Wiley Series on Mass Spectrometry

Dominic M. Desiderio and Joseph A. Loo, Series Editors



Mass Spectrometry and Stable Isotopes in Nutritional and Pediatric Research

Edited by Henk Schierbeek



Mass Spectrometry and Stable Isotopes in Nutritional and Pediatric Research

Wiley Series on Mass Spectrometry

Series Editors

Dominic M. Desiderio Departments of Neurology and Biochemistry University of Tennessee Health Science Center

Joseph A. Loo Department of Chemistry and Biochemistry UCLA

Founding Editors

Nico M. M. Nibbering (1938–2014) Dominic M. Desiderio

A complete list of the titles in this series appears at the end of this volume.

Mass Spectrometry and Stable Isotopes in Nutritional and Pediatric Research

Edited by Henk Schierbeek



Copyright © 2017 by John Wiley & Sons, Inc. All rights reserved

Published by John Wiley & Sons, Inc., Hoboken, New Jersey

Published simultaneously in Canada

No part of this publication may be reproduced, stored in a retrieval system, or transmitted in any form or by any means, electronic, mechanical, photocopying, recording, scanning, or otherwise, except as permitted under Section 107 or 108 of the 1976 United States Copyright Act, without either the prior written permission of the Publisher, or authorization through payment of the appropriate per-copy fee to the Copyright Clearance Center, Inc., 222 Rosewood Drive, Danvers, MA 01923, (978) 750-8400, fax (978) 750-4470, or on the web at www.copyright.com. Requests to the Publisher for permission should be addressed to the Permissions Department, John Wiley & Sons, Inc., 111 River Street, Hoboken, NJ 07030, (201) 748-6011, fax (201) 748-6008, or online at http://www.wiley.com/go/permission.

Limit of Liability/Disclaimer of Warranty: While the publisher and author have used their best efforts in preparing this book, they make no representations or warranties with respect to the accuracy or completeness of the contents of this book and specifically disclaim any implied warranties of merchantability or fitness for a particular purpose. No warranty may be created or extended by sales representatives or written sales materials. The advice and strategies contained herein may not be suitable for your situation. You should consult with a professional where appropriate. Neither the publisher nor author shall be liable for any loss of profit or any other commercial damages, including but not limited to special, incidental, consequential, or other damages.

For general information on our other products and services or for technical support, please contact our Customer Care Department within the United States at (800) 762-2974, outside the United States at (317) 572-3993 or fax (317) 572-4002.

Wiley also publishes its books in a variety of electronic formats. Some content that appears in print may not be available in electronic formats. For more information about Wiley products, visit our web site at www.wiley.com.

Library of Congress Cataloging-in-Publication Data:

Names: Schierbeek, Henk, 1956- editor.
Title: Mass spectrometry and stable isotopes in nutritional and pediatric research / edited by Henk Schierbeek.
Description: Hoboken, New Jersey : John Wiley & Sons, Inc., [2017] | Includes bibliographical references and index.
Identifiers: LCCN 2016040356| ISBN 9781118858776 (cloth) | ISBN 9781119341246 (epub)
Subjects: LCSH: Biomolecules–Analysis. | Mass spectrometry. | Nutrition–Research. | Pediatrics–Research.
Classification: LCC QP519.9.M3 M3158 2017 | DDC 572/.36–dc23 LC record available at https://lccn.loc.gov/2016040356

Cover image: enot-poloskun/Gettyimages

Typeset in 10/12pt, Warnock by SPi Global, Chennai, India

Printed in the United States of America

10 9 8 7 6 5 4 3 2 1

Contents

List of Contributors xvii Introduction xxi List of Abbreviations xxiii

1 Mass Spectrometry Techniques for *In Vivo* Stable Isotope Approaches 1

Jean-Philippe Godin and Henk Schierbeek

- 1.1 Introduction
- 1.2 Nomenclature for Light-Stable Isotope Changes 3
- 1.2.1 Natural Abundance 3
- 1.2.2 Tracer 3
- 1.2.3 Isotopic Ratio and Isotopic Enrichment Measurements 4
- 1.2.3.1 Delta Notation Measured by Isotope Ratio Mass Spectrometry 4
- 1.2.3.2 Expressions of Isotopic Enrichment 5
- 1.2.3.3 Normalization of Isotopic Ratio Expressed with δ Unit 6
- 1.3 Mass Spectrometry Techniques 6
- 1.3.1 Isotope Ratio Mass Spectrometry 6
- 1.3.1.1 Bulk Stable Isotope Analysis 7
- 1.3.1.2 Compound-Specific Isotopic Analysis 10
- 1.3.2 Gas-Chromatography- and Liquid-Chromatography-Based Mass Spectrometry Methods 15
- 1.3.2.1 Gas Chromatography–Mass Spectrometry 16
- 1.3.2.2 Liquid Chromatography–Tandem Mass Spectrometry 17
- 1.3.2.3 Scanning Technique in Mass Spectrometry and Tandem Mass Spectrometry *17*
- 1.3.2.4 Single Versus Triple Quadrupole Mass Spectrometry 19
- 1.3.2.5 Gas Chromatography–Mass Spectrometry and Liquid Chromatography–Mass Spectrometry Requirements for Isotopic Analysis 19
- 1.3.3 Calibration of Isotopic Measurements 24
- 1.3.4 Comparison of Gas Chromatography–Mass Spectrometry, Liquid Chromatography–Tandem Mass Spectrometry, and Gas Chromatography–Isotope Ratio Mass Spectrometry 24

٧

vi Contents

- 1.4 Choice of Mass Spectrometric Techniques and Applications to Measure Isotopic Enrichments in Metabolic Studies 26
- 1.4.1 Choice of Mass Spectrometric Technique to Measure Isotopic Enrichment 26
- 1.4.2 Applications of Mass Spectrometry Methods in Stable Isotopes Tracers Studies 28
- 1.5 Conclusion and Future Perspectives *30* References *32*

2 Stable Isotope Technology 45

- Dewi van Harskamp, Johannes B. van Goudoever, and Henk Schierbeek
- 2.1 History 45
- 2.2 Definition 45
- 2.3 Safety 46
- 2.4 Stable Isotopes and Natural Abundances 47
- 2.5 Stable Isotope Selection 48
- 2.6 Single or Multiple Label Selection 49
- 2.7 Precursor Model 49
- 2.8 Simultaneous Infusion 49
- 2.9 Infusion Techniques 50
- 2.9.1 Bolus 50
- 2.9.2 Primed Continuous Infusion 51
- 2.9.3 Staggered Infusion Technique 51
- 2.9.4 Pulse Injection Technique 52
- 2.10 Steady State 52
- 2.11 Pool Selection 52
- 2.11.1 Precursor Pool 53
- 2.12 Pool Models 53
- 2.12.1 Single Pool Model 53
- 2.12.2 Multiple Pool Model 54
- 2.12.3 Noncompartmental Modeling 54
- 2.12.4 Compartmental Modeling 55
- 2.13 Flux: Synthesis and Breakdown 55
- 2.13.1 Oxidation Rate 55
- 2.13.2 Fractional Synthesis and Absolute Synthesis 56
- 2.14 Nitrogen Balance 57
- 2.15 Doubly Labeled Water Method 57
- 2.16 Whole-body Protein Synthesis 58
- 2.17 Specific Protein Synthesis 58
- 2.17.1 Fast Protein Synthesis 59
- 2.18 Calculations 59
- 2.18.1 Protein Turnover 59
- 2.18.2 Rate of Appearance 59
- 2.18.3 Oxidation 60
- 2.18.4 First Pass Uptake 60

- 2.18.5 MIDA method 60
- 2.18.6 CO₂ Production 61
- 2.18.7 Total Body Water (TBW) 61
- 2.18.8 Total Energy Expenditure (TEE) 62
- 2.19 Considerations and Drawbacks of Isotopic Tracers 62
- 2.20 Conclusion 63 References 63
- **3** Stable Isotopes in Nutritional and Pediatric Research 67

Willemijn E. Corpeleijn and Johannes B. van Goudoever

- 3.1 Introduction 67
- 3.1.1 Stable Isotopes 67
- 3.1.2 Safety Issues 68
- 3.1.2.1 Sterility 69
- 3.1.2.2 Purity and Stability 69
- 3.1.3 Blood Sampling 69
- 3.2 Ethical Aspects 69
- 3.3 Applications of Stable Isotopes in Nutritional and Pediatric Research *70*
- 3.3.1 Energy Expenditure 71
- 3.3.2 Body Composition 71
- 3.3.3 Macronutrient Metabolism 72
- 3.3.3.1 Amino Acids 72
- 3.3.3.2 Glucose and Carbohydrate Metabolism 74
- 3.3.3.3 Essential Fatty Acids 75
- 3.3.4 Micronutrient Metabolism 76
- 3.3.4.1 Vitamins 76
- 3.3.5 Minerals and Trace Elements 77
- 3.4 Conclusion 78 References 78

Stefanie M.P. Kouwenhoven and Marita de Waard

- 4.1 Introduction 81
- 4.2 Breast Milk versus Infant Formula 81
- 4.3 Techniques to Monitor Milk Intake 82
- 4.3.1 Dose-to-Infant Technique 83
- 4.3.2 Dose-to-Mother Technique 84
- 4.4 Body Composition in Term and Preterm Infants 86
- 4.5 Amino Acid Requirement 86
- 4.6 Clinical Applications 87
- 4.6.1 The ProtEUs Study: Investigation of the Effect of an Infant Formula with an Optimized Amino Acid Composition and a Lower Protein Content 87

⁴ Early-Life Nutrition and Stable Isotope Techniques 81

- viii Contents
 - 4.6.1.1 Inclusion and Exclusion Criteria 87
 - 4.6.1.2 Subjects 88
 - 4.6.1.3 Assessment of the Study Formula Intake 89
 - 4.6.1.4 Assessment of Total Body Water (TBW) 89
 - 4.6.1.5 Tracer Protocol 89
 - 4.6.1.6 Materials and Methods 90
 - 4.6.1.7 Limitations and Sources of Errors 91
 - 4.6.1.8 Calculations 92
 - 4.7 Additional Applications 95
 - 4.7.1 Nutritional Needs of Formula-Fed Infants 95
 - 4.7.1.1 The Evaluation of Calcium Absorption in Infants 95
 - 4.7.1.2 Iron Absorption in Infants 95
 - 4.7.1.3 The Copper Level of Infant Formula 96
 - 4.7.1.4 Cholesterol Synthesis 96
 - 4.7.1.5 Other Applications 97
 - 4.8 Discussion 98
 - 4.9 Conclusion 99
 - 4.10 Future Perspectives 99 References 100
 - 5 Assessment of Amino Acid Requirement in Children Using Stable Isotopes 108

Femke Maingay-de Groof and Henk Schierbeek

- 5.1 Introduction 108
- 5.2 Nutrient Needs and Definitions *109*
- 5.3 Methods to Determine Requirements 111
- 5.3.1 Factorial Approach *111*
- 5.3.2 Nitrogen Balance 112
- 5.4 Isotopic Tracer Methods 112
- 5.5 Existing Methods to Determine Amino Acid Requirement for Neonates *114*
- 5.6 Use of the IAAO Method in the Pediatric Population *115*
- 5.7 Necessity for Performing the Study *117*
- 5.8 Biochemistry 117
- 5.8.1 Proteins and Amino Acids 117
- 5.9 Available Analytical Methods *120*
- 5.10 Clinical Application 120
- 5.10.1 Determining the Isoleucine Requirement in Neonates Using the IAAO Method *120*
- 5.10.2 Subjects 121
- 5.10.3 Study Formula 121
- 5.10.4 Tracer Protocol 123
- 5.10.5 Sample Collection *124*
- 5.10.6 Logistic Limitations and Sources of Error 124
- 5.11 Analysis and Calculations 125

- 5.12 Results 125
- 5.13 Statistical Analysis 128
- 5.14 Discussion 129
- 5.15 Conclusion 131
- 5.16 Future Perspectives *132* References *132*
- 6 Metabolism of Glutamine, Citrulline, and Arginine; Stable Isotopes Analyzing the Intestinal–Renal Axis 139

Nikki Buijs, Saskia J.H. Brinkmann, Gerdien C. Ligthart-Melis, and Henk Schierbeek

- 6.1 Introduction 139
- 6.1.1 Glutamine 140
- 6.1.2 Citrulline 141
- 6.1.3 Arginine *141*
- 6.2 Biochemistry 142
- 6.2.1 Adult Model 142
- 6.2.2 Neonatal Mammal Model 145
- 6.3 Isotopic Model 146
- 6.4 Study Design 148
- 6.4.1 Guidelines: Human Protocols, Animal Protocols, and Safety Procedures *148*
- 6.4.2 Study Population 148
- 6.4.3 Study Conditions 149
- 6.4.4 Criteria for Stable Isotope Preparations 149
- 6.4.5 Study Protocol 150
- 6.5 Mass Spectrometry Methods 151
- 6.5.1 Potential Analytical Methods 151
- 6.5.2 The Most Frequently Used Equipment and Methods 152
- 6.5.3 The Selected Method for this Application 152
- 6.5.3.1 Materials 152
- 6.5.3.2 Blood Collection 152
- 6.5.3.3 Sample Preparation 153
- 6.5.3.4 Liquid Chromatography 153
- 6.5.3.5 Mass Spectrometer Instrument Settings 154
- 6.5.3.6 Method Validation 154
- 6.6 Clinical Applications 155
- 6.6.1 Primary Goal 155
- 6.6.2 Design 155
- 6.6.3 Power Analysis 156
- 6.6.4 Subjects (Inclusion Criteria, Exclusion Criteria, Nutritional Requirements, and Physiological Requirements) 156
- 6.6.5 Experimental Design, Tracer Selection, Tracer Infusion Protocols, Sample Collection, and Treatment *156*
- 6.6.6 Additional Clinical Parameters Necessary for Calculations 157

x Contents

- 6.6.7 Logistical Limitations 157
- 6.7 Calculations 158
- 6.7.1 Whole-body Rate of Appearance 158
- 6.7.2 Organ-specific Calculations 159
- 6.7.3 Splanchnic Metabolism 160
- 6.7.4 Intestinal Metabolism 160
- 6.7.5 Hepatic Metabolism *161*
- 6.7.6 Renal Metabolism *161*
- 6.8 Discussion and Future Perspectives 161
- 6.8.1 Adults 162
- 6.8.2 Infants 164
- 6.8.3 Tracer Selection 166
- 6.8.4 Analytical Techniques 166
 - References 167

7 Applications in Fat Absorption and Metabolism 175

- Dirk-Jan Reijngoud and Henkjan J. Verkade
- 7.1 Introduction 175
- 7.2 Biochemistry of Fat Absorption 176
- 7.3 Isotope Model 178
- 7.4 Study Design/Infusion Protocols 179
- 7.4.1 Guidelines for Human Protocols and Animal Protocols and Safety Procedures *179*
- 7.4.2 Studied Population 179
- 7.4.3 Studied Conditions 179
- 7.4.4 Criteria for Stable Isotope Preparations 180
- 7.4.5 Study Protocol 180
- 7.5 Analytical Equipment 181
- 7.6 Analytical Conditions 181
- 7.6.1 Materials 181
- 7.6.2 Sample Preparation 181
- 7.6.3 Chromatography Conditions 182
- 7.6.4 Mass Spectrometry Conditions 182
- 7.7 Accuracy and Precision 183
- 7.7.1 Calibration 183
- 7.7.2 Biological Reproducibility 183
- 7.8 Calculations 184
- 7.9 Clinical Applications 187
- 7.9.1 Introduction 187
- 7.9.2 Study Design *188*
- 7.9.2.1 Patients 188
- 7.9.2.2 ¹³C-Labeled Substrates 188
- 7.9.2.3 Study Protocol 188
- 7.9.3 Analytical Techniques 188
- 7.9.4 Results and Discussion 189

- 7.10 Future Perspectives 191
- 7.10.1 Delivery 191
- 7.10.2 Instrumentation 191
- 7.10.3 Metabolism 192 References 193

8 Materno-Fetal Lipid Kinetics 197

Elvira Larqué, Hans Demmelmair, and Berthold Koletzko

- 8.1 Introduction 197
- 8.2 Biochemistry of Placental Lipid Transport 198
- 8.3 Investigation of Fatty Acid Metabolism Using Stable Isotopes 200
- 8.4 Mass Spectrometry Methods 202
- 8.5 Clinical Studies with Fatty Acids Labeled with Stable Isotopes in Healthy and Complicated Pregnancies 203
- 8.6 Calculations 207
- 8.7 Future Perspectives 209 Acknowledgments 210 References 210
- 9 Stable Isotope Applications in Human *In Vivo* Placental and Fetal Research 213 Chris H.P. van den Akker
- 9.1 Introduction 213
- 9.2 Investigation of Fetal Metabolism Using Stable Isotopes 214
- 9.3 Study Designs and Models 215
- 9.4 Infusion Protocols and Clinical Applications 216
- 9.5 Necessary Additional Clinical Parameters to be Analyzed 218
- 9.6 Necessary Analytical Mass-Spectrometry Equipment and Analytical Conditions 218
- 9.7 Calculations 219
- 9.8 Future Perspectives 222 References 222
- 10 Obesity 225

Margriet Veldhorst and Henk Schierbeek

- 10.1 Introduction 225
- 10.2 Singly and Doubly Labeled Water 226
- 10.2.1 Total Energy Expenditure 226
- 10.2.2 Body Composition 228
- 10.2.3 Study Design Using Singly or Doubly Labeled Water 229
- 10.2.4 Mass Spectrometry 231
- 10.2.4.1 Doubly Labeled Water Measurement 231
- 10.2.4.2 Sampling 232
- 10.2.4.3 High-Temperature Thermal Conversion Elemental Analyzer 232
- 10.2.4.4 Analytical Conditions 233

- xii Contents
 - 10.2.4.5 Other Potential Analytical Techniques 233
 - 10.2.5 Potential Sources of Error 233
 - 10.2.6 Calculations 234
 - 10.3 Substrate Oxidation 237
 - 10.4 Glucose Metabolism 238
 - 10.4.1 Study Design and Clinical Application 239
 - 10.5 Fat Metabolism 239
 - 10.5.1 Fat Oxidation 240
 - 10.5.2 Lipoprotein Metabolism 241
 - 10.6 Protein Turnover 242
 - 10.6.1 Study Design 243
 - 10.6.2 Clinical Applications 244
 - 10.6.3 Mass Spectrometry Methods 244
 - 10.6.3.1 Enrichment of Amino Acids 244
 - 10.6.3.2 Urea Synthesis Rate 245
 - 10.6.3.3 Albumin Synthesis 246
 - 10.7 Calculations 246
 - 10.7.1 Glucose Production 246
 - 10.7.2 Protein Oxidation 247
 - 10.7.3 Urea Production 248
 - 10.7.4 Albumin/Glutathione Synthesis 248
 - 10.8 Discussion and Future Perspectives 249 References 250
 - 11 Inborn Errors of Metabolism 258

Hidde H. Huidekoper, Frits A. Wijburg, and Ronald J.A. Wanders

- 11.1 Introduction 258
- 11.2 Stable Isotope Techniques 260
- 11.2.1 Stable Isotope Dilution 261
- 11.2.2 Stable Isotope Incorporation 261
- 11.2.2.1 Deuterated Water Method to Quantify Fractional Gluconeogenesis 262
- 11.2.2.2 Fatty Acid Oxidation from Plasma and Intracellularly Derived Triglycerides 264
- 11.2.2.3 Quantification of Whole-body Protein Synthesis, Breakdown, and Oxidation 266
- 11.3 Analytical Equipment and Methods 267
- 11.3.1 Gas Chromatography-mass Spectrometry 267
- 11.3.2 Isotope Ratio Mass Spectrometry 267
- 11.3.3 Stable Isotope Enrichment in Plasma 267
- 11.4 Study Protocol: Quantifying Endogenous Galactose Production 269
- 11.4.1 Rationale 269
- 11.4.2 Study Design 269
- 11.4.3 Experimental Design 270
- 11.4.4 Sample Analysis 270

- 11.4.5 Calculations 270
- 11.4.6 Pitfalls 271
- 11.5 Calculations 271
- 11.5.1 Stable Isotope Dilution 272
- 11.5.2 Contributions of Gluconeogenesis and Glycogenolysis to Endogenous Glucose Production 273
- 11.5.3 Calculation of Fatty Acid Oxidation from Plasma- and Intracellular-Derived Triglycerides 273
- 11.5.4 Calculating Whole-Body Protein Synthesis, Breakdown, and Oxidation 275
- 11.6 Discussion 276
- 11.7 Future Perspectives 277 References 278

12 Renal Disease and Dialysis 284

Gregorio P. Milani, Sander F. Garrelfs, and Michiel J.S. Oosterveld

- 12.1 Introduction 284
- 12.2 Total Body Water and Its Distribution 286
- 12.2.1 Principle 287
- 12.2.2 Toxicity 288
- 12.2.3 Available Methods for Deuterium Enrichment and Bromide Concentration Analysis 289
- 12.2.4 Tracer Protocols 290
- 12.3 Protein Metabolism in Chronic Kidney Disease 291
- 12.3.1 Precursor Method 292
- 12.3.2 End-product Method 292
- 12.3.3 Urea 293
- 12.4 Dialysis Metabolic Consequences and Nutrient Losses 293
- 12.4.1 Whole-Body and Skeletal Muscle Protein Studies 294
- 12.5 Primary Hyperoxalurias 295
- 12.5.1 Biochemistry 295
- 12.5.2 Stable Isotope Studies in Primary Hyperoxaluria 297
- 12.6 Clinical Applications 298
- 12.6.1 Assessment of Intra- and Extracellular Body Water and Its Distribution 298
- 12.6.1.1 Subjects 298
- 12.6.1.2 Deuterium and Bromide Dose 298
- 12.6.1.3 Specimen Sampling 299
- 12.6.1.4 Mass Spectrometry 300
- 12.6.2 Protein Metabolism in Chronic Kidney Disease 300
- 12.6.2.1 Subjects 300
- 12.6.2.2 Stable Isotope Infusion Protocols 300
- 12.6.2.3 Measurements 300
- 12.6.3 Dialysis Metabolic Consequences and Nutrient Losses 301
- 12.6.3.1 Subjects 301

- xiv Contents
 - 12.6.3.2 Stable Isotope Infusion Protocol 301
 - 12.6.3.3 Measurements 301
 - 12.6.4 Assessment of Oxalate Rate of Appearance in Primary Hyperoxaluria Type 1 301
 - 12.6.4.1 Primary Goal 301
 - 12.6.4.2 Subjects 302
 - 12.6.4.3 Experimental 302
 - 12.6.4.4 Sample Collection and Preparation 303
 - 12.6.4.5 Mass Spectrometry 303
 - 12.7 Calculations 303
 - 12.7.1 Calculation of Total Body Water 303
 - 12.7.2 Calculation of Intra- and Extracellular Body Water Volumes 304
 - 12.7.3 Calculation of Whole-Body Protein Turnover 305
 - 12.7.3.1 Calculation of the Rate of Appearance van of Leucine 305
 - 12.7.3.2 Calculation of the Leucine Oxidation 306
 - 12.7.3.3 Calculation of the Whole-Body Protein Synthesis 307
 - 12.7.4 Calculation of Phenylalanine Synthesis and Breakdown Rates 307
 - 12.7.5 Calculation of Oxalate Production Rates 307
 - 12.8 Discussion 308
 - 12.8.1 Deuterium and Bromide Dilution Techniques 308
 - 12.8.2 Protein Metabolism in Chronic Kidney Disease 309
 - 12.8.3 Dialysis Metabolic Consequences and Nutrient Losses 309
 - 12.8.4 Assessment of Oxalate Metabolism in Primary Hyperoxaluria Type 1 310
 - 12.9 Future Perspectives 310 References 310

13 Application in Oxidative Stress and Glutathione Metabolism in Preterm Infants 320

Denise Rook and Henk Schierbeek

- 13.1 Introduction 320
- 13.1.1 Oxidative Stress in Neonatology 320
- 13.2 Biochemistry/Model 321
- 13.2.1 Biochemical Pathways 321
- 13.2.2 Antioxidant Mechanisms 321
- 13.3 Guidelines and Safety Procedures 323
- 13.3.1 Human Protocols 323
- 13.3.2 Criteria for Infusion Liquids 323
- 13.3.3 Logistical Limitations 323
- 13.4 Mass Spectrometry Methods 323
- 13.4.1 Potential Analytical Methods for this Application 323
- 13.4.2 The Most Frequently Used Equipment and Methods 324
- 13.5 Materials and Methods 324
- 13.5.1 Materials 324

Contents xv

- 13.5.2 Sample Preparation 324
- 13.5.3 Analytical Conditions: LC-IRMS Conditions 324
- 13.5.4 Mass Spectrometry 325
- 13.5.5 Accuracy and Precision 326
- 13.6 Clinical Application (A Practical Example of a Study Protocol) 327
- 13.6.1 Design 327
- 13.6.2 Primary Goal 328
- 13.6.3 Power Analysis 328
- 13.6.4 Subjects 328
- 13.6.5 Experimental Design 328
- 13.6.6 Tracer Infusion Protocol and Blood Sampling 328
- 13.6.7 Measurements Necessary Additional Clinical Parameters for All Calculations 329
- 13.7 Calculations 329
- 13.7.1 Calibration and Isotopic Rearrangements 329
- 13.8 Discussion and Future Perspectives 330 References 331
- 14 Nutrient Digestion and Absorption During Intestinal Malfunction and Diseases 336
 - Margot Fijlstra
- 14.1 Introduction 336
- 14.1.1 Nutrient Digestion and Absorption in General 336
- 14.1.2 Nutrient Digestion and Absorption During Intestinal Malfunction and Diseases 337
- 14.2 Clinical Application 340
- 14.2.1 Introduction, Design, and Primary Goal 340
- 14.2.2 Materials 341
- 14.2.2.1 Rats and Housing 341
- 14.2.2.2 Chemicals 341
- 14.2.3 Experimental Procedures 341
- 14.2.3.1 Infusion Protocol 341
- 14.2.3.2 Tissue Collection 343
- 14.2.4 Analytical Methods 343
- 14.2.4.1 Mass Spectrometry 344
- 14.2.5 Calculations 344
- 14.2.5.1 Statistical Analysis 346
- 14.2.6 Results 347
- 14.2.6.1 The Mucositis Rat Model 347
- 14.2.6.2 Plasma Kinetics of IV- and ID-Infused AA 347
- 14.2.6.3 Small Intestinal Mucosa 352
- 14.2.6.4 Albumin 353
- 14.2.6.5 Liver 353
- 14.2.6.6 Thigh Muscle 353

xvi Contents

- 14.2.7 Discussion 353
- 14.2.8 Future Perspectives 357 References 357

Index 365

List of Contributors

Saskia J.H. Brinkmann

Department of Surgery VU University Medical Center Amsterdam The Netherlands

Nikki Buijs

Department of Surgery VU University Medical Center Amsterdam Amsterdam The Netherlands

Willemijn E. Corpeleijn

Department of Pediatrics AMC University of Amsterdam Amsterdam The Netherlands

Femke Maingay-de Groof

Department of Pediatrics NoordWest Ziekenhuisgroep Alkmaar The Netherlands

Marita de Waard Department of Pediatrics VU University Medical Center Amsterdam The Netherlands

Hans Demmelmair

Division of Nutrition and Metabolic Diseases Hauner Children's Hospital Ludwig Maximilian University Munich Munich Germany

Margot Fijlstra

Department of Pediatrics Beatrix Children's Hospital Groningen University Institute for Drug Exploration (GUIDE) University of Groningen Groningen The Netherlands

and

Department of Pediatrics AMC University of Amsterdam Amsterdam The Netherlands

Sander F. Garrelfs

Department of Pediatric Nephrology Emma Children's Hospital/Academic Medical Center University of Amsterdam Amsterdam The Netherlands xviii List of Contributors

Jean-Philippe Godin

Analytical Sciences Nestlé Research Center Lausanne Switzerland

Hidde H. Huidekoper

Department of Pediatrics Center for Lysosomal and Metabolic Diseases Erasmus Medical Center-University Hospital Rotterdam The Netherlands

and

Department of Pediatrics Division of Metabolic Disorders Academic Medical Center University of Amsterdam Amsterdam The Netherlands

Berthold Koletzko

Division of Nutrition and Metabolic Diseases Hauner Children's Hospital Ludwig Maximilian University Munich Munich Germany

Stefanie M.P. Kouwenhoven

Department of Pediatrics VU University Medical Center Amsterdam The Netherlands

Elvira Larqué Department of Physiology University of Murcia Murcia Spain

Gerdien C. Ligthart-Melis

Center for Translational Research in Aging and Longevity Department of Health and Kinesiology Texas A&M University College Station TX USA

Gregorio P. Milani

Department of Pediatrics Ca' Granda Ospedale Maggiore Policlinico University of Milan Milan Italy

Michiel J.S. Oosterveld

Department of Pediatric Nephrology Emma Children's Hospital/Academic Medical Center University of Amsterdam Amsterdam The Netherlands

Dirk-Jan Reijngoud

Department of Pediatrics Beatrix Children's Hospital Groningen The Netherlands

and

Center for Liver Digestive and Metabolic Diseases University of Groningen University Medical Center Groningen Groningen The Netherlands

Denise Rook

Department of Pediatrics Erasmus Medical Center University Hospital Rotterdam The Netherlands

Henk Schierbeek

Department of Pediatrics AMC University of Amsterdam Amsterdam The Netherlands

Chris H.P. van den Akker

Department of Pediatrics AMC University of Amsterdam Amsterdam The Netherlands

Johannes B. van Goudoever

Department of Pediatrics AMC University of Amsterdam Amsterdam The Netherlands

and

Department of Pediatrics VU University Medical Center Amsterdam The Netherlands

Dewi van Harskamp

Department of Pediatrics AMC University of Amsterdam Amsterdam The Netherlands

Margriet Veldhorst

Department of Pediatrics VU University Medical Center Amsterdam The Netherlands

Henkjan J. Verkade

Department of Pediatrics Beatrix Children's Hospital Groningen The Netherlands

Ronald J.A. Wanders

Department of Pediatrics Division of Metabolic Disorders Academic Medical Center University of Amsterdam Amsterdam The Netherlands

and

Department of Clinical Chemistry Laboratory Genetic Metabolic Diseases Academic Medical Center University of Amsterdam Amsterdam The Netherlands

Frits A. Wijburg

Department of Pediatrics Division of Metabolic Disorders Academic Medical Center University of Amsterdam Amsterdam The Netherlands

Introduction

High-precision mass spectrometric analyses are gaining popularity in many scientific disciplines, including metabolic kinetic studies in nutrition and pediatrics. Innovations in mass spectrometry and tracer administration techniques have made mass spectrometers the instruments of choice for the analysis of isotopic compounds. Techniques for measurements of deuterium and ¹⁸O, as well as for ¹³C isotopic analysis, have progressed. In particular, the coupling of liquid chromatography with isotope ratio mass spectrometry (LC-IRMS) has introduced new, highly sensitive analysis opportunities and opened new avenues for nutritional and pediatric research. An increasing number of researchers that use LC–IRMS in metabolic research have indicated the robustness of this technique; however, LC-IRMS is suitable for only ¹³C-isotopic measurements due to the lack of an existing LC interface for the introduction of other elements into the IRMS. A major challenge for the future, therefore, is the development of a technique that will enable the measurement of all common elements.

Although novel techniques have been developed and existing techniques have been improved, there are still new experimental disciplines left to uncover. The coupling of LC to IRMS was a major step toward further unraveling metabolic kinetics; this innovation was made feasible by the direct measurement of carbon isotopes in a wide range of low-molecular-weight compounds and macromolecules, ranging from naturally abundant to highly enriched samples. Strength of LC–IRMS lies in the straightforward analysis of underivatized components; its main drawbacks are the relatively low sensitivity (nanogram range) and its restriction to only ¹³C-isotopic samples. The low sensitivity can be a problem when measuring components in low concentrations, such as vitamins and hormones, or when samples are small, for example, in preterm infants or small rodents. Improvements in the sensitivity and robustness of LC–MS/MS systems have opened up new possibilities for studying macromolecules, such as peptides, hormones, vitamins, and small proteins, but a wide range of applications must still be developed in several disciplines using this technique.

Also, recently developed techniques, such as infrared spectroscopy for the measurement of isotopically labeled compounds, are gaining popularity in many biomedical applications. The most important advantages of these new techniques, relative to IRMS, are their low costs and simplicity. Novel developments

xxii Introduction

for these instruments are based on the wavelength-scanned cavity ring down spectroscopy (WS-CRDS analyzer). These instruments are as precise as IRMS but use less sample (e.g., when measuring ¹³C values in CO_2). This technique requires little or no sample preparation, the analysis time is short (a few minutes), and minimal skill is needed to operate the machines; however, these instruments still need to be thoroughly tested in biomedical research applications.

Even with the advances made thus far, there are still many topics in metabolic kinetic studies that have yet to be elucidated. However, the growing availability and decreasing costs of stable isotopes will make it increasingly possible to broadly explore human metabolic kinetics worldwide.

The aim of this book is to present the relevance of mass spectrometry and stable isotope methodology in nutritional and pediatric research. Applications for the use of stable isotopes with mass spectrometry cover carbohydrate, fat, protein, and specific amino acid metabolism, energy expenditure, and the synthesis of specific peptides and proteins.

The main focus of these studies is on the interactions between nutrients, endogenous metabolism within the body, and how these factors affect the health of a growing infant. Considering that the early imprinting of metabolic processes has huge effects on metabolism (and thus functional outcome) later in life, research in this area is important and is advancing rapidly.

The book should be a guideline for scientists, analytical chemists, biochemists, clinical chemists, and pediatricians, as well as for medical graduate students and lecturers involved in metabolic studies in life sciences.

This book shows the availability of modern analytical techniques and how to apply these techniques in practice and covers the entire range of available mass spectrometric techniques used for metabolic studies.

The chapters show applications of study models as well as provide detailed information about tracer administration, sampling, the selected analytical techniques, and calculations.

List of Abbreviations

δ, ‰	delta per mil			
¹³ C	carbon-13			
¹⁴ C	carbon-14			
¹⁸ O	oxygen-18			
AA	amino acids			
AAP	American Academy of Pediatrics			
ADMA	asymmetric dimethylarginine			
ADP	air displacement plethysmography			
AgNO ₃	silver nitrate			
AGT	alanine:glyoxylate aminotransferase			
AI	adequate intake			
AMS	accelerator mass spectrometry			
APCI	atmospheric pressure chemical ionization			
APE	atom percent excess			
APPI	atmospheric pressure photoionization			
ASL	argininosuccinate lyase			
ASR	absolute synthesis rate			
ASS	argininosuccinate synthase			
AUCs	area under the curves			
AV	arteriovenous			
BCAA	branched chain amino acids			
BMI	body mass index			
Br	bromine			
BSIA	bulk stable isotope analysis			
BUN	blood urea nitrogen			
BW	birth weight			
CE	cholesterol esters			
CF	cystic fibrosis			
CFA	coefficient of fat absorption			
CKD	chronic kidney disease			
Cl	chlorine			
CO_2	carbon dioxide			
СР	cerebral palsy			

xxiv List of Abbreviations

CPT2	carnitine palmitoyltransferase 2		
CRDS	cavity ring down spectroscopy		
CRI	chemical reaction interface		
CRI-MS	chemical reaction interface-mass spectrometry		
CSIA	compound-specific isotope analysis		
CTD	clinical trials directive		
CuO	cupric oxide		
CV	coefficient of variation		
DAAO	direct amino acid oxidation		
DHA	docosahexaenoic acid		
DNA	deoxyribonucleic acid		
EA	elemental analyzer		
EA-IRMS	elemental analyzer-isotope ratio mass spectrometry		
EAR	estimated average requirement		
ECF	ethyl chloroformate reagent		
ECW	extracellular water		
EGP	endogenous glucose production		
EI	electron ionization		
EL	endothelial lipase		
EOP	endogenous oxalate production		
ESI	electrospray ionization		
ESPGHAN	European Society for Pediatric Gastroenterology, Hepatology		
	and Nutrition		
ESRD	end-stage renal disease		
eV	electron volt		
FDA	Food and Drug Administration		
FFA	free fatty acids		
FFM	free fat mass		
FIA	flow injection analysis		
FIA-IRMS	flow injection analysis-isotope ratio mass spectrometry		
FID	flame ionization detector		
FPLC	fast protein liquid chromatography		
FQ	food quotient		
FSR	fractional synthesis rate		
FT-ICR-MS	Fourier transform-ion cyclotron resonance-mass		
	spectrometry		
FTIR	Fourier transform infrared spectrometry		
FWHM	full width at half maximum		
GA	gestational age		
GALT	galactose-1-phophate uridyltransferase		
GC	gas chromatography		
GC-C-IRMS	gas chromatography–combustion-isotope ratio mass		
	spectrometry		
GC-MS	gas chromatography–mass spectrometry		
GDM	gestational diabetes mellitus		

GFR	glomerular filtration rate			
GGL	glycogenolysis			
GISP	Greenland Ice Sheet Precipitation			
Glc	glucose			
GLUT2	glucose transporter 2			
Gly	glycine			
GMP	good manufacturing practice			
GNG	gluconeogenesis			
GO	glyoxylate oxidase			
GOS	galacto-oligosaccharides			
GPX	GSH peroxidases			
GR	glyoxylate reductase			
GR/HPR	glyoxylate reductase/hydroxypyruvate reductase			
GSH	glutathione			
GSSG	glutathione disulfide			
H ₂	hydrogen			
HDL	high-density lipoprotein			
HEN	high-efficiency nebulizer			
НМТ	hexamethylenetetramine			
HOG	4-hydroxy-2-oxoglutarate			
HOGA1	4-hydroxy-2-oxoglutarate aldolase			
Ht	hematocrit			
HTLC	high temperature liquid chromatography			
IAAO	indirect amino acid oxidation			
IAEA	International Atomic Energy Agency			
ICP-MS	inductive coupled plasma-mass spectrometry			
ICW	intracellular water			
IFRS	infrared spectroscopy			
IRMS	isotope ratio mass spectrometry			
IUGR	intrauterine growth restriction			
k	retention factor (chromatography term used in GC and in LC)			
KIC	alpha-ketoisocaproic acid			
KIE	kinetic isotope effect			
kV	kilovolt			
LA	linoleic acid			
LC	liquid chromatography			
LCFA	long-chain fatty acids			
LC-IRMS	liquid chromatography-isotope ratio mass spectrometry			
LDH	lactate dehydrogenase			
LDL	low-density lipoprotein			
LOD	limit of detection			
LOQ	limit of quantitation			
LPS	lipopolysaccharide			
LSC	liquid scintillation counter			
m/z	mass-to-charge ratio			

xxvi List of Abbreviations

MCAD	medium-chain acyl-CoA dehydrogenase			
min	minute			
MPE	molar percent excess			
MS	mass spectrometry			
MTX	methotrexate			
mV	millivolt			
MW	molecular weight			
Ν	chromatographic efficiency			
п	number of theoretical plates			
NAP	N-acetyl propylethyl ester			
NB	net balance			
NDIRS	nondispersive infrared spectroscopy			
NEFA	nonesterifed fatty acids			
Ni	nickel			
NICU	Neonatal Intensive Care Unit			
NMR	nuclear magnetic resonance			
NO	nitric ovide			
NO	nitrogen diovide			
NO ₂	nonovidative leucine disposal			
NDD	N pivalovl isopropyl ester			
NII O	ovygon gag			
O_2	oloigageid			
DR-T	purge and trap			
D	purge and trap			
	prospriorous			
PDC	pyrrolline-5-carboxylate			
PA DDD	Parmuc acid			
	ree Dee Beleminte			
PEI	positron emission tomography			
PEW	protein energy wasting			
PH	primary hyperoxalurias			
PHI	primary hyperoxaluria type 1			
PH2	primary hyperoxaluria type II			
PH3	primary hyperoxaluria type III			
Phe	phenylalanine			
PKU	phenylketonuria			
PL	phospholipids			
Pt	platinum			
PUFA	polyunsaturated fatty acids			
RDA	recommended dietary allowance			
REE	resting energy expenditure			
RID	retinol isotope dilution			
RMS	root mean square			
RNA	ribonucleic acid			
ROS	reactive oxygen species			
RQ	respiratory quotient			
•	· / ·			

RRT	renal replacement therapy			
Rs	resolution selectivity			
Ru	ruthenium			
SA	stearic acid			
SD	standard deviation			
SDS	standard deviation score			
sec	second			
SGLT1	sodium-dependent glucose transporter 1			
SGLT5	sodium-dependent glucose transporter 5			
SLAP	Standard Light Antarctic Precipitation			
SPME	solid-phase microextraction			
TBW	total body water			
TC/EA-IRMS	thermal conversion/elemental analyzer IRMS			
TDFHA	tridecafluoroheptanoic acid			
TEE	total energy expenditure			
TG	triglycerides			
Thr	threonine			
TIE	thermodynamic isotope effect			
TOF	time-of-flight			
TPN	total parental nutrition			
TSN	thermospray nebulizer			
TTR	tracer/tracee ratio			
Tyr	tyrosine			
U- ¹³ C	universally labeled with ¹³ C			
UHPLC	ultrahigh performance liquid chromatographic			
UL	tolerable upper intake level			
UPLC	ultra performance liquid chromatography			
V	volt			
Val	valine			
$V_{\rm CO_2}$	volume of produced carbon dioxide			
VDPB	Vienna Pee Dee Belemnite			
VLCAD	very long-chain acyl-CoA dehydrogenase			
VLDL	very low-density lipoprotein			
WBRA	whole body rate of appearance			
WHO	World Health Organization			
δ^{13} C, ‰	delta ¹³ CO ₂ per mil			

Mass Spectrometry Techniques for *In Vivo* Stable Isotope Approaches

Jean-Philippe Godin¹ and Henk Schierbeek²

¹Analytical Sciences, Nestlé Research Center, Lausanne, Switzerland
²Department of Pediatrics, University of Amsterdam, Amsterdam, The Netherlands

1.1 Introduction

Interest in the use of light-stable isotopes (i.e., Carbon-13 or ¹³C; Nitrogen-15 or ¹⁵N; deuterium or ²H; Oxygen-18 or ¹⁸O) has become widespread over the past 20 years in archeology [1], climatology [2], biochemistry [3], geochemistry [4], forensics [5, 6], and food adulteration [7, 8]. These various scientific domains share a striking commonality, which is the use of similar analytical approaches to look at the level of light-stable isotopes in various chemical components and matrices, either at natural abundance or after tracer incorporation. Among these domains, particularly in nutritional and pediatric studies, the combination of modern mass spectrometry (MS) and light-stable isotopes has been very effective for studying the effect of diet and disease on protein, carbohydrate, lipid, and energy metabolism. *In vivo* assessment of specific pathways using stable isotopes is unique and offers powerful insights about metabolic pathways and changes in metabolic fluxes in clinical studies.

In practice, once the nutritional hypothesis is defined, the clinical investigator needs to find an adequate model that can compensate for the metabolic complexity of the *in vivo* processes. It becomes obvious that the isotopic data generated has to be combined with physiological inputs, which results in information that characterizes metabolic changes and individual needs (i.e., from pregnancy [9] to elderly women [10]). As with studies in adults or in pregnant women, in pediatric studies, light-stable isotopes are used to study various metabolisms (i.e., carbohydrate, protein, lipids, and energy) [11, 12]. However, pediatric studies are limited by several parameters, such as (1) ethical and technical constraints around collecting biological fluids (i.e., breath, plasma, saliva, urine, and feces) especially in neonates and infants; (2) the low amount of biomaterial

2 1 Mass Spectrometry Techniques for In Vivo Stable Isotope Approaches

collected; (3) the invasiveness of the methods (the study protocol must be non- or semi-invasive, limiting kinetic studies and accessibility to tissues); and (4) difficulty recruiting and convincing parents to enroll their infants, limiting the number of subjects per study and increasing the pressure on the analytical precision of the method used. Consequently, the biological samples are precious and the choice of analytical technique/method is crucial. Both must be integrated in the clinical workflow from the beginning, to design fit-for-purpose analytical stable isotope approaches to deliver the clinical outcome with the expected precision to detect an effect.

The information obtained with stable isotopes in metabolic studies provides meaningful insights compared to a simple concentration measurement in blood. Briefly, stable isotope tracers allow the calculation of metabolic fluxes between organs and give a dynamic view on metabolism rather than a static one as measured by analyte concentrations [13]. For example, these tracers enable quantification of the sum of a dynamic process of several physiologic mechanisms such as carbohydrate absorption and digestion, hepatic glucose production by the liver, peripheral tissue uptake (i.e., muscle, gut, and brain), and other biochemical pathways such as the glycolysis/oxidation. To glean deeper scientific insights and decipher small effects of nutrients or to characterize phenotypes (i.e., lean, obese with or without type 2 diabetes [14]), stable isotopes offer a unique tool for better understanding glucose homeostasis compared to glycemic response [15, 16].

MS is the most versatile and comprehensive analytical technique that can be used to tackle multiple scientific questions in several fields, including physics, pharmaceutical sciences, medicine, environmental sciences, and nutrition (to mention a few). Modern MS is a common tool that is used in many laboratories, but the number of teams able to examine the incorporation and dilution of light-stable isotopes for pediatric and nutritional studies is limited. With the increased recognition of the unique metabolic information gathered from the use of light-stable isotope tracer methods in metabolic studies, MS instruments have become the field's workhorse. In parallel to MS, other techniques, such as nuclear magnetic resonance (NMR) [17], magnetic resonance spectroscopy [18], Fourier transform infrared red spectroscopy [19], or cavity ring-down spectroscopy [20] are also used to measure light-stable isotopes in various *in vivo* applications, but these techniques are less common. Typically, these instruments do not achieve the sensitivity and precision that can be obtained with MS instruments.

We focus here on modern MS approaches that enable us to examine light-stable isotope levels in organic molecules, in particular isotope ratio mass spectrometry (IRMS) and modern (organic) MS. The diversity of peripherals such as gas chromatography (GC), liquid chromatography (LC), or elemental analyzer (EA) hyphenated to MS instruments illustrates the variety of molecules to analyze. The molecules of interest in nutritional and pediatric studies are mostly amino acids, simple carbohydrates, lipids (such as cholesterol, fatty acids, and triglycerides), urea, ammonia, water, organic acids, glycerol, breath CO_2 , and macromolecules such as proteins and DNA.

The goal of this chapter is to provide a general overview and summary of the capabilities of various MS techniques in combination with light-stable isotopes for *in vivo* assessment of metabolic fluxes. It is neither a historical overview nor is it a detailed instrumental and methodological summary of all the isotopic techniques used for nutritional and pediatric studies.

1.2 Nomenclature for Light-Stable Isotope Changes

1.2.1 Natural Abundance

Many chemical elements have more than one isotope. Molecules and ions with different isotopes of the same chemical element possess slightly different physical and chemical properties. Light-stable isotopes occur naturally at abundances of approximately 1.11% for ¹³C, 0.37% for ¹⁵N, 0.20% for ¹⁸O, and 0.015% for ²H. However, isotope ratios are not constant on earth and can vary depending on the location on earth. There are some exchanges between the ocean, biosphere, and lithosphere due to kinetic and equilibrium isotope effects, leading to subtle but significant variations in nature [2]. Isotopic fractionation between light and heavy isotopes occurs when chemical reactions are not completed or when multiple products are formed, and those isotopes are unevenly distributed among the reactants and products. Isotopic fractionations can be quantitatively predicted only when the mass balances, kinetics, and equilibrium isotope effects associated with all the relevant reactions are well described [21]. For isotopic analysis, isotopic fractionation is a critical parameter to look at during chemical reactions. Rieley discussed this effect and showed that mass balance equations can be used to obtain the true isotopic abundance [22].

In plants, during photosynthesis, metabolized products become relatively depleted in ¹³C compared to environmental CO₂. A variation of the ¹³C/¹²C ratio in different plant species is observed. On the one hand, there are plants (i.e., cereal grains, rice, sugar beets, and beans) that only use the three-carbon pathway (C3-plants) for carbon fixation, and they have a ¹³C/¹²C ratio (expressed as δ^{13} C) of about –28‰ VPDB (Vienna Pee Dee Belemnite). On the other hand, C4-plants (i.e., corn, millet, sugar cane, and many grasses) also use C4 carbon fixation and are more enriched in ¹³C. Their ¹³C/¹²C ratio (δ^{13} C) is about –13‰ VPDB [23].

In clinical studies, the variation of natural isotopic abundances due to diet can lead to subtle variations that may increase the variability of the study results. It is therefore recommended that during a clinical study with stable isotopes, subjects should follow clear instructions about diet and lifestyle [24].

1.2.2 Tracer

In the last few decades, the use of light-stable isotopes was preferred to radioisotopes for biomedical and metabolic studies, as they lacked radiation emission and are safer to handle. This is particularly relevant for the pediatric population,

4 1 Mass Spectrometry Techniques for In Vivo Stable Isotope Approaches

where the use of radioisotopes is extremely limited for safety reasons. Several different stable isotope tracers can be safely administered to children. For example, [¹⁵N]-glycine and [1-¹³C]-leucine were simultaneously administered in preterm infants for measuring whole-body protein turnover [25, 26]. Cogo *et al.* infused [¹³C]-palmitic acid and [²H₃]-leucine for 3 h and [²H₅]-glycerol for 5 h to measure protein turnover and lipolysis in critically ill children who were 10 years old [27]. This concept of multiple tracer administration is only achievable if the samples are analyzed with MS or NMR instruments.

As defined by Wolfe and Chinkes, a tracer is "a compound that is chemically and functionally identical to the naturally occurring compound of interest (tracee) but is distinct in some way that enables detection" [28]. ¹³C and ¹⁵N tracers are commonly employed to trace amino acids, whereas, by design, lipids and small carbohydrates can be artificially enriched with ¹³C, deuterium, or both. Therefore, many components labeled with light-stable isotopes (i.e., tracers) have been produced and are now commercially available. Deuterium-labeled tracers are generally the cheapest of the light-stable isotope tracers. The major drawback, however, is that deuterium atoms are labile (i.e., exchangeable with unlabeled and surrounding hydrogen atoms). Deuterium-labeled water (heavy water) is an excellent tracer for measuring total body water (and body composition) and, when associated with 18-Oxygen $({}^{2}H_{2}{}^{18}O)$, allows for the assessment of total energy expenditure (TEE) [29-31], among other applications. Although there is a widespread use of the double-labeled water method, the availability of water enriched with ¹⁸O at 10 at% or 98 at% (as isotopic purity) is low due to its limited worldwide production, making it very expensive (about 10 times higher than deuterium-enriched water). Furthermore, the reactivity of oxygen with many other components makes it very challenging to manufacture ¹⁸O tracers.

1.2.3 Isotopic Ratio and Isotopic Enrichment Measurements

Of note, there is no single expression of isotopic enrichment in metabolic studies, as reported by Wolfe and Chinkes [28]. Expressions will vary with the mass spectrometers used (IRMS instruments vs organic mass spectrometers), the level of variation in the isotopes, and the metabolic models used to assess the final clinical outcomes.

1.2.3.1 Delta Notation Measured by Isotope Ratio Mass Spectrometry

The abundances of isotopic ratios, such as ¹³C/¹²C, ¹⁸O/¹⁶O, ²H/¹H, and ¹⁵N/¹⁴N, are always measured relative to the isotope ratio of a specifically selected reference material. The reference standard materials are VPDB for carbon [32], Vienna Standard Mean Ocean Water (VSMOW or VSMOW2) for oxygen and hydrogen, and laboratory air for nitrogen [33]. Since these primary reference materials are quite limited or do not exist anymore, other easily accessible international stable isotope reference materials are also commercially available from the International Atomic Energy Agency (IAEA, Vienna, Austria) in different isotopic values.

 δ values are unitless numbers such as the isotope ratios itself, but due to the small differences measured, δ values are usually expressed in parts per thousand,

per mil, or ‰ (equation 1.1).

$$\delta, \% = \left[(R_{\text{sample}} - R_{\text{standard}}) / R_{\text{standard}} \right] \times 1000, \tag{1.1}$$

where R is the ratio between the minor (heavier) isotope of the element to the major (lighter) isotope (i.e., ${}^{13}C/{}^{12}C$).

Of note, most organic components at natural abundance are depleted in the heavy isotope form relative to the reference standard, leading to negative δ values.

In some metabolic applications that use labeled water (i.e., ${}^{2}H_{2}O$) to measure body composition or use double-labeled water (i.e., ²H₂¹⁸O) to assess total energy expenditure, the parts per million (ppm) unit is also reported. In this case, the transformation is as follows (equation 1.2):

$$ppm = (1,000,000/(1 + (1/(((\delta^2 H/1000) + 1) \times 0.00015576)))), \quad (1.2)$$

where δ^2 H is the per mil ²H with respect to the international reference VSMOW or VSWOW2. The factor 0.00015576 is the $^{2}H/^{1}H$ ratio of VSMOW [34].

1.2.3.2 Expressions of Isotopic Enrichment

In metabolic studies, once the tracer has been administered, the tracer-to-tracee ratio (TTR) is commonly used to report the isotopic enrichment. Alternative units reported in peer-reviewed papers are atom percent excess (APE, %) or molar percent excess (MPE, %). These units represent the amount of tracer as a ratio of the sum of tracer and tracee. As described by Wolfe and Chinkes [28], the tracer and tracee are indistinguishable from a metabolic point of view but distinguishable by using MS, measuring different isotopologues (i.e., components differing only in their isotopic composition such as $[1^{-13}C]$ -leucine vs $[1^{-12}C]$ -leucine). TTR is calculated based on mass spectrometer data using the following formula (equation 1.3):

$$TTR = (r_{sa} - r_{bk}) \times (1 - A)^n,$$
(1.3)

where r_{sa} is the ratio of tracer/tracee in the sample (after administration of the tracer), $r_{\rm bk}$ is the ratio of tracer/tracee in a background sample (before administration of the tracer), "A" is a skew correction factor that varies with the isotope, and "n" is the number of labeled atoms. For the ¹³C tracer, A is 0.0111, whereas for the 15 N tracer, A is 0.0037, as A is equal to the natural abundance of the element.

Finally, TTR can also be transformed into MPE or into APE using equations (1.4) and (1.5):

APE,
$$\% = TTR/(1 + TTR) \times 100$$
, (1.4)

MPE,
$$\% = APE \times n(C_{\text{total}})/n(C_{\text{labeled}}),$$
 (1.5)

where C_{total} is the total number of carbons in the molecule of interest and C_{labeled} is the number of carbons labeled in the molecules.

The APE and MPE expressions are similar when no extra carbons are added to the compound of interest as in liquid chromatography-isotope ratio mass spectrometry (LC-IRMS). However, in gas chromatography-combustion isotope ratio mass spectrometry (GC-C-IRMS), the compounds are mostly

6 1 Mass Spectrometry Techniques for In Vivo Stable Isotope Approaches

derivatized, implying that the additional carbon needs to be taken into account to obtain the enrichment of the intact molecule [35].

Of course, there are other possible transformations of isotopic enrichments that can be used in specific metabolic models. As example, for measuring the fractional synthesis rate (FSR) or the absolute fractional synthesis (ASR) in muscle after infusion of a stable isotope tracer (i.e., with ${}^{13}C_6$ -phenylalanine), the isotopic ratio of phenylalanine extracted from muscle biopsy, as measured by IRMS (i.e., $\delta^{13}C$, ‰), can be transformed into a TTR value using equation (1.6):

TTR, % = 0.0112372 × (0.001 × δ_{sa} + 1) × 100, (1.6)

where δ_{sa} is the isotopic abundance (IRMS data) of the sample [36].

To calculate the isotopic enrichment using gas chromatography-mass spectrometry (GC-MS) or liquid chromatography-mass spectrometry (LC-MS), the baseline unlabeled sample and labeled samples (after administration of the tracer) are subtracted (as described by Wolfe) or can be assessed using a mathematical matrix of mass isotopomer distribution, as reported by Fernandez *et al.* [37], to determine the true isotopomer distribution.

1.2.3.3 Normalization of Isotopic Ratio Expressed with δ Unit

In order to calibrate raw δ values to international references so that interlaboratory comparisons can be carried out, it is crucial to transform raw δ values (data from the IRMS instrument) into normalized δ values for accurate and comparable isotopic determination. In this context, a specific protocol (known as isotopic normalization) needs to be put in place during isotopic analysis. The requirements for isotope normalization have increased dramatically not only with the commercialization of new technology to compare technique performance but also due to the broad types of applications and the increasing number of laboratories that can carry out isotopic analysis. Paul *et al.* described different approaches to normalize isotopic ratios [38]; normalization with two or more certified standards produces less errors than normalization is not mandatory (but advised), since an excess of isotopic enrichment (see Section 1.2.3.2) is the appropriate way to express results.

1.3 Mass Spectrometry Techniques

The basic principle of MS is to produce ions from organic molecules, to separate these ions by their mass-to-charge ratio (m/z), and to detect them qualitatively and quantitatively by their respective m/z and abundance. As schematically represented in Figure 1.1, different options exist to measure light-stable isotopes with MS.

1.3.1 Isotope Ratio Mass Spectrometry

The measurement of natural isotopic abundances and tiny variations of isotopic enrichments in organic molecules requires a very specific technique known



Figure 1.1 Typical elements (i.e., separation mode, ion source, analyzer, and detector) used to measure light-stable isotopes in metabolic studies.

as IRMS. The isotope ratio mass spectrometer, initially developed by Nier, is based on a multicollector magnetic sector mass spectrometer [39]. The theory and practice of IRMS are reviewed in detail elsewhere [40, 41] and will not be reviewed here. Briefly, the isotope ratio mass spectrometer is made of several modules, such as a tight-electron impact ion source, a magnetic sector, and several Faraday cups to simultaneously monitor several ions. To determine small differences in isotopic ratios, parameters such as sensitivity, signal stability, and counting statistics are key parameters that enable high-precision measurements [42]. The IRMS device, or the so-called "gas-IRMS," is designed to measure the isotope ratio of light-stable isotopes, such as ¹³C, ¹⁵N, ¹⁸O, ³⁴S, and ²H, of organic molecules that were previously transformed into gases, such as CO₂, N₂, CO, SO₂, and H₂. Continuous-flow-IRMS is the most common approach (as opposed to the dual isotope system with off-line conversion of organic molecules), due to the ease of sample transformation. Several interfaces are used to produce these gases. High-precision isotopic analysis of solid and liquid bulk samples is achieved using an EA or thermal conversion-elemental analyzer (TC/EA) coupled to an IRMS device for measurement of the ¹³C, ¹⁵N, ²H, and ¹⁸O isotopes, whereas GC and LC conjugated to an IRMS device allow for measurement of the isotopic ratio of specific compound(s) after chromatographic separation.

1.3.1.1 Bulk Stable Isotope Analysis

Bulk analysis of ¹⁵N, first demonstrated by Preston and Owens in 1983, is based on bulk isotopic analysis [43]. Its principle is straightforward since the bulk sample (i.e., powder or liquid) is weighed in a tin capsule that is introduced into a heated combustion interface through an autosampler (i.e., a carousel). Within the heated furnace, the organic bulk material is transformed into gases (i.e., CO₂ and N₂). These gases are carried out in a flow of helium gas stream and introduced into a heated reduction furnace where nitrous oxides are converted into N₂ (Figure 1.2). Then, any excess O₂ and water are removed before introducing the helium stream into the IRMS ion source. By design, the EA-IRMS measures ¹³C and ¹⁵N isotopic abundances. The isotopic precision of EA-IRMS, expressed as standard deviations (SD) of δ , is lower than 0.3‰ for ¹³C and ¹⁵N isotopes for sample amounts greater than 50 nmol of an element, or an amount of nitrogen (as urinary urea and ammonia after adequate processing) from 30 to 150 µg.

1 Mass Spectrometry Techniques for In Vivo Stable Isotope Approaches



Figure 1.2 Schematic representation of an elemental analyzer for EA-IRMS coupling. *Source*: Muccio and Jackson [44]. Reproduced with permission of Royal Society of Chemistry.

To examine the ²H and ¹⁸O isotopic ratios of bulk samples, the oldest approach was based on the cryodistillation of biological samples to produce H₂ gas, followed by a reduction with catalyzers (i.e., zinc and platinum), whereas for ¹⁸O isotope determination, the produced CO gas was equilibrated overnight with unlabeled CO₂ present in the water solution. These processes were time consuming and required large volumes of sample. However, in the 2000s, a new commercial system became available to both measure isotopes with smaller amounts of material and utilize an automated system. In this case, the organic material was not combusted but quantitatively pyrolyzed (at 1420 °C in a glassy carbon reactor within a TC/EA) to produce H₂ and CO gases that were introduced into the ion source of the IRMS device through a helium stream as the carrier gas. Technically, the ability to measure the ${}^{2}H/{}^{1}H$ ratio in a helium (He; m/z 4) stream was challenging, due to the large He peak in the ion source. There is a little overlap of this high abundant peak onto the m/z 3 Faraday cup collector. Because of the high intensity of the helium peak in comparison to the intensity of the ²H/¹H peak, this contributed significantly. The solution was to add a retardation lens into the m/z 3 Faraday cup collector. Moreover, H_3^+ is formed in the ion source, caused by the reaction $H_2^+ + H_2 \rightarrow H_3^+ + H^{\bullet}$. This also contributes to the ²H/¹H peak but can be accounted for by the so-called H₃⁺ factor. Practically speaking, the H₃⁺ factor needs to be assessed daily to obtain precise and accurate isotopic ratios [40]. In this case, the IRMS device is equipped with such specific collectors and is able to accurately measure both ²H and ¹⁸O isotopes (Figure 1.3). Interestingly, the system allows for the simultaneous detection of both isotopes in the same run, limiting the final volume drawn from the patient and increasing the analytical throughput (typical run time is lower

8



Figure 1.3 Typical TC-EA/IRMS chromatogram with H_2 and CO peaks after injection of water sample.

10 1 Mass Spectrometry Techniques for In Vivo Stable Isotope Approaches

than 6 min per sample). The isotopic precision of the TC/EA-IRMS is about 2.0‰ for δ^2 H and 0.3‰ for δ^{18} O. This system is particularly relevant in pediatric studies, where only a small volume of biological fluid (i.e., urine, blood, or saliva) is available.

Finally, a third bulk stable isotope analysis (BSIA) approach was developed for breath ¹³CO₂ isotopic enrichment. Analytically, this is accomplished by a combination of headspace sampling and loop injection onto a GC column capable of resolving different gases, such as CO₂ and N₂, connected to an IRMS device (GC-IRMS). In these conditions, the combustion furnace is off. The analytical measurement per se is very straightforward and the isotopic precision is lower than 0.3‰ for δ^{13} C. This method, known as the ¹³C-breath test [45], allows for the determination of specific clinical outcomes, such as the presence of *Helicobacter pylori* after ingestion of labeled urea or measurements of fat digestion and gastric emptying [46–48].

1.3.1.2 Compound-Specific Isotopic Analysis

One common feature of BSIA and compound-specific isotopic analysis (CSIA) is the use of helium as a carrier gas to transport the targeted gases (i.e., CO_2 , N_2 , H_2 , and CO). However, with CSIA, a chromatographic separation of the targeted compound is carried out prior to the transformation of the organic molecules into gases. The separation can be performed either by GC or LC.

1.3.1.2.1 Compound-Specific Isotopic Analysis with Gas Chromatography–Isotope Ratio Mass Spectrometry

This approach was first coined "isotope ratio monitoring-GCMS" by Matthews and Hayes [49], but today is named continuous-flow-isotope ratio mass spectrometry (CF-IRMS). One of the first technical considerations of CSIA by GC is to reliably convert online organic molecules into gases while maintaining the chromatographic separation and resolution achieved on the GC column. Combustion interfaces (for ¹³C and ¹⁵N applications) used after GC separation were developed in the early 1980s, whereas pyrolysis furnace applications (for ²H and ¹⁸O) were built in the 1990s (Table 1.1). In contrast to IRMS, which is a highly specialized mass spectrometer, the GC system used for GC-IRMS coupling is a standard commercial and generic instrument. Most GC methods are applicable to isotopic measurements in terms of analytical conditions, with helium (He) as the carrier gas.

Principle of Gas Chromatography Combustion Isotope Ratio Mass Spectrometry For measuring either the ¹³C or ¹⁵N isotopic ratios of selected components, GC-C-IRMS fits the purpose. Briefly, after adequate derivatization of polar compounds, the derivatized components are injected into a capillary gas chromatographic column with an autosampler. Individual compounds are carried by a helium stream and separated chromatographically according to their volatility and their interaction with the stationary phase. Then, the helium carrier introduces the compounds into a combustion furnace. This consists of

Light-stable isotopes	Natural abundance (%)	Instrument	Isotopic precision, SD(δ , ‰)	Typical sensitivity (nmol) ^{a)}
² H	0.015	GC-P-IRMS	2-5	10-50
¹³ C	1.11	GC-C-IRMS	0.1 - 0.3	0.1-5
¹⁵ N	0.37	GC-C-IRMS	0.3-0.7	1 - 10
¹⁸ O	0.20	GC-P-IRMS	0.3-0.6	4 - 14

 Table 1.1 Typical light-stable isotopes used in metabolic studies and characteristics of IRMS instruments hyphenated to gas chromatography for measuring light-stable isotopes.

a) Sensitivity expressed in nanomole of the analyzed element injected to get a precision close to the value listed in this table.

Source: Sessions [40]. Reproduced with permission of John Wiley and sons.

a ceramic tube, typically with an inner diameter of 0.5 mm, with metal wires (CuO/NiO/Pt), which is heated to 940 °C, where each compound is converted into CO₂, water, and nitrogen oxide (NOx) gases. In order to get rid of these NOx gases, a reduction furnace (heated to 650 °C and containing Cu and Pt wires) is installed in series, where nitrogen oxide gases are transformed into N₂O and NO₂. Water is removed by a Nafion[®] water trap, and finally a small fraction of the gases (in the helium stream) is introduced into the IRMS ion source (Figure 1.4). The remainder of the gas stream is diverted to the atmosphere via a split. By design, the IRMS can only accept a maximum of 0.4 mL/min of helium carrier gas [41].

Principle of Gas Chromatography Pyrolysis Isotope Ratio Mass Spectrometry For measuring deuterium and Oxygen-18 in compounds after a chromatographic separation, a pyrolysis furnace is used instead of a combustion furnace. The pyrolysis furnace is heated to 1400 °C [50]. At this temperature, organic components are transformed into H₂ and CO gases when oxygen is present. The high temperature for pyrolysis requires a high-purity Al₂O₃ (alumina) reactor tube. At such a temperature, alumina tubes are sensitive and leaks may develop over time. Within gas chromatography-pyrolysis isotope ratio mass spectrometry (GC-P-IRMS), alumina tubes have to be replaced more often than reactors used in combustion systems (for ¹³C or ¹⁵N). In addition, many users and suppliers recommend conditioning the pyrolysis reactor from time to time with injections of organic solvent or via backflushing with CH_4/He gas. This likely prevents deposits of carbon inside the alumina tubes, which decreases its efficiency [51]. One additional difference with the combustion interface is the absence of Nafion membranes, since pyrolysis of organic compounds does not produce water. Of note, halogen atoms induce contaminants for the pyrolysis process and memory effects generated may impact the accuracy and precision of deuterium isotopic measurements produced by GC-P-IRMS [52].