1

Mass Spectrometry Techniques for *In Vivo* Stable Isotope Approaches

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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

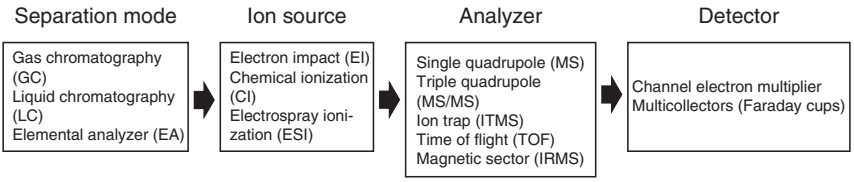

Figure 1.1 Typical elements (i.e., separation mode, ion source, analyzer, and detector) used to measure light-stable isotopes in metabolic studies.

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.

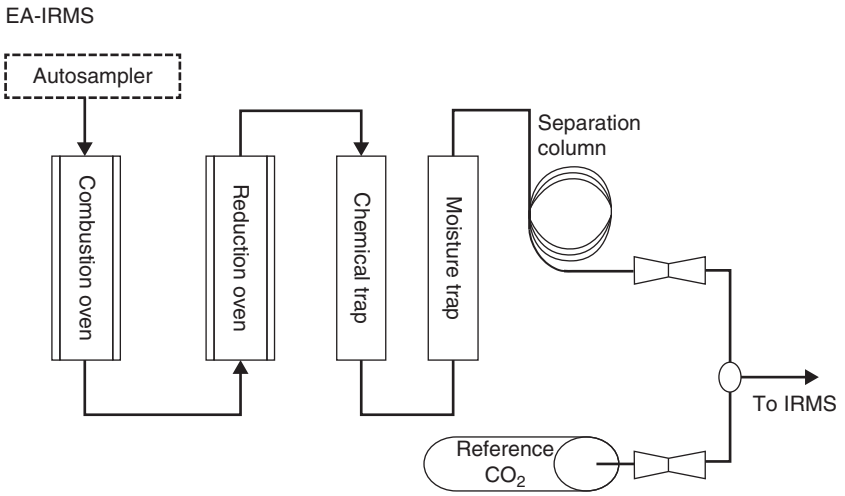

Figure 1.2 Schematic representation of an elemental analyzer for EA-IRMS coupling. *Source*: Muccio and Jackson [44]. Reproduced with permission of Royal Society of Chemistry.

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

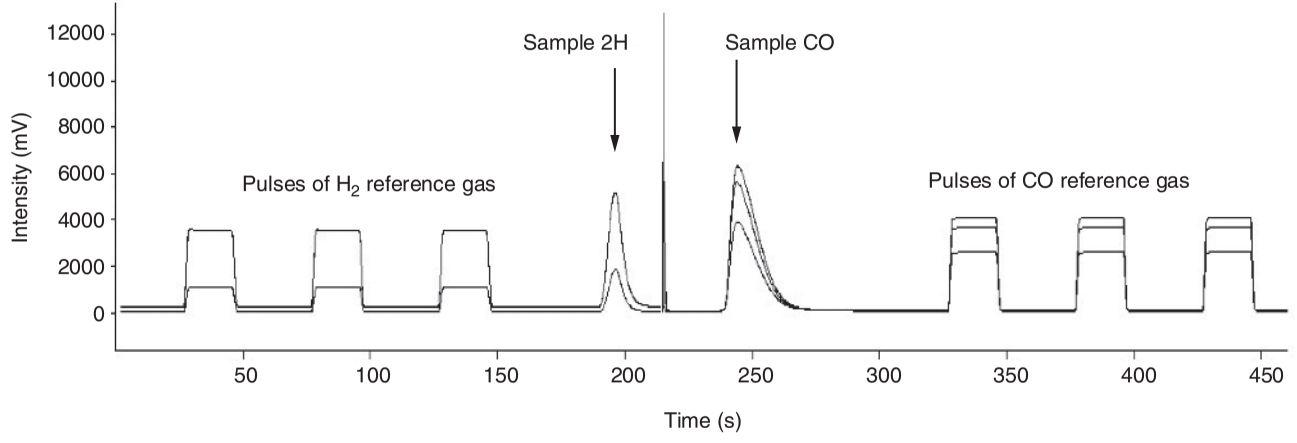

Figure 1.3 Typical TC-EA/IRMS chromatogram with H_2 and CO peaks after injection of water sample.

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].

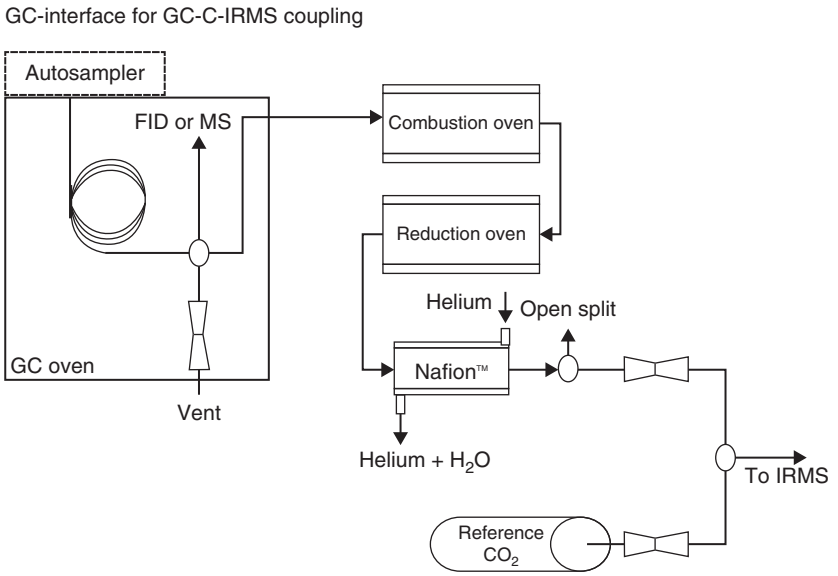

Figure 1.4 Schematic representation of GC interface for GC–C-IRMS coupling. *Source*: Muccio and Jackson [44]. Reproduced with permission of Royal Society of Chemistry.

Sample Preparation and Gas Chromatography Separation The fundamental aspect of GC is to separate the different components and to provide baseline chromatographic resolution between peaks [53]. This is of paramount importance for precisely and accurately measuring isotope ratios, such as in doping applications [54]. High-resolution chromatographic columns may separate molecules with different isotope contents, meaning that a component with one deuterium or one Carbon-13 atom will be chromatographically separated from its nonlabeled counterpart. This factor can be a real chromatographic issue when complex matrices are analyzed; therefore, adequate peak integration and background signals are important parameters to consider [55]. In such circumstances, fast GC–C-IRMS [56] and two-dimensional GC-IRMS [57, 58] may not only improve chromatography separation but also may have other challenges for isotopic abundance determination. There are only a few papers reporting such approaches, and none were metabolic or pediatric studies.

For high-precision isotopic analysis in biological fluids (i.e., plasma, urine, and saliva) or tissue (i.e., muscle), regardless of the targeted isotopes, several critical steps need to be considered. The most important ones are sample preparation and gas chromatographic separation, although isotopic standardization and processing are also critical [59]. Sample preparation may sound trivial for many analytical chemists using classical mass spectrometers, but for high-precision isotopic analysis, due to the low specificity of IRMS and the possible isotopic fractionation that may occur, these steps require careful attention. Meier-Augenstein [53] provided an extensive review of the conditions

for ^{13}C and ^{15}N isotopic analysis by GC with detailed protocols. Briefly, a typical GC sample preparation protocol implies (1) an isolation step(s) of the targeted component from complex matrices (i.e., plasma, muscle), (2) the choice of an internal or external standard with a known isotopic ratio, and (3) a derivatization step to ensure that the targeted molecule is volatile. During these steps, it is important to pay close attention to isotopic mass discrimination so as to avoid isotopic fractionation of the target component [22]; if not, this may affect the accuracy and precision of the approach and increase the error in the final results. Although isotopic fractionation is not a major issue for an *in vivo* tracer approach (as opposed to areas such as forensic sciences), careful attention to the choice of the reagents of derivatization is recommended in order to obtain reliable, robust chromatographic conditions and to limit the isotopic dilution from large and unnecessary atoms from the derivative itself. For example, silylation reagents are very popular in GC–MS for derivatization of polar groups, such as hydroxyl, amino, and thiol groups. However, for GC–C-IRMS, silylation reagents that add trimethylsilyl (TMS) or *tert*-butyl-dimethylsilyl (tBDMS) groups bring a large number of additional carbons into the targeted component, affecting the final isotopic ratio of the targeted derivatized component. It may also produce siliceous deposits on the oxidation catalyst, reducing its surface area and gas flow through the combustion reactor [60]. For amino acid isotopic analysis, optimization of the derivatization conditions is important, as described by Corr *et al*. [61], and various derivatives have been used. Of note, most of them were applied for ^{13}C and ^{15}N isotopes, a few for deuterium, and none for ^{18}O. The absolute isotopic abundance of free plasma ^{15}N amino acids varies, either due to the sample preparation method (via ion-exchange chromatography) and/or due to amino acid metabolism, which affects the $^{15}N/^{14}N$ isotopic ratios (i.e., deamination and transamination, as discussed by Metges and Petzke [62]). When deuterated labels are used, loss of one or more isotopic labels due to hydrolysation techniques is very common. One of the most popular derivatizing techniques for amino acids is using an alkyl chloroformate reagent. Several papers have described these techniques [63–68]. For small carbohydrates, different analytical strategies were developed to optimize the sample preparations. For example, Jackson *et al*. proposed a common derivative suitable for both GC–MS and GC–C-IRMS, allowing for precise measurements of deuterium and ^{13}C atoms with a glucose alkylboronate derivative [69]. Other analytical strategies, such as online solid-phase microextraction with GC–C-IRMS, were also optimized to reduce isotopic fractionation and applied in a clinical study that looked at acetate and butyrate isotopic enrichments in plasma samples [70].

1.3.1.2.2 Compound-Specific Isotopic Analysis with Liquid Chromatography–Isotope Ratio Mass Spectrometry

For decades, several developments have attempted to couple LC and IRMS (LC–IRMS) [71]; however, the technical challenge was to quantitatively transform, without any isotopic discrimination, organic molecules solubilized in a buffer (mostly organic with reverse-phase chromatography) into CO_2 gas and

to then extract the gas from a liquid into a helium stream that was introduced into the ion source of the IRMS. Progress was only achieved in 2004 when a commercial interface was developed that facilitated LC coupling with IRMS (LC–IRMS) [72].

The interface to couple the LC and IRMS, proposed by Thermo Finnigan (named LC Isolink®) or Elementar (named LiquiFace®), is based on the chemical wet oxidation of organic molecules to produce CO_2 gas in aqueous solution within a heated reactor (at 99 °C), where phosphoric acid, sodium peroxodisulfate, and the LC eluent are mixed together. Then, the inorganic eluent containing CO_2 is carried through a separation unit where CO_2 gas is extracted and selectively transferred into a helium stream. Water is removed from the gas bypassing through two Nafion membranes, and finally the He stream enriched with CO_2 gas is introduced into the IRMS ion source (Figure 1.5). By design, some level of oxygen is also introduced into the ion source, leading to some detrimental effects on the lifetime of the filament and on isotopic accuracy and precision [73, 74]. A new interface has been described recently, which has the ability to measure both ^{13}C and ^{15}N isotopes. It is based on a modified high-temperature combustion total organic carbon analyzer and the proof of concept was reported by measuring caffeine samples [75].

Compared to GC–C-IRMS, the LC–IRMS interface is less versatile in terms of potentially analyzable isotopes, since only the $^{13}C/^{12}C$ isotopic ratio can be routinely determined. On the other hand, LC–IRMS is unique in that it has two modes of isotopic analysis: one via an LC column to separate components to

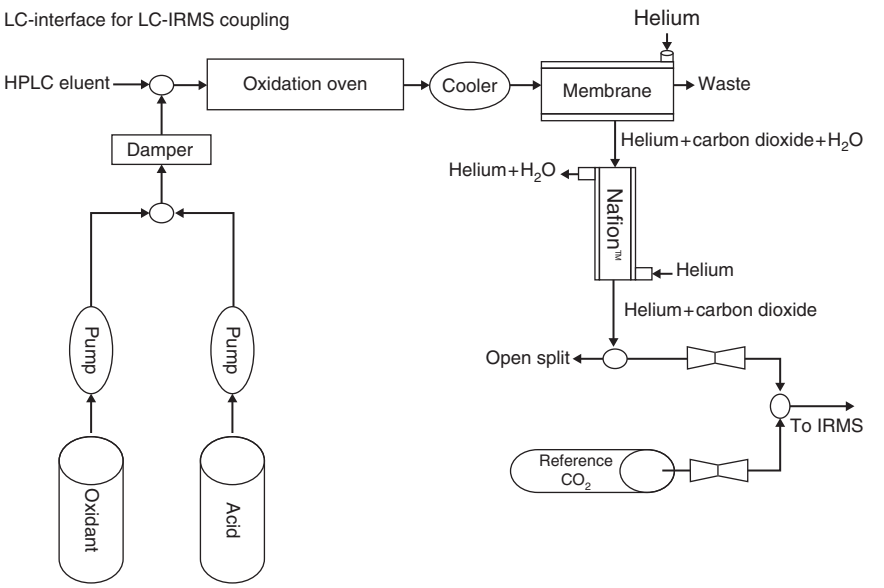

Figure 1.5 Schematic representation of LC interface for LC–IRMS coupling. *Source*: Muccio and Jackson [44.]. Reproduced with permission of Royal Society of Chemistry.

obtain their individual $^{13}C/^{12}C$ ratios and a second mode of isotopic analysis named flow-injection analysis (FIA) [76]. This mode of isotopic analysis is for both low-molecular-weight and high-molecular-weight components (i.e., albumin, insulin) [77], and is straightforward and similar to EA-IRMS for hydrosoluble molecules (solubilized in aqueous nonorganic buffer). The main advantage of FIA-IRMS, compared to EA-IRMS, is its sensitivity. The amount of material required for analysis is a 100 times less.

By design, LC–IRMS is only suitable for high-precision isotopic analysis of hydrosoluble molecules, such as amino acids, volatile fatty acids, alcohols, some phenolic acids, some simple carbohydrates, nucleotides, peptides, and proteins [78]. To analyze these components, the key features of the analytical methods must be compliant with the following analytical constraints: (1) only inorganic buffers can be used; (2) only water-soluble components can be analyzed; (3) the total flow rate (i.e., LC column plus the flow rate of acid and oxidant) must be lower than 700 µL/min; and (4) the acidic mobile phase achieves more efficient CO_2 extraction from the liquid.

One theoretical advantage of LC versus GC is the sample preparation method, which is simpler in LC. With LC separation, the components are first solubilized in a buffer and then separated without the need for derivatization. However, the LC peak widths obtained under these conditions are larger than those found using GC separations. To achieve reliable isotopic measurement, baseline separation of the molecule of interest is mandatory and therefore careful optimization of the chromatographic efficiency and selectivity (i.e., the column length, the stationary phase of the LC column, and the temperature of the LC column and buffer) is necessary [71, 79, 80]. The isotopic precision of LC–IRMS is close to that of GC–C-IRMS and will vary by the conditions used; for example, for underderivatized amino acids in plasma, SD($\delta^{13}C$) was about 0.05–0.5‰, whereas the isotopic accuracy ranged from 0.05‰ to 0.9‰. For simple carbohydrates, as reported by Moerdijk-Poortvliet *et al*., the isotopic precision and accuracy is better for LC–IRMS compared to GC–C-IRMS (i.e., 0.1 vs 0.7‰ for LC–IRMS and GC–C-IRMS, respectively, at natural abundance) [81]. The analytical constraints described above, however, make isotopic analysis by LC–IRMS quite complex and have limited its development and application in metabolic studies.

1.3.2 Gas-Chromatography- and Liquid-Chromatography-Based Mass Spectrometry Methods

An advantage of organic mass spectrometry (MS or MS/MS) over IRMS is the possibility for assessing the intramolecular position of a labeled atom in a molecule. Therefore, with the advanced use of stable isotopes and the development of GC–MS as a standard analytical tool in the 1970s, it became possible to determine the rate of transamination *in vivo* and the rate of protein turnover using doubly labeled ^{15}N,^{13}C-leucine (^{15}N,^{13}C-Leu) [82]. Overall, the benefits of MS were substantial and allowed researchers to combine several tracers in the same study, providing the investigator with the possibility of obtaining additional

scientific insights with a reduced number of subjects. In the last few decades, with the development of increasingly user-friendly software, high-performance mass spectrometers are relatively easier to operate and are robust if they are correctly maintained.

1.3.2.1 Gas Chromatography–Mass Spectrometry

GC–MS is a well-established technique, available in many laboratories for the quantitative determination of small molecules (i.e., 50–700 Da). It is able to distinguish different isotopes in labeled substrates in a wide range of components.

After chromatographic separation, neutral molecules are eluted from the GC column and are ionized (charged positively or negatively) in the ion source, before finally traveling in the mass spectrometer. The efficacy of the ionization is an important factor to take into account in GC–MS. The ions are formed within the ion source by either electron ionization (EI) or chemical ionization (CI). EI is a more harsh technique, leading to extensive fragmentation. Briefly, in the ion source, neutral molecules are bombarded with electrons at 70 eV of energy, and once an electron collides with neutral molecules, a radical cation ($M^{+\cdot}$) or anion ($M^{-\cdot}$) is formed (also called molecular ion). When the molecular ion retains a large amount of energy, it may be fragmented to produce cations or neutral fragments that cannot be observed in the mass spectrum. The fragmentation pattern of the parent molecule depends on the strength of the molecular bonds. The lower energy bonds will require less energy to break and will thus break more often than higher energy bonds. The resulting fragmentation pattern, as observed in mass spectra, is highly reproducible. For GC–MS, libraries with reference spectra are available, aiding in the identification of unknown compounds.

Mass spectra obtained with EI can be used for identification purposes; the drawback is that molecular ions are generally not abundant (low intensity). Thus, there is increased risk of background interference at lower m/z values compared to CI. However, EI is a highly robust and a very reproducible ionization technique. CI is a much milder ionization technique than EI and therefore less fragmentation of the parent molecule occurs. This technique requires that a gaseous mixture consisting of a reagent gas (i.e., methane, ammonia) and the sample molecules are present simultaneously in the ion source in a molar ratio of approximately 1000:1. In this configuration, the neutral molecule is not ionized by the electron but rather by the ionized bulk of reagent gas. The $[M + H]^+$ signal is often observed, but adduct formation (based on the reagent gas used) is also possible (e.g., $[M + NH4]^+$ can be found when using ammonia as a reagent gas). This leads to higher masses than are typically observed with EI. The intensity of the base peak (i.e., the most intense peak) of the mass spectrum is typically higher with CI than EI, as the majority of the ions will not undergo fragmentation. The signal strength for this specific m/z value will be greater than the base peak in the mass spectrum obtained with EI, resulting in increased sensitivity. One drawback of CI is that it is less reproducible, as the introduction of ionization gas shows small variations. In addition, in CI mode, the GC–MS ion source must be cleaned more frequently [83, 84].

1.3.2.2 Liquid Chromatography–Tandem Mass Spectrometry

Liquid chromatography–tandem mass spectrometry (LC–MS/MS) techniques are often used to analyze compounds that are nonvolatile and thermally unstable. With the development of atmospheric ionization technique in the mid-1980s and more specifically electrospray ionization source (ESI), LC–MS/MS has almost become a routine technique such as GC–MS. The ESI can continuously evaporate liquid solvent and has become an important technique for use as an interface for LC–MS techniques. As liquid solutions flow out of the analytical column, a high voltage is applied to the solution, creating a fine spray of highly charged droplets. Simultaneously, dry nitrogen gas and heat are applied to the charged droplets to aid the evaporation of the solvent at atmospheric pressure [85–87].

ESI is a soft ionization process that allows for the observation of intact molecules that are multiple or single charged. The number of charges on a particular molecule depends on several factors such as structure, size, composition, solvents used in LC, and instrument parameters. In general, single-, double-, or triple-charged molecules are produced for small molecules (<2000 Da), while multiple charges are produced for larger molecules (>2000 Da). In addition, the ability to produce multiple-charged molecular ions makes it possible to observe very large molecules (e.g., a large variety of polymers, proteins, and DNA fragments) using an instrument with a relatively small mass range.

LC–MS/MS also allows for the sensitive detection of single-charged low-molecular-weight molecules (e.g., amino acids, drugs, and metabolites) [86]. Furthermore, molecular ionization processes can result in positively and negatively charged ions. For example, protonated, ammonium, and alkali adducts are generally observed in the mass spectra for positively charged molecules, while deprotonated molecules are observed for negatively charged compounds [88]. Generally, positive ion detection is used for molecules with functional groups that readily accept a proton (such as amines), and negative ion detection is used for molecules with functional groups that readily lose a proton (such as carboxylic acids). Although most molecules will be initially ionized in the positive ion mode, successful analysis cannot always be accomplished in positive ion mode. Therefore, negative ion mode can be advantageous since it sometimes generates less chemical background noise compared to positive ion mode [87].

1.3.2.3 Scanning Technique in Mass Spectrometry and Tandem Mass Spectrometry

The most convenient method of mass scanning is full mass range scanning. In this mode, the mass spectrometer scans a wide range of masses surrounding the predicted target. It is typically used to analyze unknown compounds or as a first step to check the efficacy of the sample preparation. In this mode, it is possible to identify peaks according to the obtained mass spectra. Libraries exist in which compounds can be identified from their mass spectra. Therefore, to check the specificity of the method, it is possible from the full scan results to monitor if there are interferences coeluting with the compound of interest and that have similar m/z.

Another scanning mode frequently used in GC–MS (or in LC–MS) equipped with a single quadrupole is selective ion monitoring (SIM mode). In this mode, a specific m/z value is monitored during chromatographic separation. More time is spent on collecting ions of this specific m/z value than in the scanning mode. Therefore, sensitivity is enhanced up to three orders of magnitude (depending on the mass range). This is a useful mode of analysis for the quantitation of target components, even when they are not chromatographically resolved from other compounds.

A triple quadrupole instrument (QqQ or tandem MS) is equipped with three separate and successive quadrupoles [89]. The addition of two extra quadrupoles opens up possibilities for a variety of experiments. Scanning modes include product, neutral loss, parent, and selected reaction monitoring (SRM). The mode used is based on the type of information needed. The second quadrupole of the triple quadrupole operates as a collision cell (q2). While the entire mass spectrometer operates under vacuum, a small amount of inert gas (typically nitrogen, argon, or helium) is let into the collision cell. The ions passing through the first quadrupole (Q1) and entering the second quadrupole (Q2) collide with these molecules, which results in fragmentation.

Q1 and the last quadrupole (i.e., Q3) can be independently operated in both scanning and SIM modes. Ions in Q1 are named precursor ions, while those in Q3 are product ions. A triple quadrupole is tandem MS in space, as the analysis of the precursor ions and product ions are carried out in separate compartments of the instrument.

When Q1 is set to let through (a) specific m/z value(s) and Q3 is in scanning mode, this is called product scan. This mode can be used to elucidate the structure of a compound of interest. When Q1 is in scanning mode and Q3 set to let through (a) specific m/z value(s), this is called precursor scan. In this mode, compounds can be found that produce specific ions during fragmentation (e.g., ions typical for a specific functional group). When both quadrupoles operate in scan mode, scanning with a fixed offset, this is called neutral loss scan. Similar to parent scan, this operation mode can be used to study the loss of specific functional groups upon fragmentation. This mode can be used for functional groups that do not produce a specific ion, but that instead lead to the loss of a neutral fragment.

The operating mode of interest for enrichment analysis is the multiple reaction monitoring (MRM) mode. Another term is SRM; these names are interchangeable. In this mode, a specific ion is selected in Q1. After fragmentation in the collision cell, a newly formed fragment is selected in Q3.

The linear ion trap mass spectrometry (ITMS) device is another common type of mass spectrometer that is often used for isotopic analysis. It consists of a quadrupole or a spherical trap that capture ions. It can also be used as a selective mass filter [90–92]. The quadrupole or spherical trap captures ions in a stable oscillating trajectory that depends on the potential and the m/z ratio of the molecular ions. During detection, the potential is altered to produce instabilities in the ion trajectories, resulting in ejection of the ions in order of increasing m/z ratio. The ITMS device is also capable of triple quadrupole-like

fragmentation. The precursor ion is selected in the trap, where an inert gas is introduced for fragmentation. In the next step, the product ions are ejected from the trap for detection. In addition, fragmentation of the product ions can occur several times (MS^n) by keeping the product ions inside the trap and repeating the fragmentation procedure. ITMS differs from QqQ instruments in the consecutive entrance of the ion beam rather than a continual entrance in the mass analyzer. A triple quadrupole is MS/MS in space, as the analysis of the parent ions and product ions are in separate compartments of the instrument, whereas ITMS is MS/MS in time.

1.3.2.4 Single Versus Triple Quadrupole Mass Spectrometry

In SIM mode (used in single quadrupole MS), there is a possibility that coeluting compounds produce a similar fragment as the fragment that was selected for the compound of interest. The chance of having compounds coeluting with the same MRM transition is very small, resulting in a higher selectivity and specificity. Therefore, less compound separation and less stringent sample preparation are necessary. MRM has a number of advantages over SIM. In addition to increased selectivity, analysis of MRM transitions also results in increased sensitivity. Noise levels are lower, as the probability of unintentional fragments passing through both quadrupoles is very low. The lower limit of quantification (LLOQ) is defined as the amount of sample with a signal-to-noise ratio (S/N) higher than 10. Thus, the decreased noise level observed with MRM makes it possible to measure fragments at lower abundances. The upper limit of quantification (ULOQ) is typically caused by saturation of the detector. Saturation is expected to occur at abundances comparable to those in single quadrupole measurements. Therefore, in addition to the advantage of a decreased LLOQ, a similar ULOQ for both devices will result in an increased linear dynamic range for the triple quadrupole, as compared to the single quadrupole. In practice, triple quadrupole outperforms single quadrupole at low enrichments or low abundances; the reduced noise levels observed with triple quadrupole, in particular, will positively affect the results. However, the device is more expensive, and method development involves several additional steps, as compared to single quadrupole. Proper collision energies and product fragments need to be found to measure the isotope labels.

1.3.2.5 Gas Chromatography–Mass Spectrometry and Liquid Chromatography–Mass Spectrometry Requirements for Isotopic Analysis

In MS, fragmentation of the labeled molecules (tracers) can result in the loss of stable isotope labeled atoms, so understanding fragmentation patterns is important for appropriately monitoring the fragments that contain the isotopic information in order to perform accurate isotopic enrichment measurements.

1.3.2.5.1 Technical Requirement for Isotopic Analysis by Gas Chromatography–Mass Spectrometry

The main features of the GC-based MS approach for assessing isotopic enrichment are as follows: (1) high sensitivity in terms of the amount of material

needed, as compared to IRMS (typically, pg levels are enough); (2) only molecules with molecular weights lower than approximately 700 Da are amenable to GC separation; and (3) in SIM mode, the limited accuracy and precision of isotopic abundance allow for the reliable determination of isotopic enrichment higher than 0.5 atom% excess for ^{13}C and ^{15}N. A typical example of GC–MS isotopic data is reported in Figure 1.6.

One of the key requirements of GC–MS is the derivatization step used to produce volatile components. Technically, this step may add some complexity to isotopic determination. Indeed, in complex matrices (i.e., urine and plasma), the chromatographic separation or the choice of the derivative that includes silicon (Si), boron (B), or a large number of carbon or nitrogen atoms may interfere with the isotopic pattern. The consequence is an erroneous mass isotopomer determination that decreases measurement precision, especially at low isotopic enrichment. Therefore, a rule of a thumb for the choice of the derivative used in GC-based methods for measuring isotopic enrichment is to select small derivatizing groups without Si and B atoms and to choose appropriate ionized fragments containing low interferences, as reported by Antoniewicz *et al*. [93, 94].

1.3.2.5.2 Technical Requirement for Isotopic Measurement by Liquid Chromatography–Tandem Mass Spectrometry

The single quadrupole (i.e., for LC–MS) is rarely used to measure isotopic enrichment in biological fluids, and only a few attempts have been reported [95]. The lack of precision to measure isotopic enrichments also reflects its low presence in laboratories as compared to the LC–MS/MS.

For tandem MS (i.e., LC–MS/MS), the operating mode of interest for isotopic enrichment analysis is the SRM.

For isotopic analysis by ITMS, both the full-scan and SIM modes are commonly used. However, for accurate isotopic enrichment analysis, the best results are obtained by using the full-scan mode rather than the SIM mode. In the SIM mode, ions are isolated in a waveform, which means that the focus is on ions in the center of the selected mass range. Since the SIM mode is designed to isolate a narrow range of ions or a single mass, the various isotopes should be measured individually to determine TTRs. On the other hand, in the full-scan mode a range of ions are collected and detected, regardless of the ions at the extremes of the selected mass range. As a result, in the full-scan mode, the ions are collected and detected in a single step, resulting in more accurate TTRs. In addition to the full-scan mode, enhanced resolution (e.g., zoom mode) is recommended for accurate enrichment analysis, since isotopic peaks are baseline-separated. In addition, the enhanced resolution may also be beneficial separating coeluting matrix interferences.

In ITMS, fragmentation of fragile ions during the isolation of the precursor ion in the SRM mode results in a loss of isolated ion intensity. To obtain adequate ion intensity of fragile precursor ions in the SRM mode, a wider ion isolation width is required. However, the increased isolation width significantly diminishes the

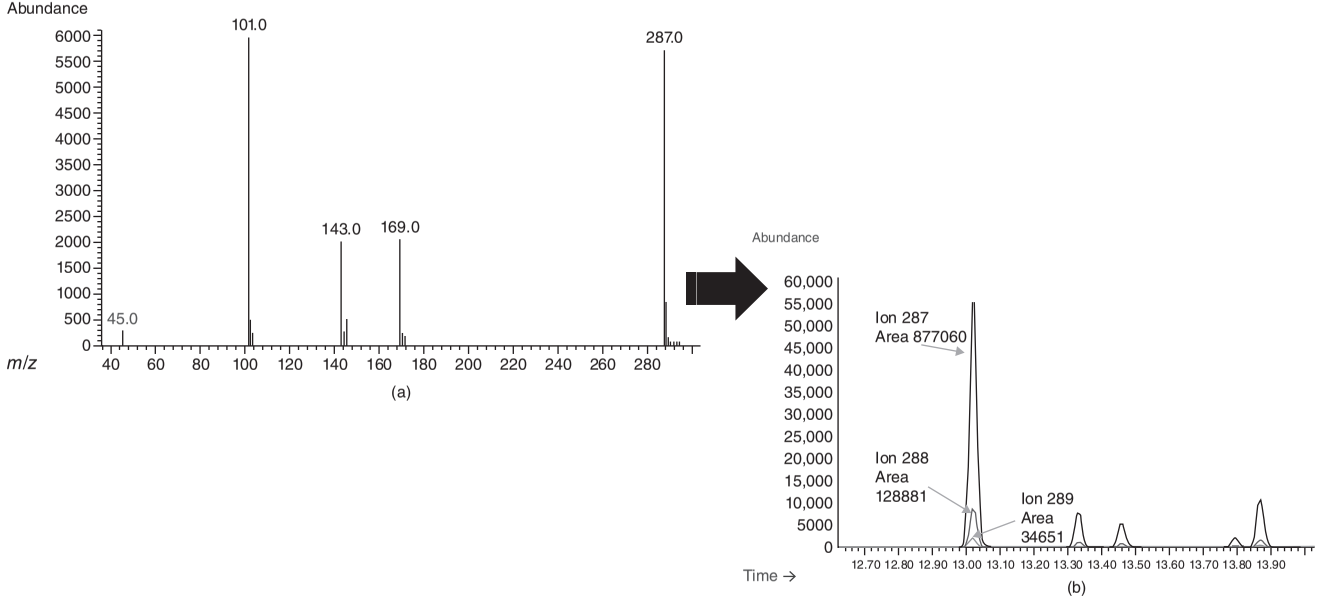

Figure 1.6 (a) The mass spectrum of di-o-isopropylene acetate derivative of glucose obtained from 100 μL of plasma after GC separation. (b) The monitoring of ions M, M + 2, and M + 6 (*m/z* 287, 289, 293, respectively) in time.

selectivity of the SRM transitions, which is a serious problem for samples that contain complex matrices [96]. As stated before, it is important to understand the fragmentation pattern in order to properly use fragments containing the stable isotope. Several applications have been reported that relate to the stable isotope enrichment of amino acids in plasma with ITMS analysis using enhanced resolution [97, 98]. Using ITMS, derivatization of amino acids enables estimation of isotopic enrichments down to 0.005% TTR [97].

MS resolution is an important issue in ITMS as well as QqQ analysis, since the isotopic peaks should be baseline-separated for accurate isotopic enrichment. In most cases, the sensitivity is compromised with both techniques so as to improve the resolution [99]. In general, QqQ provides higher sensitivity (at least 20-fold), better linear dynamic range (QqQ 10^5-10^6; ITMS 10^4-10^5), and better repeatability (QqQ (5–9%); ITMS (12–16%)) than does ITMS. In contrast, ITMS has better resolution (QqQ varying from 0.07 to 1 $\Delta m/z$, ITMS varying from 0.05 to 0.1 $\Delta m/z$) [99].

The ability to separate these different isotopes gives new opportunities for tracer studies. When running a study with a ^{15}N-labeled tracer, more accurate ratios can be obtained when the ^{15}N tracer is separated from the ^{13}C naturally occurring isotope. Fourier Transform Mass Spectrometers such as the ion cyclotron Fourier transform (ICR-FT) and orbital trapping FT systems give sufficient resolution power to separate these different isotopes. ICR-FT, however, lacks sufficient dynamic range to properly support these experiments, making orbital trapping FT-based instruments the current state-of-the-art instrumentation to use for these experiments.

Orbital trapping MS (Figure 1.7) uses a central electrode around which ions move in stable trajectories in an electrical field [100]. The axial motion is proportionally related to the m/z values. Frequencies are measured via the outer electrode of the device and separated via fast Fourier transform and calculated as m/z values. Resolutions of up to 450,000 (FWHM) at m/z 200 are easily obtained in a routine, bench-top instrument. An orbital trapping detector can be combined with other mass spectrometers to give hybrid instruments (Quadrupole-Orbitrap, Ion Trap-Orbitrap, or Tribrid-(Quadrupole-Ion Trap-Orbitrap)). This makes it possible to use this very-high-resolution instrument in combination with SRM/MRM, SIM, and full-scan methodologies.

Although the sensitivity of QqQ in the SRM and SIM modes is much better, in the full-scan mode ITMS provides higher sensitivity. Since most ITMS devices use automatic gain control, they are sensitive to coeluting peaks because the ion storage times are influenced by coeluting substances, which could affect sensitivity. Consequently, in ITMS, chromatography can play a crucial role in some cases by separating coeluting matrix interferences from the analyte of interest. To obtain accurate isotopic measurements in QqQ analysis, the SRM mode should be used to improve accuracy, selectivity, and sensitivity [101].

For many decades, the role of chemical derivatization was strictly limited to GC–MS, where it was used to enable the analysis of nonvolatile analytes [102]; however, this approach is now also used in LC–MS/MS to increase sensitivity

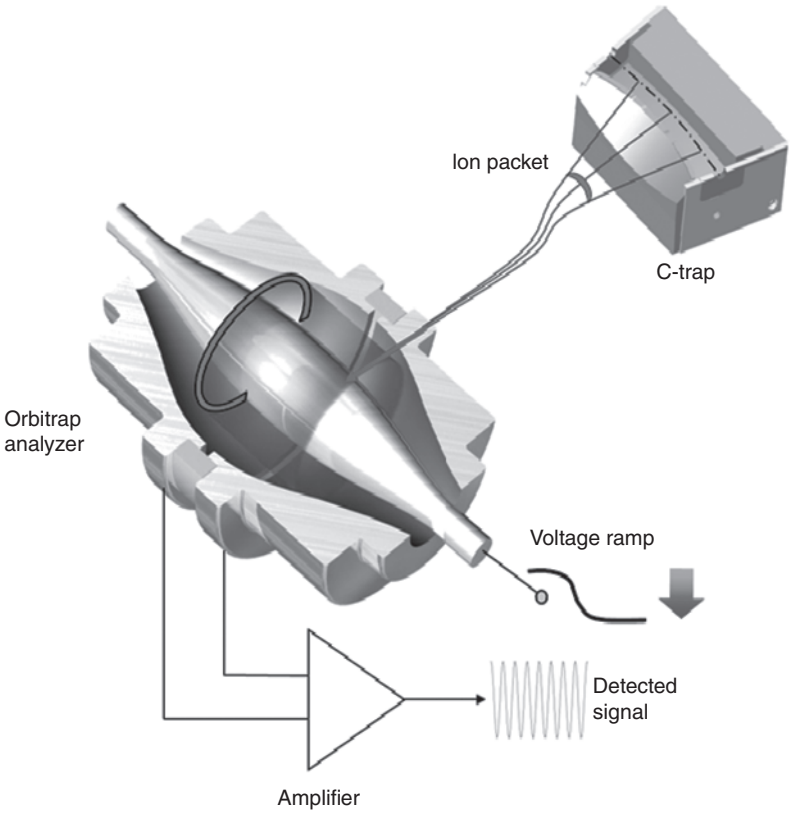

Figure 1.7 Schematic view of an Orbitrap mass trapping device. *Source*: Thermo Fisher Scientific, https://en.wikipedia.org/wiki/Orbitrap#/media/File:OrbitrapMA%26Injector.png. Used under CC BY-SA 3.0, https://creativecommons.org/licenses/by-sa/3.0/.

(i.e., ionization response), improve retention behavior, and improve extraction, followed by quantitation. As in GC–MS, derivatization in LC–MS/MS can also impact mass isotopomer distribution, and adequate methods development is needed to avoid interferences for some mass transitions. For example, Meesters *et al*. reported that, by applying the recommended method associated with the commercially Phenomenex EZ:faast amino acid kit, the TTRs of phenylalanine and tyrosine showed higher values than the theoretical ones (i.e., 2.97% was observed for natural $m+5$ at m/z 299.3, as compared to 0.003%, which is the theoretical natural enrichment at $m+5$ for phenylalanine). After optimization, the method allowed the measurement of low deuterated TTR enrichment (LOD about 0.1 TTR%) for phenylalanine and tyrosine in 25 µL of plasma sample [103]. Similarly, Nakamura reported ^{15}N isotopic analysis of 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC)-amino acid derivative mass isotopomers [104], whereas Zabielski *et al*. used an isobutyl

ester derivative of phenylalanine to measure low ^{13}C isotopic enrichment in muscle by LC–MS/MS. Interestingly, in this work, the authors showed that the LC–MS/MS instrument offered superior S/N at low isotopic enrichment for ^{13}C-labeled phenylalanine (0.0097 ± 0.0003 MPE for the interassay on 5 days), while requiring a smaller sample size (0.8 μg of tissue equivalent injected) and providing better precision (CV about 3% for low isotopic enrichment), as compared to GC–C-IRMS or GC–MS /MS applications [105].

1.3.3 Calibration of Isotopic Measurements

The most accurate method to correct for all possible sources of isotopic fractionation is to perform an internal calibration with an isotopically calibrated component (i.e., a secondary isotopic standard that is traceable to an international reference standard) with a chemical structure similar to the compound of interest. In these conditions, the standard follows exactly the same pathways and isotopic correction can be applied. This ideal situation does not occur very often with biological fluids, due to the complexity of matrices to analyze and the difficulty in finding appropriate isotopically calibrated compounds.

The alternative solution is to carry out an external calibration with an isotopically calibrated standard; this can be applied after every few samples to monitor and correct for any bias that results in isotopic drift. Werner and Brand referred to this as the identical treatment principle [106]. Such an approach, with adequate isotopic normalization, is important for antidoping studies [54, 107] but less critical for metabolic applications.

In order to demonstrate the ability of one specific method (no matter which MS instrument is used) to quantify the amount of isotopologues, the most common approach is to prepare several different mixtures of exactly known amounts of labeled compound with unlabeled compound, so as to cover the range of isotopic values expected in the study (keeping the total concentration of the tracer and tracee relatively constant). Then, by plotting the theoretical and measured isotopic abundances (in TTR or APE, based on the application and instrument) along the x-axis and y-axis, respectively, an accuracy curve is obtained. This approach allows researchers to demonstrate the method's ability to determine the accuracy of the isotopic enrichments in a specific range of abundances. Ideally, the slope of the curve is close to 1.0, showing that the accuracy of the method fits with its purpose.

1.3.4 Comparison of Gas Chromatography–Mass Spectrometry, Liquid Chromatography–Tandem Mass Spectrometry, and Gas Chromatography–Isotope Ratio Mass Spectrometry

An important parameter when comparing the performance of MS instruments is the isotopic precision of the MS methods. As described by MacCoss *et al*. [108], for single quadrupole and tandem MS, the precision of the ion-current ratios can be predicted from the ion statistic associated with different conditions (i.e., sample size, number of ions monitored, fragment intensity, derivative type, and

number of labeled atoms). It means that the relative error of TTR measurement is considerably larger for a low isotopic enrichment. However, as demonstrated by MacCoss *et al*., small TTRs can be measured with greater precision using multiple-labeled tracers for isotopic enrichment lower than 2 MPE.

A trick used in both MS and MS/MS for analyzing low enrichments of multiple-labeled tracers is to decrease the difference in signal intensities. A less abundant, naturally occurring isotopologue of the trace is chosen to represent the tracee abundance. This isotopologue should contain fewer isotope labels than the tracer. The natural abundance ratio of the isotopologue that was chosen is constant, independent of the tracer enrichment. Then, the enrichment is analyzed as the ratio of the tracer and the isotopologue, and the true enrichment is calculated. This results in a decreased difference between the peak intensities, as the signal of the isotopologue chosen to represent the tracee is significantly lower than the signal that normally would have been chosen. Lower enrichments can be analyzed with more precision than when using the traditional approach. However, the pitfall is that both the tracer and tracee may contribute to the signal that is used for the tracee, leading to less accurate results; in addition, this approach is only possible if the tracer is heavily labeled. This approach was first developed by Patterson *et al*. to look at protein synthesis, using [$^{2}H_5$]-phenylalanine as a tracer [109]. For fluxomics analysis, the ability to measure isotopologues is essential and varies between mass spectrometers [110], and the uncertainty of such measurements or the error propagation on final outcomes should be considered [111].

With IRMS, by design, the isotopic precision is much greater with a precision at ±0.00001% within a range of isotopic measurement between 0.0001% and 0.5%. Therefore, high-precision stable isotope analysis is only achievable with IRMS, and the information obtained can be used to characterize tiny variations of isotopes either at natural abundance or after a dose of tracer. Consequently, IRMS and MS/MS cannot compete with each other at natural abundance level changes but can for higher changes in isotopic measurements (Table 1.2).

Several authors compared the performance of GC–C-IRMS, LC–MS/MS, and GC–MS /MS when measuring muscle FSR using $^{13}C_6$-phenylalanine [83, 105]. According to these studies, when measuring low ^{13}C-isotopic enrichments in muscle, LC–MS/MS is a reliable alternative to GC-C-IRMS in terms of reproducibility, precision, and as the technique that uses the lowest amount of material. In terms of limit of detection, Meesters *et al*. found that LC–MS/MS has a detection limit of 0.01% TTR for phenylalanine and tyrosine in human plasma after administration of several amino acid tracers (i.e., [L-ring-$^{2}H_5$]-phenylalanine, [L-ring-$^{2}H_4$]-tyrosine, and [L-ring-$^{2}H_2$]-tyrosine) to look at whole-body protein turnover [112]. Interestingly, GC–MS and GC–MS /MS are comparable to GC–C-IRMS only if the tracers are labeled with multiple stable isotopes. If single-labeled compounds are chosen, this will create problems, as the natural background will lead to a high M + 1 peak [101, 105]. Bornø *et al*. indicated that the use of GC–MS /MS, compared to GC–MS, benefits from an increased *S*/*N*, but a drawback is the loss of signal because of the additional fragmentation

Table 1.2 Typical specifications of MS instruments hyphenated with GC or LC separation for tracer-metabolic studies.

	GC–IRMS	LC–IRMS	GC–MS	LC–MS/MS
Sample introduction	Injection of liquid	Injection of liquid	Injection of liquid	Injection of liquid
Sample transformation	Derivatization	No derivatization	Derivatization	Both
Interface	Combustion or pyrolysis to produce CO_2	Chemical oxidation to produce CO_2	EI or CI ionization modes to get ions	ESI or APCI to get ions
Mass analyzer	Magnetic sector	Magnetic sector	Quadrupole	Quadrupole-ion trap-time of flight
Detector	Faraday cups	Faraday cups	Electron multiplier	Electron multiplier
Isotopes	^{13}C, ^{15}N, ^{2}H, ^{18}O	^{13}C	^{13}C, ^{15}N, ^{2}H, ^{18}O	^{13}C, ^{15}N, ^{2}H
Range of measurable isotopic enrichment (APE)	0.0005–1	0.0005–1	0.05–100	0.009–100
Isotopic precision[a], for an average at 0.009–0.01 MPE	0.0008	–	0.0025	0.0003

a) Isotopic precision (or SD, intradays measured on 5 days) after administration of $^{13}C_6$-Phe in healthy subjects. Isotopic enrichment was measured in muscle sample. Isotopic measurements were carried out using the heptafluorobutyryl isobutyl ester as and isobutyl ester derivatives for GC-based MS methods (GC–MS, GC–MSMS, and GC–C-IRMS) and LC–MSMS, respectively. Ions monitored were M + 2 and M + 6 for GC–MS, GC–MS/MS, and LC–MS/MS in SIM and SRM modes [105].

in the collision cell. For optimized GC–MS/MS, a derivative that shows less fragmentation in the ion source is beneficial. CI would be the better choice because of the reduced fragmentation, but it suffers from higher variability [113].

1.4 Choice of Mass Spectrometric Techniques and Applications to Measure Isotopic Enrichments in Metabolic Studies

1.4.1 Choice of Mass Spectrometric Technique to Measure Isotopic Enrichment

Overall, the choice of a mass spectrometric technique for measuring isotopic enrichment in metabolic studies will depend on several factors:

- The questions being asked (i.e., the clinical outcome, the pathways to study, and the final isotopic precision required to observe an effect).

- The tracer administration (i.e., continuous infusion, bolus dose, and steady state or not).
- The available facilities (i.e., the analytical devices, the number or volume of samples available, isotopic precision needed, the number of samples, and the analytical throughput and cost).

Hypothesis-based research is the gold standard for using stable isotope tracers in a clinical study. Thus, once the clinical outcome (or pathways) to assess is foreseen (i.e., cholesterol absorption, total energy expenditure, muscle protein synthesis, and hepatic glucose production), the investigator will choose the tracer based on its selectivity to the part of the metabolism or pathways in which the tracer is involved.

Tracers are generally administered as either primed continuous infusion or via a bolus dose. In general, with the continuous infusion model (i.e., steady-state conditions), the dose of isotope tracer(s) is tailored in order to be measured in plasma samples (after its dilution by the tracee) by GC–MS. Knowing that the limit of isotopic enrichment is about 0.2–0.5% for GC–MS, the aim is to obtain a reasonable isotopic enrichment that can be determined with a good isotopic precision. Typically, an enrichment of about 5 MPE in plasma is for the goal. Under the same conditions, IRMS must be considered if tracer oxidation (measured in the breath sample with the $^{13}CO_2$ release) is of interest to assess the clinical outcome. As reported by Matthews and Bier [114], during *in vivo* labeled amino acid administration for studying protein metabolism, after infusion of [1-^{13}C]-leucine for a few hours at a sufficient rate to enrich the free leucine pool to several %, the tracer is diluted as follows: (1) about 20- to 50-fold in plasma by the existing tracee (free amino acid), (2) about a factor of 2- to 50-fold more in other amino acids by transfer of labeled atoms (especially for ^{15}N atom via transamination), (3) about 200- to 1000-fold due to breakdown into urinary urea and ammonia (as an end product of protein metabolism), (4) about 10,000-fold in CO_2 to reflect oxidation of the tracer among other macronutrients, and (5) about 1000-fold or more in amino acids bounded in novel synthesized proteins. The first two dilution steps can be measured by GC–MS or LC–MS/MS, step 3 can be measured by EA-IRMS and GC–C-IRMS, step 4 by GC–C-IRMS, and step 5 by GC–C-IRMS, LC–IRMS, or LC–MS/MS in some circumstances (Figure 1.8).

When using a bolus dose of the tracer, the tracer dose will change the kinetics; therefore, the precision will vary with its level of enrichment. For nonsteady–state conditions (i.e., determination of whole-body protein turnover after meal intake with a dual stable isotope approach [115]), the model requires the determination of the concentration and isotopic enrichment of the product (i.e., KIC) in the same plasma sample. In this specific case, GC–MS or LC–MS/MS is more appropriate for measuring both outputs.

Finally, the MS choice is also driven by the amount of sample available and the number of samples to analyze. During methods development or implementation, such considerations need to be taken into account to obtain a sensitive, precise, and high-throughput MS method to deliver reliable isotopic data.

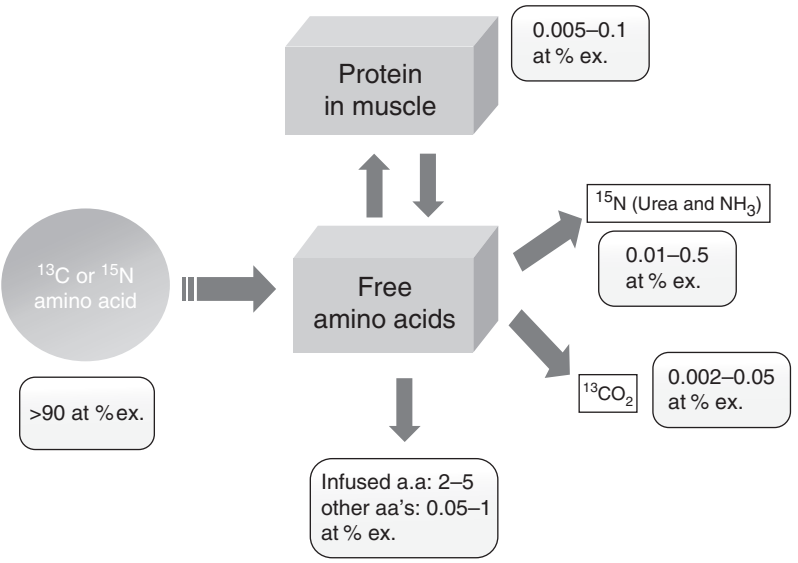

Figure 1.8 Approximate *in vivo* isotopic dilution and incorporation of labeled amino acid tracer. *Source*: Modified from Matthews *et al*. [114].

The choice of the labeling position within a molecule is of utmost importance, both in terms of targeted pathways and MS analysis. In some experiments, the unique information on the mechanisms and regulation of metabolic pathways are embedded in some specific fragments of the molecules. A typical example is measuring the rate of gluconeogenesis. As reported by Landau *et al*., after ingestion of D_2O, the contribution of gluconeogenesis to glucose production is equal to the ^{2}H-labeling ratio in carbons 5 and 2 of glucose [116]. Analytically, these isotopic enrichments are measured after successive and tedious chemical transformation of glucose into hexamethylenetetramine [117, 118] or via alternative protocols [119, 120].

1.4.2 Applications of Mass Spectrometry Methods in Stable Isotopes Tracers Studies

Ever since Schoenheimer and Rittemberg used D_2O to study fat metabolism [121], the use of stable isotope tracers has proven to be an efficient tool to obtain quantitative information about *in vivo* metabolic processes [114, 122–124]. The literature on the applications of stable isotope tracers using MS is too extensive for a full review; thus, a few key examples will be reported here.

One typical application of EA-IRMS in pediatric studies is determining whole-body protein turnover using a bolus dose of [^{15}N]-glycine by measuring the urinary end product of protein metabolism (i.e., urea and ammonia) [125, 126]. In addition to the traditional uses of tracer methods to look at specific metabolic pathways, there is increased interest in noninvasive approaches to

estimate protein intake in newborn infants. For instance, EA-IRMS studies have shown that the isotopic content of hair and fingernails from newborn babies closely approximate those of their mothers [127–129] and are correlated with the isotopic content of the protein sources (animal or vegetal) eaten by their mothers [130].

The typical applications of TC/EA-IRMS are to measure TEE and/or body composition (total body water, fat-free mass, and fat mass), by administering doubly labeled water (i.e., water enriched with deuterium and ^{18}O) or water enriched with either deuterium or ^{18}O to infants or children [29, 131–135]. D_2O was also used as a tracer to determine milk intake in infants using TC/EA-IRMS [136]. TEE is also a good approximation of food intake, as reviewed by Trabulsi and Schoeller [137].

In pediatric studies, LC–IRMS was successfully used to study glucose metabolism as a straightforward method for measuring both the concentration and the isotopic enrichment of glucose in plasma [138]. Another target component studied by LC–IRMS was glutathione (GSH) synthesis after adequate tracer administration. By sampling plasma samples, Schierbeek *et al*. developed an LC–IRMS method to determine isotopic enrichment of both the precursor (glycine) and the product (GSSG) [139, 140].

Metabolic studies using GC–P-IRMS in combination with D_2O as a tracer have focused on measuring cholesterol absorption [141], deuterium incorporation into fatty acids [142], amino acid incorporation to measure protein synthesis [143], retinol production [144], and gluconeogenesis [119].

Another classical metabolic application is to look at oxidation of tracers (i.e., ^{13}C-amino acids, ^{13}C-lipids, and ^{13}C-carbohydrates) that are either infused intravenously or administered orally. This represents direct oxidation of the ^{13}C tracer into $^{13}CO_2$ in muscle tissue. Breath sampling is quite challenging in neonates, whereas in children (and adults) the sampling process is easier [145–148].

As reviewed by Brunengraber *et al*. [149], measurements of mass isotopomer distribution, mainly by GC–MS, have greatly increased the applications of light-stable isotopes for studying the regulation and pathways of nutrient metabolism (i.e., absorption and metabolism of amino acids, glucose metabolism and recycling, glycerol production and its cycling in liver, and the contribution of gluconeogenesis to glucose production), measuring synthesis rates for polymeric molecules (e.g., protein or DNA), or measuring lipogenesis or cholesterol synthesis [149–152]. One specific stable isotope tracer ([6,6-$^2H_2$]-glucose) was widely used with GC–MS to study glucose metabolism in particular, so as to assess the magnitude of glucose production. This concept is well illustrated by the minimal model for assessing *in vivo* insulin sensitivity [153, 154].

With LC–MS/MS, Castro-Perez *et al*. measured the *in vivo* kinetics of cholesterol and cholesterol ester after D_2O administration in rats by high-resolution LC–MS/MS, using an atmospheric pressure photo ionization source [155]. Meesters *et al*. studied the concentration and *in vivo* synthesis of short-chain fatty acids using LC–MS/MS [156]. Persson *et al*. looked at isotopic enrichment of fatty acids using LC–MS/MS [157]. Blachnio-Zabielska *et al*. measured the

incorporation of [U-^{13}C]-palmitate into diacylglycerol species extracted from rat skeletal muscle [158]. Wilkerling *et al*. measured low isotopic enrichment of Phe (0.01–0.5 MPE) in chicken liver samples using LC–MS/MS [159].

To perform ^{13}C-metabolic flux analysis for metabolic engineering and metabolic investigation at a specific tissue level (i.e., liver), GC–MS/MS is the technique of choice, as reported by Jeffrey *et al*. and Choi *et al*., for the complete positional isotopomer distribution of aspartate and glutamate [160, 161].

In contrast to targeted analytical approaches, in the era of –omics, different efforts were made to merge –omics with stable isotope tracer approaches. Thus, tracer-metabolomics based on isotope ratio measurements has the potential to link flux measurements (initially developed in cells and biomass) and classical *in vivo* tracer-metabolic approaches for gaining new insights. Several reports indicate tracer-metabolomics approaches have been developed [162–169], though such an approach has yet to be used in pediatric populations.

1.5 Conclusion and Future Perspectives

A wide variety of mass spectrometers is available nowadays and, in principle, all can be used to determine isotopic enrichments. IRMS still provides the highest attainable precision and accuracy for measuring isotopic ratios at natural abundance or after tiny variations of isotopic abundance. Therefore, IRMS, connected to appropriate peripherals, still has a place in laboratories for *in vivo* metabolic applications. Of note, LC–IRMS (due to its design and principle) is still limited to a few applications and its role in pediatric or nutritional studies is quite narrow.

For *in vivo* studies with stable isotope tracers in which the amount of sample is low and the level of isotopic enrichment (and precision) measured varies, LC–MS/MS provides adequate data and is a promising analytical technique. It combines good sensitivity, selectivity, and detection limits, allowing for the measurement of isotopes in various components if adequate tracers are used.

The development of new and more sophisticated MS techniques (with high-resolution MS) opens up new avenues for overcoming existing analytical shortcomings in investigating metabolic kinetics. Orbital trapping MS is one such novel technique and is used to distinguish between different isotopes in one single molecule. When the separation of isotopes within one enrichment ratio is desired, a very high resolution is necessary to separate the different isotopes. For the A1/A0 ratio, the following isotopes can be present: ^{13}C, ^{17}O, ^{2}H, ^{15}N, and ^{33}S (Figure 1.9 and Table 1.3). When looking at the A2 isotopes, the combinations are also present, such as 2-^{13}C, [1-^{13}C–1^{15}N].

The ability to separate these different isotopes creates new opportunities for tracer studies. When applying a ^{15}N tracer study, much more accurate ratios can be obtained when the ^{15}N tracer is separated from the ^{13}C naturally occurring isotope.

Although human clinical study samples are limited to biofluids, a logical next step in the field will be integrating multiple stable isotope tracers with low

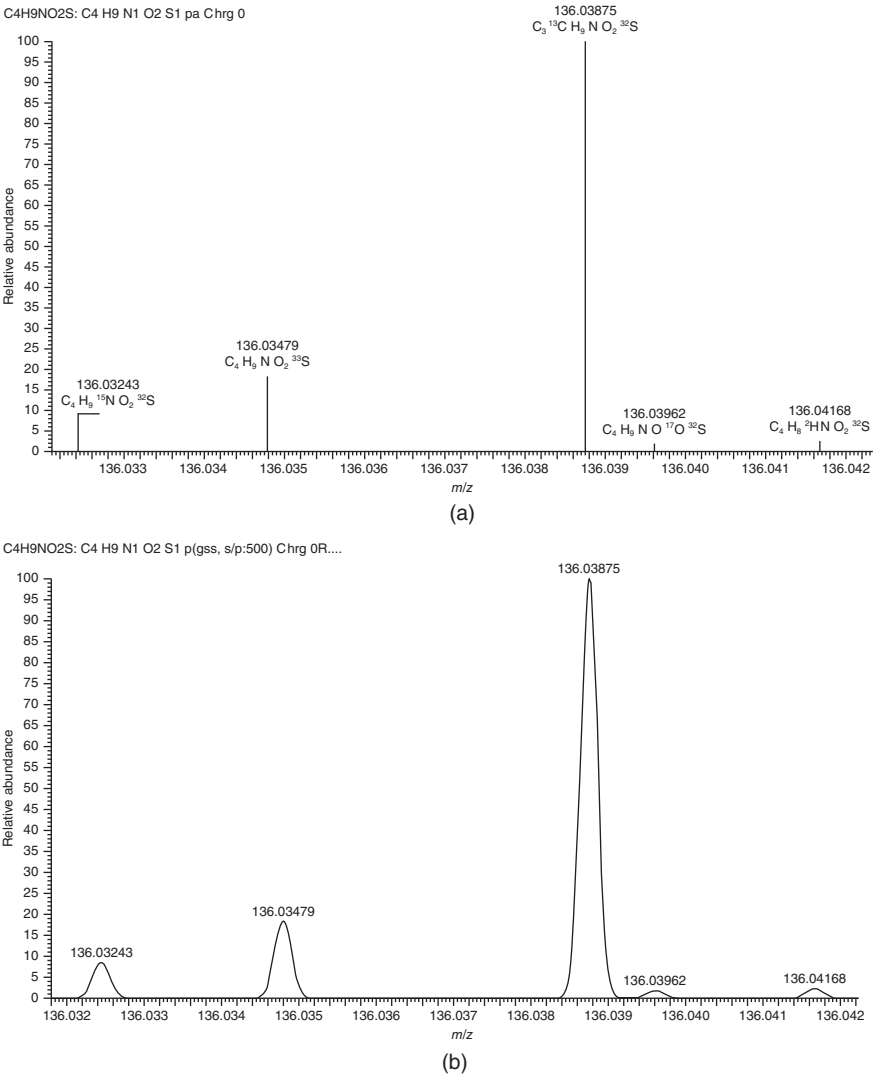

Figure 1.9 (a) Example of the fine isotope distribution of the A+1 cluster for a homocysteine molecule. The ^{33}S isotope has an abundance of 18% of the ^{13}C isotope. (b). The fine isotope distribution of A1 cluster labeled with the specific isotope species and with the natural occurring relative abundances.

amounts of biological materials to model long-term dynamic changes and loss of homeostasis.

Integrating such perspectives in the design of clinical studies will require developing appropriate modeling tools, as well as a very good understanding of physiology, the conditions of validity of the metabolic models used, and analytical techniques. Therefore, it is imperative to continue developing MS approaches to

Table 1.3 Natural abundances for the A1 fine isotope cluster.

Composition	*m/z*	Relative
C_4 H_9 [15]N O_2 [32]S	136.03243	8.54
C_4 H_9 N O_2 [33]S	136.03479	18.51
C_3 [13]C H_9 N O_2 [32]S	136.03875	100.00
C_4 H_9 N O [17]O [32]S	136.03962	1.76
C_4 H_8 [2]H N O_2 [32]S	136.04168	2.39

provide an accurate and precise isotopic picture that depicts the physiological events, and to answer the myriad of unanswered questions. This is only possible if close collaborations between analytical chemists, nutritionists, biologists, and statisticians continue.

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