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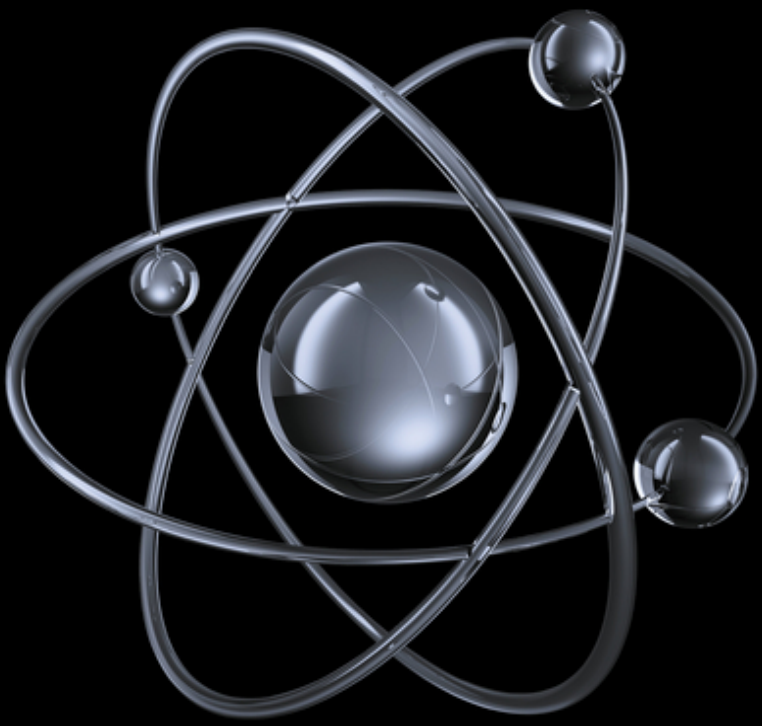

Mass Spectrometry and Stable Isotopes in Nutritional and Pediatric Research

Edited by Henk Schierbeek

WILEY

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Contents

List of Contributors

Saskia J.H. Brinkmann
Department of Surgery
VU University Medical Center Amsterdam
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
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

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 ^{18}O, as well as for ^{13}C 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 ^{13}C-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 ^{13}C-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

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 ^{13}C 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
CP	cerebral palsy

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
HMT	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

MCAD	medium-chain acyl-CoA dehydrogenase
min	minute
MPE	molar percent excess
MS	mass spectrometry
MTX	methotrexate
mV	millivolt
MW	molecular weight
N	chromatographic efficiency
n	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 oxide
NO_2	nitrogen dioxide
NOLD	nonoxidative leucine disposal
NPP	*N*-pivaloyl isopropyl ester
O_2	oxygen gas
OA	oleic acid
P&T	purge and trap
P	phosphorous
P5C	pyrroline-5-carboxylate
PA	palmitic acid
PDB	Pee Dee Belemnite
PET	positron emission tomography
PEW	protein energy wasting
PH	primary hyperoxalurias
PH1	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_{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

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Mass Spectrometry Techniques for *In Vivo* Stable Isotope Approaches

Jean-Philippe Godin[1] *and Henk Schierbeek*[2]

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

1.1 Introduction

Interest in the use of light-stable isotopes (i.e., Carbon-13 or ^{13}C; Nitrogen-15 or ^{15}N; deuterium or ^{2}H; Oxygen-18 or ^{18}O) 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

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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 ^{13}C, 0.37% for ^{15}N, 0.20% for ^{18}O, and 0.015% for ^{2}H. 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 ^{13}C compared to environmental CO_2. A variation of the $^{13}C/^{12}C$ 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 $^{13}C/^{12}C$ ratio (expressed as $\delta^{13}C$) 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 ^{13}C. Their $^{13}C/^{12}C$ ratio ($\delta^{13}C$) 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,

where the use of radioisotopes is extremely limited for safety reasons. Several different stable isotope tracers can be safely administered to children. For example, [^{15}N]-glycine and [1-^{13}C]-leucine were simultaneously administered in preterm infants for measuring whole-body protein turnover [25, 26]. Cogo *et al*. infused [^{13}C]-palmitic acid and [$^{2}H_3$]-leucine for 3 h and [$^{2}H_5$]-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]. ^{13}C and ^{15}N tracers are commonly employed to trace amino acids, whereas, by design, lipids and small carbohydrates can be artificially enriched with ^{13}C, 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 ^{18}O 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 ^{18}O 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 $^{13}C/^{12}C$, $^{18}O/^{16}O$, $^{2}H/^{1}H$, and $^{15}N/^{14}N$, 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).

$$\delta, ‰ = [(R_{sample} - R_{standard})/R_{standard}] \times 1000, \quad (1.1)$$

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., $^{2}H_2O$) to measure body composition or use double-labeled water (i.e., $^{2}H_2{}^{18}O$) 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):

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

where $\delta^{2}H$ is the per mil ^{2}H 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):

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

where r_{sa} is the ratio of tracer/tracee in the sample (after administration of the tracer), r_{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 ^{13}C 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):

$$\text{APE}, \% = \text{TTR}/(1 + \text{TTR}) \times 100, \quad (1.4)$$

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

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

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):

$$\text{TTR}, \% = 0.0112372 \times (0.001 \times \delta_{sa} + 1) \times 100, \tag{1.6}$$

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 carried out with only one. In most metabolic tracer studies, isotopic 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

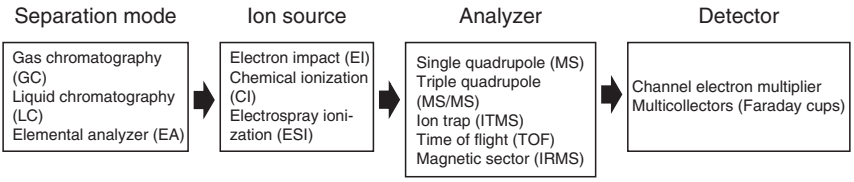

Figure 1.1 Typical elements (i.e., separation mode, ion source, analyzer, and detector) used to measure light-stable isotopes in metabolic studies.

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 ^{13}C, ^{15}N, ^{18}O, ^{34}S, and ^{2}H, of organic molecules that were previously transformed into gases, such as CO_2, N_2, CO, SO_2, and H_2. 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 ^{13}C, ^{15}N, ^{2}H, and ^{18}O 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 ^{15}N, 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., CO_2 and N_2). 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 N_2 (Figure 1.2). Then, any excess O_2 and water are removed before introducing the helium stream into the IRMS ion source. By design, the EA-IRMS measures ^{13}C and ^{15}N isotopic abundances. The isotopic precision of EA-IRMS, expressed as standard deviations (SD) of δ, is lower than 0.3‰ for ^{13}C and ^{15}N 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.

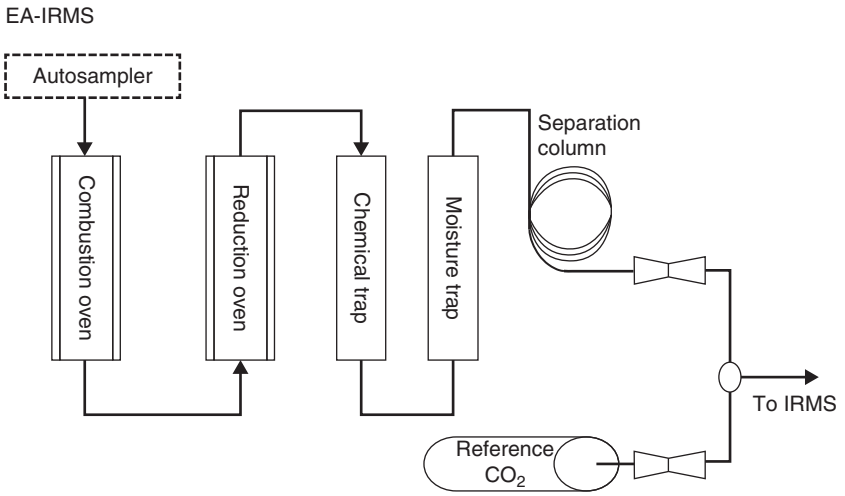

Figure 1.2 Schematic representation of an elemental analyzer for EA-IRMS coupling. *Source*: Muccio and Jackson [44]. Reproduced with permission of Royal Society of Chemistry.

To examine the ^{2}H and ^{18}O isotopic ratios of bulk samples, the oldest approach was based on the cryodistillation of biological samples to produce H_2 gas, followed by a reduction with catalyzers (i.e., zinc and platinum), whereas for ^{18}O isotope determination, the produced CO gas was equilibrated overnight with unlabeled CO_2 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 H_2 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 $^{2}H/^{1}H$ 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 $^{2}H/^{1}H$ peak but can be accounted for by the so-called H_3^+ factor. Practically speaking, the H_3^+ 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 ^{2}H and ^{18}O 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

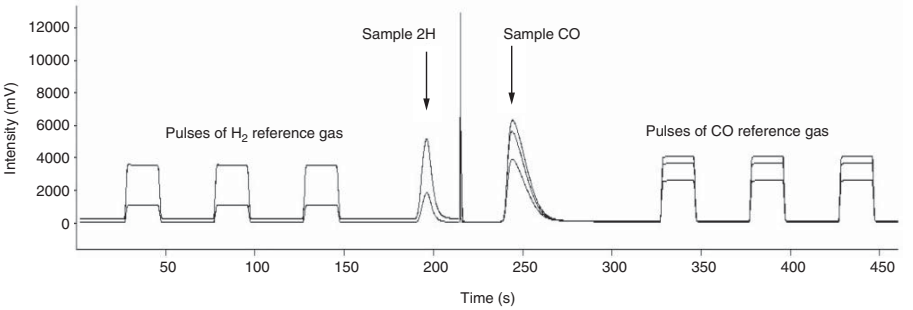

Figure 1.3 Typical TC-EA/IRMS chromatogram with H_2 and CO peaks after injection of water sample.

than 6 min per sample). The isotopic precision of the TC/EA-IRMS is about 2.0‰ for δ^2H 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 $^{13}CO_2$ 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 CO_2 and N_2, 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 ^{13}C-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 ^{13}C and ^{15}N applications) used after GC separation were developed in the early 1980s, whereas pyrolysis furnace applications (for ^{2}H and ^{18}O) 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 ^{13}C or ^{15}N 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

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.

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

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 CO_2, 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 N_2O and NO_2. 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 H_2 and CO gases when oxygen is present. The high temperature for pyrolysis requires a high-purity Al_2O_3 (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 ^{13}C or ^{15}N). 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].