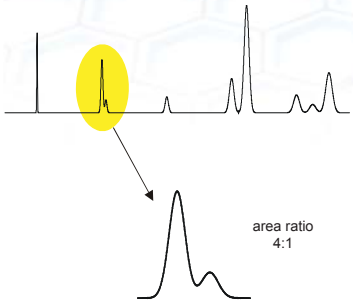
Integration and Baselines

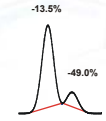


How should these peaks be integrated?

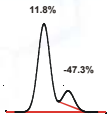
Integration and Baselines

Known area ratio: 4:1

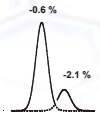
Resolution: 1.0



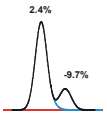
Valley - Baseline



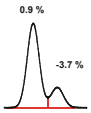
Skimmed Rider



Peak Deconvolution

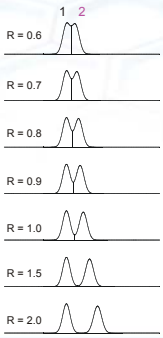


Exponentially Skimmed Rider



Vertical - Baseline

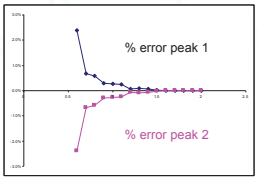
Peak Resolution and Bias



$R_s = \Delta t_r$ / ave peak width

$R_s = [N^{1/2}/2] \cdot [(\alpha-1)/(\alpha+1)] \cdot [k'_{ave}/(1+k_{ave})]$

efficiency selectivity retention



Take home message: If possible, fully separate the constituents in either the chromatographic or detection domains

Watch and learn

Method Development for Liquid Chromatography
19:28 to the end

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Ionization

- After optimization of the MS parameters based on the LC parameters and standard solutions, you need to reevaluate the analysis using a natural matrix, and assess the matrix effects for each analyte.

$$\text{Matrix Factor (MF)} = \frac{\text{Peak response in presence of matrix ions}}{\text{Peak response in mobile phase}}$$

MF = 1	indicates no matrix effects
MF < 1	indicates ion suppression
MF > 1	indicates ion enhancement

Ionization

- Depending on your calibration scheme, you might want to see no matrix effects
- If you are able to have matrix matched calibrants, you will be less effected by ion suppression/enhancement, unless the suppression doesn't allow for quantitation detection of the analyte(s)
  - Sample preparation techniques can be used to reduce the matrix
  - Column chemistry can be changed to reduce coelution compounds

Checking for Potential Interferences

- Blank – Run injection of only the solvent used to resuspend samples look to see if there is any signal for either the isotopically labeled analyte and the unlabeled analyte
- Internal Standard – Look to see if there is any signal for the unlabeled analyte
- Reference Compound – Look to see if there is any signal for the isotopically labeled analyte
- Matrix Blank – Complete sample preparation without adding the internal standard and look to see if there is any signal for the isotopically labeled analyte
- Method Blank – Complete sample preparation without any sample or internal standard and look to see if there is any signal for either the isotopically labeled analyte and the unlabeled analyte

Known Interferences

- Could be an isotope or a similar compound that can not be distinguished be the mass spectrometer
- Use standards to assess the chromatography and make sure the interfering compounds are well resolved

Unknown Interferences

- Could be a compound found naturally in the matrix or produced during the sample preparation that can not be distinguished be the mass spectrometer
- Monitor control material results, peak shape and compound ratios in samples, monitor qualitative ion transitions
- If a level seems abnormally high or abnormally low, and you have time, run the sample using a different column chemistry to remove the coeluting compound
- Adjust the MS parameters and change the ion transition(s) monitored
- If necessary, adjust the sample preparation technique to remove the interference(s)

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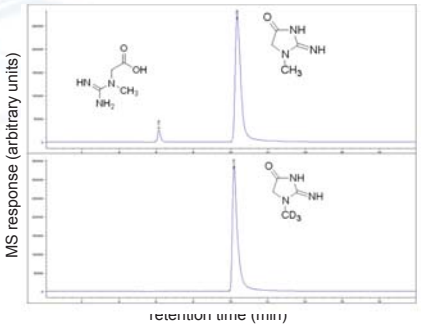
Creatinine Interferences

- Known Interference Example: Creatine
  - For GC-MS, creatine must be separated from the creatinine before derivatization, since the reaction products are the same.
    - Ion-exchange chromatography: The resin was slurry packed into 20 cm × 10 mm columns using water. The volume of resin in each column was 5 mL. Each column was washed with 150 mL water. The samples were added to the columns; each vial was rinsed 3 times. The creatine was eluted with 75 mL water. This fraction was discarded. The creatinine was eluted with 75 mL of 1.0 M NH4OH. This fraction was collected in 24/40 flat-bottomed round-bottom flasks.
  - For LC-MS, creatine can undergo a water loss during ionization, creating a possible interference for the detection of creatinine. The compounds must be separated by LC prior to detection.

Creatinine Interferences

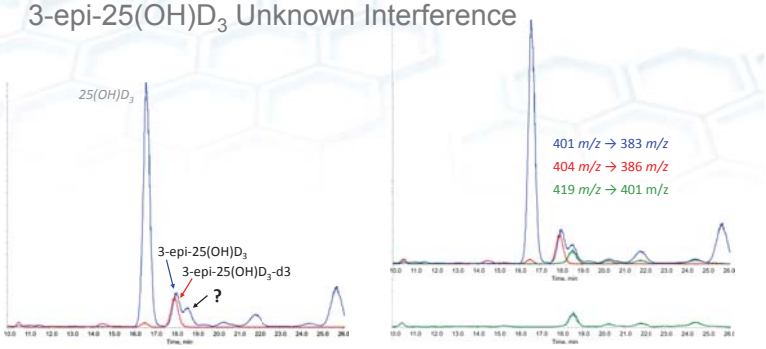
- LC-MS chromatograms of creatinine, creatine, and creatinine-d_3 in diluted SRM 3667 Creatinine in Frozen Human Urine.
- Creatine, a possible MS interferent due to water loss in the MS source, is well separated from creatinine prior to detection.



Vitamin D Interferences

- Known Interference Example: Isotopes, 3-epi-25(OH)D vs 25(OH)D
  - There are studies that show the bioavailability of the isomers are not the same, therefor separate detection of each isomer is important. Currently, only 25(OH)D_3 and 25(OH)D_2 are used in the determination of total 25(OH)D serum levels and the epimers need to be fully separated by the chromatography to remove bias in the quantitation
- Unknown Interference Example: storage/preparation, Blood bag interference
  - During routine analysis of serum samples, an unresolved peak was observed with the 3-epi-25(OH)D_3
  - Through further inspection and high resolution MS analysis, it was determined that the compound was from the collection process of the blood and had another ion transition that could be used to detect its presence.

3-epi-25(OH)D_3 Unknown Interference



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Watch and learn

Trouble shooting LC Instrumentation and Methods

NIST MATERIAL MEASUREMENT LABORATORY

Evaluating and Expressing Measurement Uncertainty

Antonio Possolo

July 22nd, 2016

SIM Clinical Measurement Workshop

NIST

Outline

Outline

References

- A. Possolo (2015) *Simple Guide for Evaluating and Expressing the Uncertainty of NIST Measurement Results*, NIST Technical Note 1900
  http://dx.doi.org/10.6028/NIST.TN.1900
- B. N. Taylor and C. E. Kuyatt (1994) *Guidelines for Evaluating and Expressing the Uncertainty of NIST Measurement Results*, NIST Technical Note 1297
  http://physics.nist.gov/Pubs/guidelines/TN1297/tn1297s.pdf
- T. Lafarge and A. Possolo (2015) The NIST Uncertainty Machine, *NCLSI Measure Journal of Measurement Science*, 10(3): 20–27

Outline

Creatinine in Serum — References

- P. Stokes and G. O'Connor (2003) Development of a liquid chromatography-mass spectrometry method for the high-accuracy determination of creatinine in serum *Journal of Chromatography B* 794: 125–136
- N. G. Dodder, S. S.-C. Tai, L. T. Sniegoski, N. F. Zhang, and M. J. Welch (2007) Certification of creatinine in a human serum reference material by GC-MS and LC-MS *Clinical Chemistry* 53(9): 1694–1699

Creatinine in Serum

$$\text{MassFracCreat} = \frac{\text{AreaCreatSample} \times \text{MassIntStd}}{\text{AreaIntStd} \times \text{RRF} \times \text{MassSample}}$$

INPUTS	
AreaCreatSample	Area Creatinine Sample
MassIntStd	Mass Internal Standard Sample
AreaIntStd	Area Internal Standard Sample
MassSample	Mass Sample

OUTPUT	
MassFracCreat	Mass Fraction Creatinine Sample

Creatinine in Serum — RRF

$$\text{RRF} = \frac{\text{AreaCreatCal} \times \text{MassIntStdCal}}{\text{AreaIntStdCal} \times \text{MassCreatCal}}$$

INPUTS	Value	Std. Unc.	DF
AreaCreatCal	$7.925\,871 \times 10^{+5}$	$4.162\,320 \times 10^{+4}$	7
MassIntStdCal	$3.512\,884$	$2.301\,860 \times 10^{-3}$	3
AreaIntStdCal	$8.218\,838 \times 10^{+5}$	$2.486\,830 \times 10^{+4}$	7
MassCreatCal	$3.558\,677$	$6.817\,934 \times 10^{-2}$	3

OUTPUT	Value / µg/g	Std. Unc. / µg/g	DF
RRF (GUM)	$9.519\,450 \times 10^{-1}$	$6.051\,330 \times 10^{-2}$	11.4
RRF (GUM-S1)	$9.532\,161 \times 10^{-1}$	$7.965\,566 \times 10^{-2}$	11.4

Creatinine in Serum

MEASUREMENT EQUATIONS

$$\text{RRF} = \frac{\text{AreaCreatCal} \times \text{MassIntStdCal}}{\text{AreaIntStdCal} \times \text{MassCreatCal}}$$

$$\text{MassFracCreat} = \frac{\text{AreaCreatSample} \times \text{MassIntStd}}{\text{AreaIntStd} \times \text{RRF} \times \text{MassSample}}$$

RRF = Relative Response Factor

Creatinine in Serum

$$\text{RRF} = \frac{\text{AreaCreatCal} \times \text{MassIntStdCal}}{\text{AreaIntStdCal} \times \text{MassCreatCal}}$$

INPUTS	
AreaCreatCal	Area Creatinine Calibrant
MassIntStdCal	Mass Internal Standard Calibrant
AreaIntStdCal	Area Internal Standard Calibrant
MassCreatCal	Mass Creatinine Calibrant

OUTPUT	
RRF	Relative Response Factor

Relative Response Factor (RRF)

DEGREES OF FREEDOM (1/3)

- Why do we need the degrees of freedom (11.4) for the output quantity (RRF)?
  - Because RRF will play role of input quantity in measurement equation for mass fraction of creatinine in sample, **and** is determined by quantities whose associated uncertainties are qualified by degrees of freedom
- Where do these degrees of freedom come from?
  - Combination of degrees of freedom of inputs that determine value of RRF

Relative Response Factor (RRF)

DEGREES OF FREEDOM (2/3)

- How do we compute these degrees of freedom?
  - Welch-Satterthwaite approximation
    Using standard uncertainties and degrees of freedom associated with input quantities (GUM G.4)

```
require(metRology)
RRF.nu = welch.satterthwaite(
           ui=c(AreaCreatCal.u, MassIntStdCal.u,
                AreaIntStdCal.u, MassCreatCal.u),
           df=c(AreaCreatCal.nu, MassIntStdCal.nu,
                AreaIntStdCal.nu, MassCreatCal.nu))
```

  - Maximum likelihood estimation
    Using very large ($> 10^7$) Monte Carlo sample from distribution of RRF

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Relative Response Factor (RRF)
DEGREES OF FREEDOM (3/3)

- What's the idea behind all this?
  - GUM's evaluation of $u(y)$ relies on approximation $y \approx c_1 x_1 + \cdots + c_n x_n$ for some coefficients $\{c_j\}$
  - If inputs were Gaussian random variables, then so would be the output. If inputs are Student's t random variables, output will *not* be Student's t
  - But a Student's t may still provide a good approximation to y's distribution: need to find "right" number of degrees of freedom for this approximant

Creatinine in Serum — Sample Mass Fraction

$$\text{MassFracCreat} = \frac{\text{AreaCreatSample} \times \text{MassIntStd}}{\text{AreaIntStd} \times \text{RRF} \times \text{MassSample}}$$

INPUTS	Value	Std. Unc.	DF
AreaCreatSample	$6.902\,806 \times 10^{+5}$	$9.646\,364 \times 10^{+3}$	11
MassIntStd	3.531 904	$9.043\,918 \times 10^{-3}$	6
AreaIntStd	$7.643\,268 \times 10^{+5}$	$7.932\,141 \times 10^{+3}$	11
RRF	$9.514\,679 \times 10^{-1}$	$6.051\,330 \times 10^{-2}$	11.4
MassSample	$4.369\,950 \times 10^{-1}$	$2.899\,449 \times 10^{-3}$	5

OUTPUT	Value / µg/g	Std. Unc. / µg/g	DF
MassFracCreat (GUM)	7.667 736	0.508 300	32.1
MassFracCreat (GUM-S1)	7.700 564	0.518 967	9.2

NIST Uncertainty Machine
SPECIAL FEATURES

- Sample drawn from distribution of y can be downloaded for further analysis in other software environments
- Graphical and tabular output also can be downloaded
- Implements novel method to evaluate relative contributions of identified sources of uncertainty, based on Monte Carlo results

  Lafarge & Possolo (2015)

Creatinine in Serum RRF
NIST UNCERTAINTY MACHINE — INPUT

NIST Uncertainty Machine

uncertainty.nist.gov

User's Manual available for download from same page

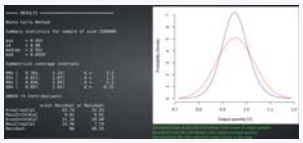
- Applicable to *measurement equations* $y = f(x_1, \ldots, x_n)$ where f is fully specified function and inputs do not depend on output
- Standard uncertainty $u(y)$ evaluated using
  - Gauss's formula (GUM Equation (13))
  - Monte Carlo Method (GUM-S1)

NIST Uncertainty Machine
REQUIRED INPUTS

$y = f(x_1, \ldots, x_n)$

- For each input must specify:
  - Measured value x_j
  - Standard uncertainty $u(x_j)$
  - Probability distribution with mean x_j and standard deviation $u(x_j)$
    - Any additional parameters will also have to be specified (for example, number of degrees of freedom for Student's t)

Creatinine in Serum RRF
NIST UNCERTAINTY MACHINE — OUTPUT (Monte Carlo)

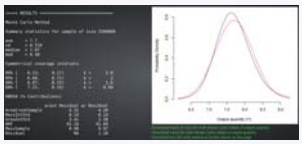


Creatinine in Serum RRF
NIST UNCERTAINTY MACHINE — OUTPUT (Gauss's Formula)

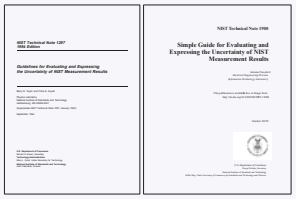
```
ANOVA (% Contributions)

                    w/out Residual  w/ Residual
AreaCreatCal             63.74        32.92
MassIntStdCal             0.01         0.01
AreaIntStdCal            21.26        10.98
MassCreatCal             14.98         7.74
Residual                    NA        48.35

Gauss's Formula (GUM's Linear Approximation)

      y = 8.952
   u(y) = 0.86

                  SensitivityCoeffs Percent.u2
AreaCreatCal               1.2e-06      68.099
MassIntStdCal              2.7e-01       0.011
AreaIntStdCal             -1.2e-06      23.000
MassCreatCal              -2.7e-01       9.100
Correlations                    NA       0.000
```


Creatinine in Serum Mass Fraction
NIST UNCERTAINTY MACHINE — INPUT

Creatinine in Serum Mass Fraction
NIST UNCERTAINTY MACHINE — OUTPUT (Monte Carlo)



NIST Measurement Results



NIST Technical Note 1900

Simple Guide for Evaluating and Expressing the Uncertainty of NIST Measurement Results

Outline

Creatinine in Serum Mass Fraction
NIST UNCERTAINTY MACHINE — OUTPUT (Gauss's Formula)

```
ANOVA (% Contributions)

                    w/out Residual  w/ Residual
AreaCreatSample            4.31         4.26
MassIntStd                 8.14         8.14
AreaIntStd                 2.41         2.38
RRF                       92.16        91.09
MassSample                 0.98         0.97
Residual                    NA          1.16

Gauss's Formula (GUM's Linear Approximation)

      y = 7.67
   u(y) = 0.508

                  SensitivityCoeffs Percent.u2
AreaCreatSample            1.1e-05         4.40
MassIntStd                 2.2e+00         0.15
AreaIntStd                -1.0e-05         2.50
RRF                       -8.1e+00        92.00
MassSample                -1.0e+01         1.00
Correlations                    NA         0.00
```


Outline

Simple Guide
MEASUREMENT

- Measurement is an experimental or computational process that,
- by comparison with a standard,
- produces an estimate of the true value of a property of a material or virtual object or collection of objects, or of a process, event, or series of events,
- together with an evaluation of the uncertainty associated with that estimate, and
- intended for use in support of decision-making

Outline

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Simple Guide — Uncertainty

MEANING

- Uncertainty is the condition of being *uncertain* (unsure, doubtful, not possessing complete or fully reliable knowledge)
  - Also a qualitative or quantitative expression of the degree or extent of such condition

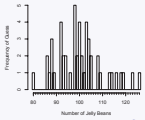
*It is a subjective condition because it pertains to the perception or understanding that **you** have of the object of interest*

Simple Guide — Measurement Uncertainty

- Measurement uncertainty is the doubt about true value of measurand that remains after making a measurement
- Measurement uncertainty is described fully and quantitatively by probability distribution on set of values of measurand

Simple Guide — Probability distribution

- Measurand: Number of jelly beans in the jar
- Frequency distribution of students' estimates
  - Depicts dispersion of measurement results
  - Characterizes students' collective uncertainty



Random Variables

- Random variable is a mathematical model for unknown value of a quantity that has a probability distribution as an attribute
  - All quantities about whose values there is uncertainty can be modeled as random variables
    - Even if the quantity value is fixed (but unknown)
    - Irrespective of whether they relate to *chance* events

Outline

Simple Guide — Probability

PROBABILITY DISTRIBUTION

- Probability distribution is mathematical object that may be visualized by analogy with distribution of mass in region of space

  Oil paint on canvas applied with painter's palette knife
  - Thickness of coating is uneven
  - Total mass of paint represents unit of probability
  - Mass of paint on subset of canvas represents probability of subset
- Probability may be interpreted in any one of many different ways: two common interpretations are
  - Subjective degree of belief (*credence*)
  - Long-run frequency

Probability Distributions & Random Variables

EXAMPLE

EXAMPLE — ATOMIC WEIGHT OF BORON

- Random variable $A_t(B)$ describes atomic weight of boron in sample known to come from one of main commercial sources in US, Turkey, Chile, Argentina, or Russia
- Probability density gives probability of $A_t(B)$'s value being in any given interval as area under curve

Outline

Simple Guide — Procedure (1/4)

MEASUREMENT & MEASUREMENT MODEL

- Define measurand
- Formulate measurement model
  - Measurement equations
    Measurand is function of inputs
  - Observation equations
    Measurand is function of parameters of probability distributions of inputs
    - EXAMPLE Observed rupture stress of alumina coupon has Weibull probability distribution, and expected rupture strength (measurand) is known function of Weibull shape and scale parameters

Simple Guide — Procedure (2/4)

INPUTS

- Observe or estimate values of inputs
- Evaluate associated uncertainties
  - Possolo & Elster (2014) Evaluating the uncertainty of input quantities in measurement models *Metrologia*, 51(3): 339–353
  - Elicitation of expert knowledge (MATCH)

optics.eee.nottingham.ac.uk/match/uncertainty.php

Simple Guide — Procedure (3a/4)

UNCERTAINTY EVALUATION — Types

- Bottom-up (Uncertainty Budget + GUM)
- Top-down (Interlaboratory study)

UNCERTAINTY EVALUATION — Modes

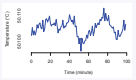
- Measurement Equation — NIST Uncertainty Machine
- Observation Equation — Custom statistical methods

Simple Guide — Measurement Models

- Measurement Equation for temperature t measured using PRT

$$t = t_0 + (R/R_0 - 1)/\alpha$$

- Observation Equation for mean temperature τ of thermal bath, using readings taken every minute during period of 100 min



WHITE NOISE

$\varepsilon_1, \varepsilon_2, \ldots$ independent Gaussian RVs with mean 0 and standard deviation σ

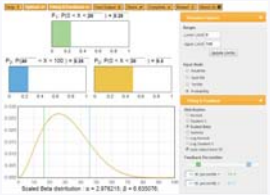
$$t_i = \tau + \varphi_1(t_{i-1} - \tau) + \varphi_2(t_{i-2} - \tau) + \varepsilon_i$$

Uncertainty Elicitation Tool (MATCH)

With 50 % probability, length of part lies between 10.07 mm and 10.15 mm, and otherwise is as likely to be below 10.11 mm as above

Uncertainty Elicitation Tool (MATCH)

Proportion of alite in cement clinker as likely to be below 30 % as above. The other quartiles are 20 % and 40 %



Simple Guide — Procedure (3b/4)

UNCERTAINTY EVALUATION

Measurement Equations

- If inputs and output are scalar quantities, use NIST Uncertainty Machine (NUM, uncertainty.nist.gov)
- If inputs are scalar quantities and output is vectorial quantity, use results of Monte Carlo method produced by NUM using suitable statistical analysis software
- If either output or some inputs are qualitative, use custom version of the Monte Carlo method

Simple Guide — Procedure (3c/4)

UNCERTAINTY EVALUATION

Observation Equations

- Use appropriate statistical method, ideally selected and applied in collaboration with a statistician
  - EXAMPLE

- Data: Rupture stresses of sample of alumina coupons
- Model: Weibull probability distribution
- Measurand: Expected rupture strength
- Statistical Method: Maximum likelihood estimation

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Simple Guide — Procedure (4/4)

- **Measurement Result** Provide estimate of measurand and report evaluation of associated uncertainty:
  - Standard uncertainty (for scalar measurands), or analogous summary of dispersion of values attributable to measurand (for non-scalar measurands)
  - Coverage region Set of possible values of measurand that, with specified probability, is believed to include true value of measurand
  - Probability distribution for value of measurand, characterized either analytically (exactly or approximately) or by suitably large sample

Outline

HCFC 22 — Measurement

- GC-MS applied to air in sample cylinder and to air in lot standard in close temporal proximity produces instrumental indications S for sample and L for lot standard
- Measurement based on ratio $r = S/L$

SAMPLE	RATIO	SAMPLE	RATIO
CC412019	0.9893637	CC416173	0.9655671
CC412019	1.0192335	CC416173	0.9717108
⋯	⋯	⋯	⋯

HCFC 22 — Measurement

CHALLENGE

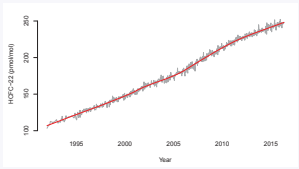
- Translate ratios into amount-of-substance fractions traceable to the International System of Units, and qualify them with evaluation of measurement uncertainty

Halocarbons in Air (SRM 1722)

- Chlorodifluoromethane ($CHClF_2$) HCFC 22 ~ 240 pmol/mol
- Propellant and refrigerant with very high global warming potential (1810 times greater than CO_2) and unacceptably high ozone depletion potential

- 18 cylinders filled with northern continental air at Niwot Ridge, Colorado (NOAA)

Niwot Ridge — NOAA Historical Data



HCFC 22 — Measurement

APPROACH

- Make measurements of calibration standards with amount fractions of HCFC 22 that are traceable to SI and that include range of amounts in SRM
  - Each standard has certified amount fraction of HCFC 22 qualified with statement of measurement uncertainty
- Build analysis function: given instrumental indications for a cylinder in SRM, produces estimate of amount fraction of HCFC 22 in cylinder
- Evaluate uncertainty associated estimate

Calibration & Analysis — Concept

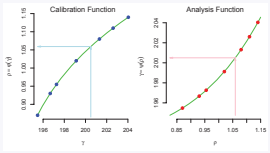
Calibration Data

- Amounts-of-substance fractions of measurand in several standards $c_1, \ldots, c_n$ and associated uncertainties $u(c_1), \ldots, u(c_n)$
- Corresponding instrumental responses $r_1, \ldots, r_n$ and associated uncertainties $u(r_1), \ldots, u(r_n)$

Analysis

- Use calibration data to build *analysis function* that, given instrumental responses, produces estimates of amount-of-substance fractions of measurand in cylinders

Calibration & Analysis — Illustration



- γ True amount-of-substance fraction
- ρ True ratio of instrumental responses
- φ Calibration function
- ψ Analysis function

Errors-in-Variables — Concepts

- Observed ratios for the standards differ from true ratios owing to measurement error: $r_j = \rho_j + \delta_j$
- Measured amount-of-substance fractions for the standards differ from true fractions owing to measurement error: $c_j = \gamma_j + \varepsilon_j$
- To build a model for relationship between ratios $\{r_j\}$ and amount-of-substance fractions $\{c_j\}$ must take into account errors in both variables
- Model for analysis function typically is polynomial of low degree

HCFC 22 — Calibration

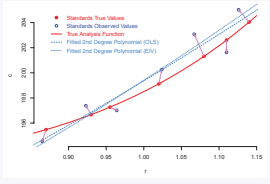
REFERENCES

- ISO 6143:2001 *Gas analysis — Comparison methods for determining and checking the composition of calibration gas mixtures*
- F. R. Guenther and A. Possolo (2011) Calibration and uncertainty assessment for certified reference gas mixtures, *Analytical and Bioanalytical Chemistry* 399: 489–500

HCFC 22 — Calibration Data

STANDARD	r	c pmol/mol	$u(c)$ pmol/mol
FF14687	0.9124537	215.00	0.80
FF14687	0.8951557	215.00	0.80
⋯			
AAL073358	0.9204826	221.50	3.00
AAL073358	0.9209551	221.50	3.00
⋯			
FF4266	0.9638986	231.22	0.60
FF4266	0.9635659	231.22	0.60
⋯			
FF23619	1.0231399	241.80	0.90
FF23619	1.0243111	241.80	0.90
⋯			
FF23624	1.0479179	256.84	0.63
FF23624	1.0736115	256.84	0.63
⋯			

Errors-in-Variables — Illustration



HCFC 22 — Calibration & Analysis

- *Calibration function* φ maps amounts-of-substance fraction (c) into ratios (r) for standards: $r = \varphi(c)$
- *Analysis function* $\psi = \varphi^{-1}$ maps ratios into amounts-of-substance fractions $c = \psi(r)$

Errors-in-Variables — Computation

Calibration Data & EIV Criterion

- Amount-of-substance fractions $\{c_j\}$ and associated uncertainties $\{u(c_j)\}$ for calibration standards
- Corresponding ratios of instrumental indications $\{r_j\}$ and associated uncertainties $\{u(r_j)\}$
- Find ψ that minimizes

$$S(\psi, \rho_1, \ldots, \rho_n) = \sum_{j=1}^{n} \left[\left(\frac{c_j - \psi(\rho_j)}{u(c_j)} \right)^2 + \left(\frac{r_j - \rho_j}{u(r_j)} \right)^2 \right]$$

Example

If analysis function is straight line, $\psi(r) = \beta_0 + \beta_1 r$, and there are $n = 7$ calibration standards, then $S(\psi, \rho_1, \ldots, \rho_n)$ is a function of $2 + n = 9$ variables

Errors-in-Variables — Refinement

- In most of our gas mixture calibrations, standard uncertainties associated with ratios of instrumental indications $\{u(r_j)\}$ are based on rather small numbers of degrees of freedom
- We use version of optimization criterion $S(\psi, \rho_1, \ldots, \rho_n)$ described by Guenther & Possolo (2011), which recognizes this limitation

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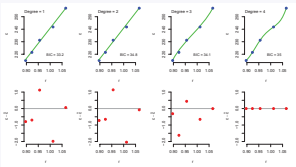
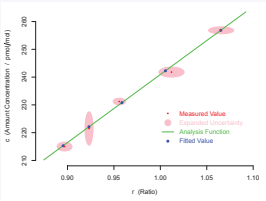
HCFC 22 — Model Selection

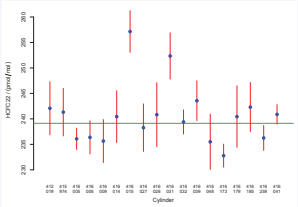


- Rely on graphical examination of residuals and on model selection criterion (BIC)

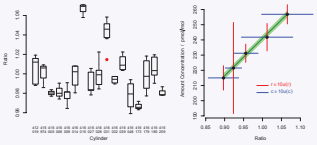
HCFC 22 — Analysis Function



HCFC 22 — Cylinder Values & Expanded Unc.



HCFC 22 — Overview



HCFC 22 — Uncertainty Evaluation

MONTE CARLO METHOD

- Repeat many times:
  - Perturb calibration data to obtain $\{c_j^*\}$, $\{r_j^*\}$, $\{u(r_j^*)\}$
  - Build analysis function ψ^* using perturbed data
  - Use ψ^* to compute amount-of-substance fractions corresponding to sample cylinder ratios

- Summarize results

NIST Consensus Builder

Antonio Possolo

July 22nd, 2016

SIM Clinical Measurement Workshop

NIST

NIST Consensus Builder

Degrees of Freedom

CIPM (2014)

- Uncertainties evaluated at level of one standard uncertainty
- Information must be given on number of effective degrees of freedom required for proper estimation of level of confidence
  - Number of degrees of freedom
    - Conveys reliability of associated evaluation of measurement uncertainty
    - Expresses extent of underlying evidentiary basis

NIST Consensus Builder

Purpose — Consensus Value

- Combine measurement results obtained by different laboratories or by application of different measurement methods, into consensus estimate
- Qualify consensus estimate with evaluation of measurement uncertainty that captures
  - Stated uncertainties associated with individual measured values
  - Additional component of uncertainty uncovered when measured values are intercompared
    ➡ dark uncertainty

Purpose — Degrees of Equivalence

- Differences between measured values and consensus value, and associated expanded uncertainties
  $(D_1, U_{95\,\%}(D_1)), \ldots, (D_n, U_{95\,\%}(D_n))$
- Differences between pairs of measured values, and associated expanded uncertainties
  $(B_{1,2}, U_{95\,\%}(B_{1,2})), \ldots, (B_{n-1,n}, U_{95\,\%}(B_{n-1,n}))$

NIST Consensus Builder — Inputs

- Lab names (REQUIRED)
- Measured values $x_1, \ldots, x_n$ (REQUIRED)
- Standard uncertainties $u_1, \ldots, u_n$ associated with measured values (REQUIRED)
- Numbers of degrees of freedom $\nu_1, \ldots, \nu_n$ standard uncertainties are based on (OPTIONAL)
- Coverage probability (REQUIRED)

Principles

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Principles (1/4)

P1 No measurement result should be set aside except for substantive, documented cause

- Graphical and statistical detection of anomalous results, consistency checks (Cochran's Q), and heterogeneity indices (Higgins & Thompson's I^2) are useful but should be advisory, not decisional

Principles (2/4)

P2 No measured value should dominate consensus value simply because associated measurement uncertainty is much smaller than uncertainties associated with other measured values being combined

Approaches

Prescriptive Approaches CCPR-K2.c-2003

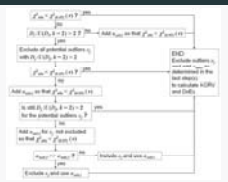


Figure 10-1: Flowchart to visualise procedure for achieving consistency

Principles (3/4)

P3 Participating laboratories and measurement methods should be selected and characterized sufficiently well to warrant belief that measured values, taken as a group, are roughly centered at true value of measurand

Principles (4/4)

P4 Statistical procedure used for data reduction should be determined only after substantive data selection, and exploratory analysis of measurement results

- DerSimonian-Laird
- Hierarchical Bayes
- Linear Pool

Prescriptive Approaches Cox (2002, 2007)

- Procedure A
  - Special case of DerSimonian-Laird
  - NICOB selects it automatically when no heterogenity is detected
- Largest Consistent Subset (LCS)
  - Violates P1
  - Other shortcomings reviewed by Toman & Possolo (2009, 2010: ACQUAL 14, 15)

Model-Based Approaches 1/2

Laboratory Effects Model

- $x_j = \mu + \lambda_j + \varepsilon_j$

  x_j Valued measured by lab $j = 1, \ldots, n$
  μ Measurand
  λ_j Effect of lab j
  ε_j Measurement error for lab j

Model-Based Approaches 2/2

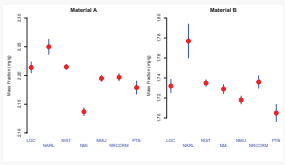
Mixture Model

- $f = \sum_{j=1}^{n} w_j \phi_j$

  f Probability density of measurand
  ϕ_j Probability density for lab j
  w_j Weight of lab j
  - ϕ_j Gaussian or Student's t

CCQM-K6

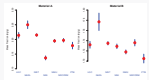
CCQM-K6 Data



- Cholesterol in human serum: samples from two frozen human serum pools

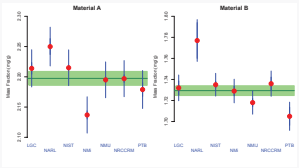
CCQM-K6 Data

- NARL and NMi were set aside in computation of KCRV for Final Report
- NARL because it did not participate in pilot study
- NMi because it was deemed to be an outlier

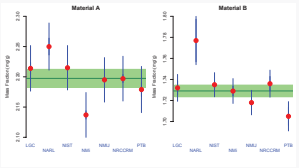


- Both will be included here to illustrate effects of measured values far from the others

CCQM-K6 DerSimonian-Laird Results

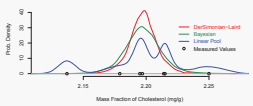


CCQM-K6 Bayes-Gelman Results



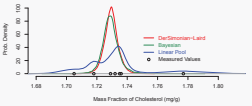
CCQM-K6 — Material A Consensus

PROCEDURE	CONSENSUS	STD. UNC.	EXP. UNC. (95 %)
DerSimonian-Laird	2.1974	0.0115	0.0235
Bayesian	2.1974	0.0149	0.0300
Linear Pool	2.1982	0.0335	0.0663



CCQM-K6 — Material B Consensus

PROCEDURE	CONSENSUS	STD. UNC.	EXP. UNC. (95 %)
DerSimonian-Laird	1.7294	0.0047	0.0095
Bayesian	1.7291	0.0055	0.0112
Linear Pool	1.7332	0.0222	0.0502



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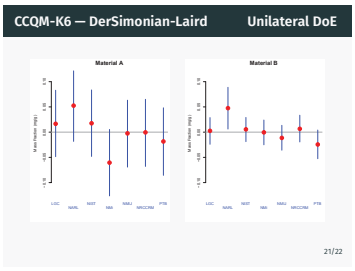

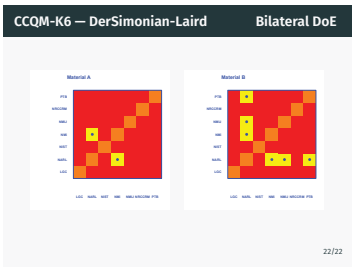

Other biomarkers

2016 SIM Clinical Measurement Course
Jeanice Brown Thomas, Johanna Camara, Susan Tai

NIST National Institute of Standards and Technology U.S. Department of Commerce

MATERIAL MEASUREMENT LABORATORY

Introduction

- In addition to creatinine, cholesterol, and glucose, NIST provides measurement services for other biomarkers
  - Additional classic blood chemistry
    - Urea, uric acid, glycerides
  - Nutritional markers
    - Pyridoxal 5'-phosphate (vitamin B_6), tocopherols, retinol, beta-carotene, 25-hydroxyvitamin D, fatty acids
  - Hormones
    - Cortisol, progesterone, testosterone

Determination of Pyridoxal 5'-Phosphate (PLP) in SRM 3950 Vitamin B_6 in Frozen Human Serum

- Vitamin B_6 status associated with multiple disease states
  - Cardiovascular disease, stroke, hypertension
- PLP is the major circulating form of vitamin B_6 and the most common direct measure of this vitamin in serum or plasma
- Lack of reference materials and methods to allow comparison of multiple measurement methods and capabilities of different laboratories
- NIST developed an ID-LC-MS/MS method for the quantification of PLP in serum in order to assign values to the new SRM 3950 Vitamin B_6 in Frozen Human Serum
- PLP certified values were the result of combining values from NIST ID-LC-MS/MS with Centers for Disease Control and Prevention (CDC) LC-fluorescence values

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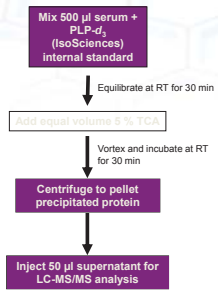
NIST ID-LC-MS/MS Method for PLP



LC-MS/MS Parameters

Instrumentation: Waters 2795 Separations Module liquid chromatograph coupled with a Micromass Quattro Ultima triple-quadrupole mass spectrometer

Column: Halo C18 column (4.6 x 150 mm, 2.7 µm), MAC-MOD Analytical, Inc. (Chadds Ford, PA)

Flow rate: 0.5 ml/min at 25 °C

Mobile phase: 0.4 % acetic acid in water (A) and 0.4 % acetic acid in acetonitrile (B)

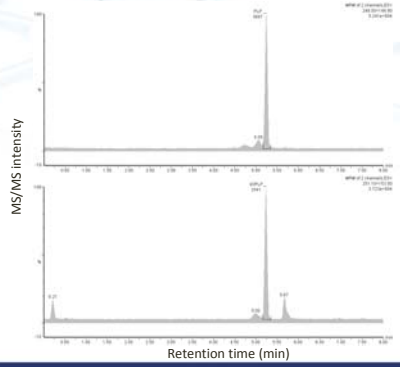
Gradient:

Time (min)	% A	% B
0	100	0
0.2	100	0
6.0	30.5	69.5
6.1	0	100
6.6	0	100
6.7	100	0
8.0	100	0

MS/MS settings: ESI+; capillary, 2.5 kV; cone, 40 V; source temperature, 140 °C; desolvation temperature, 420 °C; cone gas flow, 87 L/hour; desolvation gas flow, 605 L/hour, ion energy, 3.0; entrance, 30; collision, 20; exit, 30

Johanna Camara

ID-LC-MS/MS of SRM 3950



National Institute of Standards & Technology

Certificate of Analysis

Standard Reference Material® 3950

Vitamin B_6 in Frozen Human Serum

Table 1. Certified Concentration Values for Pyridoxal 5'-Phosphate

	Concentrations		
	(ng/g)	(ng/mL)[a]	(nmol/L)[b]
Level 1	4.49 ± 0.15	4.59 ± 0.16	18.6 ± 0.6
Level 2	8.81 ± 0.29	9.00 ± 0.29	36.4 ± 1.2

[a] Mass concentrations were calculated from mean fractions using the following measured serum densities: Level 1, 1.02215 g/mL and Level 2, 1.02138 g/mL. The uncertainty in the serum density measurements was incorporated in values that are reported relative to units of volume.
[b] Molar concentrations were calculated from mass concentrations using the relative molecular mass 247.14 g/mol.

Determination of Folates in Candidate SRM 3949 Folate Vitamers in Frozen Human Serum

- NIST currently offers SRM 1955 Homocysteine and Folate in Frozen Human Serum
  - Certified values for 5-methyltetrahydrofolate (5-mTHF) and reference values for folic acid
  - These values are based on ID-LC-MS/MS methods at both NIST and the CDC
    - Nelson, BC *et al*. Anal Biochem. 2004;325:41-51.
    - Nelson, BC *et al.* Anal Chem. 2005;77:3586-93.
    - Pfeiffer, CM *et al*. Clin Chem. 2004;50:423-32.
- The CDC and the NIH Office of Dietary Supplements have expressed interest in a new folate SRM that reflects currently encountered ranges

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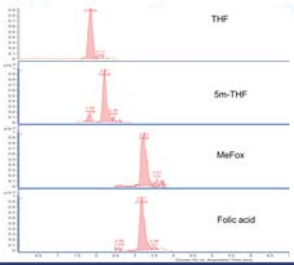
Goals for SRM 3949 Folate Vitamers in Frozen Human Serum

- 3 levels of SRM
- Each level certified for 5-mTHF and folic acid
  - Endogenous concentrations of 5-mTHF and folic acid , not diluted or spiked
- 1 level would also possess reference values for additional folate metabolites
  - Tetrahydrofolate (THF), 5-formyltetrahydrofolate (5-fTHF), 5,10-methenyltetrahydrofolate (5,10-methenylTHF), methyl folinate oxidation product (MeFox)
  - Spiking likely needed to achieve desired levels; these are often undetectable

CDC ID-LC-MS/MS method at NIST

- Fazili Z *et al*. Anal Bioanal Chem. 2013;405:4549-60.
- $^{13}C_5$-labeled folate internal standards are available (Merck & Cie)

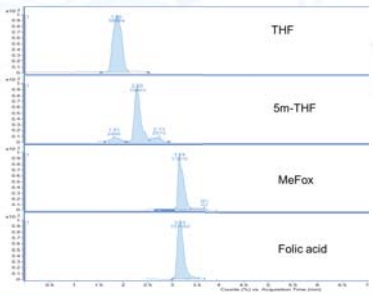
Level 1



Jeanita Pritchett and Yasmine Daniels

CDC ID-LC-MS/MS method at NIST

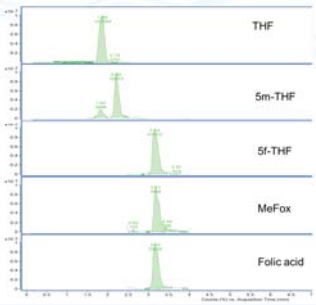
Level 2



Jeanita Pritchett and Yasmine Daniels

CDC ID-LC-MS/MS method at NIST

Level 3



Jeanita Pritchett and Yasmine Daniels

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Current State of Candidate SRM 3949 Folate Vitamers in Frozen Human Serum

- Single-donor serum from 15 donors was screened at the CDC by ID-LC-MS/MS for all 5 folates + MeFox
  - ***Zia Fazili***
- NIST was able to create a blending protocol which should achieve the desired concentrations of 5-mTHF and folic acid in all 3 SRM levels without dilution or spiking
- Some minor folates required spiking to be detectable
- Measurements at NIST and the CDC by ID-LC-MS/MS to obtain final data for certified and reference values

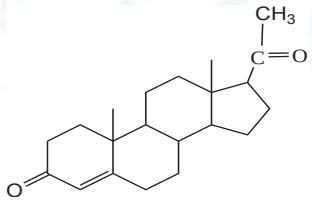
Higher-order RMP for Hormones in Serum and Urine

Analyte	Conc. ng/mL	Method	Reference
T4	50-110	LC-MS LC-MS/MS	Clin Chem 2002, 48, 637 Clin Chem 2005, 51, 161
T3	0.5-2	LC-MS/MS	Anal Chem 2004, 76, 5092 Clin Chem 2005, 51, 2303
Cortisol	30-230	LC-MS LC-MS/MS	Anal Chem 2004, 76, 1008
Estradiol	<0.01-0.35 (F) 0.01-0.04 (M)	LC-MS/MS	Anal Chem 2005, 77, 6359
Progesterone	0.15 to 25 (F) <0.05-0.3 (M)	LC-MS/MS	Anal Chem 2006, 78, 6628
Testosterone	0.2-0.75 (F) 3-10 (M)	LC-MS/MS	Anal Bioanal Chem 2007 388, 1087-1094
Norandrosterone (in urine)	2 (threshold)	LC-MS/MS	Anal Chem 2006, 78, 3393

Higher-order RMPs for Hormones

Analyte	Ref Compound	Internal Standard	Control CRM (serum-based)
T4	CRM (IRMM)	T4-$^{13}C_6$ or T4-d_5	N/A
T3	CRM (IRMM)	T3-$^{13}C_9$	N/A
Cortisol	SRM 921	Cortisol-d_3	IRMM 192, 193, SRM 971
Estradiol	Sigma	Estradiol-d_3	IRMM 576, 577, 578
Progesterone	Sigma	Progesterone-$^{13}C_2$	IRMM 347, SRM 971
Testosterone	CRM (NMIA)	Testosterone-d_3	SRM 971
19-NA	CRM (NMIA)	19-NA-d_4	N/A

Structure of Progesterone



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LC/MS/MS Method for Progesterone in Serum

- Progesterone is light sensitive
  - Prepared standards and samples with minimal exposure to light
  - incandescent light at reduced intensity
- Low concentrations in serum
  - Female serum:
    - ~ 0.15 to ~ 25 ng/mL (non-pregnancy)
    - ~ up to 230 ng/mL (pregnancy)
  - Male serum:
    - <0.05 to 0.3 ng/mL
- LC/MS/MS
  - Strong product ions from transitions at *m/z* 315 → 97 and m/z 317 → 99

Reference Compound and Internal Standard

Reference compound: Sigma lot # 065k0171

Direct method

qNMR: 99.6% [98.09, 99.98] (U_{95})

Indirect method

GC-FID: 99.7% ± 0.5%

DSC: 99.7% ± 0.09%

TGA: <0.01%

Karl Fischer: 0.024% ± 0.018%

Internal Standard : Progesterone-$^{13}C_2$

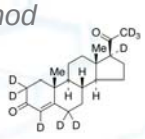
CCQM-K63b
Summary of Methods for Progesterone

		Primary Standard		
Lab	Method	Supplier	Purity assessed by	Internal Standard
CENAM	GC/MS	Sigma	Self	Cortisol-d_3
KRISS	ID-LC/MS/MS	Sigma #065k0171	NIST	Progesterone-$^{13}C_2$
LGC	ID-LC/MS/MS	Candidate LGC RM 1891	Self	Progesterone-$^{13}C_2$
NIM	ID-GC/MS	Dr. Ehrenstorfer, Germany	Supplier	Progesterone-$^{13}C_2$
NIST	ID-LC/MS/MS	Sigma #065k0171	Self	Progesterone-$^{13}C_2$
NMIA	ID-GC/MS & ID-LC/MS/MS	Dr. Ehrenstorfer, Germany	Self	Progesterone-$^{13}C_2$
NMIJ	ID-GC/MS	NMIJ-purified material	Self	Progesterone-$^{13}C_2$
PTB	ID-GC/MS	Sigma # 065k0171	Supplier	Progesterone-$^{13}C_2$

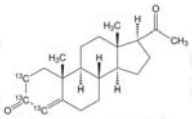
ID/MS as a *Primary Method*

Considerations when implementing isotope dilution:

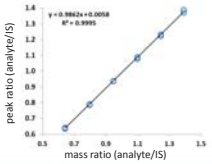
- Natural isotope effects
- Choice of labeled internal standard (d vs ^{13}C)
- Non-equilibration
- Chemical impurities of internal standards
- Instrument calibration errors

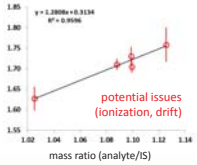


Progesterone-d_9
subject to
intramolecular rearrangement



Progesterone-$^{13}C_3$





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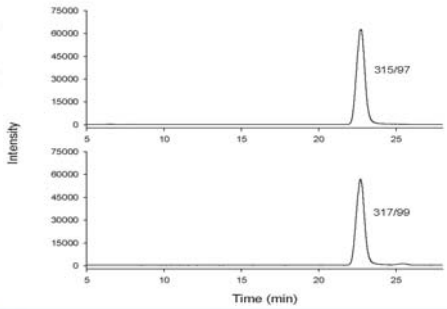
Higher-order RMP for Progesterone in Serum by LC-MS/MS

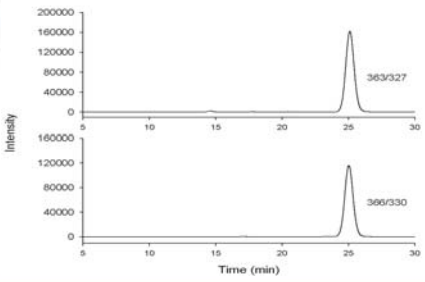
- Isotope dilution using progesterone-$^{13}C_2$
- Equilibration at room temperature for 1 h
- pH adjusted to 9.8, then liquid/liquid (hexane) extraction to isolate progesterone from serum matrix. Absolute recovery of progesterone averaged 89 %
- LC/MS/MS analysis for progesterone
  - Instrument: Applied Biosystems API 4000.
  - Monitor the transitions at *m/z* 315 → 97 and *m/z* 317 → 99
  - LC column: Zorbax Eclipse XDB-C18 column (3.5 µm)
  - Mobile phase: water-acetonitrile with acetic acid
  - LOD (S/N ~ 3): 1.8 pg

S. Tai, B. Xue, and M. Welch. Anal. Chem. 2006, 78, 6628-6633.

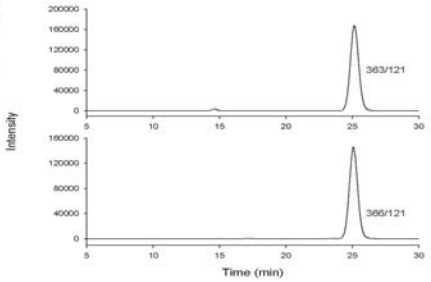
LC-MS/MS Chromatograms for Progesterone Material A: Female Serum



LC-MS/MS Chromatograms for Cortisol Transitions of 363/327 and 366/330



LC-MS/MS Chromatograms for Cortisol Transitions of 363/121 and 366/121



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The NIST Micronutrients Measurement Quality Assurance Program (MMQAP)

- Program was coordinated in 1984 by NIST Analytical Chemistry Division and the National Cancer Institute (NCI) Division of Cancer Prevention and Control for laboratories that measure fat- and water-soluble vitamins and carotenoid compounds in serum/plasma
- Program was needed to ensure the long-term reliability of the measurements made while studying the possible cancer chemoprevention role of these analytes

Program Scope

NIST Provides:

- Tools for Comparability Assessment
  - Interlaboratory Comparison Exercises
    - Frozen or freeze-dried sera are sent to laboratories for analysis.
    - Results are subsequently returned to NIST for data tabulation and evaluation
    - NIST sends feedback to laboratories evaluating results and performance
  - Standard Reference Materials (SRMs) and Control Materials
    - Are used to help validate methods and for quality assurance when assigning values to in-house control materials
    - Play a significant role in helping to improve inter-laboratory precision
  - Performance Database
    - Is maintained over time to help laboratories monitor their measurement repeatability and comparability.
- Methods Development
  - NIST liquid chromatographic (LC) methods
  - Various LC methods used by QA participants
- Workshops and Tutorials
- Site Visits and Consultations

Why Do Laboratories Participate in the MMQAP?

Laboratories participate in the QA program to:

- Improve measurement comparability.
- Obtain reliable data needed to make accurate clinical and health-care decisions

Program Focus Areas

- *Existing Intercomparison Studies:*
  - Fat-soluble vitamins, carotenoids, and other micronutrients in serum
- *Past (no Intercomparison Studies conducted):*
  - Ascorbic acid in serum and plasma
  - Catechins in green tea and serum
  - Selenomethionine in serum and plasma
  - Vitamin K_1 in serum and plasma
  - Difluoromethylornithine in serum and plasma
  - Oltipraz in serum and plasma
  - 4-(Hydroxyphenyl) retinamide in serum and plasma
  - Glycyrrhetinic acid in serum and plasma

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Interlaboratory Comparison Studies are Currently Directed Toward the Measurement of:

Core analytes reported: Retinol, α-tocopherol, γ/β-tocopherol, *trans*- and total β-carotene, and ascorbic acid

Additional analytes reported: Retinyl palmitate, δ-tocopherol, total *cis*-β-carotene, *trans*- and total α-carotene, *trans*- and total lycopene, total α- and β-cryptoxanthin, total lutein, total zeaxanthin, coenzyme Q_{10}, phylloquinone, and 25-hydroxyvitamin D

Reference Measurement Procedures and JCTLM

2016 SIM Clinical Measurement Course

Jeanita S. Pritchett, Susan Tai, and Ashley Beasley Green

NIST National Institute of Standards and Technology U.S. Department of Commerce

MATERIAL MEASUREMENT LABORATORY

Standardization of Clinical Measurements

- Clinical measurements provide medical information for patient care
  - Health care providers use clinical measurements to make medical decisions for patients
- Therefore, high accuracy and standardization of clinical analytes is imperative for high quality medical practice
- Significant step toward achieving high quality and traceable measurements is via reference measurement procedures and reference materials
- Joint Committee for Traceability in Laboratory Medicine (JCTLM)
  - Plays a significant role in the standardization and global harmonization of clinical analytes
  - Establishes a database of available higher-order reference materials, available higher-order reference measurement procedures and reference measurement laboratories for laboratory medicine

Clin Biochem Rev. 2007 Aug; 28(3): 105–114.

Joint Committee on Traceability in Laboratory Medicine (JCTLM)

JCTLM

- International consortium established in 2002 and sponsored by the **International Bureau of Weights and Measures (BIPM)**, the **International Federation for Clinical Chemistry and Laboratory Medicine (IFCC)** and the **International Laboratory Accreditation Cooperation (ILAC)**
- JCTLM developed and maintains database of:
  - Available **Certified Reference materials (CRMs)** and **Reference Measurement Procedures (RMPs)** that can be used by manufacturers to meet the traceability requirements of the EC Directive
  - Laboratories worldwide providing **Reference Measurement Services (RMSs)** for the value-assignment of calibrators for the in vitro diagnostic (IVD) industry

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New Regulatory Requirement: EU IVD Directive

A New Driver:
EU IVD Directive went into effect in 2003
It affects U.S. IVD industry that exports to EU

Stated Purpose of Directive
- Eliminate trade barriers *within Europe* by ensuring access to the entire EU market with one single product approval (CE Mark)

Essential Requirements
- IVD Calibrators and/or control materials must be traceable to *"standards of a higher order"*
  - –nationally/internationally recognized *certified reference materials*

This Directive recognizes the importance of certified reference materials in reducing inter- and intra-laboratory variability

Joint Committee on Traceability in Laboratory Medicine (JCTLM)

- **JCTLM-Working Group (WG) on Traceability: Education and Promotion**
  - Purpose: To produce and promote educational materials to demonstrate the value of traceability in laboratory medicine as a means to reduce between method variability in the interests of improved clinical outcomes and patient safety
- **JCTLM-Working Group 1 (WG1): Reference Materials and Reference Procedures**
  - Purpose: To establish a process for identifying, reviewing against agreed criteria, and publishing, list(s) of Higher Order Certified Reference Materials and Reference Measurement Procedures required for industry compliance with the EC IVDD
- **JCTLM-Working Group 2 (WG2): Reference Measurement Laboratories**
  - Purpose: To establish criteria and processes for listing reference measurement services of laboratories

JCTLM Review Process

- Review teams of experts review CRMs, RMPs and RMSs for entry into database for the following analyte categories:
  - Blood gases
  - Coagulation factors
  - Electrolytes
  - Metabolites and substrates
  - Non-electrolyte metals
  - Nucleic acids
  - Vitamins and micronutrients
  - Blood grouping
  - Drugs
  - Enzymes
  - Microbial serology
  - Non-peptide hormones
  - Proteins

Relevant ISO Standards for Higher-Order CRMs and RMPs

- **ISO 17511:** *In vitro* diagnostic medical devices -- Measurement of quantities in biological samples -- Metrological traceability of values assigned to calibrators and control materials
- **ISO 15193**: *In vitro* diagnostic medical devices -- Measurement of quantities in samples of biological origin -- Requirements for content and presentation of reference measurement procedures
- **ISO 15194**: *In vitro* diagnostic medical devices -- Measurement of quantities in samples of biological origin -- Requirements for certified reference materials and the content of supporting documentation
- **ISO 15195**: Laboratory medicine -- Requirements for reference measurement laboratories
- **ISO 18153**: *In vitro* diagnostic medical devices -- Measurement of quantities in biological samples -- Metrological traceability of values for catalytic concentration of enzymes assigned calibrators and control materials

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Definitions of Reference Materials

- **Reference Material (RM):** material, sufficiently homogeneous and stable with reference to specified properties, which has been established to be fit for its intended use in measurement or in examination of nominal properties [VIM:1993, 5.13]
- **Certified Reference Material (CRM)**: reference material, accompanied by documentation issued by an authoritative body and providing one or more specified property values with associated uncertainties and traceabilities, using valid procedures [VIM:1993,5.14]
- **Standard Reference Material (SRM)**: ***Certified Reference Material (CRM)*** issued by the National Institute of Standards and Technology (NIST)
  - Homogeneous, stable material well-characterized for one or more chemical and/or physical properties
  - Assist laboratories worldwide in validating analytical measurements of chemical composition

NIST Clinical Diagnostic SRMs

- **For calibration/traceability**
  - **17 Pure, Crystalline Standards**
    - SRM 911c Cholesterol
    - SRM 914a Creatinine
    - SRM 918b Potassium Chloride (Clinical)
  - **2 Solutions (ethanol)**
    - SRM 2972 25-Hydroxyvitamin D2 and D3 Calibration Solutions
    - SRM 2972a Vitamin D Calibration Solutions
- **For method validation (to improve accuracy and comparability)**
  - **20 Serum/Plasma Materials**
    - SRM 1951c Lipids in Frozen Human Serum
    - SRM 1955 Homocysteine and Folate in Frozen Human Serum
    - SRM 956c Electrolytes in Frozen Human Serum
  - **8 Urine Material**
    - SRM 3668 Mercury, Perchlorate, and Iodide in Frozen Human Urine
    - SRM 3667 Creatinine in Frozen Human Urine

SRM 2972 25-Hydroxyvitamin D2 and D3 Calibration Solutions

SRM 972a Vitamin D Metabolites in Frozen Human Serum

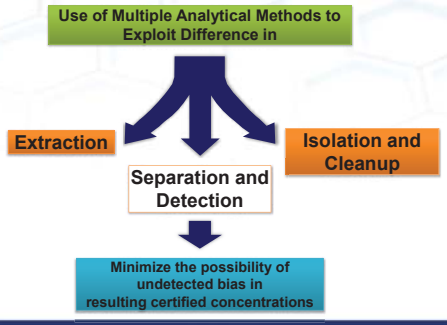
SRM 2669 Arsenic Species in Frozen Human Urine

Modes Used at NIST for Value-Assignment of Reference Materials for Chemical Composition

NIST Special Publication 260-136:
Definition of Terms and Modes used at NIST for Value-Assignment of Reference Materials for Chemical Measurements

	Certified Value	Reference Value	Information Value
1. Certification at NIST Using a Primary Method (Definitive Method or Reference Measurement Procedure) with Confirmation by Other Methods	✓		
2. Certification at NIST Using Two Independent Critically-Evaluated Methods	✓	✓	
3. Certification/Value-Assignment Using One Method at NIST and Different Methods by Outside Collaborating Laboratories	✓	✓	✓
4. Value-Assignment Based On Measurements by Two or More Laboratories Using Different Methods in Collaboration with NIST		✓	✓
5. Value-Assignment Based on a Method-Specific Protocol		✓	✓
6. Value-Assignment Based on NIST Measurements Using a Single Method or Measurements by an Outside Collaborating Laboratory Using a Single Method		✓	✓
7. Value-Assignment Based on Selected Data from Interlaboratory Studies		✓	

Independent Analytical Methods Approach for Certification of Organic Constituents in SRMs



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Certification of Creatinine in Human Serum for SRM 967

Material for SRM 967
- L1: unspiked pooled serum from postmenopausal females
- L2: spiked pooled serum from postmenopausal females

Reference Compound
- SRM 914a creatinine pure compound

Internal Standard
- Creatinine-$^{13}C_2$ for ID/GC-MS
- Creatinine-d_3 for ID/LC-MS

Methods
- ID-GC/MS definitive method coupled with ion exchange chromatography
- ID-LC/MS higher-order RMP coupled with protein precipitation

Measurement Protocol
- Three sets on 3 separate days
- Duplicate aliquots from each vial (2 vials for GC/MS, 3 for LC/MS)
- Control: SRM 909b (serum-based), Levels I and II

Certification of Creatinine in Human Serum for SRM 967
SRM 909b as controls

Method	Set	SRM 909b Level I (mg/dL)	SRM 909b Level II (mg/dL)
GC/MS	1	0.629	5.282
	2	0.631	5.294
	3	0.627	5.279
	Mean ± SD	0.629 ± 0.002	5.285 ± 0.008
LC/MS	1	0.635	5.267
	2	0.627	5.270
	3	0.635	5.289
	Mean ± SD	0.632 ± 0.005	5.275 ± 0.012
Certified Value[1]		0.6355 ± 0.0062	5.287 ± 0.060
% Difference, GC/MS		1.0	0.03
% Difference, LC/MS		0.50	0.22

[1] The uncertainty of the certified value is given as the expanded uncertainty.

Reference: Clin Chem 53(9), 1694-1699 (2007).

VIM Definition of a Reference Measurement Procedure

"Measurement procedure accepted as providing measurement results **fit for their intended use** in assessing measurement trueness of measured quantity values obtained from other measurement procedures for **quantities of the same kind**, in calibration, or in characterizing reference materials" [VIM:1993, 2.7]

- **In simpler terms, a RMP is a measurement procedure which:**
  - Provides measurements which have been thoroughly assessed for bias
  - Has been validated to measure what it is intended to measure
  - Provides the results that we need

Uses of Reference Measurement Procedures (RMPs)

- Value-assignment of certified reference materials (CRMs)
- Comparison of routine assays
- Assessment of the performance characteristics of routine assay systems (instrumentation and reagents)
- Detection of analytical biases on quantities in routine samples

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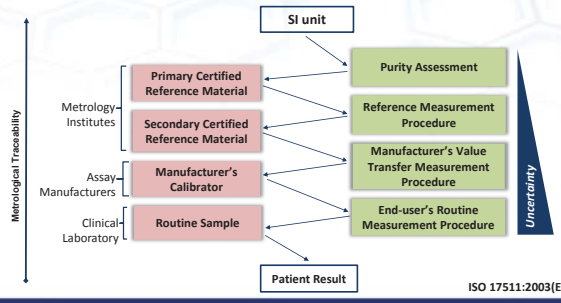
The Role of RMPs in Measurement Traceability



ISO 17511:2003(E)

Higher-order RMP Recognized by JCTLM

- **Accuracy**
  - Purity assessment of primary standard
  - Recovery of added analyte
  - Comparison with certified value of CRM
- **Repeatability (3 independent sets)**
  - Within-set CV
  - Between-set CV
- **Interference Testing**
  - Structural analogues of metabolites
  - Structure analogues of synthetic compounds
- **Uncertainty Estimation**
- **Inter-laboratory Comparison**

Reference: ISO 15193

Elements of a Reference Measurement Procedure

ISO Guide 15193:2009
In vitro diagnostic medical devices Measurement of quantities in samples of biological origin Requirements for content and presentation of reference measurement procedures

Element	Type	Subclause in this International Standard
Title page	Mandatory	—
Contents list	Optional	—
Foreword	Optional	—
Warning and safety precautions	Mandatory	4.2
Introduction	Optional	4.3
Title of reference measurement procedure	Mandatory	—
Scope	Mandatory	4.4
Normative references	Optional	—
Terms, definitions, symbols, and abbreviated terms	Optional	4.5
Measurement principle and method	Mandatory	4.6
Check list	Optional	4.7
Reagents	Mandatory	4.8
Apparatus	Mandatory	4.9
Sampling and sample	Mandatory	4.10
Preparation of measuring system and analytical portion	Mandatory	4.11
Operation of measuring system	Mandatory	4.12
Data processing	Mandatory	4.13
Analytical reliability	Mandatory	4.14
Special cases	Optional	4.15
Validation by inter-laboratory comparisons	Mandatory	4.16
Reporting	Mandatory	4.17
Quality assurance	Mandatory	4.18
Bibliography (Annex)	Optional	4.19
Dates of authorization and revision	Mandatory	4.20

Elements of a Reference Measurement Procedure *[ISO 15193:2009]*

Mandatory Descriptive Elements for RMPs:

- Title
- Forward
- Warning and safety precautions
- Scope
  - *Type of materials to which the RMP will be applied*
  - *Objective of the RMP*
  - *Limits for values*
  - *Interferences*
- Reagents (description and use)
- Apparatus (description, preparation and use)
- Principle and method of measurement
- Sampling and Samples:
  - *Pre-analytical factors that influence measurement*
  - *Sample storage*
  - *Sample preparation*
- Data processing
- Analytical performance
- Inter-laboratory validation
- References
- Dates of authorization and revision

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JCTLM-Approved NIST Reference Measurement Procedures

59 RMPs in Total
28 RMPs in Organic

- **Toxic elements in whole blood/serum/urine:**
  - RNAA/INAA (As, Cd, Co)
  - ID/ICP-MS (Cd, Pb, Hg)
- **Nutritional elements/electrolytes in whole blood/serum:**
  - RNAA/INAA (Cu, Zn)
  - AA (Ca)
  - FAAS/FAES (Li, K, Na)
  - ID/ICP-MS (Ca, Mg, K, Na)
  - ID/TIMS (Ca, Cl, Li, Mg, K)
  - Coulometric titration (Cl)
  - Gravimetry (Na)
- **Metabolites/biomarkers in serum:**
  - ID/GC-MS (creatinine, glucose, homocysteine, total cholesterol, total glycerides, triglycerides, urea, uric acid)
  - ID/LC-MS (creatinine, homocysteine)
  - ID/LC-MS/MS (homocysteine)
- **Antiepileptic drugs in serum:**
  - ID/LC-MS/MS (lamotrigine, phenobarbital, phenytoin, topiramate)
- **Thyroid and steroid hormones in serum:**
  - ID/LC-MS (cortisol, total thyroxine)
  - ID/LC-MS/MS (cortisol, 17b-estradiol, norandrosterone, progesterone, testosterone, total triiodothyronine)
- **Vitamin metabolites in serum:**
  - ID/LC-MS (methyltetrahydrofolic acid)
  - ID/LC-MS/MS (folic acid, 25-hydroxyvitamin D2, 25-hydroxyvitamin D3, methyltetrahydrofolic acid)

Challenges of Designing Pooled and Spiked Samples

2016 SIM Clinical Measurement Course

Jeanice Brown Thomas, Johanna Camara, David Duewer, Margaret Kline

NIST National Institute of Standards and Technology U.S. Department of Commerce

MATERIAL MEASUREMENT LABORATORY

General Points

- Native levels of analytes achieved through pooling are generally preferred to material altered through spiking or dilution
  - Homogeneity, commutability
- Spiking has been acceptable for specific analytes; verified by study
- Some native levels can be achieved through choosing correct donors
  - i.e., hormone levels differing in male versus female serum
- Endogenous levels of some nutritional analytes can be affecting by specifying that donors receive certain foods/dietary supplements prior to sample collection

SRM 3949 Folate Vitamers in Frozen Human Serum

- NIST originally provided SRM 1955 Homocysteine and Folate in Frozen Human Serum
  - Input from experts at the U.S. Centers for Disease Control and Prevention (CDC) indicated a new material should reflect the elevated levels of folic acid and 5-methyltetrahydrofolate (5-mTHF) they were observing in U.S. population samples
  - CDC also indicated the need for a material which was value assigned for additional minor folates, which can be measured by LC-MS/MS and contribute to total folate calculations
- The new SRM 3949 contains 3 levels of folic acid and 5-mTHF; one level was also supplemented for some minor folates (often below detection in real samples)
- The high level for folic acid and 5-mTHF was achieved by supplementing 5 donors with 400 µg folic acid 1 hour prior to blood draw
- Folate values were assigned by combining data from CDC ID/LC-MS/MS and NIST ID/LC-MS/MS

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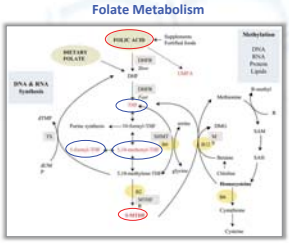
Example: SRM 3949 Folate Vitamers in Frozen Human Serum

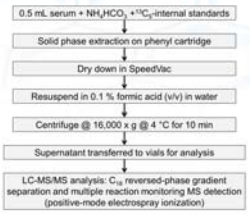
- New folate SRM that reflects current clinical-relevant ranges
- 3 levels of SRM with each level certified for 5-methyltetrahydrofolate (5-mTHF) and pteroylglutamic acid (PGA), or folic acid
  - Endogenous concentrations of 5-mTHF and folic acid, not diluted or spiked
- 1 level would also possess reference values for additional folate metabolites
  - Tetrahydrofolate (THF), 5-formyltetrahydrofolate (5-fTHF), 5,10-methenyltetrahydrofolate (5,10-methenylTHF), methyl folate oxidation product (MeFox)
  - Spiking likely needed to achieve desired levels; these are often undetectable

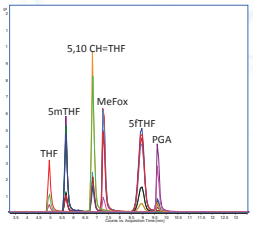


Partnered with the Centers for Disease Control (CDC)

Technical Contacts: *Johanna Camara, Mark Lowenthal, Jeanita Pritchett*

Example: NIST-CDC Folate ID/LC-MS/MS Method





- ID/LC-MS/MS Parameters:
  - Agilent 1290 LC coupled to an Agilent 6460 triple quad
  - Column: Zorbax SB-C18 (2.1 x 150 mm; 3.5 µm particle size); 35 ºC
  - Mobile Phase: (A) 0.1 % formic acid in H_2O (B) 0.1 % formic acid in ACN; gradient separation
  - $^{13}C_5$-labeled folate internal standards are available (Merck & Cie)

Serum profiles from 15 donors (ID/LC-MS/MS)

Donor	PGA* (nmol/L)	5-mTHF* (nmol/L)	THF* (nmol/L)	5-fTHF (nmol/L)	5,10-methenylTHF* (nmol/L)	MeFox* (nmol/L)
A	1.8 ± 0.4	22 ± 1.3	0.72 ± 0.3	<LOD	<LOD	0.32 ± 0.0
B	1.0 ± 0.2	33 ± 0.5	0.57 ± 0.1	<LOD	<LOD	2.98 ± 0.0
C	4.1 ± 0.3	16 ± 1.1	0.38 ± 0.1	<LOD	<LOD	1.70 ± 0.0
D*	14.1± 2.0	36 ± 1.3	1.27 ± 0.3	<LOD	<LOD	0.39 ± 0.0
E	0.6 ± 0.2	8 ± 0.6	0.59 ± 0.3	<LOD	<LOD	0.80 ± 0.1
F*	21.3 ± 3.0	72 ± 3.8	2.23 ± 0.3	<LOD	0.6 ± 0.1	0.53 ± 0.0
G*	1.6 ± 0.4	16 ± 2.1	0.50 ± 0.1	<LOD	<LOD	0.50 ± 0.1
H	1.8 ±0.5	26 ± 1.0	0.86 ± 0.5	<LOD	<LOD	1.53 ± 0.1
I	1.0 ± 0.3	27 ± 1.2	0.76 ± 0.1	<LOD	<LOD	0.67 ± 0.0
J	2.2 ± 0.5	46 ± 2.0	0.86 ± 0.3	<LOD	<LOD	3.22 ± 0.2
K	0.4 ± 0.2	5 ± 1.4	0.25 ± 0.1	<LOD	<LOD	0.13 ± 0.1
L	1.2 ± 0.2	32 ± 2.9	0.92 ± 0.0	<LOD	<LOD	1.49 ± 0.1
M	0.7 ± 0.1	17 ± 2.9	0.42 ± 0.0	<LOD	<LOD	1.43 ± 0.3
N*	11.6 ± 0.4	39 ± 1.6	0.37 ± 0.2	<LOD	<LOD	1.29 ± 0.1
O*	32.3 ± 2.7	35 ± 0.4	1.70 ± 0.3	<LOD	0.3 ± 0.1	2.74 ± 0.0

* Donor supplemented with 400 µg folic acid 1 hour prior to blood draw

SRM blending protocol to achieve targets

NIST uses contract clinical research laboratory for the collection and pooling of sera

Level		Target concentration (nmol/L) PGA	5-mTHF	THF	MeFox
1		1 ± 0.5	10 ± 5	na	na
		10 ± 4	50 ± 5	na	na
		5 ± 3	30 ± 5	5 ± 3	5 ± 3

Level	Combine donors	Theoretical concentration (nmol/L) PGA	5-mTHF	THF	MeFox
1	C, E, G, K, M	1.5	13.2	na	na
2	D, F, J, L , N	9.4	44.55	na	na
3	A, B, H, I, O	6.7	28.1	0.9	1.6

Level		CDC ID/LC-MS/MS concentration (nmol/L) PGA	5-mTHF	THF	MeFox
1		1.6 ± 0.1	16.0 ± 0.3	na	na
2		9.19 ± 0.5	49.2 ± 2.2	na	na
3		6.50 ± 0.45	32.7 ± 1.16	0.62 ± 0.14	1.92 ± 0.06

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SRM 967a Creatinine in Frozen Human Serum

- SRM 967a contains an adult normal and adult high level of creatinine; creatinine values assigned based on NIST ID/LC/MS RMP
- Cannot obtain a high level from healthy donors; high level is associated with kidney dysfunction
- High level was achieved by spiking crystalline creatinine into normal serum
- A commutability study was performed for the original SRM 967 in collaboration with the National Kidney Disease Education Program
- Both levels of SRM 967 were commutable with routine clinical lab methods based on enzymatic or chemical reactions

SRM 2378 Fatty Acids in Frozen Human Serum

- New SRM designed with three levels representing different fatty acid profiles
  - Level 1- donors taking fish oil supplements (1000 mg/day for one month prior to donation)
  - Level 2- donors taking flaxseed supplements (1000 mg/day for one month prior to donation)
  - Level 3- donors not taking fish oil or flaxseed supplements
- Fatty acid values were assigned by combining data from CDC ID-GC-MS and NIST ID-GC-MS

SRM 972a Vitamin D Metabolites in Frozen Human Serum

- The original SRM 972 was prepared with pooled donor serum, with some levels being artificially augmented
  - Level 2 was normal human serum diluted with horse serum to obtain a lower level 25(OH)D_3
  - Level 3 was spiked to obtain equivalent concentrations of 25(OH)D_2 and 25(OH)D_3
- The diluted and spiked levels of SRM 972 were not commutable with several routine assays
- Newer SRM 972a was also designed with four levels containing different combinations of vitamin D metabolites
  - Level 1-Level 3: all concentrations achieved by pooling donors
  - Level 4: spiked to create a high level of 3-epi-25(OH)D_3; cannot be achieved by pooling

Design of the SRM 968 Serum Series:

Fat-soluble Vitamins, Carotenoids, Cholesterol in Human Serum

SRM 968 3 Levels 1989	**SRM 968a** 3 Levels 1991	**SRM 968b** 3 Levels 1995	**SRM 968c** 2 Levels 1999	**SRM 968d** 2 Levels 2008	**SRM 968e** 3 Levels 2010	**SRM 968f** 2 Levels In progress
5 analytes	12 analytes	15 analytes	21 analytes	12 analytes	17 analytes	??
Lyophilized	Lyophilized	Lyophilized	Lyophilized	Liquid frozen	Liquid Frozen	Liquid Frozen
Spiked Blended	Spiked Blended	Spiked Blended	Spiked Blended	Blended	Blended	Blended

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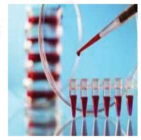
SRM 968e: Fat-soluble Vitamins, Carotenoids, and Cholesterol in Human Serum

Sample Design Considerations



- Identify the sample type (whole blood, plasma, serum, liquid-frozen, lyophilized) and source/supplier (blood banks or other sources).
- Determine the required storage conditions (-20 ˚C, -70 ˚C, -80 ˚C).
- Identify the analytes of interest and establish target values.
  This may require:
  Sample prescreening
  Dosing through feeding studies

CERTIFICATE OF ANALYSIS

NO TEST METHOD CAN PROVIDE TOTAL ASSURANCE THAT HEPATITIS B VIRUS, HUMAN IMMUNODEFICIENCY VIRUS, HEPATITIS C VIRUS, OR OTHER INFECTIOUS AGENTS ARE ABSENT. THUS, ALL BLOOD PRODUCTS SHOULD BE HANDLED AT THE BIO-SAFETY LEVEL 2 AS RECOMMENDED BY THE CDC/NIH MANUAL: BIO-SAFETY IN MICROBIOLOGICAL AND BIOMEDICAL LABORATORIES, FOR POTENTIALLY INFECTIOUS HUMAN SERUM OR BLOOD SPECIMENS.

EACH DONOR UNIT SUPPLIED TO YOUR FACILITY HAS BEEN TESTED ACCORDING TO FDA GUIDELINES FOR THE DETECTION OF HEPATITIS B SURFACE ANTIGEN, ANTIBODIES TO HIV AND HCV, HIV-1 RNA, AND HCV RNA. EACH DONOR HAS BEEN TESTED ACCORDING TO FDA GUIDELINES FOR SYPHILIS. ALL UNITS/DONORS YEILDED NON-REACTIVE/NEGATIVE RESULTS FOR EACH TEST PERFORMED. ALL BLOOD IS COLLECTED IN THE UNITED STATES OF AMERICA IN FDA LICENSED CENTERS AND TESTED WITH FDA APPROVED TEST KITS.

TEST	MANUFACTURER / METHOD
HBsAg	ABBOTT EIA
ANTI - HIV 1/2	ABBOTT EIA
ANTI - HCV	ABBOTT EIA
HIV-1 RNA	NGI or ROCHE NAT/PCR
HCV RNA	NGI or ROCHE NAT/PCR
SYPHILIS	ASI RPR

INTERSTATE BLOOD BANK, INC.

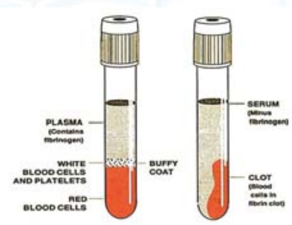
BY:

SHIP DATE:

Plasma versus Serum

Anticoagulated

Plasma is the liquid, cell-free part of blood, that has been treated with anti-coagulants.



Clotted

Serum is the liquid part of blood after coagulation. It is free of clotting factors such as fibrinogens.

Serum = plasma - fibrinogen

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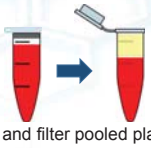
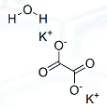
Conversion of Plasma to Serum

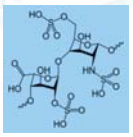


- Thaw, centrifuge, and filter pooled plasma by vacuum through Whatman 541 filter paper.
- Allow plasma to mix overnight at 4 °C. On the following day, filter each pool a second time using the same process. At this time test each pool for clot formation.
- If there is evidence of clotting, refreeze the serum at -20 °C or below overnight and repeat the process until there is no evidence of clotting.

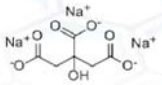
Examples of Anti-coagulants used for Plasma



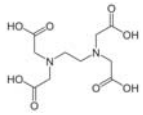
Potassium oxalate



Heparin (Lithium)

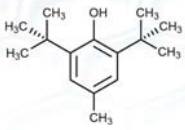


Sodium citrate

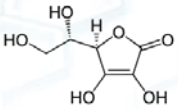


Ethylenediaminetetraacetic acid or EDTA

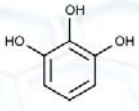
Commonly Used Antioxidants



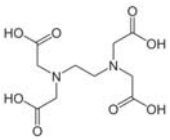
Butylated hydroxytoluene or BHT



Ascorbic Acid



Pyrogallol



Ethylenediaminetetraacetic acid or EDTA

Plasma versus Serum Recommendations

Serum

- Preferred for analysis because it maintains homogeneity over time
- Has less cryo-precipitants after thawing than plasma
- Some analyte concentrations in serum are found to be greater than those in plasma due to the greater protein content of plasma

Plasma

- After being stored frozen, may form precipitants which can interfere with subsequent pipetting and with the quantitative analysis of analytes
- May not maintain homogeneity over time

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Blending versus Spiking of Serum Pools

- Blending is preferred when possible.
- Spike only if the analyte is adequately soluble in the spiking solution. For example, retinol and tocopherol readily dissolve in ethanol. Therefore, the spiking solution(s) can be directly spiked into the serum pool. However, beta-carotene does not sufficiently dissolve in ethanol and cannot be spiked in the serum. The beta-carotene solution will be inhomogeneous throughout the matrix.
- Establish your analyte target values before the serum pools are blended and/or spiked.

Preparing a Spiked Solution from a Liquid Spike

$$[\text{Analyte}]_{\text{Final}} = \frac{[\text{Analyte}]_{\text{Spike}} \times \text{Volume}_{\text{Spike}} + [\text{Analyte}]_{\text{Sample}} \times \text{Volume}_{\text{Sample}}}{\text{Volume}_{\text{Sample}} + \text{Volume}_{\text{Spike}}}$$

http://www.handymath.com/cgi-bin/spikeh3.cgi?submit=Entry

Recommended Method for Preparing a Solution

Gravimetry is our preferred method for preparing solutions.

- Better accuracy (less bias, more precise)
- Mass concentration can be converted to mass fraction via measured density

$$[\text{Analyte}]\frac{\mu g}{g} = \frac{[\text{Analyte}]\frac{\mu g}{mL}}{\text{Density}\frac{g}{mL}}$$

Storage and Handling

Challenge	Effect
Exposure to light	Direct sunlight and artificial light cause some analytes to decompose
Exposure to air	Some analytes are readily destroyed by oxidation in the presence of air
Exposure to heat and metal ions (such as copper and iron)	Cause oxidation and analyte decomposition

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Storage and Handling Recommendations

- Store samples(s) in amber vials or in nontransparent containers
- Minimize exposure to air
- Minimize exposure to light; work in subdued lighting
- Store sample(s) at sub-ambient temperatures (-20 ˚C, -70 ˚C, -80 ˚C) and in the dark
- After pooling and blending, minimize repeated thawing and refreezing of serum samples

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