High-precision ^{13}C isotopic analyses in life sciences by gas and liquid chromatography coupled to isotope ratio mass spectrometry

Godin, Jean-Philippe

UNIVERSITÉ DE GENÈVE
Section des sciences pharmaceutiques
Laboratoire de spectrométrie de masse du vivant

FACULTÉ DES SCIENCES
Professeur G. HOPFGARTNER

CENTRE DE RECHERCHE NESTLÉ
Département des Sciences Bio-Analytiques

DR L. FAY

High-Precision ^{13}C Isotopic Analyses in Life Sciences by Gas and Liquid Chromatography Coupled to Isotope Ratio Mass Spectrometry

THÈSE

présentée à la Faculté des sciences de l'Université de Genève
pour obtenir le grade de Docteur ès sciences, mention interdisciplinaire

par

Jean-Philippe GODIN

de

Melleroy (France)

Thèse N° 3947

Vers-Chez-Les-Blanc (Suisse)
Atelier de reprographie du Centre de Recherche Nestlé
(Avril, 2008)

UNIVERSITÉ
DE GENÈVE

FACULTÉ DES SCIENCES

Doctorat ès sciences
mention interdisciplinaire

Thèse de *Monsieur Jean-Philippe GODIN*

intitulée :

"High-Precision ^{13}C Isotopic Analyses in Life Sciences by Gas and Liquid Chromatography Coupled to Isotope Ratio Mass Spectrometry"

La Faculté des sciences, sur le préavis de Messieurs G. HOPFGARTNER, professeur ordinaire et directeur de thèse (Section des Sciences pharmaceutiques – Laboratoire de spectrométrie de masse du vivant), L. B. FAY, docteur et co-directeur de thèse (Nestlé Research Center – Lausanne, Suisse), R. ROBINS, docteur (Université de Nantes – Laboratoire d'Analyse Isotopique et Electrochimique – Nantes, France), et C. GUILLOU, docteur (Bureau Européen des Vins, Alcools et Boissons Spiritueuses – Ispra, Italie), autorise l'impression de la présente thèse, sans exprimer d'opinion sur les propositions qui y sont énoncées.

Genève, le 30 janvier 2008

Thèse - 3947 -

Le Doyen, Jean-Marc TRISCONE

N.B. - La thèse doit porter la déclaration précédente et remplir les conditions énumérées dans les "Informations relatives aux thèses de doctorat à l'Université de Genève".

Nombre d'exemplaires à livrer par colis séparé à la Faculté : - 7 -

REMERCIEMENTS

Je souhaite tout d'abord exprimer ma profonde reconnaissance au Docteur Laurent-Bernard Fay qui m'a proposé de réaliser une thèse au sein de son département au Centre de Recherche Nestlé. Je tiens aussi à le remercier de m'avoir fait confiance tout au long de cette période ainsi que pour ses conseils éclairés, son soutien et sa patience.

Je souhaite également associer à ces remerciements le Professeur Gérard Hopfgartner qui m'a fait l'honneur d'encadrer cette thèse à l'Université de Genève. Je lui témoigne ma profonde gratitude pour sa disponibilité, son soutien, la confiance et ses connaissances qu'il m'a transmis.

Je remercie tout particulièrement le Docteur Claude Guillou pour l'attention portée à ce travail en acceptant de faire partie de ce jury.

Je tiens également à remercier le Docteur Richard Robins de me faire l'honneur de juger ce travail.

J'adresse en particulier mes remerciements au Docteur Sunil Kochhar responsable du groupe «Metabolomics & Biomarker» pour la grande confiance qu'il m'a accordée pendant cette période.

Je remercie le Docteur Alastair Ross pour sa disponibilité et son écoute lors de nos fructueuses discussions. De grands remerciements vont également à Anne France Mermoud et Marylin Cléroux pour leur aide apportée sur divers projets Nestlé et pour m'avoir quotidiennement supporté pendant ces années. Je leur exprime ici ma profonde sympathie.

Je voudrais bien sûr remercier l'ensemble de mes collègues du groupe «Metabolomics and Biomarker» travaillant au centre de Recherche Nestlé pour leur sympathie et leur disponibilité.

Je voudrais également remercier Cynthia Parker pour la relecture de mes différents articles et le Docteur Sean Austin pour ces remarques pertinentes.

Je remercie le Docteur Emmanuel Varesio pour ses conseils distillés à la demande ainsi que les Docteurs Emmanuel Bourgogne et Luc-Alexis Leuthold pour leur enthousiasme lors de mes visites à l'Université de Genève.

Diverses collaborations ont également été établies lors de ce travail. J'aimerais donc remercier les Docteurs Christiane Obled et Isabelle Papet (INRA Clermont-Ferrand, France), les Docteurs Michael Krummen et Dieter Juchelka (Thermo Fischer, Bremen, Allemagne), le Docteur Klaus Kaiser (Spectronex, Suisse), les Docteurs Denis Breuillé, Magali Faure, Martin Kussmann, Elizabeth Prior (Nestlé), le Professeur Denis Barron (Nestlé) et enfin Laure Poquet et Marie-Claude Compondu (Nestlé).

Je souhaiterais remercier mes parents qui m'ont toujours encouragé et sans qui ce travail n'aurait pu être possible.

Je souhaiterais également exprimer mes chaleureux remerciements à Ludovica, la femme qui partage ma vie, pour son aide si précieuse, son indulgence pendant ces années et sa présence quotidienne à mes côtés. Sans toi, ce travail n'aurait pu être possible.

Enfin je dédie ce travail de thèse à mes deux petits amours Matilde et Giulia nées pendant ce travail de thèse.

Merci à tous…

A Matilde et à Giulia

TABLE OF CONTENTS, LIST OF FIGURES AND TABLES AND ABBREVIATIONS

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

SYMBOLS & ABBREVIATIONS

$AgNO_3$: Silver nitrate

AMS: Accelerator Mass Spectrometry

APE: Atom Percent Excess

APCI: Atmospheric Pressure Chemical Ionisation

APPI: Atmospheric Pressure Photoionsation

Br: Bromine

BSIA: Bulk Specific Isotope Analysis

CHF: Swiss Franc

Cl: Chlorine

CO_2: Carbon dioxide

CRI: Chemical Reaction Interface

CRI-MS: Chemical Reaction Interface-Mass Spectrometry

CuO: Cupric oxide

CSIA: Compound Specific Isotope Analysis

CV: Coefficient of variation

DNA: Deoxyribonucleic acid

EA: Elemental Analyzer

EA-IRMS: Elemental Analyzer-Isotope Ratio Mass Spectrometry

ECF: Ethyl choloroformate reagent

EI: Electron impact

ESI: Electrospray

eV: electron Volt

FDA: Food Drug Administration

FIA: Flow Injection Analysis

FIA-IRMS: Flow Injection Analysis-Isotope Ratio Mass Spectrometry

FID: Flame Ionisation Detector

FSR: Fractional Synthesis Rate

FWHM: Full Width at Half Height Maximum

FT-ICR-MS: Fourier Transform-Ion Cyclotron Resonance-Mass Spectrometry

GC: Gas Chromatography

GC-C-IRMS: Gas Chromatography-Combustion-Isotope Ratio Mass Spectrometry

GC-MS: Gas Chromatography-Mass Spectrometry

GMP: Good Manufacturing Pratice

H_2: Hydrogen

HEN: High Efficiency Nebulizer

HTLC: High Temperature Liquid Chromatography

IAEA: International Agency Energy Atomic

IRMS: Isotope Ratio Mass Spectrometry

ICP-MS: Inductive Coupled Plasma-Mass Spectrometry

k: Retention factor (chromatography term used in GC and in LC)

KIE: Kinetic Isotope Effect

kV: kilo Volt

LC: Liquid Chromatography

LC-IRMS: Liquid Chromatography-Isotope Ratio Mass Spectrometry

LC-C-IRMS: Liquid Chromatography-Combustion-Isotope Ratio Mass Spectrometry

LC-CRI-MS: Liquid Chromatography-Chemical Reaction Interface-Mass Spectrometry

LC-CRI-IRMS: Liquid Chromatography-Chemical Reaction Interface-Isotope Ratio Mass Spectrometry

LOD: Limit of Detection

LSC: Liquid Scintillation Counter

Min: Minute

MPE: Molar Percent Excess

MS: Mass Spectrometry

mV: milli Volt

MW: Molecular Weight

***m/z*:** mass over charge

n: Number of replicates

N: Chromatographic efficiency

NAP: N-acetyl propylethyl ester

NO_2: Nitrogen dioxide

NPP: N-pivaloyl isopropyl ester

Ni: Nickel

O_2: Oxygen gas

P: Phosphorous

PDB: Pee De Belenmite

PET: Position Emission Tomography

Pt: Platinum

P&T: Purge and Trap

RNA: Ribonucleic acid

rms: root mean square

Rs: Chromatographic resolution

Ru: Ruthenium

SD: Standard Deviation

sec: Second

SPME: Solid Phase Micro Extraction

Thr: Threonine

TOF: Time-of-Flight

TIE: Thermodynamic Isotope Effect

TSN: Thermospray nebuliser

U-^{13}C: Universally labeled with ^{13}C

UPLC: Ultra Performance Liquid Chromatography

V: Volt

Val: Valine

VPDB: Vienna Pee De Belemnite

δ^{13}C, ‰: Delta per mil

α: Chromatographic selectivity

^{13}C: Carbon 13

^{14}C: Carbon 14

^{18}O: Oxygen 18

ABSTRACTS AND SCIENTIFIC COMMUNICATIONS

THESIS ABSTRACT

Stable isotope mass spectrometry has proved to be a valuable tool for measuring ^{13}C isotopic ratio at natural abundance and for unravelling small isotopic enrichments in many applications, including life science research. The state-of-the-art method to assess $^{13}C/^{12}C$ isotopic ratio for a specific compound in complex biological samples is still gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS). However, as the volatility and the polarity of the compounds, as well as the isotopic dilution and the kinetic isotopic effect, are complex issues for the GC separation, an alternative LC separation method coupled to the IRMS device (LC-IRMS) is presented in this work. Although the idea of combining the LC with isotope ratio mass spectrometry was proposed over 10 years ago, the interface allowing LC-IRMS coupling was only commercialized in 2004. We reviewed in this thesis all the work performed in the area, based on the LC separation for ^{13}C isotopic analyses. This work indicates that currently more and more developments based on LC separation coupled, among others, to IRMS mass spectrometers are performed to measure ^{13}C isotopic ratio of non-volatile compounds.

We have started our experimental work by using the state-of-the-art method, GC-C-IRMS, for the simultaneous analyses of several amino acids. We extended the use of the N(O,S)-ethoxycarbonyl ethyl ester derivative (adding 5 extra carbons) to valine and threonine in the same GC run using a polar stationary phase column. We also measured the isotopic ratio of other amino acids eluting during the same run with good precision, using both standard and gut mucin samples.

We have subsequently shown that isotopic analyses of standard macromolecules (peptides, insulin and albumin) by flow injection analysis (FIA-IRMS) gave accurate values (lower than 1‰) if compared to isotopic analyses performed by the elemental analyzer-IRMS (EA-IRMS). The work hightlighted that the amount of compound needed in FIA mode was approximately 1000 fold less compared to the amount used with the EA-IRMS instrument.

Additionally despite the fact that the LC-IRMS is sensitive to organic buffers and to the LC flow rate, we have developed a reliable LC-IRMS method for underivatised valine. By comparing isotopic ratio values acquired with LC-IRMS and GC-C-IRMS, we reported an isotopic bias associated to the above-mentioned GC derivative (approximately -2‰ measured between $\delta^{13}C$ values at -12 to 70‰ for valine). We showed that both methods gave similar results for standard valine and for valine extracted from bound bone-proteins.

We also demonstrated that APE, Atom%, and $\delta^{13}C$ values were not directly comparable between both methods while MPE values were.

Furthermore, to overcome the impossibility of using an organic buffer, the high temperature liquid chromatography was coupled to the IRMS (HTLC-IRMS). Typically, at 150°C water strength is increased and is equivalent to a mixture of 50/50% methanol and water. Thus, the on-line high-precision ^{13}C isotopic analyses of phenolic acids (*p*-coumaric acid, its sulphate, glucuronide conjugates and ferulic acid) were reported by LC-IRMS at 170°C. In these conditions, the use of high temperature in isothermal conditions (170°C) does not affect $\delta^{13}C$ values when compared to FIA-IRMS performed at ambient temperature. However, the temperature gradient affected the CO_2 background due to the column bleed and the isotopic precision and accuracy were also deteriorated. Despite such drawbacks, the HTLC approach seems promising for the measurement of high-precision isotopic ratio in isothermal conditions.

Finally, we have applied LC-IRMS to measure *in vivo* production of glucose in plasma. We report here a new reliable method for measuring ^{13}C glucose isotopic ratio using 100µL of plasma. SD ($\delta^{13}C$) at 0.5‰ over time points taken over few hours allows the use of this method in a clinical study.

This thesis work has shown that the LC-IRMS is a valuable tool for measuring high-precision ^{13}C isotopic ratios of a wide range of water soluble and non-volatile molecules.

RÉSUMÉ DE THÈSE

La spectrométrie de masse de rapport isotopique (SMRI) est un outil parfaitement adapté à la mesure isotopique du carbone 13 dans les sciences du vivant. Etant donné les contraintes (de dilution isotopique et de fractionnement isotopique) associées à l'analyse par chromatographie gazeuse liée à une interface de combustion (CG-C) couplée à la SMRI (CG-C-SMRI), une nouvelle ère s'ouvre avec la récente commercialisation de la CL-SMRI (2004). Dans ce contexte, suite à l'analyse isotopique par CG-C-SMRI, principalement sur la valine et la thréonine, avec le dérivé N(O,S)-ethoxycarbonyl ethyl ester, nous présentons dans ce travail l'analyse isotopique par CL-SMRI sur diverses molécules.

Typiquement, l'un des avantages de ce nouveau couplage est la mesure de rapports isotopiques directe sans chromatographie. Ainsi, l'analyse isotopique sur des petites molécules et des macromolécules a pu démontrer qu'un gain substantiel (environ 1000 fois) est envisageable par rapport à l'analyseur élémentaire-SMRI.

Malgré les contraintes chromatographiques de la CL-SMRI (liées à l'absence d'éluant organique et au débit limité en CL), une nouvelle méthode est présentée pour l'analyse de la valine. Par comparaison avec les valeurs mesurées en CG-C-SMRI, un biais isotopique a été reporté (équivalent à 2‰) et uniquement les valeurs exprimées en MPE (excès molaire d'enrichissement isotopique) sont équivalentes entre ces deux techniques.

Pour s'affranchir de l'absence d'éluant organique en CL, la chromatographie liquide à haute température (CLHT) a été couplée à la SMRI. Ainsi, des rapports isotopiques d'acides phénoliques ont été mesurés à 170°C et comparés à des mesures effectuées à température ambiante. Bien que le gradient de température génère une dérive de la ligne de base affectant la précision et la justesse des rapports isotopiques, la CLHT ne détériore pas les rapports isotopiques si l'on travail avec une isotherme à très haute température. De plus, elle présente des intérêts majeurs pour l'élution de composés polaire en phase inverse.

Enfin l'analyse isotopique par CL-SMRI du glucose plasmatique mesurée avec une précision de 0.5‰ est reportée et démontre sa possible utilisation dans une étude clinique.

Ce travail montre que la CL-SMRI est un outil complémentaire à la CG-C-SMRI et adapté à la mesure des rapports isotopiques du carbone 13 de composés hydrosolubles et non-volatiles.

SCIENTIFIC COMMUNICATIONS

Only peer-reviewed articles related to this PhD work are reported here.

PEER-REVIEWED PAPERS

"Hyphenation of High Temperature Liquid Chromatography and Isotope Ratio Mass Spectrometry (HTLC-IRMS): a new approach for measuring ^{13}C isotopic ratios of organic and phenolic acids" by Jean-Philippe Godin, Gérard Hopfgartner and Laurent-Bernard Fay *Submitted,* **2008**

"Liquid and Gas chromatography coupled to Isotope Ratio Mass Spectrometry for the determination of ^{13}C Valine isotopic ratios in complex mixture" by Jean-Philippe Godin, Denis Breuillé, Christiane Obled, Isabelle Papet, Henk Schierbeek, Gérard Hopfgartner and Laurent-Bernard Fay *in press in Journal of Mass Spectrometry*, **2008**

"Liquid Chromatography combined to Mass Spectrometry for ^{13}C isotopic analyses in Life Science Research" by Jean-Philippe Godin, Gérard Hopfgartner and Laurent-Bernard Fay *Mass Spectrometry Reviews*, **2007**; 26:751

"Novel method for measurement of glutathione kinetics in neonates using liquid chromatography coupled to isotope ratio mass spectrometry" by Henk Schierbeek, Frans te Braake, Jean-Philippe Godin, Laurent-Bernard Fay and Johannes B. van Goudoever *Rapid Communication Mass Spectrometry,* **2007**; 21: 2805

"Determination of ^{13}C isotopic enrichment of valine and threonine by GC–C–IRMS after formation of the N(O,S)-ethoxycarbonyl ethyl ester derivatives of the amino acids" by Jean-Philippe Godin, Magali Faure, Denis Breuille, Gérard Hopfgartner, Laurent-Bernard Fay *Analytical Bioanalytical Chemistry*, **2007**; 388: 909

"Isotope ratio monitoring of small molecules and macromolecules by liquid chromatography coupled to isotope ratio mass spectrometry" by Godin J-P, Hau J., Fay LB and Hopfgartner G. *Rapid Communication Mass Spectrometry,* **2005**; 19: 2689

ORAL COMMUNICATIONS

"Corrélations expérimentale et théorique entre les mesures isotopiques relatives et absolues du carbone 13 par chromatographie gazeuse et liquide couplées à un spectromètre de masse de rapport isotopique" by Jean-Philippe Godin, Gérard Hopfgartner and Laurent-Bernard Fay, *Journées Jeunes Chercheurs de la SFIS*, Lyon (France), October **2007**

"High temperature liquid chromatography- isotope ratio mass spectrometry; a perfect match for isotopic analysis of organic acids in plasma" by Jean-Philippe Godin, Gérard Hopfgartner and Laurent-Bernard Fay, *HPLC 2007*, Ghent (Belgique), June **2007**

"Couplage chromatographie liquide-spectromètre de masse isotopique: développement et applications" by Jean-Philippe Godin, Gérard Hopfgartner and Laurent-Bernard Fay, *IRMS User meeting Thermo,* Avignon (France), May **2006**

"Analyses isotopiques par chromatographie liquide-spectrométrie de masse isotopique : passé et présent" by Jean-Philippe Godin, Gérard Hopfgartner and Laurent-Bernard Fay, *Journées Jeunes Chercheurs de la SFIS*, Paris (France), October **2005**

"Compound Specific Isotope Analysis of underivatised amino acids by LC-IRMS for tracer application", by Jean-Philippe Godin, Gérard Hopfgartner and Laurent-Bernard Fay, *Isotopes 2005,* Bath (UK), June **2005**

"Analysis of ^{13}C non-volatile compounds by Liquid Chromatography-Isotope Ratio Mass Spectrometry" by Jean-Philippe Godin, Gérard Hopfgartner and Laurent-Bernard Fay, *Riggi Meeting*, (Switzerland), November **2004**

POSTER PRESENTATIONS

"Hyphenation of High Temperature Liquid Chromatography with Isotope Ratio Mass Spectrometry (HTLC-IRMS)" by Jean-Philippe Godin, Gérard Hopfgartner and Laurent-Bernard Fay at *55th ASMS*, Indianapolis, (USA), June **2007**

"$^{13}C/^{12}C$ isotopic analysis of biological samples by irm-LC/MS" by Jean-Philippe Godin, Gérard Hopfgartner and Laurent-Bernard Fay. *17th IMSC*, Pragues (Czech Republic) August **2006**

"Nouvelle méthode isotopique pour évaluer la synthèse protéique avec un couplage HPLC-IRMS" by Jean-Philippe Godin, Gérard Hopfgartner and Laurent-Bernard Fay, 4ème *Journées de la Société Française d'isotopes stables*, Nantes (France) **2006**.

"Optimisation of the on-line hyphenated High Performance Liquid Chromatography coupled to High-precision Isotope Ratio Mass Spectrometry" Jean-Philippe Godin, Gérard Hopfgartner and Laurent-Bernard Fay, *21th LC/MS, Montreux symposium*, November **2004**

CHAPTER 1

Liquid and gas chromatography combined with mass spectrometry for ^{13}C isotopic analyses

Keywords: mass spectrometry, stable isotopes, GC-C-IRMS, LC-IRMS, LC-MS

1 LIQUID AND GAS CHROMATOGRAPHY COMBINED WITH MASS SPECTROMETRY FOR ^{13}C ISOTOPIC ANALYSES

The main part of this chapter has been published in ***Mass Spectrometry Reviews****, 2007, 26: 751-774 by Jean-Philippe Godin, Gérard Hopfgatner and Laurent-Bernard Fay. Some new sections have been added.*

1.1 MASS SPECTROMETRY

The principle of mass spectrometry can be divided into four different functions as reported in Figure 1. The fifth function is focused on the sample introduction mainly performed by the gas chromatography (GC) and liquid chromatography (LC) as separation techniques.

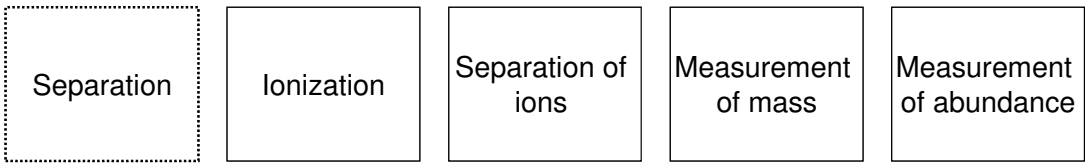


Figure 1: Illustration of the four main function of a mass spectrometer with the fifth element focused on the analytical separation performed by GC and LC

In mass spectrometry, the ionization is a fundamental process as it results in the formation of either positive or negative ions depending on the ionization method. During the ionization process, fragmentation may also occur. A range of ionization techniques is available and is listed in Table 1 (together with the usage and the typical ions formed). The ionization techniques include electron impact (EI), electrospray (ESI), atmospheric pressure chemical ionization (APCI), desorption electrospray ionization, MALDI, atmospheric pressure photoionization (APPI). Then the ions are separated according to their mass over charge ratio (*m/z*), typically with 1 digit. In certain cases, the *m/z* is measured with higher accuracy (3-4 significants

digits). Finally, the measurement of the ion abundance, based on the peak area or peak height leads to semi-quantitative or quantitative information. The sensitivity is typically in the picomole or femtomole range. The mass detection of a molecule by mass spectrometry is conditioned by the capacity of the analyte to be ionized. Apart from the chemical properties of the analytes, the eluent flow and its composition, the sample matrix, and the ionization source, all influence the ionization process (Siuzdak, 2006).

In order to separate the ions, mass spectrometry manufacturers made a significant of progress in the last decade with various configurations and with the introduction of new types of analyzers. There are the quadrupole MS, the triple quadrupole MS, the quadrupole linear ion trap (Hopfgartner et al., 2004), the time of flight (TOF), the ion trap, the Fourier Transform-ion cyclotron resonance MS (FT-ICR-MS) and the magnetic sector instrument.

Table 1: Ionisation techniques available in mass spectrometry

Ionisation source	Acronym	Event	Typical ions	Amenable to LC	Applications
Electron ionization	EI	Electron transfer	$M^{+\cdot}$	very limited (GC easy)	lmw
Chemical ionization	CI	Proton transfer	$[M+NH_4]^+$	very limited (GC easy)	lmw
Electrospray	ESI	Evaporation of charge droplets	$[M+H]^+/[M+nH]^{n+}$	excellent	lmw, macromolecules
Atmosperic pressure chemical ionization	APCI	Corona discharge and transport of proton	[M+H]+	excellent	lmw
Atmosperic pressure photochemical ionization	APPI	Photoexcitation / transfert of proton	$[M^+]/[M+H]+$	excellent	lmw
Matrix-assisted laser desorption/ionization	MALDI	photon absorption/ transfert of proton	[M+H]+	possible	lmw, macromolecules

lmw: low molecular weight molecule

The performance of the mass spectrometers in combination with the ionization technique can be defined by several intrinsic parameters which are the mass resolving power (or resolution), the mass accuracy, the linear dynamic range, and the sensitivity as described in Figure 2. The quadrupole and the ion trap devices are very robust and offer a mass resolution of 4000 and accuracy at approximately 100 parts per million (ppm). The TOF has a mass resolving power of 8000-15000 FWHM with a mass accuracy of 5-10ppm. The FT-ICR MS can reach a resolving power of higher than $1x10^6$. They can also provide accurate mass measurement at 1-2ppm (Siuzdak, 2006). The Orbitrap device can also reach a resolving power of higher than $1x10^6$ with mass accuracy at 1-5ppm (Hu, 2005).

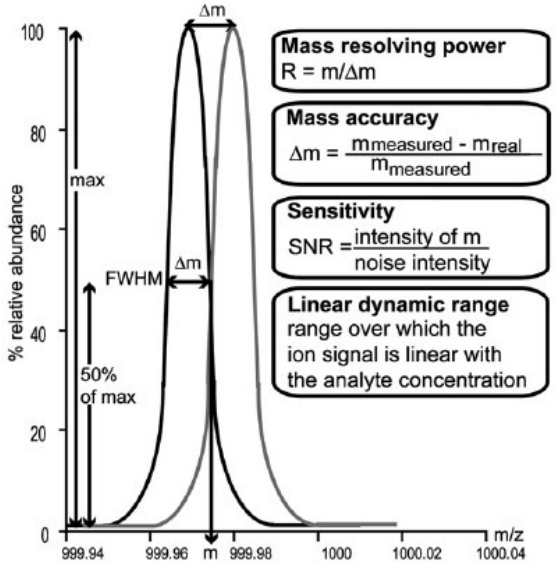


Figure 2: Parameters used to describe the performance of the mass spectrometers

The mass resolving power or the resolution (m/Δm) corresponds to the mass (m) at the apex of the mass signal and Δm is the width at x% height (typically 50%) of this mass signal, designated by the FWHM (full width at half height maximum).The mass accuracy is described by the ratio between the mass error (difference between measured and exact mass) and the theoretical mass, often represented as parts per million (ppm). 100ppm of mass accuracy corresponds to a theoretical mass of 1000 with a measured mass at 999.9. The sensitivity is described by the ratio between the intensity level of the mass signal and the intensity level of the noise. The linear dynamic range is described as the range of linearity of the ion signal measured as a function of the analyte concentration (Moco et al., 2007).

Mass spectrometry (MS) has become an essential analytical tool in modern life science reserach, due not only to its sensitivity but also to the total information delivered by this technique as in the proteomics (Kussmann, 2007) and metabolomics areas (Dettmer et al., 2007). Typically, mass spectrometers allow quantitation of molecules as well as the structural determination of organic molecules via the determination of molecular weight and the study of fragmentation patterns. The usage of ^{13}C labeled compound in mass spectrometry is mainly used as an

internal standard or to obtain metabolite information. Additionally, in other scientific areas (geochemistry, ecology, food chemistry, forensic and sport) mass spectrometry is also used to obtain the precise determination of stable isotope ratios of exogenous or endogenous molecules.

1.2 STABLE ISOTOPES

1.2.1 INTRODUCTION

The name “isotope” was proposed by the British chemist F. Soddy, taking its origin from the Greek words “isos”, which means equal, and “topos”, which means place. The reality of isotopes was debated almost during one century from Prout who postulated their existence in 1815 to J.J. Thomson who revealed experimentally in 1913 the mass spectrum of neon by showing a peak at mass 22 accompanying the expected one at mass 20 using the first mass spectrometer (then called a parabola spectrograph). In the same period, at the Cavendish Laboratory in Cambridge, FW. Aston completed building his first mass spectrograph (now mass spectrometer) that he reported in 1919. Subsequent improvements in the instrument led to the development of another instruments employing electromagnetic focusing to identify 212 naturally occurring isotopes. In 1922, Aston received the Nobel Prize in Chemistry. In parallel, JJ. Dempster of the University of Chicago also improved the resolution and developed the first electron ionization source, which ionized volatilized molecules with a beam of electrons. Very soon after, H.C. Urey was awarded the Nobel Prize in chemistry (1934) for the discovery of deuterium oxide. Different groups of researchers launched the idea of using stable isotopes in kinetic and dynamic investigations. Thus, studies on fat metabolism in mice with deuterium were performed by R. Schoenheimer and D. Rittenberg (Young & Ajami, 1999). This was the first isotopically-based recognition of the turnover of body constituents.

Stable isotopes are elements sharing the same place in the periodic table (Table 2). They have the same chemistry, i.e. the same number of protons, but they are different in their atomic mass due to different number of neutrons. Common organic elements such as H, C, N and O have a stable isotope counterpart such as ^{2}H, ^{13}C, ^{15}N and ^{18}O. Moreover, each of these elements has also a corresponding radioactive

isotope. Their masses are not constant but change during radioactive disintegration with emission of other particles.

For example, carbon has two stable isotopes ($^{12}_{6}C$ and $^{13}_{6}C$) of ~ 98.89% and 1.11% natural abundance, respectively and two radioactive isotopes as ^{14}C and ^{11}C. ^{11}C decays 100% by positron emission to ^{11}B (boron). It has a half-life of 20.3min and it is mainly used in position emission tomography (PET) which is an imaging technique in medicine. The ^{14}C decays into ^{14}N (nitrogen) and it has a half-life of 5730±40 years.

Table 2: Isotopes used in metabolic studies (Rennie, 1999)

Element	Stable isotope	Natural abundance	Radioactive
H	^{2}H	0.02%	^{3}H
C	^{13}C	1.1%	^{14}C
N	^{15}N	0.37%	^{13}N*
O	^{18}O	0.04%	^{11}O*

** no long-lived radioisotopes of these elements*

1.2.2 STABLE ISOTOPE TRACER PREPARATION FOR CLINICAL STUDY

1.2.2.1 "Research chemical" vs. drug

Stable isotope tracers (^{13}C, ^{15}N and ^{18}O) do not have a specific pharmacological action and thus can be treated as their corresponding natural compound. For instance, we have approximately 2000mg/kg (as dried material) of ^{13}C in our body (Abramson, 2001).

The stable isotope tracers usually carry the label "*research chemical, not intended for human use*". However, stable isotope tracers are widely used in clinical studies with administration to volunteers. This apparent contradiction can be explained by regulatory aspects, which influence the tracer preparation for human studies. For drugs (meaning compounds changing the physiological or metabolic behaviour of individuals if they are taken in sufficient dose), the producer (pharmaceutical companies) must receive the permission from authorities (i.e. FDA, Food and Drug Administration in USA) or other national agencies. In order to obtain this approval, they need safety and toxicology dossiers with the clinical studies. Moreover for the production of this drug, they also need to be compliant with various norms like ISO (International Standard Operation) norm or Good Manufacturing Practice (GMP)

guidelines. Guaranteeing such compliance results in 10 fold increase price compared to the price of the corresponding research chemical (see Table 3 for prices of tracers). For this reason, the supplier prefers to keep the tracer as *research chemical* (Vogt, 1997).

For the sample preparation of tracer, a widely accepted rule is that the pharmacist is legally responsible for that. The price for preparing and testing one tracer solution (5mL in sealed ampoule) of [6,6]-^{2}H glucose, ^{13}C glucose and $NaH^{13}CO_3$ is approximately 40CHF without the cost of the tracers and 46CHF for a U-^{13}C glucose solution of 100mL (quotation 2007).

Table 3: Prices of natural compounds and their ^{13}C stable isotope counterpart

Stable isotope	Amount	Price*
glucose	1kg	82.8
[1-^{13}C] glucose	1g	192.5
[U-^{13}C] glucose	1g	770.0
valine natural	1g	15.6
[1-^{13}C] valine	1g	233.2
caffeine	100g	29.6
[$^{13}C_3$] caffeine	1g	1122

** The price (2005) of stable isotope tracers is from Euriso-Top (France) and for other compounds is from Sigma Aldrich (Switzerland).*

1.3 ISOTOPIC ENRICHMENTS

1.3.1 RELATIVE ISOTOPIC ENRICHMENT

The ^{13}C isotopic enrichment is achieved by determining the ratio of the heavy (^{13}C atom) to the light (^{12}C atom) isotopes in the sample. The isotopic abundance of a sample is always calculated relative to a reference. The variation in isotopic ratio is so small at natural abundance that it often occurs around the third or fourth significant figure after the decimal point. Therefore, variation of isotopic ratio the δ ^{13}C value is expressed in per mil (‰). The result of this calculation is a relative $\delta^{13}C$ calibrated against the international standard.

The calculation is performed according to the following formula (Equation 1):

$$\delta^{13}C_{\text{sample}}\ (‰) = [(R_s / R_{st}) - 1] \times 1000 \qquad (\textit{Equation 1})$$

R_s is the ratio of $^{13}C/^{12}C$ in the sample and R_{st} is the corresponding ratio measured for the international standard used (for carbon, R_{st}=0.0112372 ± 0.0000009). The material used as the international reference for the carbon was Pee Dee Belemnite (PDB, a carbonate from South Carolina). This reference material is no longer available but has now been replaced by another carbonate with the same isotopic ratios for ^{13}C and ^{18}O isotopes. The scale has remained the same and was named VPDB (i.e. Vienna PDB) (Coplen et al., 2006; Slater et al., 2001; Werner & Brand, 2001). The practical advantage of using $\delta^{13}C$ (‰) notation are that small variations leading to the modification of the fifth digit after the decimal point is simpler to tackle. As example, a sample with $\delta^{13}C$= -1‰ corresponds to a $^{13}C/^{12}C$ ratio of 0.0112260. A negative $\delta^{13}C$ value shows that the molecule is depleted in the heavy isotope relative to the standard. On the other hand, a positive $\delta^{13}C$ value shows that the molecule is enriched in the heavy isotope relative to the standard.

For metabolic investigations, it is more convenient to use the atom fractions, namely Atom Percent (AP or A%, Equation 2) and then Atom Percent Excess (APE). The formula for the calculation of atom percent is:

$$AP = \left[\frac{100 \times R \times \left(\left(\delta^{13}C/1000\right)+1\right)}{1+R\times\left(\left(\delta^{13}C/1000\right)+1\right)}\right] \qquad (\textit{Equation 2})$$

where R is the ratio of ($^{13}C/^{12}C$) of the International Standard of VPDB, (R_{VPB}=0.0112372).

1.3.2 ABSOLUTE ISOTOPIC ENRICHMENT

The Atom Percent Excess (APE) represents an absolute measurement of the isotopic enrichment. APE is then defined as AP (sample) minus AP (background). APE can be subsequently transformed in MPE (Molar Percent Excess) using the following formula: MPE = APE x ($C_{Total}/C_{labeled}$), where C_{Total} is the total number of carbon atoms in the molecule and $C_{labeled}$ is the number of labeled carbon atoms within the molecule.

To illustrate the equivalence of APE versus δ ^{13}C value measured, the δ ^{13}C notation described previously measures relative differences in ‰, whereas APE notation measures absolute differences in %. Thus, the variation of δ ^{13}C of 1‰ means a variation of 0.001APE (Meier-Augenstein, 1999b) representing a factor of 1000 as the difference between both values.

Moreover, in many metabolic studies, the most relevant information to be obtained is the ratio of the isotopically labeled tracer versus the unlabeled compound: this ratio is often expressed as the tracer-tracee ratio, or TTR. Practically, in MS, the area of the corresponding peaks is used to calculate the TTR. This ratio can then be converted to % of enrichment expressed as Molar Percent Excess (MPE) (Wolfe, 1992) according to Equation 3.

$$MPE = TTR / (1 + TTR) \qquad \textit{(Equation 3)}$$

1.3.3 STANDARD REFERENCES

IAEA distributes (www.IAEA.org) a number of reference materials at natural abundance or labeled reference such as NBS22 (oil at -29.74‰), IAEA-CH6 (sucrose at -10.4‰), IAEA-309A (U-^{13}C glucose at 93.9‰) or IAEA-CO-9 (barium carbonate at -47.1‰) (Coplen et al., 2006).

1.3.4 ACCURACY AND PRECISION OF ISOTOPIC MEASUREMENT

The isotopic accuracy is the measure of how close the experimental value is to the “accepted reference value”. It is a measure of the systematic error (see Figure 3). In tracer measurement, the isotopic accuracy originating from the analytical instrument as well as the sample preparation is assessed by plotting a curve (theoretical excess of isotopic enrichment versus the measured one) using samples corresponding to a known amount of unlabeled material mixed with a known amount of labeled material with a known isotopic enrichment. Thus, the measured isotopic data are corrected to obtain the accurate isotopic values.

The isotopic precision is determined by the replicate measurement of one sample (Figure 3). It is generally assessed by calculating the standard deviation (SD). The SD ($\delta^{13}C$) is in the range of 0.1-0.3‰ for high-precision ^{13}C measurement performed by the gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS).

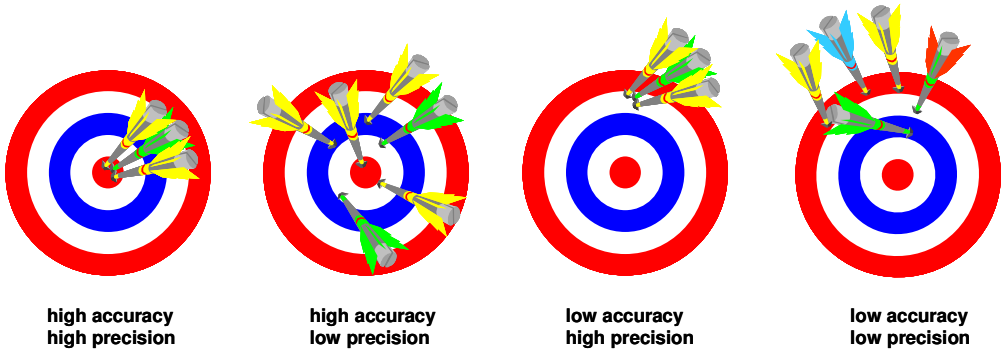


Figure 3: Illustration of the accuracy and precision of the measurement

1.4 STABLE ISOTOPES OFFER UNIQUE INSIGHTS

Due to the intrinsic property of IRMS to detect small variations of the isotopic ratio, the analysis of stable isotopes by IRMS has a broad range of applications for compounds at natural abundance and for labelled compounds. The tracer methods (with labeled compound) find applications in nearly every field of life sciences (medicine, biology, physiology, nutrition, toxicology and biotechnology). The common issues for all these isotope tracer studies concern the possibility to trace the entity of interest, called the tracee, which may be a compound, or an atom. An ideal tracer has the same physical, chemical or biological properties as the tracee but it presents some unique characteristics that enable its detection in the system where the tracee is also present.

1.4.1 $^{13}C/^{12}C$ ISOTOPIC RATIO AT NATURAL ABUNDANCE

Isotope ratios are not constant on earth. Carbon compounds are exchanged between oceans, biosphere and lithosphere (Ghosh & Brand, 2003). Thus due to the kinetic and equilibrium isotope effects, isotopic ratio presents subtle but significant variations in the nature.

1.4.1.1 In plant

During photosynthesis, metabolized products become depleted in ^{13}C compared to CO_2. Typically dependant on the plant species, assimilation and diffusion processes of the CO_2 produce isotope fractionation in the ^{13}C isotopic ratio. Plants (cereal grains,

rice, tobacco, sugar beets and beans) that form a 3-carbon phosphoglyceric acid as the first product of carbon fixation according to the Calvin cycle (C3-plant) have a $\delta^{13}C$ approximately at -28‰ VPDB. On the other hand, plants that photosynthesize dicarboxylic acid by the Hatch-Slack mechanism (C4-plant, corn, millet, sugar cane and many grasses) are more enriched in ^{13}C and have a $\delta^{13}C$ approximately at -13‰ VPDB (Asche et al., 2003).

1.4.1.2 In animal and human physiology

Although less known, in human and animal physiology, isotopic ratio may also provide valuable information: the carbon isotope in food ingested by animals (or humans) is generally reflected in the metabolized products. It is then possible to discriminate animals which have different diets enriched in C3-plants or C4-plants by analyzing teeth and bones (Stott & Evershed, 1996). Based on the same principle, for humans, it is noteworthy that Europeans are lighter in carbon than Americans due to the fact that the American food comes from maize, a C-4 plant whereas European diet is more based on plants produced by the C-3 photosynthetic cycle. Sea food is heavier in carbon 13 than other food thus making Japanese people heavier in carbon than Europeans but lighter than Americans.

More recently, different studies have shown that the isotopic composition of metabolites depends on the diet as well as on the isotopic fractionation during metabolism. The biochemical pathways are regulated in response to environmental factors and isotope ratios may therefore shift in response to these modifications through the modification of fluxes (Brenna, 2001). If such modifications are known, the measurement of isotopic ratios could be used to determine the physiological state of an organism or a volunteer. Physiologically, the study of the ^{13}C isotopic abundance of different tissues (spleen, liver, brain, pancreas, kidney, muscles, etc…) as bulk analyses was studied in 76 healthy human tissue samples and six cancerous tissues was shown that the $\delta^{13}C$ values in tissue fall between -21 and -26‰ with a mean near -23‰. No apparent trend was detected according to the tissue type, and between the healthy vs cancerous tissues (Lyon & Baxter, 1978). However, a recent study of isotopic analyses (^{13}C and ^{15}N) of hair from American volunteers was investigated with patients suffering from eating disorders (anorexia, boulimia versus a control group). This study showed that with adequate statistical treatment, 80% of accuracy was reached to predict which patients had an eating disorder (Hatch et al.,

2006) thus showing a good ability for diagnosis. Other works carried out on cirrhosis showed that ^{15}N abundances of amino acids in hair was affected by this disease whereas hair ^{13}C abundances did not differ from healthy subjects (Petzke et al., 2006). Other work was related to nutritional stress of pregnant women (Fuller et al., 2005).

1.4.2 INFORMATION AVAILABLE FROM STABLE ISOTOPE TRACER EXPERIMENTS

Stable isotope tracer experiments can convey two sorts of information: how fast a specific metabolic process is running and what the products of the processes are. Thus, the speed of incorporation of an isotopic label in a molecule gives information on the synthesis, absorption and oxidation rate of that metabolite. The position of the label in the molecule can give information on the biosynthetic pathway of the molecule (Hellerstein & Neese, 2000).

The principles of the kinetic analysis from isotopic labeling experiments have been described in detail by Wolfe (Wolfe, 1992). Relatively recent examples in biomedical applications of stable isotope tracer studies are in the field of obesity (Dolnikowski et al., 2005), insulin sensitivity (Clapperton et al., 2002) lipogenesis (Bederman et al., 2004), brain (Ando & Tanaka, 2005), liver (especially hepatic gluconeogenesis (Jeukendrup et al., 1999) and synthesis of mucins and mucoproteins (Faure et al., 2002). Generally, stable isotope tracer studies are associated to many different compounds such as carbohydrates, proteins, DNA, amino acids, lipids, short chain fatty acids and cholesterol (Wolfe, 1992) (Stellaard & Elzinga, 2005).

As illustrated in Figure 4, the tracer is added to a particular body pool at a constant rate and samples are taken at defined intervals. The tracer is then equilibrated throughout the pool, enabling the rate of flux of the tracer and its tracee to be simply calculated. The movement of the metabolite through the compartment (flux) is calculated as the rate of infusion of tracer divided by the isotopic enrichment of tracee in the steady state. Additionally, there are variety of ways of applying the tracer and to determine the pools to be sampled and also many different ways in which the resulting information can be analysed mathematically (Wolfe, 1992).

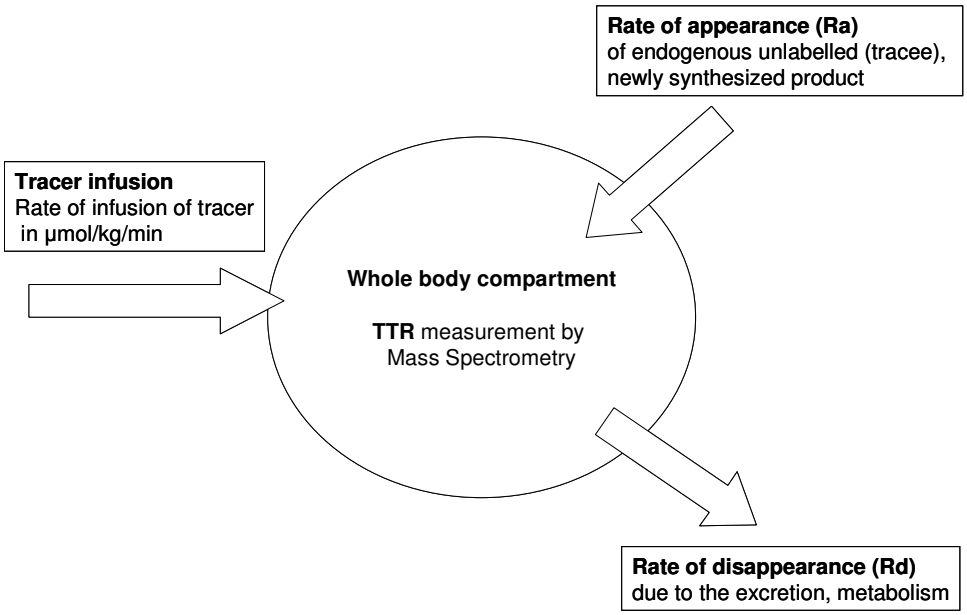


Figure 4: Principle of whole body synthesis rate determination using stable isotopes

As an example, using the constant infusion method, as long as sufficient samples are taken, this technique provides information on the degree of labeling of macromolecule such as proteins allowing calculation of its fractional synthesis rate (FSR). Thus, by measuring the increase of isotopic enrichment of the macromolecule over the time and by measuring the isotopic enrichment of the precursor at the steady state, the FSR can be calculated as described in Figure 5.

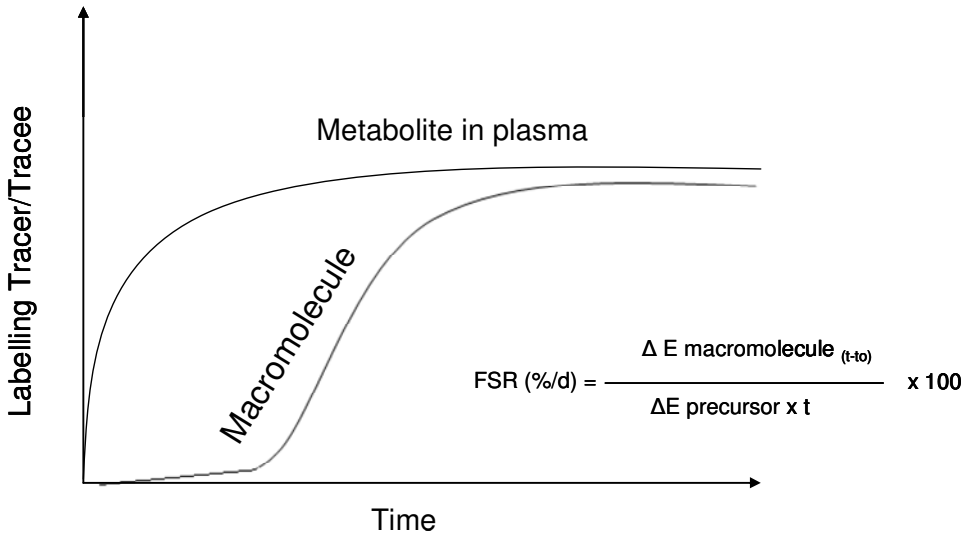


Figure 5: Principle of the measurement of protein synthesis rate

Whatever the studies and the context of tracer studies, the dose of stable isotope is tailored to the working range and to the limit of detection of the device. For instance, for assessing the protein turnover (FSR) with a labeled amino acid as tracer, the isotopic enrichment of the amino acid in proteins at the end of the infusion period depends on the turnover rate of the protein. In the case of proteins with a high turnover rate, only a few hours of tracer infusion are required to reach enrichment values higher than 1MPE. Thus, routine GC-MS or LC-MS methodologies can be used. However, for muscle proteins with a mean FSR of 1.15%/day only have an enrichment of 0.01MPE after infusion of few hours. More generally, most proteins have isotopic enrichments below 0.5MPE (Rennie, 1999). Thus, the traditional methodology used for analyzing such low isotopic enrichment is to isolate the specific protein-bond tracer from a purified protein (50-500µg). To do so the protein is cleaved into free amino acids and the isotopic enrichment measured using IRMS coupled to GC or LC.

1.5 ^{13}C ISOTOPIC MEASUREMENTS BY MASS SPECTROMETRY

Research in the field of stable isotopes is governed by various concepts affecting measurement of the isotopic ratio such as equilibrium partitioning and physical processes (e.g. evaporation and adsorption) (Criss, 1999). Moreover, all natural reactions (especially enzymatic reactions) and “artificial” chemical reactions are known to fractionate isotopes, thus leading in the long term to measurements of different isotopic compositions (Lichtfouse, 2000). This “isotopic fractionation” phenomenon enables recording of biogeochemical changes or tracing geographical origin of bio-molecules (Hayes, 2001). Isotopes can be used as natural or artificial tracers to follow the behaviour of organic molecules in complex media such as living organisms and ecosystems.

Nowadays, stable isotopes are used in many scientific disciplines, such as ecobiology (Moens et al., 2005), geochemistry (Craig, 1953; Hayes et al., 1990), drug manufacturing (Jasper et al., 2004), forensic science (Benson et al., 2006), doping control (Ayotte et al., 2001; Saudan et al., 2005), authenticity assessment (Hor et al., 2001), environmental chemistry (McRae et al., 2000; Schmidt et al., 2004), and even in art (Fortunato et al., 2005). In the domain of life sciences, the commercial

availability of labeled precursors has increased the number of stable isotope applications in various fields such as: medicine (Hellerstein, 2004), nutrition (Dolnikowski et al., 2005), toxicology (Turner, 2006), metabolic studies (Berthold et al., 1991; Mahmoud et al., 2004), and in quantitative proteomics (Doherty et al., 2005; Doherty & Beynon, 2006; MacCoss et al., 2005; MacCoss & Matthews, 2005; Wu et al., 2004).

The use of stable isotopes is preferred to radioisotopes for biomedical and metabolic studies due to their safe handling, the lack of radiation emission and their stability (Koletzko et al., 1997; Vogt, 1997). This is particularly relevant for studies in the pediatric and obstetric fields, as well as in long term studies in adults (Demmelmair et al., 1997), where the use of radioactive isotopes has limitations due to safety concerns as well as precautions for eliminating contaminated waste. On the other hand, the use of stable isotopes requires expensive mass spectrometers and trained staff. This is also the case for an accelerated mass spectrometer system used to measure small doses of radioactive isotopes (Jackson et al. 2001; Brown et al. 2006). However, this is not the case for liquid scintillation counting (LSC), used to measure higher doses of radioactive isotopes which do not require mass spectrometry. This may also explain why stable isotopes don't replace radioisotopes in all applications.

Mass spectrometry is today considered to be the best technique to measure isotopic ratios and enrichments because it is the most precise, sensitive and accurate for analysis of most elements in the periodic table. However, for light elements (e.g. C, H, N and O) with a high ionization energy (higher than 11eV), the most suitable devices for the ^{13}C isotopic ratio acquisition are the isotope ratio mass spectrometer (IRMS) and the atmospheric pressure mass spectrometer (molecular MS). Typically, the analytical figure of merit for isotopic precision of IRMS is in the order of 10^{-4}. For the molecular MS it is in the order of 10^{-2}. This means that the absolute $^{13}C/^{12}C$ ratio determination in molecular MS in full scan mode can not be lower than 2% and in the single ion monitoring mode (SIM) the performance is in the range of 0.005%. Using the same scale, the IRMS isotopic precision measured is a few parts per thousand (between 0.0001 - 0.0003%). This IRMS value is usually expressed relative to a standard, using the term "delta" ($\delta^{13}C$) in ‰. These analytical figures of merit illustrate on the one hand the "high-precision" domain covered by the IRMS, and on the other hand, the domain of "low-precision" covered by using the molecular MS, as illustrated in Figure 6. It is worth highlighting that the two devices do not measure the same species: the IRMS measures the $^{13}C/^{12}C$ isotopic ratio after conversion of organic molecule into ionized CO_2, whereas molecular MS measures isotopomer

ratios of ionized molecules. In addition, the selectivity in complex mixtures is on the one hand only performed by the separation method using IRMS and on the other hand for molecular MS the mass spectrometer can also be considered. This implies that baseline resolution of analytes is mandatory with IRMS and co-elution of compounds is a minor issue in MS. Thus, the nature and structure of the compounds impacts the sample preparation. Commonly, most of the IRMS applications concern determinations of natural abundance variations and metabolic studies using stable isotope-enriched tracers. For tracer studies, the ranges of precision of both molecular MS and IRMS techniques are complementary because they measure isotopic enrichment in various physiological pools presenting different isotopic dilutions and different ranges of isotopic values (Montigon et al., 2001; Wagenmakers, 1999).

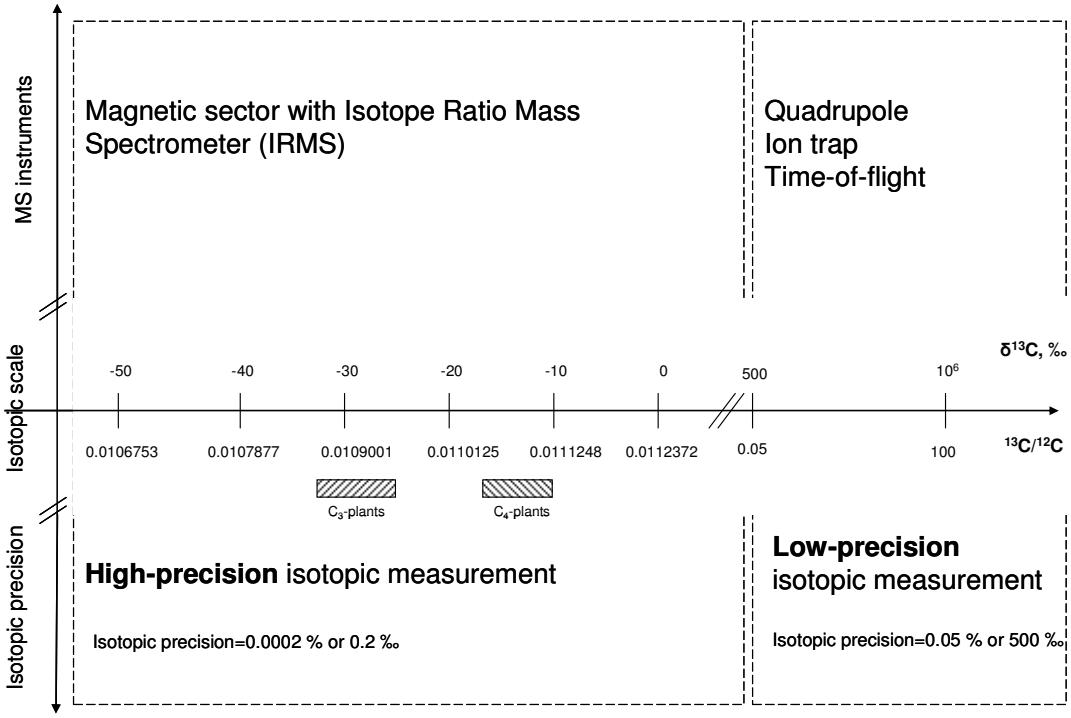


Figure 6: MS instruments used to measure $^{13}C/^{12}C$ isotopic ratio over a wide range of carbon isotope ratios observed in nature and for artificial tracer enrichment.

In figure 6, $\delta^{13}C$ scale and the corresponding isotope ratios are shown. This scheme classifies instruments according to their isotopic precision: IRMS measures high-precision isotopic values (isotopic precision equivalent to 0.0002% for LC-IRMS

device) and molecular MS (with quadrupole mass filter, ion trap and time-of-flight) measures isotopic enrichment with an isotopic precision around 0.05 %.

The coupling of LC with molecular MS (LC-MS) has grown rapidly since its first development in the 1970's. The LC-MS technique is now established as a workhorse in many laboratories. The sensitivity of detection can reach very low levels. For ADME (Absorption Distribution Metabolism Excretion) investigations or for environmental studies, the sensitivity level is in the nanomolar range and for proteomics analyses a few femtomoles of peptide can be routinely detected. For "low-precision" isotopic measurement, LC-MS has also reported ^{13}C isotopic ratio measurements. In the same manner, the LC-ICPMS technique is interesting for the analysis of hetero-elements (metallic or non metallic such as Pt, Ru, Cl, Br, S, P) covalently bond to carbon for metabolic, pharmacokinetics and other studies (Lobinski et al., 2006). For "high-precision" ^{13}C isotopic ratio measurements, GC coupled to IRMS (i.e. GC-combustion-IRMS with its acronym GC-C-IRMS) is still the state of the art. Although the idea of combining LC to IRMS was proposed over 10 years ago, two issues have always limited the association of LC with magnetic sector instruments. These are: 1) an efficient step for solvent removal and 2) for analyte transformation into CO_2 gas without any isotopic effect. Due to these limitations, the interface enabling LC coupled with IRMS was only commercialized in 2004 (Krummen et al., 2004)[1] and ^{13}C isotopic analysis carried out with ICP-MS have demonstrated reliable ^{13}C isotopic measurement at natural abundance only since 2003 (Luong & Houk, 2003). Moreover, a large number of compounds and matrices can be targeted using stable isotope-based methods. The chemical diversity of the targeted molecules (from macromolecules to small molecules, from hydrophobic to hydrophilic, and from acidic to basic molecules) in complex biological samples poses a number of challenges to the analytical chemist. The main challenge is to quantitate $^{13}C/^{12}C$ isotopic ratio for compounds at natural abundance or for ^{13}C labeled compounds, with enough precision and accuracy to unravel fine differences between samples, according to the design or the context of the study.

The above requirements were the driving force for the instrumental development of interfaces able to cope with complex matrices, low amount of target compound(s) and variable isotopic enrichments. In this context, the development of a new tool such as LC coupling, complementary to GC coupling, with mass spectrometers for measuring ^{13}C isotopic ratio is crucial. As the $^{13}C/^{12}C$ ratio quantification can be carried out with various instruments (molecular MS and IRMS), all possible couplings

with LC need to be considered. This paper summarizes the most important aspects of this new field of stable isotope research. It introduces the instrumentation, the analytical capabilities and a summary of stable isotope work conducted with these instruments.

[1] Different acronyms were used to describe the coupling between LC and IRMS. Three different acronyms describe the three interfaces developed since the 90's. The moving wire-IRMS was the first system reported using the acronym "LC-C-IRMS" (as a reference to the "GC-C-IRMS" principle). The acronym "moving transport-IRMS" will be used in this review to name the LC-C-IRMS as well as the recent improvements. Later, the "LC-CRI-IRMS" acronym describing the chemical reaction interface coupled to IRMS CRI-IRMS was introduced and we will use this also. Finally, the commercialized system was described using the generic acronym "irm-LC/MS" to mimic the term "irm-GC/MS" also used to indicate the GC-C-IRMS device. In the present review, the generic acronym "LC-IRMS" will describe the commercialized wet oxidation interface system currently available.

1.6 ^{13}C ISOTOPIC ANALYSES WITH IRMS AND MOLECULAR MS

The fundamental aspects of IRMS and MS have been reviewed many times. Only relevant points, within the scope of ^{13}C isotopic analyses, will be outlined here. ^{13}C isotopic measurements have been tackled with various mass spectrometers (see Table 4); meaning different sample ionization processes (electron impact and electrospray) as well as different analyzers to separate the resulting ions such as a magnetic sector, a quadrupole mass filter, a time-of-flight and an ion trap.

1.6.1 ISOTOPE RATIO MASS SPECTROMETRY (IRMS)

The theory and practice of IRMS has been reviewed in detail elsewhere (Brand, 1996; Brenna et al., 1997; De Laeter & Kurz, 2006; Sessions, 2006; Werner & Brand, 2001) and will be only briefly outlined in this review. Nier (Nier, 1947) was the first to build a magnetic sector mass spectrometer specifically designed for assessing isotope ratios with "high-precision" (De Laeter & Kurz, 2006). The IRMS device or so-called gas IRMS is dedicated to the analysis of light stable isotopes such as ^{13}C, ^{15}N, ^{18}O, ^{34}S and ^{2}H which are then transformed respectively into CO_2, N_2, CO, SO_2, and H_2 gases and finally introduced into the mass spectrometer ion source (Figure 7). In order to measure isotopic ratio with "high-precision", two inlet systems allow fast introduction

of a reference gas into the ion source as well as of the gas produced after the analyses by GC, elemental analyzer (EA) or LC devices. Subsequently, the gaseous samples collide with an electron beam in a high vacuum area and then the molecules lose electrons to produce positive ions. The IRMS sensitivity for CO_2 can be described by the number of ions collected per input of molecule; the absolute sensitivity is around one ion per 800-1200 molecules in dual inlet mode.

These ions are accelerated through a flight tube in an electromagnetic field. Finally, they are separated according to their *m/z* ratio and counted by a multiple Faraday cup collector to cancel fluctuations in the ion beam intensity. The accelerated ions enter the magnetic field and are separated along radial paths based on their mass-to-charge ratio and their kinetic energy. The radius (r) of the path traced by an ion in the magnetic field is related to its mass (M), its charge (q), the potential of the source (V) and the magnetic field flux density (B) according to Equation 4:

$$r = \sqrt{\frac{2MV}{qB^2}} \qquad \textit{(Equation 4)}$$

A unique feature of the magnetic sector IRMS is the trapezoidal peak shape produced by selection of the ion source (entrance) and collector (exit) slit widths. As the ion beam is scanned across the exit slit of the mass spectrometer, the ion current increases until the entire width of the ion is contained inside the collector. The resulting flat-top peak ensures that the measured ion current does not change if there are small fluctuations of parameters such as in the accelerating potential or in the magnetic flux density.

As suggested by the name isotope ratio mass spectrometry, more than one ion is measured simultaneously in order to determine isotopic ratio. An IRMS has 6-8 collectors for measuring several ion currents. This configuration has several advantages: any change in the total ion current affects the signal in all-detectors and fluctuation in the production of ions, or stability of the ion current are not a limiting factor.

For ^{13}C measurements, the faraday cups allow simultaneous measurement of three ions at *m/z* 44, 45 and 46. These ions represent the different isotopomers $^{12}C^{16}O^{16}O$ (*m/z* 44), $^{13}C^{16}O^{16}O$ and $^{12}C^{17}O^{16}O$ (*m/z* 45), and $^{12}C^{16}O^{18}O$ (*m/z* 46) (Werner & Brand, 2001). Then the peak area of each isotopomer is measured and finally transformed into $^{13}C/^{12}C$ ratio using *m/z* 44, 45 and 46. The *m/z* 46 isotopomer is

measured for assessing the ^{18}O isotope content from which the ^{17}O content is calculated to obtain the true ^{13}C value measured for *m/z* 45 (Santrock et al., 1985).

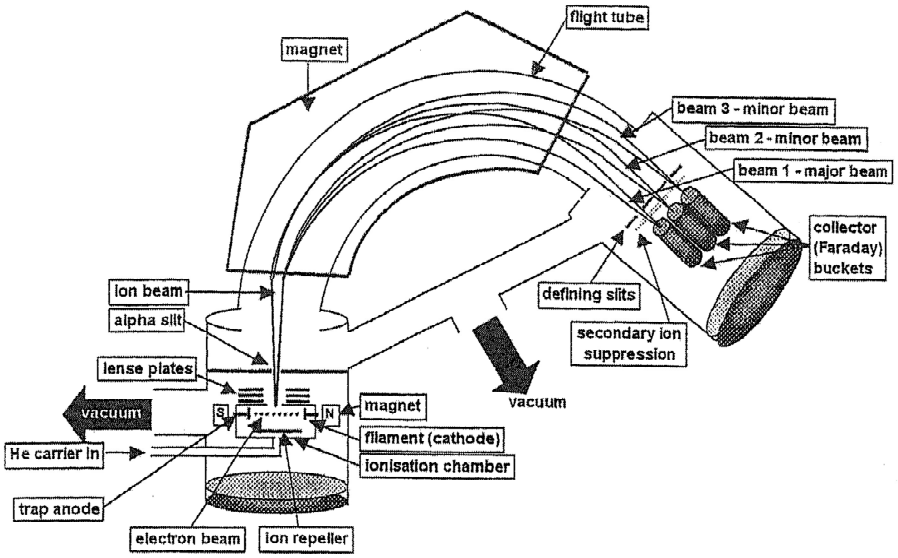


Figure 7: Isotope Ratio Mass Spectrometer device (Lees & Kelly, 2003)

Table 4: Specifications of MS instruments (IRMS, MS and ICP-MS) using LC separation for measuring ^{13}C isotopic ratio

	Moving wire-IRMS	LC-CRI-IRMS	LC-IRMS	LC-ICP-MS	LC-MS	LC-CRIMS
Sample introduction	Injection of liquid	Injection of liquid	Injection of liquid	Injection of liquid	Injection of liquid	Injection of liquid
Sample transformation	Yes, removal of solvent with heater, combustion and oxidation of the molecules into CO_2	Yes, nebulisation of the solvent, addition of reactant gas which transform molecules into CO_2	Yes, wet chemical oxidation of molecules into CO_2	Yes, transformation of the molecules in $^{12}C+$ and $^{13}C+$	No transformation of molecules. Ions molecule measured	Yes, nebulisation of the solvent, addition of reactant gas which transform molecules into CO_2
Interface	Moving wire, capillaries, furnaces	Universal interface, thermospray nebulizer, reactant gas (O_2)	Acid and oxidant reagents, capillaries	Inductive plasma	Electrospray (ESI), positive or negative mode	Electron impact
Mass analyzer	Magnetic sector	Magnetic sector	Magnetic sector	Two quadrupoles	Quadrupole-Ion trap-TOF	Quadrupole
Detector	Multicollectors (3 faradays cups)	Multicollectors (2 faradays cups)	Multicollectors (3 faradays cups)	Electron multiplier	Electron multiplier	Electron multiplier
Measurable isotopic enrichment in APE	-0.05 to 1 APE (a)	-0.05 to 1 APE (a,b)	-0.05 to 1 APE (a)	Natural abundance	0.05 to 100 APE	0.5 to 100 APE
Isotopic precision (c)	0.0002 atom % (d)	0.0005 atom %	0.0002 atom %	Not Applicable	0.05 atom %	0.05 atom %
Sample size (e)	nmol	nmol	nmol	Hundreds of nmol	pmol-fmol	pmol-fmol

a): Typical range of ^{13}C isotopic enrichment corresponding to a $\delta^{13}C$ of -50 to 1000‰. Nevertheless IRMS instrument is able to measure down to -0.05APE and up to 1APE. But such depleted samples are rare in nature and such highly enriched samples are isotopically diluted to belong to the range cited below to avoid inaccurate isotopic value.
(b): As mentioned in this review, the CRI interface produced interference molecules which interfered with m/z 46 leading to inaccurate isotopic ratio at natural abundance. Therefore, isotopic analyses at natural abundance are possible but the accuracy might be non suitable for specific applications.
(c): The precision reported here is only suitable for high-precision isotopic measurement with a sample size higher than 0.5-5nmole of compound injected. IRMS instrument can also measure femtomole of molecule if hundreds of ‰ of precision is needed.
(d): Isotopic precision reported for 10nmol of carbon injected; for lower amount of carbon (< 1nmol of carbon), the isotopic precision is increased to 0.001atom%.
(e): The sample size is given for low molecular weight molecules. Therefore, this number can be decreased to pmol levels for IRMS instrument if macromolecules are studied

1.6.2 MOLECULAR MASS SPECTROMETRY (MS)

Molecular MS instruments are used for a broad range of applications, including analysis of complex biological molecules or matrices (Hopfgartner & Bourgogne, 2003). Most of the studies reported to date on ^{13}C isotopic analysis were carried out with MS using GC as the sample introduction, with electron impact as the ionization mode and a quadrupole filter as the mass analyzer (Hernandez-Perez et al., 2002; Montigon et al., 2001; Reeds et al., 1997; Slater et al., 2002). Using GC-quadrupole MS, the accuracy and precision for isotopic labeled molecules reported was 0.5 APE (or 500‰ using the same scale than the IRMS device) (Meier-Augenstein, 1999). The principal advantages of conventional GC-MS are its ease of use and the lower sample size requirement (Table 5). Additionally, low isotopic enrichment (0.005%) using highly substituted tracers containing at least three ^{13}C labeled atoms were also measured using GC-MS (Patterson, 1997). Nevertheless, more and more developments using LC-MS for measuring ^{13}C isotopic enrichment in metabolism studies are reported with an electrospray ion source (ESI). The soft ionization of ESI in combination with a quadrupole filter, a time-of-flight or an ion trap, require lower sample size than with the IRMS device and the isotopic precision measured by LC-MS is in the same range as with GC-MS. In addition, for molecular MS instruments, increasing the amount of labeled compound does not increase the limit of detection. The limit of detection for [3,4-^{13}C] cholesterol was 15 times lower using GC-C-IRMS than in GC-MS (Guo et al., 1993). Therefore, the feature of such mass spectrometers makes them an attractive tool for ^{13}C isotopic analysis in the range of 0.005 to 100% of ^{13}C enrichment.

Table 5: Sensitivity for ^{13}C isotopic ratio measurement based on GC separation

Mode	GC-MS	Off-line IRMS	GC-C-IRMS
Min sample size	1 pmol	1µmol	1 nmol
Detection limit (APE)	0.5	0.001	0.0005
Precision (APE)	0.5	0.0005	0.0001

1.7 ISOTOPE RATIO MONITORING

1.7.1 CONTINUOUS FLOW-IRMS

Continuous flow-isotope ratio mass spectrometry (CF-IRMS) is the main tool used to monitor isotopic ratios using a separation technique combined with an helium stream to transport the CO_2 gas into the IRMS source. It consists of three sections; the sample separation device, the interface and the isotope ratio mass spectrometer. According to the complexity of the samples, and the type of analysis required, two different approaches are available: the bulk isotopic analysis (BSIA) and the compound specific isotopic analysis (CSIA). The elemental analyzer coupled to IRMS (EA-IRMS) and GC coupled to IRMS are the two main instruments used nowadays. With GC introduction, the major hurdle was the development of interfaces allowing quantitative transformation while keeping the chromatographic resolution of the molecules. GC hyphenated to IRMS through different interfaces (with different performances, see Table 6) analyzes various elements such as carbon, nitrogen, oxygen and deuterium. The combustion interface was developed in the 1980's for ^{13}C and ^{15}N and later (1990's) the pyrolysis interfaces were developed for ^{2}H (Hilkert et al., 1999) and ^{18}O (Begley & Scrimgeour, 1997) either for bulk or compound specific analyses.

Table 6: Characteristics of major light stable isotopes measured by GC-IRMS (Sessions, 2006)

Isotopes	Sample gas	Interface	Ref. standard	Precision[a] (‰)	Sensitivity[b] (nmol)
$^{2}H/^{1}H$	H_2	Pyrolysis	Water (VSMOW)	2-5	10-50
$^{13}C/^{12}C$	CO_2	Combustion	Carbonate (VPDB)	0.1-0.3	0.1-5
$^{15}N/^{14}N$	N_2	Combustion	Air (AIR)	0.3-0.7	1-10
$^{18}O/^{16}O$	CO	Pyrolysis	Water (VSMOW)	0.3-0.6	4-14

a: Precision (SD) reported for isotopic measurement performed with GC-IRMS
b: Amount of compound needed to reach the precision of the left column

Nevertheless, with the commercialisation of the LC-IRMS tool, the way of performing the bulk or the compound specific ^{13}C isotopic analysis has changed: both types of analyses (CSIA and BSIA) can now be performed using the same LC-IRMS instrument for water-soluble compounds (Figure 8).

For the bulk analysis (which is the most widespread application), the entire sample is either oxidized or combusted producing CO_2.

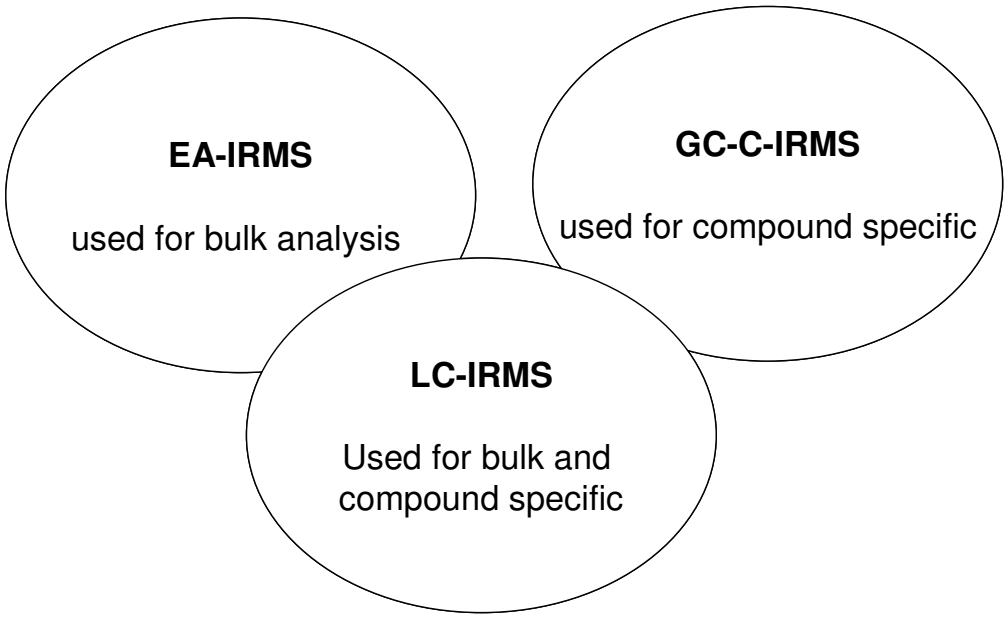


Figure 8: Isotopic ratio monitoring of available devices for bulk and compound specific isotopic analyses

1.7.2 ^{13}C COMPOUND SPECIFIC ISOTOPE ANALYSIS

For ^{13}C compound specific isotope analysis (CSIA), the targeted compound is separated from the matrices and then transformed into CO_2 either through the combustion or oxidation processes used in GC-C-RMS and LC-IRMS, respectively.
The term "Compound Specific Isotope Analysis" (CSIA) was coined by M. Schoell, in the late 1980's and refers specifically and exclusively, to GC coupled to IRMS for high-precision isotopic analysis. CSIA became feasible with the work of Matthews and Hayes (Matthews & Hayes, 1978) who introduced the combustion furnace with Pt and CuO as the oxygen source after the work of Sano. Sano used a GC-MS equipped with a catalytic combustion unit to monitor urinary metabolites after ingestion of ^{13}C aspirin (Sano et al., 1976). Currently different interfaces are commercially available to measure isotopic ratio of elements other than carbon (Sessions et al., 2006). The minimum sample size or limit of detection (LOD) used with IRMS is usually reported in nanograms (ng) of element (carbon atom) or

amplitude of CO_2 signal associated to an isotopic precision (standard deviation) (Jochmann et al., 2006). In addition, theoretically the IRMS precision is statistically limited by the number of counts from the minor ion beam indicating that certain amplitude of signal is needed to measure isotopic ratio of carbon. Therefore, with an efficiency (ions/molecules) of 10^{-4}, theoretically to obtain a standard deviation of 0.1 ‰, 0.31 nanomoles of carbon is needed (Asche et al., 2003).

Whatever the configuration (EA, GC or LC), the amount of gases entering into the IRMS ion source is limited to approximately 0.4mL/min. Therefore for each system, it requires an open split as at such high gas flow rate (i.e. within the elemental analyzer the flow rate is ~100 mL/min), the split ratio is huge. This affects the sensitivity by reducing the amount of sample available for the electron impact ionization. This explains the different amounts of compound needed between the LC-IRMS and the EA-IRMS. Thus, within the GC and LC instruments (with a flow rate between 1-2mL/min), the split ratio is minor and the sensitivity is less affected compared to the EA-IRMS.

1.8 GAS AND LIQUID CHROMATOGRAPHY SEPARATIONS

Ideally, the ultimate goal of the separation method for high-precision isotopic analyses is to perform a sensitive, precise and accurate measurement of a small variation of isotopic enrichment. For that, the classical ways to introduce the sample are either using the gas chromatography (GC) or liquid chromatography (LC) instruments.

1.8.1 GAS CHROMATOGRAPHY (GC)

In the myriad of applications involving the analyses of volatile and semi-volatile compounds, gas chromatography (specifically gas-liquid chromatography) is probably the best method of separation. For non-volatile compounds, derivatization is mandatory. This step is useful to improve the volatility, change the polarity, and convert labile compounds to more thermally stable derivatives. This step can also be meaningful to eliminate the peak tailing with polar compounds, and to improve the detectability of compounds in complex matrices. The compounds that require derivatization include carbohydrates, polyols, thiols, polycarboxylic acids, acids, lipids,

and polyfunctional compounds such as amino acids. The GC technique involves a sample being vaporized and injected into the head of the chromatographic column. The sample is transported through the column by the flow of an inert, gaseous mobile phase. The column itself contains a liquid stationary phase which is adsorbed onto the surface of an inert solid. The retention factor (k) depends on the vapour pressure of the compounds at the column temperature and on the affinity of the components for the stationary phase.

The critical parameter in developing a method for gas chromatography is the resolution (Rs, see Equation 5) required for performing the measurement between two species (characterized by their retention times T_1 and T_2, and their peak widths at the baseline, W_1, W_2) with a non specific detector (such as FID or IRMS devices).

$$Rs = \frac{2 \times (T_1 - T_2)}{W_1 + W_2} \qquad (Equation\ 5)$$

Chromatographic resolution is an interplay of the efficiency, selectivity and retention (see Equation 6).

$$Rs = \sqrt{\frac{N}{4}} \left(\frac{\alpha - 1}{\alpha} \right) \left(\frac{k}{k+1} \right) \qquad (Equation\ 6)$$

where N is the column efficiency expressed as the theoretical plate number, α is the selectivity factor, and k is the solute partition ratio (or retention factor).

The resolution and the time required for the chromatographic separation are functions of several interrelated column and operational parameters. Major factors influencing the separations are the column internal diameter, column length, the type of stationary phase and its film thickness, the carrier gas velocity and the column temperature.

GC columns are also characterized by their theoretical plate number, N (which is dimensionless); also called the column efficiency calculated from a chromatogram under isothermal conditions using the relationship described in Equation 7.

$$N = 5.54 \times \left(\frac{Tr}{W_h} \right)^2 \qquad \textit{(Equation 7)}$$

where W_h is the width of the peak at half height and Tr is the retention time.

In general, the column efficiency increases proportionally with the length of the column. It is often meaningful to use the theoretical plate height, H, calculated by dividing the length of the column by its number of theoretical plates (Equation 8).

$$H = \frac{L}{N} \qquad \textit{(Equation 8)}$$

The GC technique is always referred to as a high-resolution chromatographic separation technique. The theoretical plate number is used to describe the general quality of the GC columns without any reference to specific separations. Thus, it does not indicate the resolving power of the column directly; however, it does indicate its ability to produce sharp and narrow peaks. For a typical GC column (for a DB5 column, 50m x 0.32mm, 0.2µm of film thickness, with helium as carrier gas heated at 270°C), N is approximately 150000.

1.8.2 LIQUID CHROMATOGRAPHY (LC)

When the goal of the method is the separation of non-polar, neutral, ionic, ionisable and/or thermolabile compounds, the liquid chromatography (LC) method is interesting to implement. Table 7 shows some basic characteristics of LC methods according to the targeted compounds.

Moreover, according to the characteristic of internal diameter (i.d.) of the LC columns, the LC flow rate and the injection volume are not similar. Typically, for a 4.6mm i.d. column, the volume of the column is 4.1mL, the optimum flow rate is 1mL/min, the injection volume is 100µL. For a 2.0mm i.d. column, the volume is 783µL with an optimum flow rate at 0.2mL/min and with an injection volume at 19µL (Abian J., 1999). As reported for the GC, the column plate number (N) is an important characteristic of a column. For the LC column packed with particles of 5µm diameter having a length of 25cm, N is approximately 17000-20000.

Table 7: Characteristics of LC methods

Method	Description and column	Targeted compounds
Reversed phase LC	- Water-organic mobile phase - C18, C8, cyano, phenyl	First choice for most samples, neutral, nonionized compounds that dissolve in water-organic mixture
Ion exchange LC	- Water and organic mobile phase with a buffer to control the pH - Cation or anion exchange columns	Ionic and ionisable compounds, especially for cations and bases
Normal phase LC	- Organic solvent (Hexane, alcohol) - Cyano, diol, amino and silica columns	First choice for lipophilic samples that do not dissolve well in water-oragnic solvent. Well adapted for isomer separations and preparative scale LC

In order to explain and understand the nature of the equilibration process occurring in chromatography (gas or liquid), the Van Deemter equation (or its simplified equation, see Equation 9) can be used. According to this equation, there is an optimum flow rate (u) for which H is a minimum and N is a maximum (Snyder et al., 1997).

$$H = A + \frac{B}{u} + Cu \qquad (Equation\ 9)$$

where each of the three terms is attributed to a particular type of diffusion or mass transfer process and u is the linear velocity of the mobile phase in LC and the carrier gas in GC.

The eddy diffusion term A: this parameter is attributed to the zone dispersion due to diverse paths of the solute molecule through the column packing. This term is independent of the flow rate but dependant on the quality of the packing and the particle size of the packing.

The longitudinal diffusion term B: This term describes the dispersion o the zone due to molecular diffusion in the carrier gas in an axial direction (from the zone centre). This term contributes to the zone dispersion and is inversely proportional to the flow rate.

The mass transfer term C: This term describes the behaviour of the solute molecules that are constantly and reversibly transferring from the mobile phase to the stationary phase. This transfer is not instantaneous; time is required for the molecules to pass (by diffusion) through the mobile phase to reach the interface and to enter into the stationary phase. Those molecules close to the stationary phase enter it immediately, whereas those molecules some distance away will find their way to it some time later. Since the mobile phase is continually moving, during this time interval, those molecules that remain in the mobile phase will be swept along the column and dispersed away from those molecules that were close and entered the stationary phase immediately. Thus the C term is inversely proportional to the diffusivity of the solute (Ds) in the stationary phase and proportional to the diffusivity of the solute in the mobile phase (Dm).
All of these three terms need to be minimized to obtain a sharper peak in order to maximise the column efficiency (N).

Currently, there is a general trend in LC methods development to increase the throuput of the LC system without degrading the resolution. In order to speed up the LC separation, the knowledge of the Van Deemter curve shows that it is possible to reduce the analysis time by increasing the flow rate using small particle size (< 2µm). Nevertheless this support generates higher backpressure in the system which limits this approach to specific LC instruments (with the Ultra Performance Liquid Chromatography, UPLC) and LC columns (Mazzeo et al., 2005). Another important approach is to use monolithic columns to improve the mass transfer (C term) and the efficiency of the separation (with a high flow rate but without generating a high back pressure) (Guiochon, 2007).
A new trend in LC separation is to heat the mobile phase and the column at high temperature (HTLC) (Guillarme et al., 2004). This decreases the viscosity and subsequently increases Dm. Thus, the Van Deemter curve is more flattened when the temperature is increased allowing work at a higher flow rate without build up of excessive pressure. The HTLC principle has other advantages such as that the dielectric constant of water is decreased with the temperature meaning that the eluting strength of water is increased for reversed stationary phase applications. Thus, heated aqueous buffer can replace the organic buffer. This can be performed only if an efficient oven is used for heating both the column and the mobile phase.

1.9 GAS CHROMATOGRAPHY COUPLED WITH IRMS

Gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS) was introduced in the early 1990's more precisely during the 11th International Mass Spectrometry Conference held in Bordeaux (France) in 1988. In many cases, this technology has surpassed the classical (or off-line) approaches in isotopes determination mainly due to its throuput in terms of sample preparation which is less tedious and laborious than before. Additionally, the isotopic precision met by the GC-C-IRMS was adequate for studying variation of isotope ratios at natural abundance for a sample size of one nanomole per element. This represents an improvement compared to the off-line sample analysis. Thus, the GC-C-IRMS is now the workhorse within many laboratories working with high-precision ^{13}C isotopic measurement for compound specific isotopic analysis.

1.9.1 HOW DOES THE GC-C-IRMS WORK?

The derivatised mixture is injected into a capillary gas chromatography column with an auto-sampler (Figure 9). Individual compounds are then separated according to their volatility, interaction with stationary phases and are then introduced in a constant stream of helium into the combustion furnace (ceramic tube, typically 0.5 mm inner diameter with metal wires (CuO/NiO/Pt) heated at 960°C where each compound is converted into CO_2, H_2O and nitrogen oxide gases (N_xO_y). Then N_xO_y gases are converted to N_2 after a passage through the reduction furnace (containing pure Cu wires) heated at 650°C. This step is fundamental since N_2O or NO_2 might produce *m/z* 44, *m/z* 45 and *m/z* 46 in the ion source interfering with the measurement of the $^{12}CO_2$ and $^{13}CO_2$ isotopic ratios. However, these furnaces can introduce a small peak broadening and isotopic fractionation. Water is then removed by a water trap (permeable membrane of sulfonated fluoropolymer Nafion®). Finally, a small fraction of gases is introduced into the IRMS ion source with electron impact mode of ionization. The rest of the gas stream is diverted to the atmosphere via a split.

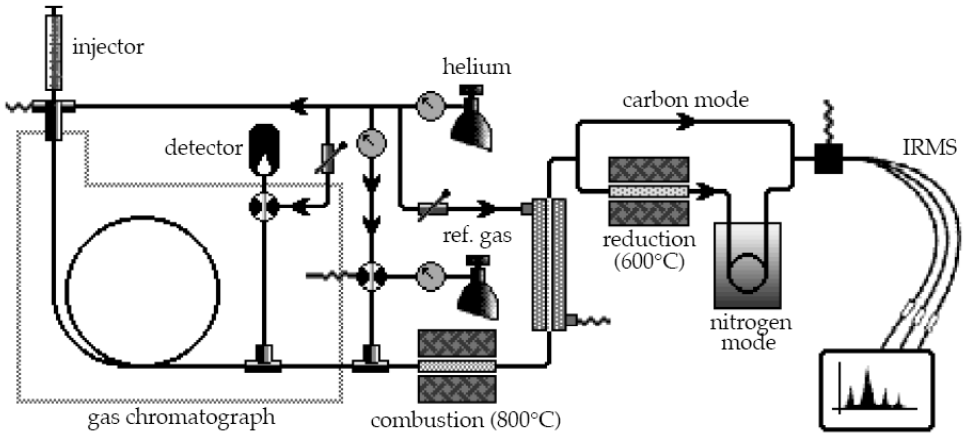


Figure 9: Set up of conventional GC-C-IRMS instrument (Meier-Augenstein, 2004)

1.9.2 ISOTOPIC FRACTIONATION IN GC-C-IRMS

The IRMS is designed to follow subtle variations of stable isotopes in natural processes; it also detects changes induced by physical, chemical or biological processes inducing isotopic fractionation. To overcome isotopic fractionation, the process involved must be quantitative (meaning that the substrate is completely transformed in the product). If this is not possible, the isotopic fractionation must be reproducible to correct for it. Two main mechanisms can explain isotopic fractionation: On one hand, the kinetic isotopic effect (KIE) which is the result of changes in the bond strength resulting in different reaction rates for the bond (Rieley, 1994). The lighter isotope is more reactive, hence its concentration is more important in reaction products and the reactants are enriched with the heavier isotopes. The most significant isotopic effect is the primary isotope effect where a bond containing the isotope of interest is broken or formed in the rate determining step of the reaction.

On the other hand, isotopic fractionation can also be explained by the thermodynamic isotope effect (TIE) which is related to the energy state of a system. The TIE is associated with differences in physico-chemical properties such as infrared absorption, molar volume, vapour pressure and boiling point which are all related to vibration energy levels. Typically, this isotopic fractionation may be observed during the peak integration. In the GC-C-IRMS, on average *m/z* 45 signal precedes the *m/z* 44 by 150ms (Brand, 1996). This time shift depends on the GC conditions such as the polarity of the stationary phase. It means that to obtain an accurate isotopic ratio it is mandatory to integrate correctly both signals and for two

adjacent peaks to have baseline separation. This chromatographic effect is not caused by a vapour pressure effect but rather on the different solute/stationary phases interactions (with the Van der Waals dispersion forces leading to an earlier elution of ^{13}C atom versus the ^{12}C atom). This is caused by the lower molar volume of the heavier compound. This is explained by the reduction of the bond length (and an increase of bong strength) between ^{13}C-H and ^{12}C-^{13}C and ^{12}C-H and ^{12}C-^{12}C, respectively (Meier-Augenstein, 2002).

Finally, some of the GC parameters such as the flow conditions in the injector, the split/splitless mode of injection, as well as the amount of compounds injected, might also produce isotopic fractionation (Glaser & Amelung, 2002; Meier-Augenstein et al., 1996; Schmitt et al., 2003).

1.9.3 SAMPLE PREPARATION IN GC-C-IRMS

With the commercialization of the GC-C-IRMS tool, it is now possible to perform CSIA of a single molecule in a mixture after appropriate derivatisation to obtain volatile compounds (Meier-Augenstein, 2004). The derivatisation is an important step with the GC-C-IRMS to obtain reliable ^{13}C isotopic ratio measurements. Thus, the basic rules to follow are: 1) the addition of as little carbon as possible; 2) use of a relatively fast reaction; 3) good separation of the targeted compound from the rest of compounds present in the sample; 4) no interference with the by-products; 5) stable end-product and 6) reproducible isotopic ratio to obtain control of the isotope effect (KIE).

For GC-C-IRMS measurement performed at natural abundance, in order to circumvent all the derivatisation issues (isotopic dilution and KIE), mass balance equations and calibration curves allow assessment of the correct $\delta^{13}C$ value (Brenna et al., 1997). For such calculations, the stoechiometry of the reactions involved and the $\delta^{13}C$ value of the reagents used for the derivatization must be determined using other hyphenated-IRMS techniques such as EA-IRMS. However, using EA-IRMS, the possible “isotopic bias” induced by the chemical impurities of the chemical reagents not detected by GC-C-IRMS but observed by EA-IRMS, can also lead to statistical differences between the two techniques. In order to avoid the derivatization procedure needed with GC-C-IRMS, an alternative approach was developed: it consists of the chemical reduction of non-volatile compounds such as amino acids or fatty acids into volatile ones such as amino (Zaideh et al., 2001) or fatty alcohols (Corso et al., 1998). Nevertheless, this solution does not allow the direct

measurement of the $\delta^{13}C$ value of the primary compound before chemical reduction. For tracer studies, since the outcome is generally the calculation of the excess of isotopic enrichment, using the GC-C-IRMS, only "absolute" isotopic measurement is used. This mode of calculation is less prone to error than the "relative" determination of isotopic enrichment at natural abundance.

The chromatographic separation of compounds by GC is also characterized by its inability to directly analyze thermolabile compounds and macromolecules without significant efforts in terms of sample preparation. The ^{13}C isotopic analysis of an intact glutathione derivative (tripeptide with a MW of 307Da) by GC-MS is one of the few exceptions (Capitan et al., 1999). The isotopic analysis of macromolecules can be performed indirectly by acidic or basic hydrolysis into monomers or sub-units, then derivatized and analyzed by GC-C-IRMS. Such conditions might slightly alter isotopic composition of the released monomers. Amino acids derived from acidic hydrolysis of proteins showed an isotopic fractionation around -1.3 to 1.6‰ due to incomplete hydrolysis (Jim et al., 2003). This indirect way to analyze macromolecules is commonly used in tracer studies when a ^{13}C tracer is administrated and then slowly incorporated into macromolecules. Using this method it is possible to measure the synthesis rate of proteins (Faure et al., 2002) (Smith & Rennie, 1996), triglycerides (Sidossis et al., 2004), lipoproteins (Chan et al., 2004), RNA (Grimble et al., 2000) and DNA (Collins et al., 2003). However, if macromolecules need to be analyzed intact, EA-IRMS is the technique of choice. In this case, the applications are more oriented towards isotopic measurement of macromolecules at natural abundance. The down side is that EA-IRMS requires high amounts of material to obtain reliable results; a few hundreds of µg of compound per analysis are mandatory for accurate and precise $\delta^{13}C$ measurements. For instance, 700µg were used to perform a biosynthetic study of the origins of adult human haemoglobin expressed in E. coli, yeast and human blood based on ^{13}C and ^{15}N isotopic analyses (Apostol et al., 2001).

1.10 INTERFACES USED TO COMBINE LC WITH IRMS AND MOLECULAR MS INSTRUMENTS

As outlined previously, the targeted molecules cannot always be analyzed directly. They need further physico-chemical transformation to obtain species compatible with the IRMS and MS instruments (either CO_2 gas in a stream of helium for IRMS, or

ionized molecules in solution for molecular MS). These steps are performed using specific interfaces as illustrated in Figure 10. In order to perform high-precision isotopic measurements with IRMS and ICP-MS magnetic sector instruments, several conditions must be fulfilled. With IRMS, the interfaces used (moving transports, chemical reaction interface and wet oxidation interface) between LC and IRMS must quantitatively convert all the organic molecules into CO_2 gas. It means also that one picomole (pmol) of protein will produce more CO_2 than one pmol of a lower molecular weight molecule without affecting the $^{13}C/^{12}C$ ratio. On the other hand, with the ICP-MS, the ICP interface must convert all the organic molecules into ^{12}C and ^{13}C ionized atoms. Whereas, for molecular MS, the isotopic ratio as well as the specific position of labeling in the molecule is obtained through fragments.

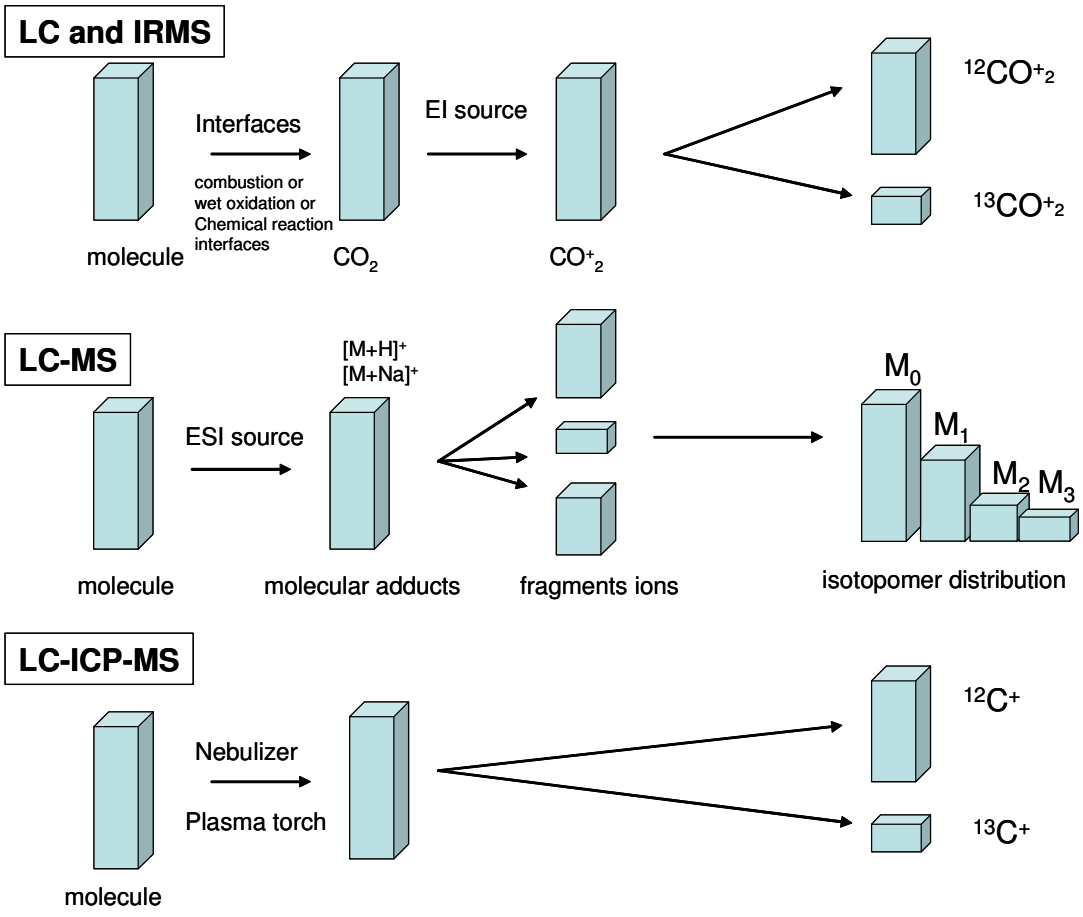


Figure 10: $^{13}C/^{12}C$ isotopic ratio determination using IRMS, MS or ICP-MS devices

According to the interfaces used, the organic molecules are transformed either as ionized CO_2 gas, ionized molecules or ionized molecular atoms. For LC-IRMS, $^{13}C/^{12}C$ isotopic ratio is measured using CO^+_2 as final species. For molecular MS, $^{13}C/^{12}C$ isotopic ratio is assessed through the isotopomer distribution in each of the fragment ions. For LC-ICP-MS, $^{13}C/^{12}C$ isotopic ratio is measured using $^{12}C^+$ and $^{13}C^+$ ionized atoms.

1.10.1 MOVING TRANSPORT ASSOCIATED TO A COMBUSTION INTERFACE

In the 1960's, the moving transport instrument was used to couple LC to flame ionization detector (FID). The LC-FID system required an efficient desolvation step prior to the introduction of the sample into the detector. The similarity between the FID and the IRMS detector in terms of constraints to remove organic solvent allows the moving transport to also be combined with the IRMS (Caimi & Brenna, 1993) (Brand & Dobberstein, 1996). The molecules present in the eluent flow were first coated (1/10 of the LC flow rate) onto the moving wire transport (which was cleaned with a cleaning furnace heated to 800°C), then the solvent was removed in a drying oven (heated at 150°C with a counter-flow of helium) and finally introduced into a combustion furnace containing CuO held between 650°C and 850°C. Compared to the GC-C-IRMS, the back-flush valves (used to remove the solvent peak) are not needed but an open split is mandatory to buffer the ion source pressure against the volume increase that occurs when samples are combusted into CO_2. The molecules were successively transformed into CO_2 gas in a combustion furnace and finally introduced into the ion source after the removal of the residual water with a Nafion® drier membrane. This system gave isotopic precision, SD ($\delta^{13}C$), around 0.5‰ by flow injection mode mode (FIA, meaning injection without any LC column as dropwise or "discrete" analysis) and 1‰ by LC for amounts greater than 50µg (Caimi & Brenna, 1993). In 1995, the sensitivity of the interface was improved by more than two orders of magnitude by changing the coating process as well as other parameters (Caimi & Brenna, 1995). A pneumatic aerosol spray was installed to atomize the liquid into an aerosol cone which was then coated onto the wire. At the end of the nebulization, desolvation and combustion processes, the amount of compound introduced into the IRMS was still relatively low due to the coating and the sampling splits: 3µg of sample produced around 10-250ng of CO_2. More recently, an advanced optimization of the process using the moving transport was reported (Sessions et al., 2005) as described in Figure 11. In comparison to the system

developed by Brand & Dobberstein (1996) this new configuration was mainly used in a drop wise mode. Several points were improved: i) a new source for the wire (with low carbon and Ni content), ii) modification of the combustion furnace (by including CuO), iii) the use of compressed air in the cleaning oven, and iv) the optimization of the wire speeds, the gas flow rates and the oven temperature. Thus, bulk isotopic analysis produced a CO_2 peak width of approx. 5sec. Using this device, the precision was better than 0.2‰ for 10nmol of C and 1‰ for samples containing lower than 1nmol of C. The isotopic accuracy was better than 0.5‰ with a significant improvement in terms of high-throughput: 1µL of sample was analyzed every 30-40sec. Nevertheless, this system suffers from factors affecting the combustion efficiency: such as the presence of inorganic salts, the temperature of the combustion furnace, the presence or not of CuO as oxidant in the combustion tube and probably also the length of the combustion tube. In addition, isotopic fractionation associated with the desorption of the molecules from the wire and the isotopic contribution of the high CO_2 background coming from the wire, from the LC stationary phase of the column (column bleeding) and the laboratory materials, also affect the $\delta^{13}C$ values. Thus, this device was described as "semi-quantitative" with respect to carbon content but quantitative and highly accurate with respect to $^{13}C/^{12}C$ isotopic ratio.

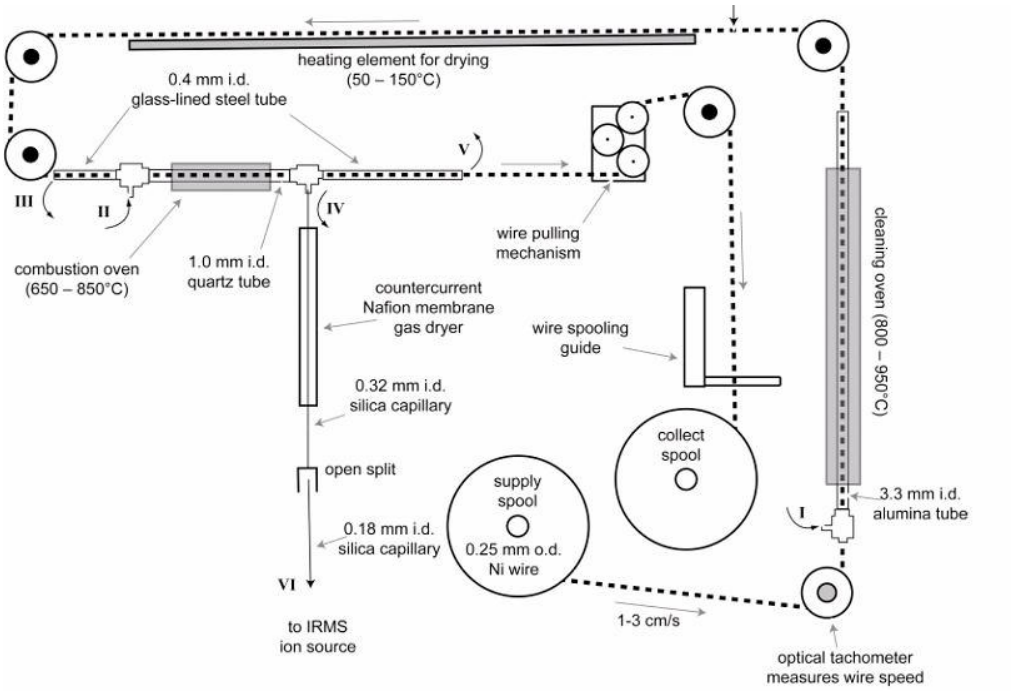


Figure 11: Schematic representation of the moving wire-IRMS (Sessions et al., 2005)

1.10.2 CHEMICAL REACTION INTERFACE (CRI)

The chemical reaction interface (CRI) was developed by Markey and Abramson in 1982. It was hyphenated to GC, LC and to an organic MS with quadrupole analyzer (Abramson, 1994) and IRMS (Teffera et al., 1996). The main feature of the CRI interface is to produce and then monitor elemental and isotopic composition (above the natural abundance) of labeled and unlabeled compounds. Historically the LC-CRI-MS was developed using a moving belt interface (Moini & Abramson, 1991) which later was replaced by a particle beam interface (Teffera et al., 1993). The key step for performing the coupling between LC and CRI-MS or CRI-IRMS was the implementation of an efficient step for solvent removal. This step is mostly performed with a thermospray nebulizer (TSN) operating at 1mL/min within a Universal Interface (UI) (McLean et al., 1996). To obtain optimal flow rates according to the internal diameter (i.d.) of the LC column, a "post column solvent make up" was used. In 2005, a new configuration was proposed with a pneumatic high efficiency nebulizer (HEN) which removes the solvent more efficiently than the TSN (Figure 12) (Jorabchi et al., 2005). The LC separation is performed with micro-flow rates between 10-100µL/min. Briefly, the CRI is built with a counter current gas diffusion cell in which the helium stream runs in the opposite direction to the particle beam emerging from the nebulizer (TSN or HEN). Then the small molecules and other minor molecules are transformed into polyatomic molecules, such as CO_2 using microwave-induced helium plasma spiked with O_2 or SO_2 as reactant gas. These two gases showed different performance for CO_2 production and filament lifetime (Abramson, 1994). Due to the instability of the CO_2 in the microwave induced plasma, the CRI also produced a substantial amount of CO, NO_2 (*m/z* 46) and $C_2H_5O^+$ (Abramson, 1994). For amounts of sample higher than 20µg, these by-products interfered with *m/z* 44, 45 and *m/z* 46 in the interface, meaning that the $^{13}C^{16}O_2$ signal (*m/z* 45) was incorrect. Therefore, the ^{13}C isotopic values were inaccurate and were associated with erroneous results (estimated as 1-2 of $\delta^{13}C$ ‰) except for compounds which did not contain O atoms, such as hydrocarbons. Therefore LC-CRI-IRMS was rarely used to determine subtle variations of $\delta^{13}C$ value at natural abundance but was more reliable for measuring $\delta^{13}C$ values of targeted compounds above the natural abundance e.g. in the case of metabolic studies.

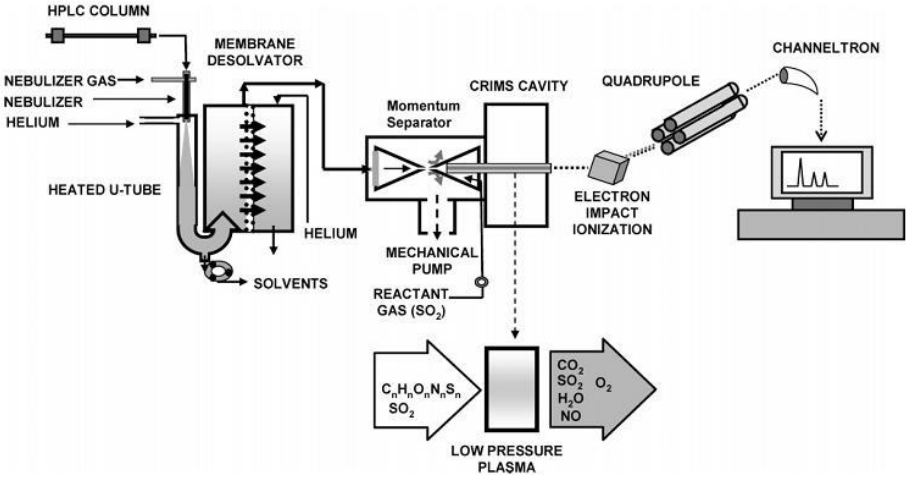


Figure 12: Schematic diagram of the instrumental setup for CRI-MS (Jorabchi et al., 2005)

1.10.3 WET OXIDATION INTERFACE

The most recent solution proposed to hyphenate LC to IRMS, is the wet oxidation interface which was commercialized in 2004 (Krummen et al., 2004). This interface is based on wet oxidation of organic molecules for producing CO_2 gas in aqueous solution. The principle of this interface is very similar to that reported for the Total Organic Carbon coupled to IRMS (TOC-IRMS) instrument (St Jean, 2003). The main improvement was the direct and straightforward on-line measurement of the CO_2 and the absence of sequential oxidation steps, needed for the TOC-IRMS. As illustrated in Figure 13, the oxidation of organic molecules into CO_2 gas is performed within a heated reactor where acid (phosphoric acid), oxidant (sodium peroxodisulfate) and LC eluent are mixed together. Then, inorganic eluent containing CO_2 is transported through a separation unit (composed of three membranes) which can selectively extract CO_2 from aqueous buffer into a counter-flow of helium. The residual water is removed from the gas through two Nafion® membranes drier. Finally, the CO_2 in the helium stream is introduced into the IRMS ion source through an open split. The quantitative oxidation of the molecules and the efficient separation unit contribute to obtain a transfer close to 100% of the CO_2 present in the eluent into the helium stream, at liquid flow rates lower than 400µL/min (Krummen et al., 2004). For higher liquid flow rates, the area of the CO_2 peak is slightly affected (Godin et al., 2005). There are several analytical constraints to produce CO_2 from organic molecules in

solution; only inorganic buffer can be used, the total flow rate must be lower than 700µL/min and an acidic pH is preferred to achieve higher extraction of CO_2 gas from the liquid (Krummen et al., 2004; McCullagh et al., 2006). In LC-IRMS, the CO_2 background produced, might originate from the column (stationary phase bleeding or organic solvent still present within the stationary phase), and/or from the presence of organic material in the mobile phase, and from the pumps themselves. Nevertheless, this background does not affect the isotopic precision and accuracy of the $\delta^{13}C$ if it is stable, as already pointed out using GC-C-IRMS instrument (Merritt & Hayes, 1994). Using LC-IRMS, the stability of the CO_2 background is measured over 5min and if the level of CO_2 signal (in mV) does not go beyond 10mV as SD and ± 0.005 as slope. The CO_2 background is then considered stable and $^{13}C/^{12}C$ isotopic measurement can be performed.

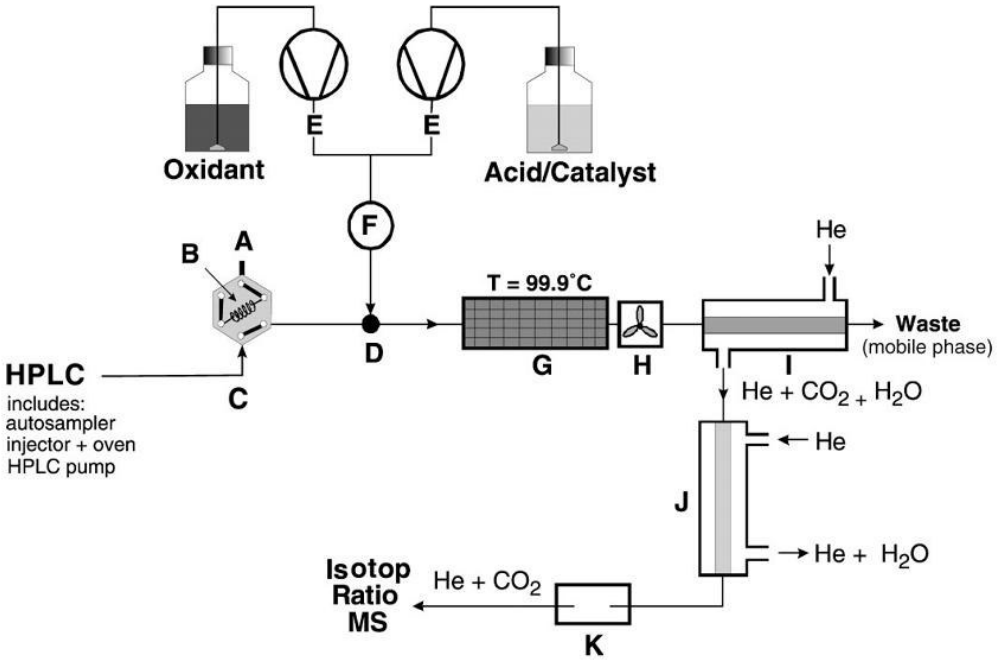


Figure 13: Principle of the LC-IRMS interface commercialized in 2004

A: Needle port; B: sample loop; C: 6-port valve; D: T-piece; E: Two head pumps; F: pulse damper; G: oxidation reactor; H: cooler; I: CO_2 separation unit; J: gas dryer; K: open split (Krummen et al., 2004)

1.10.4 ICP- MS

Inductively coupled plasma mass spectrometry is based on the generation of atoms representative of the elemental composition of the sample within a plasma torch; the liquid sample is transformed into an aerosol via a nebulizer and a spray chamber. Next, the aerosol passes through different heated zones in the plasma torch where the sample is dried, vaporized, atomized and then positively ionized. ICP-MS has an extensive dynamic range (8 orders of magnitude) and low detection limits (around ppt, i.e. part per trillion) for many elements. ICP-MS with a quadrupole mass analyzer is used for the determination of isotopic ratio at natural abundance of many elements. Nevertheless, the isotope ratio precision obtained is not better than around 2-5‰ (2 σ). To overcome this limitation as well as others, multiple collector ICPMS (MC-ICP-MS) was developed. The isotopic precision achievable with this instrument is in the range of 0.03 to 1‰/amu (2σ) depending on the elements studied (Rehkamper et al., 2004).

Using ICP-MS, the ^{13}C isotopic ratio is measured using atomic carbon species, such as $^{12}C^+$ and $^{13}C^+$ ions. Thus, as opposed to the IRMS instrument, ICP-MS does not need oxygen correction to obtain accurate carbon isotopic ratios. Nevertheless, ^{13}C isotopic analyses at natural abundance have suggested a spectral interference of $^{12}CH^+$ (*m/z* 13) producing an inaccurate isotopic ratio. In addition, the presence of organic solvent as mobile phase affects the stability of the ICP-MS by modifying the plasma ionization conditions. This leads to a reduction or an increase of the signal intensity according to the elements measured and inaccuracies of isotopic ratio if no improvements are considered. Therefore, different approaches were described to prevent this process through various optimization steps (of the carrier gas flow, or by using a membrane desolvator with the addition of oxygen, or by a reduction of the flow rate using smaller column internal diameter (Lobinski et al., 2006). However, none of these modifications have proved satisfactory. Thus, for ^{13}C isotopic analysis by ICP-MS, organic mobile phase is completely prohibited for LC separation. One solution reported was to use heated water as mobile phase to mimic organic solvent to elute organic compounds such as caffeic acid, paracetamol and phenacetin (Smith et al., 2004).

1.11 LC COUPLED TO MOLECULAR MS AND IRMS

Whatever the molecular weight and the polarity of the compounds, a large set of LC column sizes and stationary phases are commercially available. Table 8 summarizes the different LC columns, buffer and the targeted molecules analyzed for each combination presented below.

1.11.1 LC COUPLED TO MOLECULAR MS

LC-MS is now a mature technique. Many different separation approaches have been reported in many reviews and will not be covered here.

Another approach to combine LC and MS for isotopic analyses was through the CRI interface; the Abramson team used the LC-CRI-MS device to selectively detect and quantify elements (such as ^{13}C, ^{15}N and ^{2}H) in biological systems (Abramson, 1994). CRI-MS was used in combination with LC separations using reversed phase chromatography (Abramson et al., 2001; McLean et al., 1996), with ion exchange chromatography (Abramson et al., 2001) for low molecular weight compounds and size exclusion chromatography (SEC) (Lecchi & Abramson, 1998).

1.11.2 LC COUPLED TO IRMS

1.11.2.1 Sample preparation

Sample preparation and sample handling remain two critical aspects for isotopic analyses. Whatever interfaces are used to hyphenate LC and IRMS, LC separation methods present interesting features for ^{13}C isotopic analysis when compared to GC. Since polarity and volatility of compounds are not major issues in LC, the sample preparation in LC is faster as no derivatization is needed. Thus, the time saving for large series of analyses might be significant for clinical/ animal studies. Therefore, the direct measurement of $\delta^{13}C$ with LC at natural abundance might become a straightforward and simple method for low molecular weight compounds as well as for macromolecules.

Using FIA-IRMS (also described as discrete samples or dropwise analysis), the compound of interest must be either analyzed as bulk material or isolated ensuring that no other contaminating carbons are present in the final sample. Nevertheless, the key feature of FIA-IRMS versus EA-IRMS is that it requires a lower amount of

compound. Typically for FIA-IRMS, 100 to 600ng of carbon is required for low molecular weight compounds or macromolecules with an SD ($\delta^{13}C$) lower than 0.3‰ (Godin et al., 2005).

1.11.2.2 Liquid chromatography separation

Although LC has been used for many years as a separation method in analytical chemistry, currently all the compounds analyzed by GC methods for isotopic measurements cannot be readily analyzed by LC hyphenated to IRMS. For the commercial LC-IRMS instrument, the limitation may come from the difficulty to solubilise compounds in aqueous mobile phase.

1.11.2.2.1 LC columns

For moving transport-IRMS, primarily, two different types of LC stationary phases were used: reversed stationary phases (Caimi & Brenna, 1993) and normal phases in combination with organic and inorganic buffers (Caimi & Brenna, 1995). For such system, the removal of buffers was efficient, allowing the use of organic mobile phase at high flow rates (higher than 2mL/min). With the recent improvement to the moving wire-IRMS device by Sessions (Sessions et al., 2005), organic and inorganic buffers were used in both modes of analysis (FIA and LC mode). Nevertheless, the nature and the concentration of the inorganic salt significantly affected the CO_2 production efficiency which limits the buffer for LC separation.

For LC-CRI-IRMS, LC separations have been carried out with reversed phase, normal phase, ion exchange and size exclusion columns (Abramson et al., 2001) (Abramson et al., 1996). The main limitation is the column i.d. In order for the nebulizer to function correctly, a make-up solvent was added to obtain an optimal flow rate of 1mL/min (Lecchi & Abramson, 1999). Only recently with the work of Jorabchi (Jorabchi et al., 2005), lower flow rates (45µL/min for a 1mm i.d. column) were tested for LC separation of non-labeled and ^{13}C-labeled peptides.

For LC-IRMS, most of the work has been performed with ion exchange and mixed-mode columns. Due to the use of aqueous mobile phase, a reversed phase C18 column is not the primary choice of LC columns. Nevertheless, a few experiments have been reported with reversed phase columns (see Table 8).

Table 8: Examples of LC columns, buffers and molecules analyzed by LC hyphenated to IRMS and molecular MS for ^{13}C isotopic analyses

Instruments	LC columns	Buffers	Target molecules	Reference
LC-C-IRMS	Partisphere 5 Pac (4.6 mm x 12.5 cm)	acetontrile -water	Sugars	(Caimi & Brenna, 1993a)
	Supelco Si normal phase (4.6 x 150 mm)	hexane-amyl alcohol	Tocopherols, ergocalciferols	(Caimi & Brenna, 1995)
	Ultramex 3 RP C18 (4.6 x 150 mm)	acetontrile-methanol-chloroform	Retinyl acetate, tocopherols	
LC-CRIMS	Kromasil C18 (4.6 mm x 150 mm)	methanol	Testosterone	(Teffera et al., 1996b)
or		acetone-acetonitrile	Heptadecanoic and testosterone	
LC-CRI-IRMS		ammonium acetate-acetonitrile	Tirilazad mesylate	(Abramson et al., 1996d)
	Aquapore C8 (300A°), (4.6 x 150 mm)	methanol-acetonotrile	Chlorophylls	(Teffera et al., 1996b)
	Poros Hamilton (2.1 x 30 mm)	water-acetonitrile	Growth hormone	(Osborn & Abramson, 1998)
	Supleco 18S column (4.6 x 250 mm)	ammonium acetate- water-acetonitrile	Nucleosides	(Lecchi & Abramson, 1998)
	GPC 300°A pore size (4.6 x 250 mm)	triethylammonium acetate-water-acetonitrile	Nucleic acids	(Lecchi & Abramson, 1999)
	GPC 100-1000A°, (4.6 x 250 mm)	trietlyamine-formic acid, tetrahydrofurane, water	Proteins	
LC-IRMS	Alltech 700 CH (6.5 x 300 mm)	water	Sugars, honey	(Krummen et al., 2004)
	Purospher RP18 (2 x 55 mm)	water	Paracetamol, acetylsalysilic	(Krummen et al., 2004)
	Nova-Pack C18 (3.9 x 300 mm)	phosphate	Ala, Leu, Phe, Trp	(Krummen et al., 2004)
	Luna SCX (4.6 x 250 mm) and C30 RP-Aq	phosphate	Amino acids	(Godin et al., 2005)
	HyperReZ Carbohydrate H+ (300 x 8 mm)	water	Honey	(Cabanero et al., 2006)
	Reprosil-Pur C18 AQ column (4 x 250 mm)	phosphate	Volatiles fatty acids	(Heuer et al., 2006)
	Sielec Primesep A (4.6 x 250 mm)	water-sulfuric acid	Amino acids	(McCullagh et al., 2006)
LC-MS	Allsphere 3 um ODS 2 column (3.2 x 150 mm)	ammonium acetate, tetrahydrofurane-ammonium acetate, acetonitrile, tetrahydrofurane	Amino acids	(van Eijk et al., 1999)
	Luna C18 (2 x 150 mm)	formic acid, water-acetonitrile	Phenylalanine	(Wu et al., 2004b)
	Zorbax Extend C18 (2.1 x 150 mm)	water, triethylamine-acetontrile	Long-chain fatty acyl coenzyme A esters	(Sun et al., 2006)
	Aegis column C8 (0.3 x 150 mm)	acetonitrile-water-formic acid	Palmitoylcarnitine	(Guo et al., 2006)

1.11.2.2.2 Peak resolution

In LC, as in GC, the measurement of ^{13}C isotope ratio must be performed ensuring that none of the steps in the analysis alters the isotopic composition of the molecule of interest. The key factor in achieving reliable isotopic measurements is to obtain baseline separation of the molecules of interest from the other molecules in the mixture. In order to overcome the lower peak capacity in LC versus GC, the efficiency (described with N as theoretical plate number) and the selectivity (α) must be optimized during LC method development.

1.11.2.2.3 Chromatographic efficiency

To increase the LC efficiency, two approaches are possible: by increasing column length or by decreasing the particle diameter. There are limited publications that describe the addition of multiple LC columns in series to increase column length. However, the concept of the ultra performance liquid chromatography (trade mark UPLC) using LC columns with a 1.7µm particle size (with an associated back pressure up to 1000bar) was introduced (Mazzeo et al., 2005). By reducing the particle size of the column, the pressure increases rapidly (a 1.7µm column produces a pressure 27 times higher than a 5µm column of the same dimension (Nguyen et al., 2006). The reduction of particle size also leads to an improvement of the efficiency and sensitivity, as compared with classical LC (de Villiers et al., 2006). To overcome the pressure limitations when working with small particles, special LC columns were developed. However, two aspects limit the combination of UPLC with an IRMS with the commercialized interface (LC-IRMS): on the one hand, only a limited number of UPLC columns are able to work with an aqueous mobile phase. On the other hand, UPLC chromatographic separation also implies a very small peak width (only a few seconds) which means that the extra-column dead volume has to be as low as possible. Practically, with LC-IRMS instrument, the peak width can be reduced leading also to a decrease of the sensitivity by increasing the helium flow rate in the separation unit. The peak width is 60-80sec for an injection of approximately 300ng of carbon (by FIA-IRMS mode) against 5sec for FIA-CRI-IRMS instrument.

1.11.2.2.4 Chromatographic selectivity

For method development using LC-IRMS, the chromatographic selectivity is probably easier to tackle than the efficiency. Nowadays, more and more LC stationary phases

are suitable for 100% aqueous mobile phase. Indeed, the manufacturers of LC columns have overcome the "de-wetting" phenomenon (leading to irreproducible retention times) by introducing special packing material (Majors & Przybylski, 2002). With such LC stationary phases, inorganic buffers (typically water or phosphate buffer) are used. In order to overcome the difficulty of developing an LC-IRMS method without organic buffer and to increase the LC efficiency, high temperature liquid chromatography (HTLC), using temperature between 40°C-200°C, might be a promising technique. By increasing the water temperature, the dielectric constant which is also a measure of the water polarity decreases. Then at elevated temperature, water is similar, in terms of eluotropic strength, to a mixture of organic solvent and water. For instance, at 150°C, the polarity of the water is approximately equivalent to a mixture of water/methanol (50/50 %) at room temperature (Coym & Dorsey, 2004). Therefore, the use of temperature programming might replace the gradient elution of organic solvent in LC or the ramp of temperature in GC. Another interesting feature of high temperature liquid chromatography (HTLC) in molecular LC-MS is the possibility to increase the LC flow rate and thus the throughput due to the decrease of the eluent viscosity with temperature. Nevertheless, to use HTLC, LC columns need to be thermally stable particularly to prevent column bleeding over 100°C (Teutenberg et al., 2006). This approach of HTLC has proved to be very effective for several chromatographic separations (de Boer et al., 2005; Fields et al., 2001; Guillarme et al., 2004; Marin et al., 2004; Vanhoenacker & Sandra, 2006) and for specific coupling such as HTLC-FID (Guillarme et al., 2005).

1.11.2.3 Chromatographic isotope effect in LC separation

An isotope effect is a physical phenomenon which may cause isotope fractionation. This isotope effect is not directly observable. Alternatively, isotope fractionation is an observable quantity described in terms of isotopic enrichment or depletion of the heavy isotopes (Hayes, 1983). In GC-C-IRMS, typically, heavy atoms elute 100-150ms before the light atoms (Brand, 1996). In LC-IRMS, as reported in Figure 14 with an ion exchange column, the heavy ^{13}C isotope is eluted after the light one (^{12}C) (Godin et al., 2005; McCullagh et al., 2006). The chromatographic isotope effect in LC seems related either to the nature of the compounds, to the stationary phases, or to both, and needs to be carefully investigated. The review by Filer summarizes the effect of ^{13}C labeled compounds as well as other isotopes when used with LC (Filer, 1999). Additionally, as pointed out by Caimi and Brenna (1997), during separation of

methyl palmitate with preparative LC on reversed stationary phase, the ^{13}C content within a peak was around 7% higher in the front of the peak than in the tail. The same ratio was also found for un-derivatized valine using a reversed phase column with water as mobile phase (Tripp et al., 2006) and with a C18 column with water/acetonitrile (Baumann et al., 1992). Therefore, in LC the chromatographic isotope effect is not systematically identical to that observed in GC. Consequently, the chromatographic isotopic fractionation should be carefully evaluated in order to avoid substantial error in measuring ^{13}C isotopic ratio especially: i) when the peak collection or the peak trapping in bi-dimensional LC is necessary or ii) when poor chromatographic resolution is observed or iii) when the peak tailing is increased.

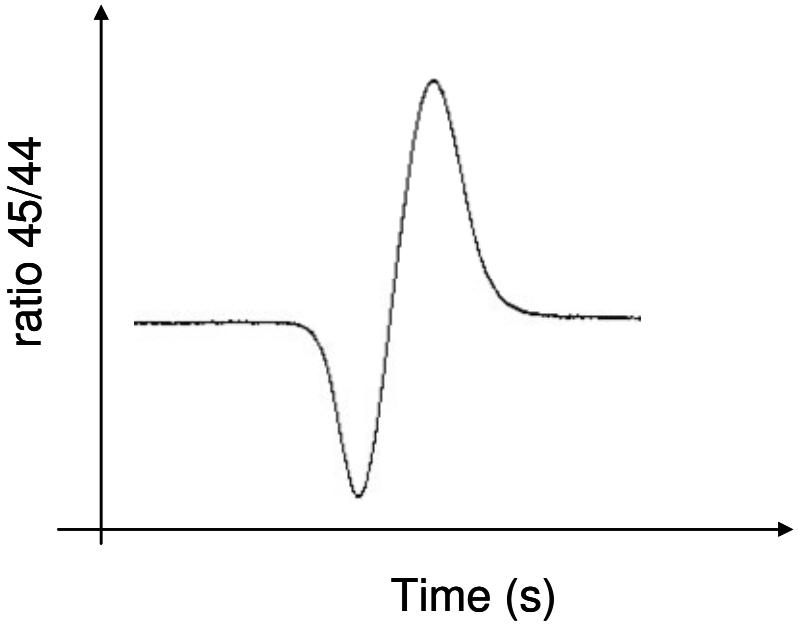


Figure 14: Chromatographic separation of isotopologues of underivatised amino acids measured by LC-IRMS using ion exchange chromatography column

In Figure 14, Luna SCX column is used with phosphate buffer (see Godin et al., 2005 for the analytical details of the LC-IRMS method). In this condition, ^{12}C atom is eluted earlier than ^{13}C atom, which is the inverse phenomenon classically observed in GC-C-IRMS.

1.11.3 ISOTOPIC CALIBRATION OF CO_2 PEAK

The isotopic calibration needs to be optimized according to the conditions, samples and to potential isotopic fractionation due to the method used. The approaches are the same as described for the GC-C-IRMS (Meier-Augenstein, 2002; Merritt et al., 1994): the use of an internal standard (I.S.) with known $\delta^{13}C$, or CO_2 pulses of calibrated reference gas are two main options (used either separately or together). In addition, with LC compared to GC-C-IRMS, the possibility of using switching valves in combination with different loops to either inject I.S. through the LC column as a second injection to be as close as possible to the target compounds or without the LC column using the FIA mode, favors the identical treatment of the reference and targeted compounds avoiding the isotopic fractionation. Nevertheless, the FIA mode suffers from the fundamental drawback that the reference gas does not correct for isotopic fractionation, which may happen with the LC stationary phases. The optimal solution is then to mix the I.S. with the sample prior to the injection.

1.12 APPLICATIONS OF LC COUPLED TO IRMS

The applications reported here using moving transport-IRMS and LC-CRI-IRMS were developed using non-commercial devices. Only those carried out with LC-IRMS were performed using a commercial interface (LC Isolink®, Thermo, Bremen, Germany). To overcome the analytical constraints of the commercial system described earlier, various strategies, as illustrated in Figure 15, may be foreseen for ^{13}C isotopic analyses of low-molecular weight compounds and macromolecules.

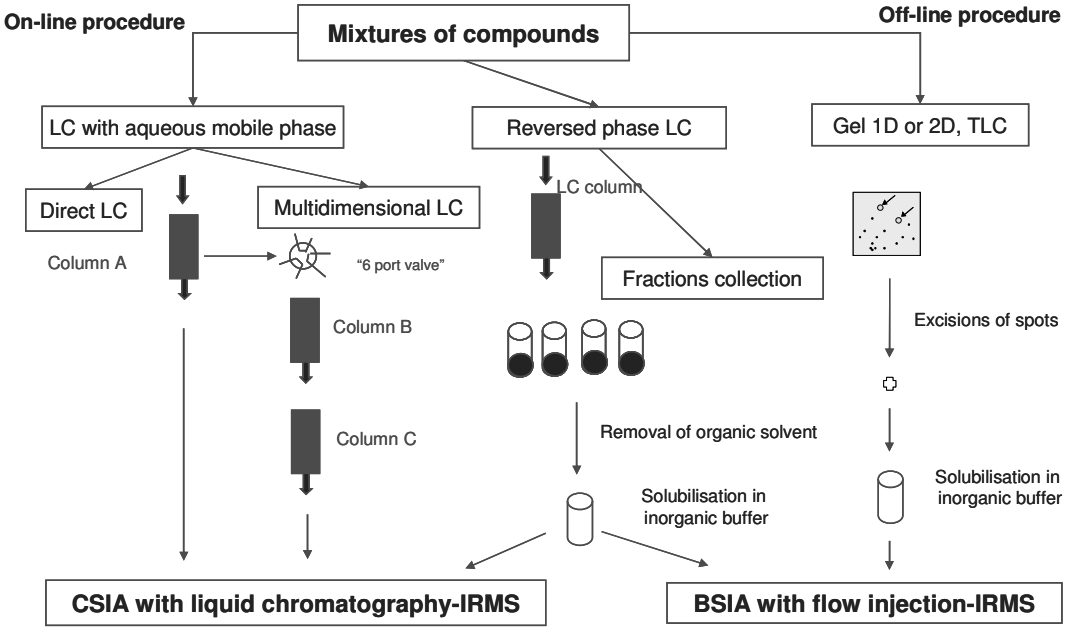


Figure 15: In view of the analytical constraints of LC-IRMS in terms of LC mobile phase, various analytical approaches have been foreseen for deconvoluting complex samples for measuring high-precision ^{13}C isotopic ratio.

In Figure 15, two distinct areas are foreseen: 1) a direct strategy based on isotopic analysis of compound in a complex mixture analyses by LC columns for performing compound specific isotope analysis (CSIA). This approach may use LC separation with either aqueous mobile phase or organic mobile phase. In the latter option, more tedious steps are needed to remove the organic solvent and finally inject the compounds in appropriate buffers. 2) an indirect strategy with an off-line step is described where the compound of interest is isolated and then injected directly in the system after appropriate choice of solubilization buffer. The latter is similar to the bulk isotopic analysis (BSIA) classically performed with EA-IRMS instruments (Douthitt, 1999). (Godin et al., 2004)

1.12.1 ISOTOPIC ANALYSIS OF LOW MOLECULAR WEIGHT MOLECULES BY IRMS

Sugars were the first molecules tested with LC coupled to IRMS. The hydrophilic nature of sugars and their ease of separation by LC columns with water as eluent make this application straightforward in terms of chromatographic separation. Indeed,

the moving transport-IRMS showed a chromatographic separation for isotopic analysis of standard fructose, sucrose and lactose: the precision, SD ($\delta^{13}C$) was assessed at 1‰ by injecting around 300µg of each compound (Caimi & Brenna, 1993). Honey samples were also analyzed by LC-CRI-IRMS (Teffera et al., 1996) and more recently its adulteration was also tackled by an LC-IRMS method (Cabanero et al., 2006). Honey is a relatively expensive natural product which contains glucose and fructose in approximately equal amounts. Honey can be then easily adulterated with cheaper sugars such as high fructose corn syrup (HFCS) and sugar derived from corn (a C_4-plant). In the work of Cabanero *et al.,* in addition to isotopic analysis of protein fraction, systematic study of $\Delta\delta^{13}C$ [fructose-glucose], $\Delta\delta^{13}C$ [fructose-sucrose], and $\Delta\delta^{13}C$ [glucose-sucrose] allowed the detection of additions of sugars (from C_3-plant, C_4-plant, iso-glucose syrup and high-fructose corn syrup) as reported in Table 9. Therefore, this new LC-IRMS method presents relevant benefits for detecting honey adulteration especially with sugars derived from C_3-plants which were impossible to detect with previous isotopic analysis. In addition, this method presents advantages in terms of limit of detection, throughput and simplicity of use.

The importance of amino acids in biology research places them among the most studied molecules by IRMS. Most of the time, amino acids are analysed by GC-C-IRMS either at natural abundance or at higher isotopic enrichment in tracer studies. Using the LC-IRMS device, LC separations of underivatized amino acids were achieved with various LC columns at natural abundance (Krummen et al., 2004; McCullagh et al., 2006) or for slightly enriched amino acids (Godin et al., 2005). The most advanced separation for underivatized amino acids was proposed recently as illustrated in Figure 16 (McCullagh et al., 2006); 15 underivatised amino acids were resolved from collagen samples and only a few of them co-eluted (such as glycine with threonine and serine with glutamine). The average SD ($\delta^{13}C$) for the 15 amino acids was 0.18‰ (n=6) and the accuracy of $\delta^{13}C$ reported was very close (lower than 0.25‰) to the one determined by FIA-IRMS. This method was applied to human and animal bone collagen hydrolysates, for paleodietary reconstruction.

Table 9: Stable carbon isotope ratios in adulterated honeys and their protein fractions measured by LC-IRMS device

	Pure honey		**10 % beet sugar added**		**20 % beet sugar added**		**5 % cane sugar added**		**10 % cane sugar added**		**5 % cane syrup added**		**10 % cane syrup added**		**10 % HFCS added**	
sample	$\delta^{13}C$ Total EA (‰)	$\delta^{13}C$ Protein (‰)	$\delta^{13}C$ Total EA (‰)	$\delta^{13}C$ Protein (‰)	$\delta^{13}C$ Total EA (‰)	$\delta^{13}C$ Protein (‰)	$\delta^{13}C$ Total EA (‰)	$\delta^{13}C$ Protein (‰)	$\delta^{13}C$ Total EA (‰)	$\delta^{13}C$ Protein (‰)	$\delta^{13}C$ Total EA (‰)	$\delta^{13}C$ Protein (‰)	$\delta^{13}C$ Total EA (‰)	$\delta^{13}C$ Protein (‰)	$\delta^{13}C$ Total EA (‰)	$\delta^{13}C$ Protein (‰)
1	-25.3	-24.9	-25.1	-24.8	-25.2	-24.8	-24.5	-24.9	-23.7	-24.9	-24.6	-24.7	-24	-24.6	-23.9	-24.9
2	-24.4	-24.3	-24.3	-24.2	-24.4	-24.2	-23.6	-24.3	-22.6	-24.3	-23.8	-24.1	-23.2	-23.9	-23.3	-24.2
3	-25.5	-25.3	-25.3	-25.2	-25.2	-25.2	-24.6	-25.3	-23.9	-25.3	-24.8	-24.9	-24	-24.7	-24.3	-25.3
Honey quality																
"AOAC" method		pure [a]	pure		pure		pure		adulterated		pure		pure		adulterated	
LC-IRMS		pure	adulterated		adulterated		adulterated		adulterated		adulterated		adulterated		adulterated	

a: pure = considered to be pure by the limit given by $\delta^{13}C$= 1‰ as difference at natural abundance between whole $\delta^{13}C$ of honey and its protein fraction.

"AOAC method" is global brand recognition and was defined as "official" by regulations. Here the AOAC method described referred to AOAC method number 991.4.

To improve the sensitivity with which a corrupted honey can be detected by IRMS, White & Winters developed an internal standardization based on ithe sotopic analysis of protein fractions in honey and sugar fractions (White JW & Winters K., 1989). A difference of $\delta^{13}C$ at 1‰ was proposed between whole honey sample and protein fraction to detect corruption. Nevertheless, the addition of C3-plant material can not be detected in honey by this technique. Using the method described by Cabanero et al., C3-plant material was detected Reprinted from Cabanero et al.

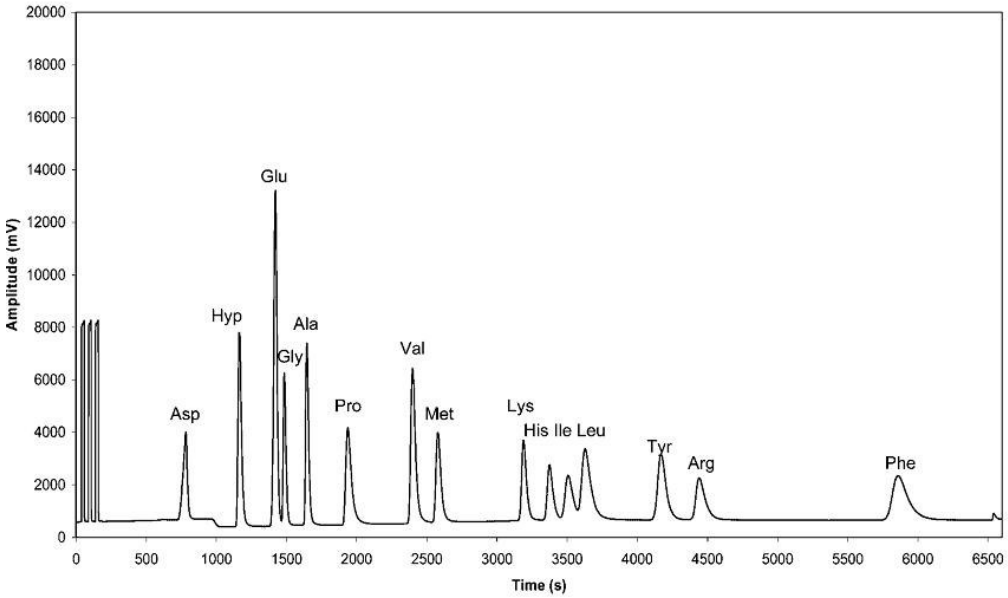
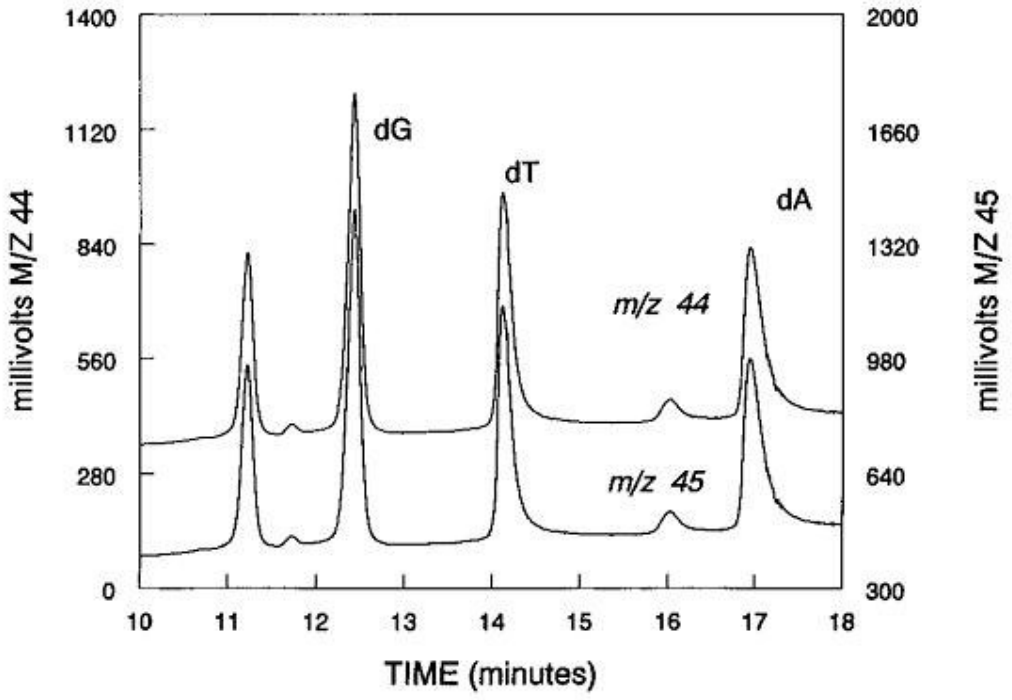


Figure 16: LC-IRMS chromatogram of underivatised amino acids

In Figure 16, McCullagh et al. (2006) demonstrated that LC-IRMS separation of 15 standard underivatised amino acids is possible with a 100 % aqueous mobile phase. A mixed mode stationary phase using a gradient elution with two inorganic buffers: 100% H_2O (A) and 0.2% H_2SO_4 (B); the gradient profile is: 0% B to 10 min, 0–100% B over 30 min, then 100% B is maintained to the end of the analysis. Flow rate is 0.7 mL/min; column, SIELC Primesep A (4.6 x 250 mm i.d.), 5µm packing; McCullagh et al (2006).

"Radioisotope" and "stable isotope" based tools were the two approaches developed for measuring deoxyribonucleic acid (DNA) synthesis for cell proliferation. For studies based on stable isotopes, various tracers have been used to assess DNA turnover such as U-^{13}C glucose, labeled nucleotides, labeled glycine or D_2O (Black & Abramson, 2003). A new method was developed for investigating DNA synthesis by LC-CRI-IRMS using ^{13}C glycine as tracer with different ratios of labeling (33 and 3.3%). This tracer was chosen because one nitrogen and 2 carbons of glycine are incorporated into purine. In parallel, to validate this approach, ^{3}H thymidine incorporation was also performed. After DNA extraction, enzymatic hydrolysis and purification, isotopic analyses of nucleosides such as dG, dT and dA (respectively deoxyadenosine, deoxythymine and deoxyguanoside) were carried out by LC-CRI-IRMS (Chen & Abramson, 1998) as illustrated in Figure 17a. To evaluate the validity of this approach, a washout of ^{13}C isotopic enrichment in dA and dG from the

hydrolysis of DNA from a cell was reported (Figure 17b). The results showed that this stable isotope approach can be a suitable complement to the radioisotope method using ^{3}H thymidine which is extensively used in cell biology. The authors also compared their results to the study performed by Macallan *et al.* (1998) carried out with $^{2}H_2$ labeled glucose (with an isotopic enrichment at 10-15% for glucose) and measured by GC-MS. Despite the high efficiency of glucose to label nucleosides, LC-CRI-IRMS with ^{13}C glycine requires 1 order of magnitude less labeled material than GC-MS with deuterated glucose.



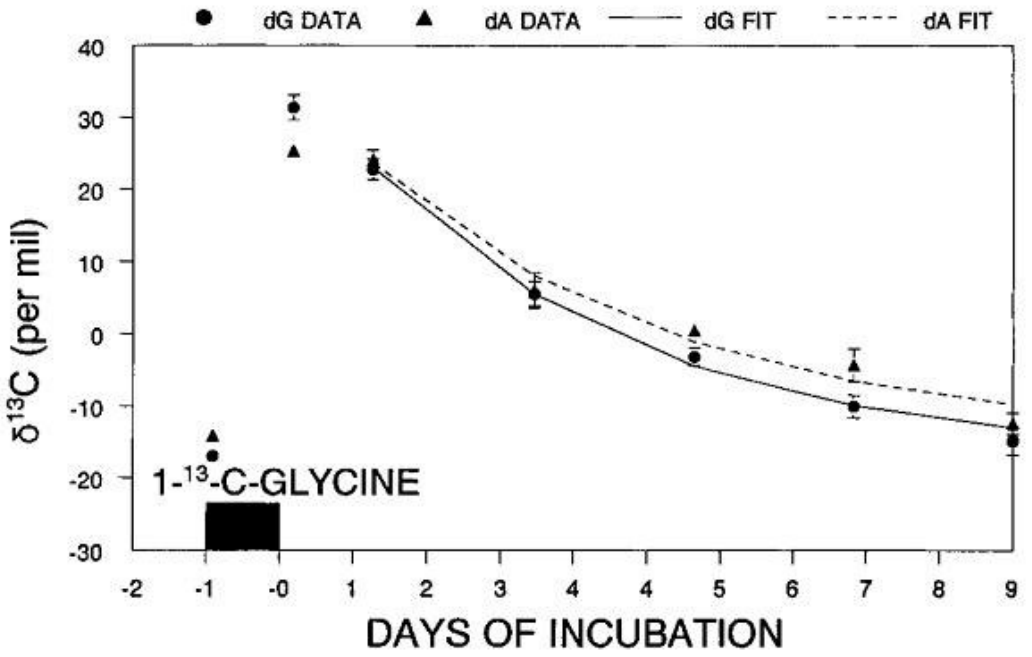


Figure 17: ***Panel A****: HPLC-CRI-IRMS chromatogram of DNA nucleosides extracted from HEP G2 cells. The peak eluting at 11.2 min is unknown. The amplifier for m/z 45 has a 100 x gain compared with m/z 44.* ***Panel B:*** *Washout of ^{13}C in dA and dG in DNA from HEP G2 cells following a 1 day pulse of labeled glycine. The points for day 0 were not used for the fit. The correlation coefficient was 0.974 for dA and 0.994 for dG. (Chen et al., 1999)*

Isotopic analysis of fatty acids remains typically an application for GC-C-IRMS since the derivatization is performed by the "methylation" reaction and leads only to a small isotopic dilution (1 carbon brought by the GC derivative against at least 10 carbons coming from the fatty acid itself) (Meier-Augenstein, 2002). Nevertheless, one experiment was performed using moving transport-IRMS with labeled linoleic acid standard (with $\delta^{13}C$ values between -28 to 83‰) using approximately 12.5µg of compound. The SD ($\delta^{13}C$) reported was 0.35‰ (Caimi & Brenna, 1993). Moreover, isotopic analyses of small organic acid salts, such as lactate, formate, acetate and propionate were also investigated after LC separation. Natural abundance ^{13}C isotopic analyses of lactate, formate, acetate, propionate and ethanol during anaerobic degradation of rice roots to model the carbon flow (Penning & Conrad, 2006) as well as volatile fatty acids in sediments (Heuer et al., 2006) have been published.

Stable isotope tracers are also a useful tool to assess bioavailability of vitamins in humans (Van Lieshout et al., 2003). In this case, GC-MS is still the analytical method of choice for analyzing such compounds as for ^{13}C labeled vitamins C (Bates et al., 2004), A (Tanumihardjo, 2000) or K (Jones et al., 2006). Nevertheless, LC methods hyphenated to IRMS also need to be envisaged for monitoring and measuring low ^{13}C isotopic enrichment of vitamins to avoid tedious and time consuming steps of sample preparation. To date α, β-tocopherol, retinyl acetate and ergocalciferol at natural abundance were analyzed using normal phase or reversed phase conditions using moving transport-IRMS (Caimi & Brenna, 1995). By comparing the $\delta^{13}C$ obtained of α tocopherol analyzed in various LC conditions against $\delta^{13}C$ obtained using off-line analysis, the SD ($\delta^{13}C$) reported was between 0.49 to 1.74‰ and the accuracy between 0.14 to 2.58‰. The noisy background and its drift over the run explained the lower accuracy and precision reported.

There is significant interest in the use of stable isotope analyses in detecting the adulteration of commercially available drug compounds as well as in controlling the quality of drug batches, or for metabolism studies (Jasper, 1999). To date a human mass balance was investigated by LC-CRI-IRMS with labeled drugs such as $^{15}N_2$-^{13}C phenobarbital (Browne et al., 1993) and ^{15}N-$^{13}C_2$ acetaminophen (Browne et al., 1998); for labeled acetaminophen, good results (in terms of sensitivity, precision and accuracy in quantitating the drug) were reported for concentrations greater than 1µg/mL in whole urine. More recently, LC-IRMS analyses (Krummen et al., 2004) on different sources of natural acetylsalicylic acid and paracetamol from various suppliers were carried out to tackle drug counterfeiting.

Other isotopic analyses have been performed using the moving transport-IRMS instrument with amino acids and cholesterol with an SD ($\delta^{13}C$) lower than 0.3‰ for more than 8nmol carbon (Sessions et al., 2005). In addition, the potential of such instrument has been used for assessing mass balance with drug compounds. In this case, labeled ^{13}C AZT and ^{14}C AZT (azidothymine) were administrated to rats and were monitored in urine and feces by LC-CRI-IRMS using FIA and by scintillation counting for radioisotope measurement (Chen et al., 1999).

1.12.2 ISOTOPIC ANALYSIS OF MACROMOLECULES BY IRMS

Moving transport-IRMS, LC-CRI-IRMS and LC-IRMS were also used to tackle high-precision isotopic analysis of macromolecules both with and without LC separation. Purified albumin, obtained from 18 different species (avian, primates, rodents,

ruminants and other), was analyzed by FIA with the moving transport-IRMS device. The amount injected was approx. 2-5µg, equivalent to 40pmol of each protein or 150nmol of carbon. The SD ($\delta^{13}C$) obtained was around 0.45‰ (across all the samples) and the accuracy of $\delta^{13}C$ value was in agreement (less than 0.4‰) with analyses performed with a dual-inlet system (Caimi & Brenna, 1996). Other macromolecules such as standard insulin and yeast RNA were also studied by FIA using LC-IRMS (Godin et al., 2005) and LC-CRI-IRMS (Sessions et al., 2005), respectively. Chlorophyll samples (from spinach and algae) were studied with both modes of introduction (FIA and LC) using moving transport-IRMS (Sessions et al., 2005) and LC-CRI-IRMS (Teffera et al., 1996). In the latter case, 2µg of testosterone with a known isotopic enrichment were used as internal standard and the isotopic precision was statistically different according to the origin of samples (with SD ($\delta^{13}C$) of 0.5‰ for n=13 using 2µg injected). These data were in agreement with data obtained previously with the moving transport-IRMS device. Other macromolecules such as a synthetic conjugate vaccine activated with U-^{13}C adipic acid dihydrazine were also analyzed with size exclusion chromatography-CRI-IRMS (Abramson et al., 2001).

High-precision ^{13}C isotopic analysis was shown to be a useful tool in forensic as well as in doping studies. To date, the distinction between endogenous and exogenous macromolecules has been investigated for recombinant human growth hormone (rhGH) using LC-CRI-IRMS (Abramson et al., 1996) (Abramson et al., 2001). The SD ($\delta^{13}C$) measured was 0.5‰ for 150pmol of rhGH (representing 2-3µg of material). To obtain accurate $\delta^{13}C$ values, albumin samples (approx. 30pmol) with known $\delta^{13}C$ (determined by off-line combustion) were injected during the same run. Various rhGH samples (one human pituitary and three synthetic products) were analyzed and characterized showing a small statistical difference for $\delta^{13}C$: -11.3‰ for the human one and -12.8‰, -10.3‰ and -18.5‰ for the synthetic products. However, the LC-CRI-IRMS sensitivity was an issue below 1µg of rhGH making the doping isotopic analyses a challenging activity for this instrument.

1.12.3 MISCELLANEOUS: ISOTOPIC ANALYSIS BY ICP-MS

Inductively coupled plasma mass spectrometry in combination with a quadrupole mass filter or a magnetic sector instrument (ICP-MS), was introduced in 1983 and has been used for measuring the isotopic ratio of many heavy elements in a multitude of applications, e.g. in nutrition to investigate metabolism and bioavailability

of inorganic elements (Courtois et al., 2003; Couzy et al., 1995; Kastenmayer et al., 1994; Kastenmayer et al., 2002), in geochemistry and in environmental studies (Beauchemin, 2006).

Papers describing the use of LC-ICP-MS for measurement of $^{13}C/^{12}C$ isotope ratio are rather scarce; $^{13}C/^{12}C$ isotopic ratio at natural abundance of glucose and tryptophan were measured in FIA mode using a prototype instrument built with two quadrupoles mass filters (twin-quadrupole) and an ion beam splitter that eliminates the noise (Luong & Houk, 2003). One quadrupole measured *m/z* 12 and the second measured *m/z* 13. As the ion beams were not equally split between both quadrupoles, an accuracy curve was plotted using a known mixture of natural glucose with a known amount of ^{13}C glucose to correct the bias observed: the mean square correlation coefficient for 6 calibration curves was 0.9996 ± 0.0002 showing excellent isotopic precision and accuracy for non-labeled and labeled glucose. In addition, the measured $^{13}C/^{12}C$ ratio at natural abundance for myoglobin was 0.0109 ± 0.0004 and 0.0111 ± 0.0002 for respectively, myoglobin and β cyclodextrin compared to a theoretical $^{13}C/^{12}C$ ratio of 0.0111.

1.13 APPLICATIONS OF LC COUPLED TO MOLECULAR MS

This section provides a brief summary of some methods and applications using molecular MS devices.

1.13.1 ISOTOPIC ANALYSIS OF LOW-MOLECULAR WEIGHT MOLECULES

In LC-MS, time-of-flight (TOF) and ion trap analyzers were also used to perform ^{13}C isotopic measurement. Historically, ^{13}C isotopic enrichments of glucose was one of the first isotopic analyses carried out. This was performed with a thermospray LC-MS (Esteban et al., 1987). Using ^{13}C glucose as internal standard, the results showed good linearity curve of glucose (10µg) with addition of 0.1 to 1% of ^{13}C glucose. When used on biological samples (glucose from human plasma), the results were consistent with the clinical expectation. More recently, a LC-TOF instrument was also used to measure isotopic enrichment of $^{2}H_5$ phenylalanine and $^{13}C_6$ phenylalanine in rabbit skin protein (Wu et al., 2004). The MS parameters were tuned to obtain only

the proton adducts without any ion fragmentation. Thus, $^{13}C_6$ phenylalanine was studied between 0.02 to 9.2% by following the area of M+1/ M+6 ions. LOD was measured at 0.05% and the reproducibility measured was 1.9% with a non-dependence of the isotopic enrichment between 0.03 to 1nmol (measurement performed with standard at 0.94% of ^{13}C isotopic enrichment). ^{13}C isotopic enrichment of 22 plasma amino acids was also assessed with an ion trap analyzer in negative ion mode. The unique aspect of this method was the sample preparation: as in GC, the amino acids were derivatized; here the derivatisation was performed with o-phthaldialdehyde (OPA) (Van Eijk Hans MH et al., 1997; van Eijk et al., 1999). The results showed that the limit of detection for ^{13}C isotopic enrichment was around 0.25% for most amino acid derivatives and 0.5% for the arginine derivative. The isotopic precision was lower than 1% and was lowest for valine (0.2%). The minimum amount to obtain accurate isotopic enrichment was 25pmol for most of the amino acid derivatives (except for the arginine which required 50pmol). Using this method, arginine was characterized by a short retention time and a lower ionization response compared to other amino acid derivatives.

One of the advantages of LC over GC is the ability to analyze polar and thermolabile compounds; $^{13}C_4$ palmitoylcarnitine and U-^{13}C palmitoylcarnitine were measured in rat muscle with an ion trap analyzer (Guo et al., 2006). The precision reported was ± 0.05MPE for standard solutions between 0.4 to 0.9MPE. After infusion of labeled palmitate (2h at 0.5µmol/kg/min), the isotopic enrichment of $^{13}C_4$ palmitoylcarnitine was measured at 0.51 ± 0.03MPE for gastrocnemius muscle and 0.43 ± 0.02MPE for tibialis anterior. The limit of detection was estimated at 0.05MPE based on the routine analysis of those compounds in rat muscle. Finally, the precision reported for biological samples showed that very low isotopic enrichment can be reliably measured with only 20mg of tissue for both isotopic measurement and concentration.

LC-CRI-MS was also used for studying drug metabolism. Radioisotope (^{14}C) and stable isotopes (^{13}C, ^{15}N) neurosteroid tirilazad (a potential inhibitor of membrane lipid peroxidation *in vitro*) were administrated in monkeys and dogs (Abramson et al., 1996d). To validate this approach, the chromatographic profiles of monkey and dog bile were acquired with the CRI-MS and with the online radioactivity monitoring (RAM). The data regarding signal/noise, sensitivity and the ability to maintain chromatographic resolution gave similar results between CRI-MS and RAM. The areas of 8 selected peaks for ^{13}C, ^{15}N and ^{14}C compounds, showed good agreement (within 95% confidence interval).

In metabolomics, concentration and profiling are used to characterize the metabolic status of biological samples (Rezzi et al., 2007; Dettmer et al., 2007). In this context, the availability of pure metabolites or stable isotope labeled internal standards is a prerequisite for quantitative analytical methods, for controlling matrix effects and for assessing analyte recovery. However, the high cost of chemical synthesis and the lack of availability of chemical standards for all the metabolites make this approach complicated and expensive. Nevertheless, *in vivo* labeling using ^{13}C atoms can be performed with direct supply of ^{13}C as a pure carbon source with $^{13}CO_2$, or U-^{13}C glucose or with more complex sources as ^{13}C leucine enriched milk proteins (Boirie et al., 1995). In this way quantification, profiling experiments, flux analysis (Hellerstein, 2004; Hellerstein & Murphy, 2004; Hellerstein & Neese, 2000) and pathways identification can be pursued by addition of standardized extracts from ^{13}C metabolomes (Birkemeyer et al., 2005) by various instruments, among them LC-MS instruments.

1.13.2 ISOTOPIC ANALYSIS OF MACROMOLECULES

For assessing pharmacokinetics of macromolecules, the production of labeled macromolecules and their specific detection might be a powerful tool for the administrative safety dossier in the pharmaceutical industry. In this context, the replacement of radioisotope (radio-iodine labeled protein) by stable isotope labeled proteins could be an interesting methodology to investigate. This type of study was performed for assessing growth hormone (rGH) pharmacokinetics in rats by injecting intravenously, “uniformly” ^{13}C and ^{15}N labeled rGH (Osborn & Abramson, 1998). Labeled rGH was produced by cell culture and gave a compound with 81% labeling. In this example, the LC-CRI-MS was used to follow labeled macromolecules and large labeled metabolites in biological fluids. In the same study, the GC-CRI-MS was used to monitor small labeled metabolites. In this work, the plasma sample was directly injected after filtration on a C_{18} HPLC column. Labeled growth hormone was detected 1hour after administration. The absolute amounts of rGH detected, varied from 66 to 825pmol in 20µL of plasma and the limit of quantification was assessed at 18ng (or 825fmol with a MW of22804Da for the rGH).

Nucleic acids and proteins (collagen, lysozyme and bovine serum albumin) were also analyzed by LC-CRI-MS (Lecchi & Abramson, 1998; Lecchi & Abramson, 1999). More recently using HEN and CRI interfaces, complex mixtures of proteins and small amounts of ^{13}C labeled peptides obtained after proteolytic digestion of chicken

lyzosine previously reduced and alkylated with ^{13}C iodoacetic acid were also analyzed using column with 1mm (i.d.). Thus, by monitoring the enrichment of $^{13}CO_2$ by LC-CRI-MS, the analyte was easily distinguished from matrix carbon as illustrated in Figure 18 (Jorabchi et al., 2005).

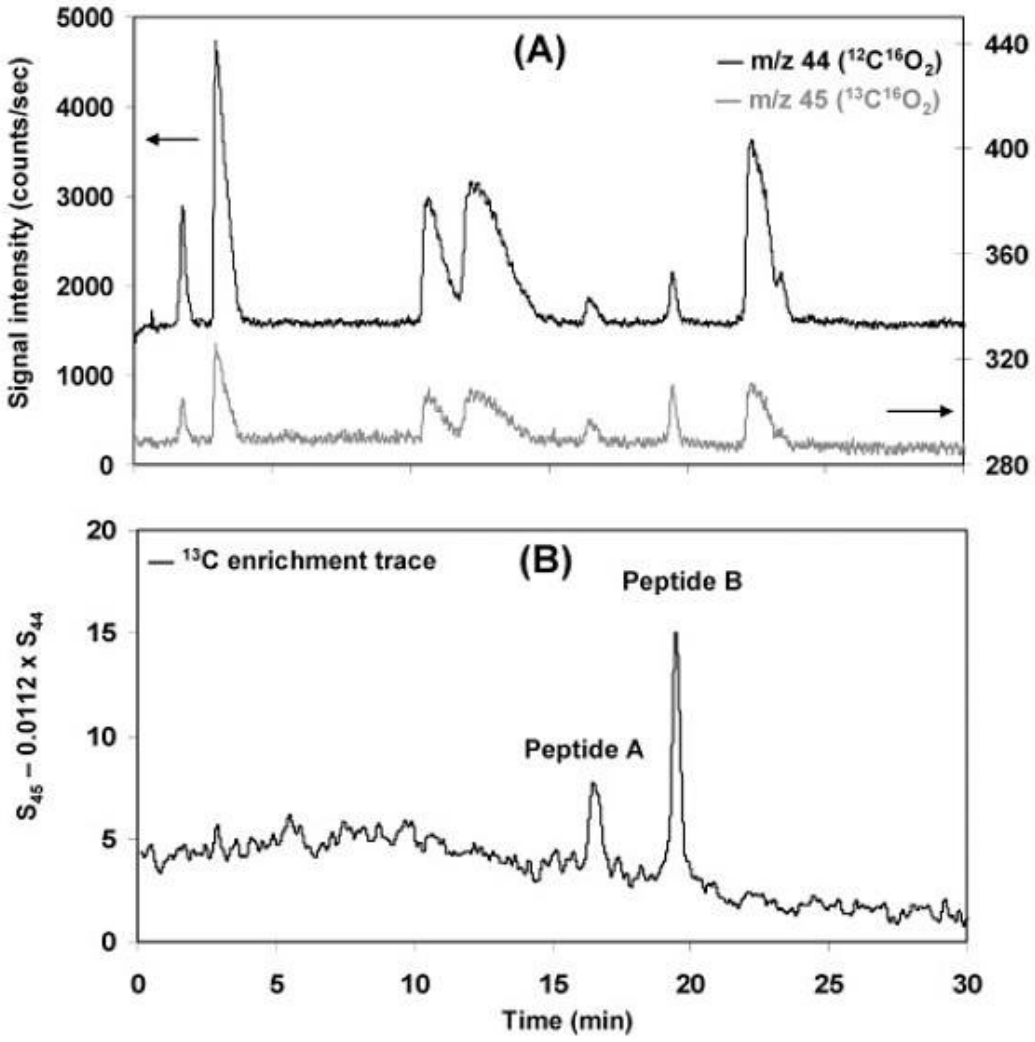


Figure 18: Chromatographic separation of a peptide mixture at 45µL/min. (A) is a LC-CRI-MS chromatogram of a peptide mixture obtained by monitoring m/z 44 ($^{12}C^{16}O_2$) and m/z 45 ($^{13}C^{16}O_2$) channels, respectively; (B) enriched-carbon-only chromatogram obtained by five-point adjacent averaging. The mixture is composed of peptide A, peptide B, unlaleled and labeled proteins after proteolytic digestion of chicken lyzosine previously reduced and alkylated with ^{13}C-iodoacetic acid) and proteins (bradykinin, angiotensin II and its analogue, insulin, horse apomyoglobyn) separated on a 1mm column (i.d.); To get the enriched chromatogram, the calculation used is

described below: Enriched $^{13}CO_2$ chromatogram = m/z 45 ($^{13}CO_2$) – 0.0119 x m/z 44 ($^{12}CO_2$). (Jorabchi et al., 2005)

LC-MS has also been used to measure ^{13}C isotopic enrichment of long-chain fatty acyl coenzyme A in muscle using a quadrupole analyzer (Sun et al., 2006). The LOD reported was 0.5% of isotopic enrichment (with an amount of 200pg injected) for U-^{13}C palmitoyl-CoA. The smallest sample size required was 15mg of tissue. Both the concentration and the isotopic enrichment were reliably measured with such an amount. Nevertheless, due to possible contamination of the sample by moisture, a range of 20-100mg of tissue was analyzed giving good sensitivity. This represents a significant improvement compared to other methods requiring at least 200-1000mg of tissue (Kalderon et al., 2002) (Kasumov et al., 2002).

A common feature in quantitative proteomics is the use of a stable isotope to tag proteins (Tao & Aebersold, 2003; Schneider & Hall, 2005). Most of these approaches are based on complete isotope substitution such that the chemical entities between control and experiments are separated by several atomic mass units (meaning also a high increase of $^{13}C/^{12}C$ ratio of peptides or proteins between control and experimental samples in a single experiment). Nevertheless, among these various strategies there is one that uses a subtle modification of isotope ratio for proteomics studies (Whitelegge et al., 2004). The tryptic peptide studied was derived from phycocyanin from control and from cell culture supplemented with ^{13}C carbonate (between 0 to 6%). Then, after sample preparation, the $^{13}C/^{12}C$ isotopic ratio of peptide containing additional 1.5% of ^{13}C were measured at 0.0199 ± 0.0002 and 0.0157 ± 0.0007 for $[M+H]^+$ respectively for MALDI-TOF and ion trap instruments. The authors showed that with data acquired by Maldi-TOF and µLC-MS-MS devices with minor modification of the $^{13}C/^{12}C$ isotopic ratio, the protein identification experiments are not compromised and several advantages were observed in terms of signal to noise ratio, identification of monoisotopic peak and width of the isotopic envelop.

1.14 CONCLUSIONS

$^{13}C/^{12}C$ isotopic ratio analyses are being used in an increasingly wide number of fields thanks to the recent developments in MS, which has become the instrument of choice for the analysis of ^{13}C isotopically labeled compounds. This review has outlined the progress made in recent years in combining LC introduction methods to MS instruments through various analyzers (such as magnetic sector, quadrupole, ion trap and time-of flight) to perform ^{13}C isotopic analysis.

The recent progress and papers published using LC-IRMS coupling have demonstrated its reliability, especially with ^{13}C isotopic analysis of amino acids in biological matrices and carbohydrates in honey samples. The future challenge for LC-IRMS is to develop other applications with 100% aqueous mobile phase for LC separations. Today, new approaches such as the high temperature liquid chromatography, specific stationary phases and multidimensional LC might provide a solution to increase the LC separation efficiency. Moreover, FIA-IRMS may prove to be an alternative technique to perform ^{13}C isotopic analyses by reducing the amount of compound analysed and by increasing the throughput compared to EA-IRMS. This is particularly interesting when expensive and/or small amounts of compound are available.

For ^{13}C isotopic analyses performed with LC-MS using TOF and ion trap mass analyzers, the results are particularly promising. They show that non-volatile compounds which were difficult to analyze by GC-MS can be targeted and detected with low LOD and isotopic enrichment (0.005%). This lower LOD and higher sensitivity would have a direct impact on the cost of tracer studies and in terms of amount of sample needed. Moreover, recent advances in the use of ^{13}C labeling for the assessment of isotope ratios might enhance the ability to accurately monitor and quantitate the dynamics involved in the metabolome and proteome.

1.15 AIM OF THE PHD WORK

The content of this manuscript is focused on high-precision ^{13}C isotopic analyses carried out essentially with LC-IRMS technique. The work described herewith aims at developing analytical methods to measure isotopic enrichments of different targeted compounds. However, in view of the analytical constrainst of the LC-IRMS interface,

different approaches will be developped either with standard compounds or with biological samples.

Typically, a ^{13}C isotopic measurement is carried out with GC-C-IRMS technique. Thus, in the second chapter, ^{13}C isotopic enrichments of amino acids by GC based methods will be illustrated. This chapter will give an overview of the possible chromatographic issues encountered when separating and measuring isotopic enrichments of several derivatized amino acids in the same chromatographic run. Thus, as the derivatization step is a crucial parameter to obtain accurate and precise isotopic ratio, the measurement by LC-IRMS of isotopic enrichments of underivatised amino acids is interesting to pursue.

As a pioneer use of the LC-IRMS interface, different analytical possibilites for measuring ^{13}C isotopic ratio for small molecules and macromolecules will be illustrated in chapter 3. In chapters 3, 4, 5 and 6 several analytical approaches will be developped for deciphering isotopic analyses in complex samples. A 2D-LC separation (using ion exchange and reversed phase chromatography) and a 1D-LC separation with an ion exchange chromatography column will be reported for underivatised amno acids in chapters 3 and 4. Specifically for Val, the isotopic enrichments obtained by LC-IRMS will be compared with GC-C-IRMS measurements. Thus, in chapter 4, the isotopic accuracy and precision will be assessed between LC and GC based IRMS techniques for labeled samples. Although the ion exchange chromatography matches the LC-IRMS analytical constraints, this mode of separation is limited to ionisable compounds. Therefore, to improve the attractivity of the LC for high-precision measurements, in chapter 5, the high temperature liquid chromatography will be explored for phenolic and organic acids. Finally, the last chromatography approach reported in this work (chapter 6) will be the use of a polymeric column for measuring isotopic enrichment of glucose in plasma.

CHAPTER 2

^{13}C isotopic analyses of amino acids by GC-C-IRMS

Keywords: Amino acids, GC-C-IRMS, ethoxycarbonyl ethyl ester,

2 ^{13}C ISOTOPIC ANALYSES OF AMINO ACIDS BY GC-C-IRMS

2.1 INTRODUCTION

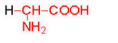
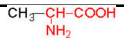
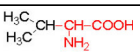
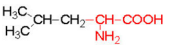
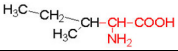
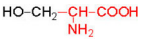
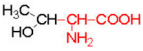
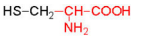
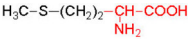
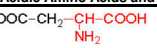
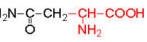
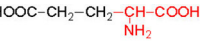
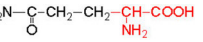
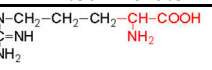
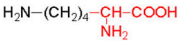
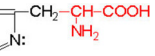
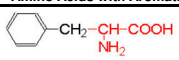
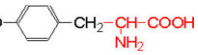
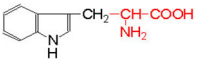
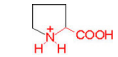
2.1.1 AMINO ACIDS

Amino acids are small and polyfunctional molecules. They are classified in different groups such as branched chain amino acids, hydrophobic amino acids and basic amino acids (see Table 10).

The amino acids which are essential vary from species to species, as different metabolisms are able to synthetize different substances. Nine amino acides are generally regarded as essential for humans: isoleucine, leucine, lysine, threonine, tryptophan, methionine, histidine, valine and phenylalanine. In addition, the amino acids arginine, cysteine, glycine, glutamine and tyrosine are considered conditionally essential, meaning that they are normally required in the diet, but must be supplied exogenously to specific populations that do not synthetize it in adequate amounts. In the case of phenylketonuria disease (PKU), individuals affected with PKU must keep their intake of phenylalanine extremely low to prevent mental retardation and other metabolic complications. However, phenylalanine is the precursor of tyrosine. Without phenyalanine, tyrosine cannot be synthetised and therefore tyrosine becomes essential in the diet of PKU patients.

The branched-chain amino acids (BCCA) refer to the amino acids having aliphatic side chains that are non-linear. These are leucine, isoleucine and valine. The concentration of these three essential amino acids makes up approximately 1/3 of the skeletal muscle in the human body and plays an important role in protein synthesis. BCCA's are currently used clinically to aid in the recovery of burn victims, as well as in supplements for athletes. BCCAs and more particularly leucine, have anabolic effects on protein metabolism by increasing the rate of protein synthesis and decreasing the rate of protein degradation in resting human muscle (Blomstrand, 2006).

Table 10: Structure, symbol and pKa of amino acids

Amino Acid	Symbol	Structure	pK_1 (COOH)	pK_2 (NH_2)	pK R Group
Amino Acids with Aliphatic R-Groups					
Glycine	Gly - G		2.4	9.8	
Alanine	Ala - A		2.4	9.9	
Valine	Val - V		2.2	9.7	
Leucine	Leu - L		2.3	9.7	
Isoleucine	Ile - I		2.3	9.8	
Non-Aromatic Amino Acids with Hydroxyl R-Groups					
Serine	Ser - S		2.2	9.2	~13
Threonine	Thr - T		2.1	9.1	~13
Amino Acids with Sulfur-Containing R-Groups					
Cysteine	Cys - C		1.9	10.8	8.3
Methionine	Met-M		2.1	9.3	
Acidic Amino Acids and their Amides					
Aspartic Acid	Asp - D		2	9.9	3.9
Asparagine	Asn - N		2.1	8.8	
Glutamic Acid	Glu - E		2.1	9.5	4.1
Glutamine	Gln - Q		2.2	9.1	
Basic Amino Acids					
Arginine	Arg - R		1.8	9	12.5
Lysine	Lys - K		2.2	9.2	10.8
Histidine	His - H		1.8	9.2	6
Amino Acids with Aromatic Rings					
Phenylalanine	Phe - F		2.2	9.2	
Tyrosine	Tyr - Y		2.2	9.1	10.1
Tryptophan	Trp-W		2.4	9.4	
Imino Acids					
Proline	Pro - P		2	10.6	

The amino acids have always been challenging compounds for the development of new analytical approaches. Typically, amino acids have been analysed by GC and LC coupled either with the FID, UV, ELSD, MS (Piraud et al., 2003) and fluorescence detectors. Amino acids are non-volatile and a derivatisation step is therefore required prior to separation by gas chromatography to reduce the polarity of the functional groups and facilitating the separation (Calder et al., 1999; Husek, 1975; Sobolevsky et al., 2003). According to the derivative used, some of the amino acids are not stable. Typically, arginine is converted to ornithine with BSTFA (Halket et al., 2005) and not derivativatised at all with N(O,S)-acylalkyl derivatives. Although not indispensable, the derivatisation step is also used in liquid chromatography with the fluorophore o-phathaldialdehyde (OPA) or 9-fluoronylmethylchloroformate (FMOC) derivatives (Van Eijk Hans MH et al., 1997) among other derivatives to enhance the signals. However, native amino acids (underivatised amino acids) are also analyzed using liquid chromatography separation techniques with various detectors (Petritis et al., 2002).

2.1.2 HIGH-PRECISION ^{13}C ISOTOPIC ANALYSES OF AMINO ACIDS

GC-C-IRMS is a highly sensitive technique, enabling rapid on-line separation and ^{13}C carbon isotope determinations of individual compounds, thus avoiding the requirement for the time-consuming off-line preparation procedures. So far, the method has been employed to probe the amino acid carbon isotope compositions of materials as diverse as meteorites (Engel & Macko, 1997), eggshells (Johnson B, 2451), hawkmoth larvae (O'Brien et al., 2002), archaeological bone collagen (Jim et al., 2003) and plasma (Menand et al., 1997). Isotopic information of individual amino acids has been also used to assess protein turnover rates (Wagenmakers, 1999).

The principal challenge to GC-C-IRMS carbon isotope analysis of amino acids relates to their derivatization. The most widely used approach for amino acids derivatisation in GC-C-IRMS analysis involves a stepwise procedure, with the esterification of the carboxyl group with an acidified alcohol and the acylation of amine, hydroxyl and thiol functions with an anhydride forming the N(O,S)-acylalkyl derivatives. Three other derivatives have been widely used for the GC-C-IRMS analysis of amino acids, namely trifluoroacetyl isopropyl ester (TFA-IP), N-acetyl-N-propyl ester (NAP) and the N-pivaloyl-isopropyl ester (NPP). Although the TFA-IP derivative has good chromatographic performance, it may still impair the efficacy of the oxidation catalyst (Shinebarger et al., 2002). For the NAP derivative, poor

resolution of certain amino acids has been reported. Finally, for the NPP derivative, the excessive amount of exogenous carbon atoms is problematic for carbon isotope determinations at natural abundance (Metges & Daenzer, 2000).

In this section, we report our experimental work by using the state-of-the-art method, the GC-C-IRMS with isotopic analysis of derivative amino acids.
We extended the use of N(O,S)-ethoxycarbonyl ethyl ester using standard solutions of amino acids and amino acids from gut mucosa samples. 11 derivative amino acids were studied, among them two (valine and threonine) above natural abundance after administration of valine and threonine as ^{13}C tracers.

2.2 DETERMINATION OF ^{13}C ISOTOPIC ENRICHMENT OF VALINE AND THREONINE BY GC-C-IRMS AFTER FORMATION OF THE N(O,S)-ETHOXYCARBONYL ETHYL ESTER DERIVATIVES OF THE AMINO ACIDS

by Jean-Philippe Godin, Magali Faure, Denis Breuille, Gérard Hopfgartner, Laurent-Bernard Fay, ***Analytical Bioanalytical Chemistry****, 2007, 388: 909.*

2.2.1 INTRODUCTION

Evaluation of protein turnover requires use of tracers, especially for evaluation of the rate of protein synthesis, which is calculated from the rate of incorporation of the tracer into proteins after bolus injection or perfusion of the tracer into the bloodstream. When these methods are used, ^{13}C isotopic enrichment of amino acids in the proteins is usually low (0.001–0.05 Atom% Excess) whereas isotopic enrichment of free amino acids in plasma is high (up to 100%, depending on the method chosen). For all these approaches, gas chromatography coupled to mass spectrometry (GC-MS) or combustion–isotope ratio mass spectrometry (GC-C-IRMS) are the two techniques of choice (Meier-Augenstein, 1999). GC-MS is the method used for measurement of the relatively high enrichment found in the free amino acid pool

whereas GC–C–IRMS is the state-of-the-art technique for assessing the ^{13}C isotopic values observed in proteins at natural abundance or at low isotopic enrichment of volatile compounds. The latter is the enrichment obtained after incorporation of the tracer into proteins from the free amino acid pool during a fixed period of time (Sessions, 2006). A few pre-requisites are mandatory for obtaining precise and accurate $\delta^{13}C$ values by GC-C-IRMS (Meier-Augenstein, 1999). Each chromatographic CO_2 peak must be baseline separated from the other peaks. For protein metabolism studies, however, the presence of at least 15 amino acids at a wide range of concentration and isotopic enrichment makes this difficult to achieve, depending on the derivative used. The number of carbon atoms added by reaction with the derivatisation reagent must be as low as possible to avoid propagation of errors generated either because of dilution of the ^{13}C atoms by the carbon atoms in the derivatisation reagent or because of derivatisation reactions (for example acetylation) which cause isotopic fractionation in the product formed (Docherty et al., 2001). Some derivatives are therefore more convenient than others for measurement of low ^{13}C isotopic enrichment, as is summarized in Table 11. The N-acetyl-N-propyl (NAP) derivative is the most frequently used, because it gives accurate and precise $\delta^{13}C$ by GC-C-IRMS, because of to its low contribution of additional carbon and its stability over time (Matthews & Hayes, 1978; Yarasheski et al., 1992). Nevertheless the NAP derivative has two main drawbacks: the two-step derivatisation procedure and coelution amino acid derivatives on apolar GC columns (Metges & Daenzer, 2000). As remarked by Demmelmair et al. (Demmelmair & Schmidt, 1993), moreover, NAP derivatisation of Arg, His, Cys, and Thr is incomplete and irreproducible. It is therefore of interest to improve the derivatisation procedure by using a simple, rapid, and reproducible derivatising reagent. An interesting alternative approach is use of the N-pivaloyl-isopropyl (NPP) ester derivative (Metges & Daenzer, 2000). After use of this reagent, however, the ^{13}C Val derivative is eluted in two peaks. Alternatives to the NAP and NPP derivatives are the N(O,S)-alkoxycarbonyl alkyl esters introduced by Husek and frequently used in GC–MS (Badoud R. et al., 1991; Husek P., 1998; Husek, 1975; Husek & Simek, 2001). The chemical reactions involved (esterification and acetylation) occur in one step only in aqueous media and are catalysed by pyridine (see Figure 19). Derivatisation takes 5min and does not affect the hydroxy group of carbohydrates. The reaction is, therefore, particularly suitable for analysis of amino acids in media enriched with sugars or in biological matrices. In GC-C-IRMS two different N(O,S)-alkoxycarbonyl alkyl ester derivatives are usually used the methyl and the ethyl chloroformate (MCF and ECF, respectively) reagents that

produce either N(O,S)-methoxycarbonyl methyl esters (Kulik et al., 1998) or N(O,S)-ethoxycarbonyl ethyl esters (Menand et al., 1997; Montigon et al., 2001). These reactions have the advantage of requiring only one step for derivatisation. The number of carbon atoms added is, moreover, reasonably low compared with the number added by use of other derivatives (the resulting isotopic dilution is comparable with that for the NAP derivative). Both derivatives (with MCF or ECF reagents) are normally used to measure $\delta^{13}C$ of only one amino acid at a time and using an apolar GC column. Preparation of the N(O,S)-ethoxycarbonyl ethyl ester was reported for Val and Gln and has recently also been developed for polynitrogenous amino acids (Asx, Glx, Lys, and His) to perform $^{15}N/^{14}N$ position-specific isotopic analysis (Sacks & Brenna, 2005).

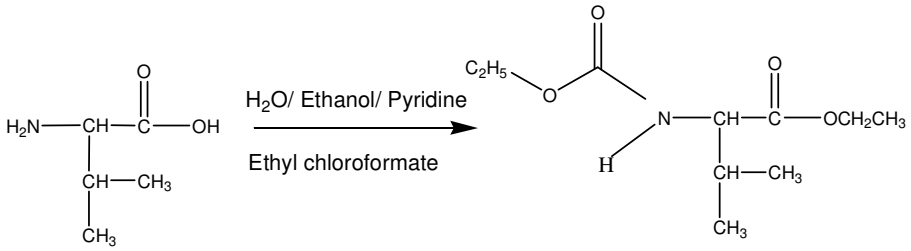


Figure 19: Reaction of derivatisation of valine into N(O,S)-ethoxycarbonyl ethyl ester derivative

In this paper we show that the N(O,S)-ethoxycarbonyl ethyl derivative is also effective for determination, in the same run, the ^{13}C isotopic enrichment of Val and Thr, by use of a polar GC column. This is important, because biological functions are increasingly being studied by an approach using several tracers. Such analysis of several tracers within the same run enables better comparison of the quantitative utilization of these amino acids for protein synthesis. We give such an example by assessing the performance of the method in the measurement of the protein fractional synthesis rate (FSR) of mucins extracted from gut mucosa using either ^{13}C Val or ^{13}C Thr tracers.

Table 11: Overview of the derivatisation methods used in nutrition or metabolic studies to measure ^{13}C isotopic enrichment of amino acids by GC-C-IRMS

Derivatisation	Steps of reaction	Carbons added for Val	Typical GC column used (a)	Comments	Reference
Ter-butyldimethylsilyl derivative (tBDMS)	1	12 extra carbons	CP-Sil 8 column (apolar)	Excessive carbon load Derivative suitable for U-^{13}C amino acids or to separate Gln with Glu and Asn with Asp	Schwenj et al.
N-heptafluorobutyryl propyl ester derivative (HFBPr)	2	7 extra carbons	DB -5 MS column (apolar)	Poisoning of combustion catalyst TFA (trifluoroacetate derivative) is suitable for Arg	Berthold et al. Yu et al.
N-Pivaloyl-isopropyl ester derivative (NPP)	2	8 extra carbons	Ultra 2 column (apolar)	No major caveats except that Val gave a double peak. Method validated for ^{13}C and ^{15}N amino acids.	Metges et al.
N-acetyl-n-propyl ester derivative (NAP)	2	5 extra carbons	Ultra 2 column (apolar)	No major caveats except that Thr with Ser, Gln with Glx, Asn with Asx are co-eluted.	Demmelnair et al.
N(O,S)-methoxycarbonyl methyl ester derivative	1	3 extra carbons	CP-Sil 19CB column (apolar) (with 7 % phenyl and 7 % cyanopropyl siloxane)	Only reported for Val. Not suitable for Arg.	Kulik et al. Reijngoud et al.
N(O,S)-ethoxycarbonyl ethyl ester derivative	1	5 extra carbons	DB-5 MS column (apolar)	Not suitable for Arg. Thr is coeluted with other amino acids on an apolar column.	Husek et al.
			DB-225 MS column (mid-polarity)	Used for Thr.	Schaart et al.

2.2.2 EXPERIMENTAL

2.2.2.1 Chemicals and reagents

Ethanol (95%v/v) and pyridine (99%) were purchased from Fluka (Buchs, Switzerland) and Merck (Darmstadt, Germany), respectively. Dichloromethane and sodium hydrogen carbonate (both 99%) were purchased from Merck. Ethyl chloroformate was purchased from Aldrich Chemie (Darmstadt, Germany). The mixture of seventeen amino acids was purchased from Sigma (St Louis, USA). Natural Val was purchased from Ajinomoto (Tokyo, Japan) and natural Thr from Sigma. [U-^{13}C] Thr was purchased from Euriso-top (St Aubin, France) and [1-^{13}C] Val from Mass Tracer (Woburn, USA).

2.2.2.2 In vivo experiment

The procedure was approved by the Institute's Ethics Committee and conducted in conformity with guiding principles for the care and use of laboratory animals. Male Sprague–Dawley rats (n=12; Iffa Credo, Saint Germain sur l'Arbresle, France) weighing 260g were housed in individual wire-bottom cages in a temperature-controlled room (22–23°C) with a 12h:12h light-dark cycle. They consumed a diet containing 12% protein (Breuille et al., 1998). The rats were divided in two groups. After an 8day acclimatization period the first group (n=6 rats) received a bolus injection of [1-^{13}C] Val (150µmol per 100g body wt, 80% MPE) and the second group (n=6) received a bolus injection of [U-^{13}C] Thr (500µmol per 100g body wt, 15% MPE), both in a lateral tail vein. After 2min, blood (200µL) was sampled from a lateral tail vein. The rats were anaesthetised with sodium pentobarbital (6mg per 100g body weight) and euthanised by blood puncture in the abdominal aorta from 35 to 53min after tracer injection, depending on the animals. After sacrifice of the rats the small intestine was rapidly isolated and washed with phosphate-buffered saline, pH 7.4. Mucosal samples were obtained by scraping, immediately frozen in liquid nitrogen, and then stored at –80°C for further analysis.

Sample preparation of mucosal samples and standards protein hydrolysates were prepared as described elsewhere and dried under a N_2 atmosphere at room temperature (Faure et al., 2002). For preparation of standards, weighed amounts of [1-^{13}C] Val and [U-^{13}C] Thr were mixed with weighed amounts of commercially available unlabeled Val and Thr. The concentration of the Val standard solutions

(natural and labeled) was approximately 1.6mmol L^{-1}. The concentrations of the natural and labeled solutions of Thr were approximately 2.8 and 0.3mmol L^{-1}, respectively. The combined mixture was dissolved in water, then dried and derivatised. This procedure was repeated to obtain standard calibration points for the accuracy curve for different ^{13}C enrichment in the range between 0 and 1.1atom% excess (APE) for Val, and 0 and 0.8APE for Thr. For the accuracy curves two sets of standard solutions were prepared for each amino acid to study accuracy in two ranges of isotopic enrichment (low and high). In the first set the five calibration points for Val were at 0, 0.022, 0.096, 0.112 and 0.225 and the five for Thr were at 0, 0.031, 0.062, 0.190 and 0.256. In the second set the six calibration points were used for both amino acids. For Val, they were at 0, 0.022, 0.112, 0.337, 0.561, and 1.115APE and for Thr, they were at 0, 0.104, 0.212, 0.325, 0.562, and 0.886APE. The standard mixture of amino acids was prepared from 70µL of the standard mixture (amino acid concentration 2.5µmol mL^{-1}) which was dried and derivatised.

2.2.2.3 Derivatisation procedures

N(O,S)-ethoxycarbonyl ethyl ester derivatisation of Val and Thr was performed after hydrolysis and purification of the samples by cation-exchange chromatography on 200 to 400mesh AG50W-X8 resin (Biorad, Richmond, USA) in accordance with Faure et al (Faure et al., 2002). Briefly, 60µL deionized water, 32µL ethanol, and 10µL pyridine were added to each dry sample. After brief vortex mixing 7µL ethyl chloroformate was added. The vials were tightly capped, vortex mixed for approximately 15sec, and left for 5min at room temperature. Subsequently, 500µL saturated sodium hydrogen carbonate solution and 2mL dichloromethane were added and the vials were thoroughly shaken. The aqueous phase was then removed and anhydrous sodium sulfate was added to the vials to remove residual water. The organic phase was then dried at 55°C under a gentle flow of nitrogen. The samples were ready to inject for GC-C-IRMS analysis after re-dissolution in 50µL ethyl acetate.

2.2.2.4 Instrumentation

The GC-C-RMS analysis was performed with a Hewlett–Packard 5890 series II gas chromatograph interfaced with a MAT252 isotope-ratio mass spectrometer (Thermo Electron, Bremen, Germany). The combustion interface consisted of a NiO/CuO/Pt combustion furnace reactor heated at 940℃ and a copper reduction furnace at 600℃. A Nafion water trap was placed between the gas chromatograph and the isotope-ratio mass spectrometer. The ^{13}C isotopic enrichment of both amino acid derivatives was determined by monitoring ions of mass *m/z* 44, *m/z* 45, and *m/z* 46 of CO_2 gas. The IRMS was operated at an accelerating voltage of 10kV. The ion source was held at a pressure of 1×10^{-6}Torr, and ions generated by electron impact at 70eV. Three faraday-cup detectors were used for simultaneous and continuous monitoring of the CO_2^+ signals for the three major ions at *m/z* 44 ($^{12}CO_2$), *m/z* 45 ($^{13}CO_2$ and $^{12}C^{17}O^{16}O$), and *m/z* 46 ($^{12}C^{18}O^{16}O$). To obtain comparable signal outputs the preamplifier feedback resistors were set at 300MΩ, 30GΩ, and 100GΩ for *m/z* 44, *m/z* 45, and *m/z* 46, respectively. The GC-MS instrument was a Hewlett–Packard 6890 series gas chromatograph, using helium as carrier gas, coupled to an MSD5975 (Agilent) quadrupole mass spectrometer. It was operated in positive chemical-ionisation mode (PCI) with methane as reactor gas.

2.2.2.5 Experimental conditions

For GC-C-IRMS analysis, the injector temperature was set at 250℃. Compounds were separated on a 60m x 0.32mm i.d., 0.25µm film thickness, DB-Wax fused-silica capillary column (J&W Scientific, Koeniz, Switzerland). Helium was used as carrier gas with a fixed head pressure of 18psi. Splitless injection was used with a purge delay of 0.8min. The deactivated liner (Agilent, USA) was changed regularly. The oven temperature was held at 70℃ for 1min after injection, then programmed at 15℃min^{-1} to 140℃ which was held for 1min, then at 3℃min^{-1} to 172℃, which was held for 2min, and finally at 2℃min^{-1} to 248℃, which was held for 5min. Ions with *m/z* of 44, 45, and 46 were monitored for determination of [1-^{13}C] Val and [^{13}C] Thr enrichment. The total run time per analysis was approximately 62min. The oxidation furnace was re-oxidized for approximatively 30min before each sequence. The GC–C–IRMS data were processed by use of Finnigan Isodat NT software. GC-MS analysis was performed to confirm the peak purity of Val and Thr and to identify the other amino acids (data not shown) in positive chemical-ionisation mode. Amino acid

derivatives were chromatographed on a 30m x 0.25m, 0.25µm film thickness, DB Wax fused-silica capillary column (J&W Scientific). The ethyl acetate layer containing derivative amino acids (1µL) was injected in split mode. The carrier gas was helium (99.996%) maintained at a column head pressure of 19psi. The oven temperature was held at 70°C for 1min after injection, then programmed at 15°C min to 140°C which was held for 1min, then at 3°C min^{-1} to 172°C, which was held for 2min, and finally at 2°min^{-1} to 248°C, which was held for 5min. The MS interface and ion-source temperatures were 280 and 220°C, and mass spectra were scanned over the range *m/z* 50 to 550Da.

2.2.2.6 Calibration and isotopic calculation

Three pulses of CO_2 reference gas were admitted to the inlet at the beginning of each run and during the run near the Val and Thr peaks for about 20sec.

Isotope ratios are expressed relative to international standards, rather than as absolute isotope values. The [^{13}C] abundance was first expressed as $\delta^{13}C$ values calibrated against the international standard (Vienna Pee Dee Belemnite, VPDB) (Slater et al., 2001).

The delta notation is defined as δ (‰) $^{13}C/^{12}C_{sample} = (R_s / R_{st} - 1) \times 1000$, where R_s is the ratio of $^{13}C/^{12}C$ in the sample and R_{st} is the ratio of $^{13}C/^{12}C$ of the international standard used. The result of this calculation is a relative delta calibrated against the international standard.

Atom % was calculated as: Atom % $= \left[\frac{100 \times R \times ((\delta 13C/1000)+1)}{1+R\times((\delta 13C/1000)+1)}\right]$, where R is the ratio of ($^{13}C/^{12}C$) of International Standard of Pee Dee Belemnite, R=0.0112372.

Atom% Excess (APE) is defined as Atom% (background) minus Atom% (sample). Data were finally expressed in MPE accounting for all carbons of the derivatised Val or Thr molecules. The formula was then MPE_{meas}=APE ($C_{total}/C_{labeled}$) where C_{total} is the total number of carbon atoms in the N(O,S)-ethoxycarbonyl ethyl ester derivative and $C_{labeled}$ is the number of labeled carbons in the molecule: For [1-^{13}C] Val derivative, MPE=10APE (*e.g.*, C_{total}=10 and $C_{labeled}$=1), and for U-^{13}C Thr derivative, MPE=2.25APE (C_{total}=9 and $C_{labeled}$=4).

2.2.3 RESULTS AND DISCUSSION

2.2.3.1 Gas chromatographic separation in GC–C–IRMS

The GC separation was performed with a polar GC column characterized by its lower temperature range compared with apolar GC stationary phases. Column cleaning is known to be crucial to avoid memory effects which might affect the precision of $\delta^{13}C$ measurements for biological samples. To assess potential cross contamination for the targeted compounds (Val and Thr) by other compounds or by release of material from the column, ten replicate injections of one in vivo sample were performed on the same day. The results revealed no cross contamination occurred under these conditions. The CV (calculated from atom% values) reported for labeled Val (1.7001 ± 0.011 atom%, n=10) was approximately 0.01% and for natural Thr (1.0790 ± 0.0006 atom%, n=10) approximately 0.06%. These CV calculated for a specific *in vivo* sample also gave the variation of the isotopic measurement of the instrument. This variation between 0.01 to 0.06% was in agreement with the variation of the instrument for the standard MCF-Val derivative (CV at 0.015%) measured by GC-C-IRMS (Reijngoud et al., 1998). Baseline separation of the peaks of interest is a primary requirement if precise (SD) and accurate $\delta^{13}C$ values are to be obtained, particularly for complex biological samples.

The length of the alkyl group within the derivative (methyl, ethyl, propyl, or isobutyl) is known to substantially affect the retention time of the amino acid derivatives in gas chromatography (Namera et al., 2002) and, depending on the polarity of the GC columns, some amino acids can coelute. By use of our approach, with a polar GC column and N(O,S)-ethoxycarbonyl ethyl ester derivatives, eleven amino acid derivatives (those of Val, Ala, Leu, Iso, Gly, Pro, Asp, Thr, Ser, Met, and Phe) were baseline resolved. As shown in these conditions Ser is coeluted with Gln. Montigon et al. (Montigon et al., 2001) have shown that Gln can be analyzed by use of an apolar GC column.

2.2.3.2 Precision of isotopic measurement

The mean precision was assessed with the Equation 1 using standard deviation (σ) of individual amino acids in a mixture (n=11 amino acids involved in the calculation): (Metges & Daenzer, 2000).

$$mean\ precision\ (‰) = \sqrt{(\sum \sigma^2 \div n)} \qquad \textit{(Equation 1)}$$

The mean precision calculated by use of equation 1 or eleven amino acids was 0.32‰. This mean isotopic precision was in the same range as the mean precision calculated by Metges et al. for NPP and NAP-derivatised amino acids (0.45 and 0.26‰, respectively) (Metges & Daenzer, 2000). Table 12 shows the results obtained from five injections of a mixture containing eleven amino acid derivatives. The SD (for individual amino acids) was between 0.1 to 0.7‰; for Val and Thr it was with 0.54 and 0.32‰, respectively.

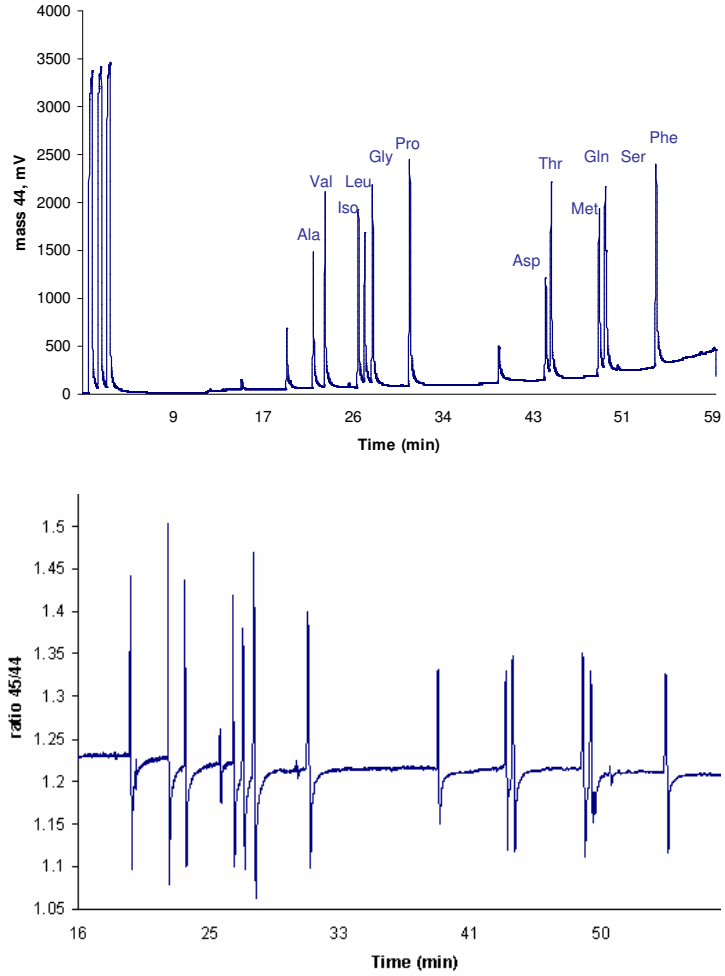


Figure 20: GC–C–IRMS chromatogram obtained from N(O,S)-ethoxycarbonyl ethyl ester derivatives of amino acid standards (1nmol of each).

In Figure 20, the amino acid derivatives were identified by GC–MS in PCI ionisation mode. The uper panel shows the CO_2 peak (m/z 44) for each. The lower panel shows the 45/44 ratio.

2.2.3.3 Inter and intra-day repeatability of ^{13}C isotopic measurement

Kinetic isotopic fractionation can occur during the acetylation and the esterification reactions when one reagent reacts non-quantitatively. Rieley discussed this effect and showed that a mass balance equation can be used to obtain the true isotopic value at natural abundance (Rieley, 1994). For the N(O,S)-ethoxycarbonyl ethyl ester derivative, five carbons from the reagents are incorporated into the derivative and might lead to irreproducible isotopic fractionation. For tracer study, at low or higher isotopic enrichment, these limitations can be overcome by studying the inter-day repeatability (Docherty et al., 2001). If incomplete derivatisation reactions lead to reproducible isotopic fractionation measurement of the excess of isotopic enrichment is not affected. The inter-day repeatability of $\delta^{13}C$ for Val and Thr standards was analysed over three different days at natural abundance and at low isotopic enrichment (respectively 0.112atom% and 0.190atom% for Val and Thr). These results are indicative of excellent inter assay repeatability of the isotopic measurement at natural abundance and acceptable inter-assay repeatability at higher ^{13}C isotopic enrichment. The associated SD on atom% was between 0.015 and 0.0003 for a range of isotopic enrichment between 1.208 and 1.987atom%. Those values, obtained for standard solutions or for biological samples, showed that inter-day and intra-day repeatability were excellent and enable metabolic investigations to be performed.

Table 12: ^{13}C isotopic ratio ($\delta^{13}C$, ‰) at natural abundance for N(O,S)-ethoxycarbonyl ethyl ester derivatives of amino acids in a mixed standard solution separated on a polar GC column.

Standard amino acids	Ala	Val	Leu	Iso	Gly	Pro	Asp	Thr	Met	Ser	Phe
rrt	0.95	1	1.14	1.16	1.19	1.35	1.91	1.93	2.12	2.15	2.36
$\delta^{13}C$	-32.47	-26.17	-28.87	-33.39	-38.25	-28.59	-39.54	-25.68	-32.84	-28.31	-34.86
	-32.55	-27.12	-28.77	-33.64	-38.59	-29.12	-39.5	-26.56	-33.47	-28.27	-35.32
	-32.1	-26.54	-28.67	-32.93	-38.74	-28.69	-39.31	-25.93	-33.66	-28.31	-35.3
	-31.89	-25.7	-28.69	-33.48	-38.76	-28.39	-39.52	-26.11	-32.61	-28.8	-34.66
	-31.89	-26.71	-28.69	-33.4	-38.76	-28.39	-39.51	-26.1	-33.5	-28.8	-34.66
Mean	-32.18	-26.45	-28.74	-33.37	-38.62	-28.64	-39.48	-26.08	-33.22	-28.5	-34.96
sd	0.31	0.54	0.08	0.26	0.22	0.3	0.09	0.32	0.46	0.28	0.33
CV (%)	1	2	0.8	0.6	1	2.2	0.2	1.2	1.4	1	0.9

Table 13: Inter-assay precision of isotope ratio measurement ($\delta^{13}C$, ‰) for the derivatives of Val and Thr at natural abundance and at low ^{13}C isotopic enrichment, using standard solutions

	Val $\delta^{13}C \pm SD$, CV %		Thr $\delta^{13}C \pm SD$, CV %	
	Natural abundance	At 0.112 Atom %	Natural abundance	At 0.190 Atom %
Day 1	-29.82 ± 0.41, 1.4	92.24 ± 0.10, 0.1	-37.99 ± 0.31, 0.8	157.36 ± 0.58, 0.4
Day 2	-29.37 ± 0.42, 1.4	92.82 ± 0.40, 0.4	-37.57 ± 0.65, 1.7	146.20 ± 0.78, 0.5
Day 3	-29.73 ± 0.28, 0.9	97.91 ± 0.40, 0.4	-38.42 ± 0.16, 0.4	162.64 ± 0.89, 0.5

2.2.3.4 Accuracy of isotopic measurement

The accuracy of the isotopic measurement was checked with a set of standard enrichment calibration points for both amino acids. The results (mean, SD, CV, and the difference expressed as Δ for theoretical APE minus measured APE for Val and Thr) are summarized in Table 14. Comparison of calculated and measured APE revealed variation from 0.0004 to 0.05APE for Val and from 0.002 to 0.15APE for Thr. Thus, isotopic accuracy is at least 0.009 APE for APE ≤ 0.11 and between 0.01 and 0.1APE for APE ≥ 0.2. For higher enrichment (1.1APE or 0.9APE), which corresponds to measured $\delta^{13}C$ values of 1046.2 ± 4.7‰ and 913.9 ± 3.2‰ for Val and Thr, respectively (as expected, because of the large difference between $\delta^{13}C$ for reference CO_2 gas and the measured CO_2 peak) the accuracy of isotopic measurement is lower, but still good, for *in vivo* samples. As remarked by Brenna (Brenna et al., 1997), the limit of the IRMS instrument in terms of accurate isotopic measurement was approximately 1000‰, meaning that, for higher values, the $\delta^{13}C$ must be diluted by adding a known amount of a natural compound with known $\delta^{13}C$ values.

For practical use, standard curves of calculated APE and measured APE were plotted to correct for isotopic fractionation of the sample preparation technique and the instrument, within two ranges of isotopic enrichment. The results (data not shown) for Val and Thr were indicative of a linear relationship between measured and calculated APE for both ranges. Between 0 and 1.12APE and 0 to 0.89APE for Val and Thr, respectively, the respective equations were $y=1.037x + 0.015$, $r^2=0.998$, and $y=1.156x - 0.008$, $r^2=0.999$. Between 0 and 0.23APE and 0 and 0.32APE for Val and Thr, respectively, the respective equations were $y=1.057x - 0.0016$, $r^2=0.999$, and $y=1.012x + 0.0017$, $r^2=0.999$.

The lower limit of enrichment measured by GC–C–IRMS in this study was at 0.02 ± 0.0005APE for both amino acids. These limits of detection were in the same range as the limit of detection reported for Gln using the same derivative (Montigon et al., 2001).

Table 14: Accuracy of isotopic measurement of standard solutions of ^{13}C Val and ^{13}C Thr derivatives by GC-C-IRMS

^{13}C Val			^{13}C Thr		
Theor. APE	APE meas. ± SD (CV) (n=3)	Δ (APE)	Theor. APE	APE meas. ± SD (CV) (n=3)	Δ (APE)
0.022	0.022 ± 0.0002 (0.9)	0.0004	0.012	0.014 ± 0.0004 (2.9)	0.002
0.112	0.115 ± 0.0004 (0.4)	0.003	0.125	0.134 ± 0.0001 (0.1)	0.009
0.561	0.599 ± 0.0087 (1.5)	0.039	0.190	0.200 ± 0.0009 (0.5)	0.010
1.115	1.169 ± 0.0051 (0.4)	0.054	0.886	1.033 ± 0.003 (0.3)	0.147

2.2.4 BIOLOGICAL APPLICATION

For tracer studies using IRMS, the accuracy of $\delta^{13}C$ is less important than the precision of $\delta^{13}C$. Thus, the possibility of following precisely the variation of the relative background of $\delta^{13}C$ is one of the main criteria for obtaining a reliable analytical method for isotopic measurement. The fluctuation at natural abundance does not go beyond 1–2‰ on a time-scale, corresponding to 0.001–0.002atom% (Goodman & Brenna, 1992). The $\delta^{13}C$ precision for four different rats and for several amino acid derivatives is reported in Table 15. The CV for most of the amino acids was below 0.2%, corresponding to an SD below than 0.002atom% (except Asp, for which the CV was 1.4%). These SD values correspond to the sum of inter-animal variability and the inter-repeatability of the measurement over several days. These data showed that isotopic precision was preserved through the different steps of hydrolysis, purification, and derivatisation of the samples and was in agreement with isotopic fluctuation of the compound at natural abundance. The $\delta^{13}C$ precision calculated for each rat and for each amino acid was, moreover, in the same range as the precision classically reported for GC-C-IRMS between 0.0002 and 0.0005atom%.

Table 15: ^{13}C isotopic enrichment (Atom%) of N(O,S)-ethoxycarbonyl ethyl ester amino acids derivative from mucosa hydrolysates

		Ala	Val	Leu	Iso	Gly	Pro	Asp	Thr	Phe
Rat 1	Atom %	1.0771	1.513	1.0723	1.0645	1.0717	1.0725	1.0732	1.0802	1.0751
	sd (n=3)	0.0007	0.0229	0.0009	0.0007	0.0007	0.0009	0.0007	0.0006	0.0024
Rat 2	Atom %	1.0733	1.0704	1.07	1.0653	1.0753	1.0694	1.1084	1.2372	1.0718
	sd (n=3)	0.0003	0.0004	0.0003	0.001	0.0004	0.0004	0.0004	0.0005	0.0003
Rat 3	Atom %	1.0736	1.0708	1.07	1.0618	1.074	1.0699	1.1004	1.0794	1.0733
	sd (n=3)	0.0006	0.0005	0.0003	0.0001	0.0002	0.0003	0.0011	0.0004	0.0023
Rat 4	Atom %	1.0733	1.0738	1.0721	1.0638	1.0705	1.0723	1.0749	1.0798	1.0741
	sd (n=3)	0.0002	0.0002	0.0002	0.0002	0.0013	0.0001	0.0005	0.0003	0.0006
	Mean (n=4)	1.0743	1.0717	1.0711	1.0639	1.0729	1.071	1.0892	1.0798	1.0736
	sd (n=4)	0.0019	0.002	0.0013	0.0015	0.0022	0.0016	0.018	0.0004	0.0014
	CV (n=4)	0.2	0.2	0.1	0.1	0.2	0.2	1.6	0.04	0.1

Most of the isotopic ratio of amino acids derivative is at natural abundance. For Val and Thr, statistics were performed with n=3 rats (using natural isotopic abundance). The higher CV obtained for Asp is explained by its low chromatographic resolution from Thr. Met and Ser were in too low concentration to obtain reliable isotopic measurement in these in vivo samples.

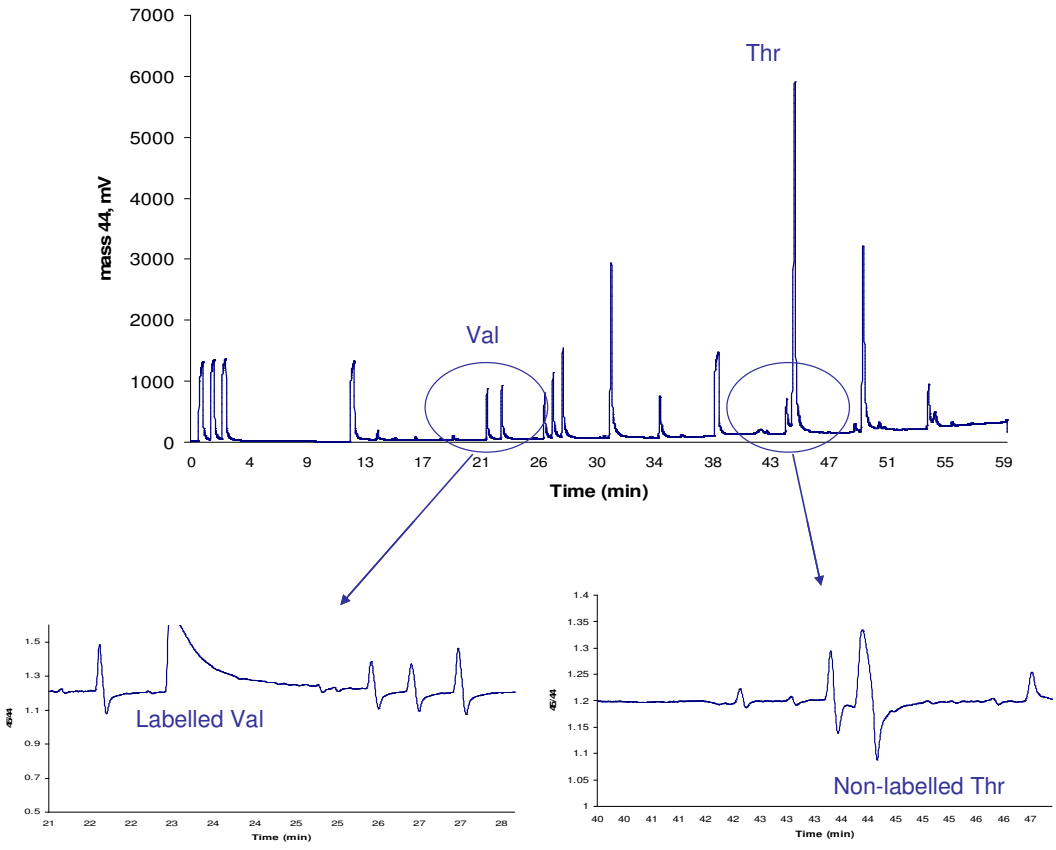


Figure 21: GC-C-IRMS chromatogram obtained from mucosa protein hydrolysate from a rat after administration of ^{13}C Val.

In Figure 21, measured ^{13}C isotopic enrichment for Val and Thr was 1.7164 ± 0.01atom% (n=3) and 1.0794 ± 0.0004atom% (n=3), respectively. The magnified areas show the 45/44 ratio for ^{13}C-labeled Val and for unlabeled Thr.

This method has been successfully used to quantify Thr utilization for protein synthesis in different plasma and intestinal compartments during acute inflammation in rats (unpublished work). Incorporation of ^{13}C-tracers into proteins was measured after an incorporation time of 15 to 50min, depending on the animals. Protein synthesis was measured from Val or Thr enrichment using two groups of rats (n=6 rats per group). For each group the ^{13}C isotopic enrichment of Val and Thr was measured in the same run; because of the design of the animal study isotopic enrichment of one amino acid was basal and for the other was higher (as illustrated in Figure 21). In the first group, for example, basal isotopic enrichment for Val was measured at 1.0726 ± 0.0007atom% and isotopic enrichment for Thr (APE) was calculated by using the basal isotopic enrichment of Thr (measured at 1.0793 ± 0.002atom%) of the second group. Thus, the time course of ^{13}C enrichment (APE) of Thr in the small intestinal mucosal proteins can be plotted as illustrated in Figure 22.

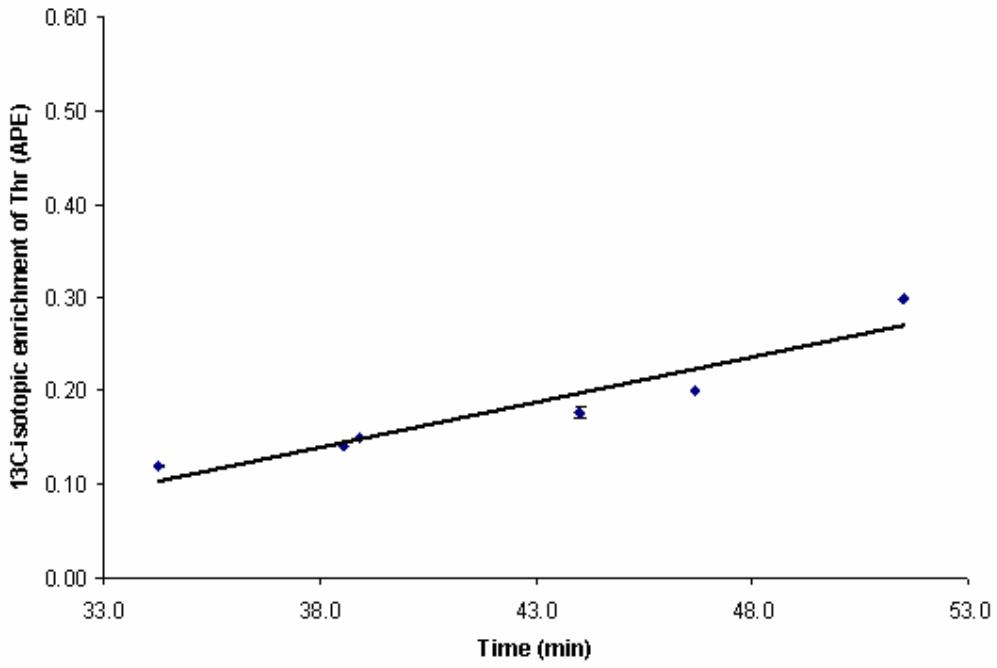


Figure 22: Time course of ^{13}C Thr enrichment after a flooding dose of ^{13}C Thr (500µmol per 100g body weight) in a lateral tail vein.

Values are means ± SD; error bars that are not visible are within the symbols. The slope for incorporation of the ^{13}C tracer into the protein was 0.0097, with r^2=0.896

2.2.5 CONCLUSIONS

This new GC-C-IRMS method enables simultaneous detection of Val and Thr using N(O,S)-ethoxycarbonyl ethyl ester derivatives and GC on a polar stationary phase. For Val and Thr the repeatability of the method was excellent, both at natural abundance and at low isotopic enrichment. The one-step derivatisation procedure using N(O,S)-ethoxycarbonyl ethyl esters derivatisation reagent is a rapid and straightforward alternative to NAP derivatisation for measurement of ^{13}C isotopic enrichment of amino acids within the tracer study. The procedure in aqueous media combined with the low isotopic dilution (identical with that for NAP derivatisation) enables precise and accurate $\delta^{13}C$ measurement. The overall isotopic precision obtained was 0.32‰ for eleven amino acid derivatives. By use of *in vivo* samples, moreover, the variation at natural background for nine amino acids and several rats revealed precision was better than 0.002atom%; this is indicative of the reliability and the suitability of this method for metabolic study.

2.3 PERSPECTIVES

20 years after its commercialization, the GC-C-IRMS device is still the workhorse in many laboratories that perform CSIA of polar and non-polar compounds.

The current trend in GC-C-IRMS developments is focused on two points:

- More and more strategies are published to reduce the number of carbon added by the derivative in GC-C-IRMS as for glucose with boronate derivative (Jackson et al., 2007) and for glutathione with the (N,S)-ethoxycarbonyl methyl ester derivative (Tea et al., 2007). The work reported previously illustrated also this aspect and the challenge to obtain a baseline separation of amino acids by GC-C-IRMS. Typically, LC-IRMS might be of great interest to overcome this issue.

- The second point is the sensitivity of the GC-C-IRMS detection. This aspect is particularly important for isotopic analyses of compounds in low concentration (µg/L). To overcome the GC-C-IRMS limitation, analyte concentrations need to be adjusted to reach the range of GC-C-IRMS detectability (1nmol of carbon on column). Thus, solid phase micro-extraction procedure (SPME) is developed for volatile compounds (Goupry et al., 2000; Blessing et al., 2007; Palau et al., 2007) and purge & trap (P&T) extraction technique GC-C-IRMS are for aqueous solution (Zwank et al., 2003). Typically, the detection limit using P&T extraction in combination with GC-C-IRMS, reached 0.25 to 5µg/L for toluene and tetrachloromethane, respectively (Zwank et al., 2003). In life science, SPME GC-C-IRMS method was developed for measuring low isotopic enrichment (lower than 1MPE) for acetate, propionate and butyrate in plasma (with 200, 15 and 4µM, respectively) in the frame of tracer study (Ferchaud-Roucher et al., 2006).

CHAPTER 3

^{13}C isotopic analyses of small and macromolecules by LC-IRMS

Keywords: albumin, insulin, amino acids, LC-IRMS, FIA-IRMS

Picture of the LC-IRMS instrument installed at the Nestlé Research Center.

From the left to the right, there are an autosampler (CTC, Switzeerland), the LC pumps (Rheos, Flux, Switzerland), a degasser (Flux, Switzerland), a Polaratherm 9000 (heater) (PatSelerity, Belgium), an LC Isolink interface (Thermo Fischer, Germany) and a MAT252 (Thermo Fischer, Germany).

3 ^{13}C ISOTOPIC ANALYSES OF SMALL MOLECULES AND MACROMOLECULES USING LC-IRMS

3.1 INTRODUCTION

The new LC-IRMS device that was so often mentioned as “an ideal tool”, has been presented in 2004 at Pittcon (USA) (see Figure 23) and was commercialized the same year by Thermo Fisher (Bremen, Germany).

Figure 23: LC Isolink® interface commercialized by Thermo Fischer (Bremen, Germany) to couple Liquid Chromatography to IRMS device for high-precision ^{13}C isotopic measurement

One way to reduce the number of carbon in the derivative would be to not derivatise at all the compound. In this context, coupling between the LC and IRMS present interesting perspectives that will be illustrated in the next section.

The LC-IRMS device allows the measurement of the isotopic ratio of water soluble and non-volatile compounds under their native form for low molecular weight molecules and for macromolecules. For that, we will use the two modes of introduction available in the instrument (the bulk and the compound specific isotope analyses through the flow injection and the LC analyses).

3.2 ISOTOPE RATIO MONITORING OF SMALL MOLECULES AND MACROMOLECULES BY LIQUID CHROMATOGRAPHY COUPLED TO ISOTOPE RATIO MASS SPECTROMETRY

*Published in **Rapid Communication Mass Spectrometry**, 2005, 19: 2689 by Jean-Philippe Godin, Jorg Hau, Laurent-Bernard Fay and Gérard Hopfgatner*

3.2.1 INTRODUCTION

The isotope ratio mass spectrometer (IRMS) is an analytical instrument with a wide range of applications in various fields such as biomedicine, metabolism, pharmacy, environment, geochemistry, food and forensic sciences.(Lichtfouse, 2000; Schmidt et al., 2004) An IRMS is a mass spectrometer designed to undertake precise determinations of small variations in isotopic abundances; it is thus particularly suitable for tracer studies at a low enrichment level (0.005 to 5 %). It is commonly based on a magnetic sector mass analyser. Light isotope ratio mass spectrometry allows the determination of isotope ratios such as $^{13}C/^{12}C$, $^{15}N/^{14}N$, $^{18}O/^{16}O$, $^{2}H/^{1}H$ and $^{34}S/^{32}S$ using e.g., CO_2, N_2, CO, H_2 and SO_2 as gases. While the coupling of IRMS to gas chromatography (GC) is a well-established technique, the combination of IRMS with liquid chromatography (LC) still remains a challenge. After chromatographic separation, it is mandatory to bring the analyte into the IRMS ion source in the gaseous state. Obtaining a reliable and reproducible conversion of organic molecules

to CO_2 is relatively straightforward when coupling GC to IRMS, but coupling LC to IRMS is much more complicated since the CO_2 has to be generated in, and extracted from, a liquid phase.

To date, three different systems for producing CO_2 from organic compounds have been developed and applied to perform compound-specific isotope analysis (CSIA). The first system for GC coupling was introduced in the mid-1970s by Sano *et al.* (Sano et al., 1976) and Matthews and Hayes (Matthews & Hayes, 1978) by addition of a combustion step immediately after the chromatographic separation. This system, GC-C-IRMS, was commercialised in the 1990s. The working principle of the GC-C-IRMS consists briefly in the production of gaseous CO_2 from an organic analyte in a combustion furnace (CuO/NiO/Pt) heated to 940°C, followed by a copper furnace at 600°C to reduce the nitrous oxide formed to N_2 (Meier-Augenstein, 1999). This combustion principle was also applied to LC-IRMS coupling (referred to as LC-C-IRMS); Caimi *et al.* (Caimi & Brenna, 1993) and Brand *et al.* (Brand W.A & Dobberstein P., 1996) explored "moving wire" and "moving belt" interfaces. The principle of both interfaces consists of depositing analytes on a moving belt after various steps of cleaning, heating and spraying. Consecutively, the analytes are oxidized to CO_2 and H_2O in a CuO/Pt furnace at 850 °C, and the CO_2 is swept into the IRMS ion source by a constant helium flow.

A second system, developed by Markey and Abramson (Abramson & Markey, 1986), is based on the use of microwave-induced plasma and is known as a "chemical reaction interface" (CRI). It was used for coupling both GC and LC to MS (McLean et al., 1996; Teffera & Abramson, 1994). The principle is that the HPLC eluent is vaporised by a pneumatically heated nebulizer; the particle stream is then carried by a helium flow through a membrane-based gas diffusion cell, and a momentum separator removes the excess of helium. After addition of reactant gas, the stream enters the microwave-powered ion source cavity. The first experiments with the HPLC-CRI-IRMS were reported by Teffera *et al.* (Teffera et al., 1996) (Abramson et al., 1996; Abramson et al., 2001).

The main difficulties of those techniques were for the moving wire technique the difficulty of used, the isotope fractionation of low molecular weight compounds in the drying stage and the low recovery of the analytes. For the CRI technique, the main difficulties were the low sensitivity and the isotope fractionation of low molecular weight compound by particle beam separation. As a consequence, neither of these two technologies has been commercially available.

The third system is derived from the well-established methodology used to determine the total organic carbon (TOC) in liquid samples based on chemical oxidation (Bisutti et al., 2004). The first experiments of coupling this technology to an IRMS were reported by St Jean in 2003 with a step by step batch processing (St Jean, 2003). One year later, Krummen *et al.* published an on-line technology based on an interface allowing the coupling of an IRMS with a TOC or an LC instruments. This new interface is based on chemical oxidation and commercialised it under the name Finnigan LC IsoLink® (Thermo Electron, Bremen, Germany) (Krummen et al., 2004). This interface is designed to convert organic compounds present in aqueous phase including inorganic buffers to CO_2. The technique uses a wet oxidation, e.g. by ammonium or sodium peroxodisulfate under acidic conditions, such as phosphoric acid. Using this interface, two modes of introduction can be used to obtain accurate $\delta^{13}C$ values: flow injection analysis mode, suitable for pure compounds (also termed "bulk-sample isotope analysis" or "BSIA by FIA-LC/MS"), and classical LC, enabling "compound-specific isotope analysis" (termed "CSIA by "irm-LC/MS").

This chapter will cover these two modes (FIA and LC coupled to IRMS) and describe the evaluation of the analytical performance of a commercially available LC interface to IRMS, in particular using biomolecules (as amino acids and macromolecules) at natural abundance and at low enrichment (for the amino acids).

3.3 EXPERIMENTAL SECTION

3.3.1 CHEMICALS AND BUFFERS PREPARATION

Phosphoric acid was purchased from Fluka (95% v/v, Buchs, Switzerland) or from Merck (99%, Darmstadt, Germany). Sodium peroxodisulfate (p.A.), ortho dihydrogen phosphate (p.A.), potassium hydrogen phosphate (p.A.), acetonitrile and methanol were purchased from Merck. Bovine insulin pancreas (28.3 USP units), non-acetylated bovine serum albumin, and the mixture of 17 amino acids were purchased from Sigma (St Louis, USA). Angiotensin III (Arg-Val-Tyr-Ile-His-Pro-Ile) was obtained from Feinbiochemica (Serva, Heidelberg, Germany). The amino acids Thr, Leu, Ile, Gly, Asn, Val, Ala, Gln, Ser, Pro, Arg, His, Lys, Tyr, Phe, were from Sigma. The [1-^{13}C]-labeled Phe, Ala and Val were purchased from Cambridge Isotope Laboratories (Innerberg, Switzerland). The [U-^{13}C] Thr was a gift from INRA

(Clermont-Ferrand, France). Deionised water was prepared using a Milli-Q system (18.2MΩ.cm).

Phosphate buffers (5mM, pH 3.3 and 150 mM, pH 4.2) were prepared with Milli-Q grade water; pH was adjusted with phosphoric acid. To decrease the ubiquitous $CO_2^{+\cdot}$ background signal *(m/z* 44, measured with a preamplifier feedback resistor of 300 MΩ), all reagents and LC eluents were thoroughly degassed in an ultrasonic bath (Digitana AG, Switzerland) at (30 ± 5)°C for (15 ± 5)min using a slight water vacuum. The concentration of the acid reagent was about 1.0molL^{-1} phosphoric acid in water, and the concentration of the sodium peroxodisulfate was about 0.5molL^{-1} in water. To avoid potential problems with the filament in the ion source due to the presence of O_2 produced by the oxidant reagent, the oxygen level (*m/z* 16, measured with a preamplifier feedback resistors of 300MΩ) was monitored each day. In our experiments, the signal for O^+ varied between 1.7 and 2.5V.

3.3.2 INSTRUMENTS

Experiments were carried out on a MAT252 IRMS (Finnigan MAT, Bremen, Germany). The IRMS was operated at an accelerating voltage of 10kV. The ion source was held at a pressure of 1 x 10^{-6}Torr, and ions generated by electron impact at 70eV. Three faraday cup detectors monitored simultaneously and continuously the $CO_2^{+\cdot}$ signals for the three major ions at *m/z* 44 ($^{12}CO_2$), *m/z* 45 ($^{13}CO_2$ and $^{12}C^{17}O^{16}O$) and *m/z* 46 ($^{12}C^{18}O^{16}O$). To obtain comparable signal outputs, the preamplifier feedback resistors were set at 300MΩ, 30GΩ and 100GΩ for *m/z* 44, *m/z* 45 and *m/z* 46, respectively (on this IRMS instrument, the full range of the three cups is 10V). Carbon dioxide from Carbogas (quality 57) was used as working reference gas, and was itself calibrated both against Iso Top CO_2 ($\delta^{13}C$= -25.40‰, Messer Griesheim, Krefeld, Germany) using the dual inlet of the MAT 252, and against NBS22 (oil, $\delta^{13}C$ = -29.73‰) using the EA-IRMS coupled to the same IRMS instrument.

An LC Isolink interface (Thermo Electron, Bremen, Germany) was coupled to the MAT252 without any modifications. The temperature of the interface reactor was set at 99.9°C. The helium (99.9998%) flow rate of the separation unit was set at 1 mLmin^{-1}. The eluent was delivered with a Rheos 2000 (Flux, Basel, Switzerland) liquid chromatography pump. This pump was carefully cleaned and purged to remove any trace of organic solvent; even the pump seals were replaced. This pump was dedicated to the inorganic buffer. Inside the LC interface, the tubes for the acid and oxidant reagents were in stainless steel (OD 1/16"). To connect the LC column to the Rheos pump and the manufactured interface, PEEK tubing and nuts were used with

0.005mm i.d. The tubes connecting the buffer bottles to the pump itself were manufactured in "No-Ox" material (1/8"x1.5 mm, Socochim, Lausanne, Switzerland). In contrast to PTFE tubing, "re-gassing" of the eluent was not observed. In addition, the reagent bottles were degassed with helium throughout the chromatographic run. The pump heads of the oxidant and acid pumps were rinsed with water at least twice a day to avoid crystallisation in the interface of buffers used at high concentration; the pump control software performed this procedure semi-automatically.

In addition to the LC interface, a Carlo Erba (Milan, Italy) 1100 CHNS elemental analyser (EA) was also coupled to the MAT252. The EA-IRMS instrument was used for control analysis of the expected isotopic values. The oxidation oven was filled with chromium (III) oxide and held at 1031°C. The reduction oven was filled with copper and held at 650°C. The GC column (Porapak PQS) was set to 60°C. The helium (99.9998%) carrier gas flow rate was adjusted to 120mLmin^{-1}. A water trap was installed and filled with magnesium perchlorate. The instrument was equipped with an autosampler for solid samples. The cycle time for one complete determination was 350sec.

The IRMS, data acquisition system and Rheos pump were controlled by an off-the-shelf PC running under Microsoft (Redmond, USA) Windows XP Professional. The IRMS instrument was controlled using Isodat 2.0SP1.43 (Thermo), while the LC pumps were controlled using Janeiro version 2.3 software (Flux). The elemental analyser was also controlled using the same Isodat software.

3.3.3 CALIBRATION AND ISOTOPIC CALCULATION

Three pulses of CO_2 reference gas were admitted in the inlet at the beginning of each run for about 20sec. The constant flow rates during this period gave these peaks flat top appearance. A level of CO_2 corresponding to (1.5 ± 0.5)V at *m/z* 44 was used to calibrate the analytes peaks.

Isotope ratios are expressed relative to international standards, rather than being reported as absolute isotope values. The $^{13}C/^{12}C$ abundance ratio was expressed as $\delta^{13}C$ values calibrated against the international standard (Vienna Pee Dee Belemnite, VPDB) (Slater et al., 2001a). The delta notation is defined as $\delta^{13}C_{\ sample}$, ‰ = $[(R_s / R_{st}) - 1] \times 1000$, where R_s is the ratio of ^{13}C in the sample and R_{st} is the ratio of the international standard used. The result of this calculation is a relative δ calibrated against the international standard.

Atom % was calculated as: Atom % = $\left[\frac{100\times R\times((\delta 13C/1000)+1)}{1+R\times((\delta 13C/1000)+1)}\right]$, where R is the ratio of ($^{13}C/^{12}C$) of International Standard of Pee Dee Belemnite, R=0.0112372. Atom% Excess (APE) is defined as Atom % (background) minus Atom % (sample).

3.3.4 HPLC PARAMETERS

For irm-LC/MS, the LC pump and the chromatography column were connected to a Rheodyne 7125 injection valve equipped with a 10µL loop. Samples were filtered through 0.2µm Nylon membrane filters (Nalgene, Waters, Switzerland). An in-line filter of 0.2µm (Vici, Schmidlin Labor, Switzerland) was also placed after the LC column to avoid particles passing into the interface. A 2D-LC method was developed for the underivatised amino acids used a strong cation exchange column (Luna SCX, 250×4.6mm, Brechbühler, Switzerland) and a reversed phase C30 column (Develosil-RP Aqueous column, 250 × 2.1mm, Brechbühler) connected in series. The columns were held at room temperature, (23 ± 2)°C. The LC flow rate was 250µL min^{-1}, and the flow rate of the acid and oxidant reagents in the LC interface was 50µLmin^{-1} each. The LC gradient was linear from 5mM KH_2PO_4 (pH 3.2) to 150mM KH_2PO_4 (pH 4.3) over 99min. The dead time of the 2D-LC system was about 735sec. For the FIA-LC/MS analyses, the eluent was simply degassed water. The flow rates used here was 300µLmin^{-1} and again 50µLmin^{-1} for the acid and oxidant reagents in the interface.

3.3.5 BIOLOGICAL SAMPLE PREPARATION AND ANALYSIS

For the animal study, male Sprague-Dawley rats (around 300 g) were obtained from Iffa-Crédo (Arbesle, Fance). They were allocated to individual cages and allowed free access to diet and water. In order to determine the *in vivo* synthesis rate of intestinal mucoproteins, the flooding dose method was used. A flooding dose (Garlick et al., 1980) of U-^{13}C Thr (10% of enrichment, 500µmol $100g^{-1}$ body weight, 99APE, Mass Trace, Woburn USA) was intravenously injected into the rats. The mucin was isolated and purified according to the method described by Faure *et al* (Faure et al., 2002). The dry samples were stored at -20°C. Subsequently, they were diluted with phosphate buffer (400µL), filtered and injected into the LC-IRMS. Originally, all the biological samples were used to validate the method and perform the isotopic analysis by GC-C-IRMS, and some samples from the same set were used in the

present study to demonstrate the applicability of the irm-LC/MS to tracer analysis. These experiments were approved by the Ethical Committee of Nestlé Research Center and by the Service Vétérinaire Cantonal (Lausanne, Switzerland).

3.4 RESULTS AND DISCUSSION

3.4.1 INFLUENCE OF LC FLOW RATE ON SIGNAL RESPONSE AND ON ISOTOPE RATIO

In IRMS, the signal recorded is a voltage that is proportional to the ion current (see Equation 1). The total peak area over time, A_{44} (using *m/z* 44), is related to the number of moles of $CO_2^{+\cdot}$ entering the ion source by the following equation (Sacks et al., 2003):

$$A_{44} = [CO_2]\frac{N_a e}{E} R_\Omega \qquad \textit{(Equation 1)}$$

where $[CO_2]$ is the number of moles of CO_2, N_a is the Avogadro number, e is the ion charge, E is the absolute sensitivity of the IRMS in molecules ion^{-1}, and R_Ω is the value of the feedback resistor in the amplifier.

Since an IRMS is a mass-flow-sensitive detector (in contrast e.g. to electrospray ionisation MS, which behaves like a concentration-dependant detector), the peak area for the same instrument depends on a number of different parameters such as i) the nature of the analyte, ii) the amount of analyte per time, iii) the efficiency of the membranes to extract the $CO_2^{+\cdot}$ from the eluent, iv) the helium flow rate within the membranes, and v) the split ratio in the open split interface. For the oxidant and the acid reagents, both reagents should be present in excess. A too-low concentration of oxidant will not be sufficient to oxidise the analyte completely, while a too-high concentration will lead to a production of gaseous O_2, which will cause deterioration of the filament.

In this work, we focus on the influence of the LC flow rate on the $CO_2^{+\cdot}$ signal with two model compounds of different molecular weight (131 and 897Da). Figure 24 shows the peak areas and peak heights of Leu and angiotensin III for different LC flow rates, obtained by flow injection analysis mode. For both model compounds, the

$CO_2^{+\cdot}$ signal shows the same trend. It can be seen that the peak height is only slightly affected by the LC flow rate. In contrast to this, the peak area decreases with increasing flow rate, mainly after 300µLmin^{-1}. This behaviour did not change if the reagent flow rate was increased from 50 to 100µLmin^{-1} (data not shown). These observations show that the $CO_2^{+\cdot}$ signal depends both on the IRMS detector and also on the interface itself. It should be noted that these LC flow rates are perfectly compatible with the use of narrow-bore HPLC columns of 2 and 3mm i.d.

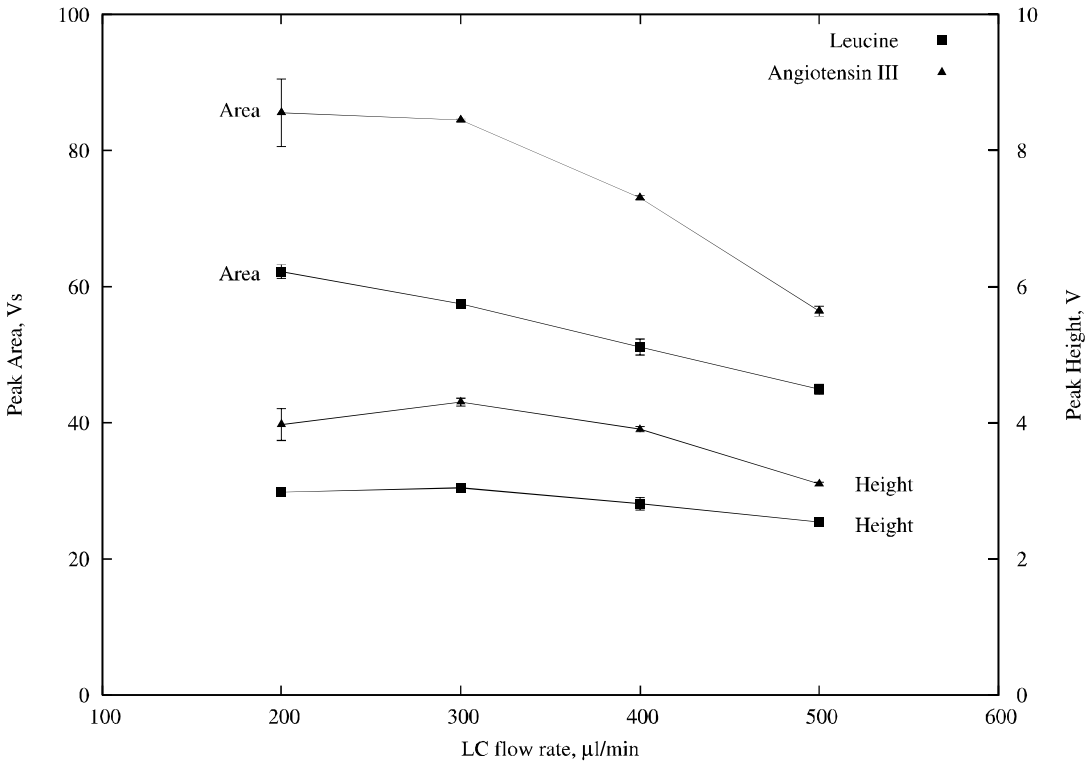


Figure 24: Relationship between $CO_2^{+\cdot}$ signal and LC flow rate in FIA-LC/MS. Data are averaged from triplicate determinations; injections were 750ng of Leu and 800ng of angiotensin III.

Using flow rates between 200 and 500µLmin^{-1}, the $\delta^{13}C$ values for Leu and angiotensin III were (-23.35 ± 0.73)‰ (n=12) and (–30.01 ± 0.91)‰ (n=12), respectively. The standard deviation (SD) is mainly due to the difference observed between low and high flow rates (200 and 500µLmin^{-1}, see Fig. 24). Careful inspection of Fig. 25 suggests that some systematic error is present that increases the deviation at low flow rates. Observing the non-corrected data in Fig. 25, the raw, non-corrected $\delta^{13}C$ values for Leu changes from –22.14‰ at 200µlmin^{-1} to –23.93‰

at 500µlmin^{-1}. Similarly, angiotensin III gives about –28.63‰ and –30.99‰ at these flow rates. Such an inaccuracy is generally regarded as unacceptable for natural abundance studies. Consequently, both a fixed flow rate and an internal standard with known isotopic composition referred as identical treatment "IT"(Werner & Brand, 2001) should be used to achieve good accuracy and precision of $\delta^{13}C$ values. Then, if an internal reference with known isotopic abundance is used in the same run (here, beet sugar, $\delta^{13}C$ =-25.38‰), the standard deviation of $\delta^{13}C$ values was considerably improved over the total range of flow rates used (Leu with $\delta^{13}C$= -23.27 ± 0.31‰, n=12); angiotensin III =-29.76 ± 0.39‰, n=12).

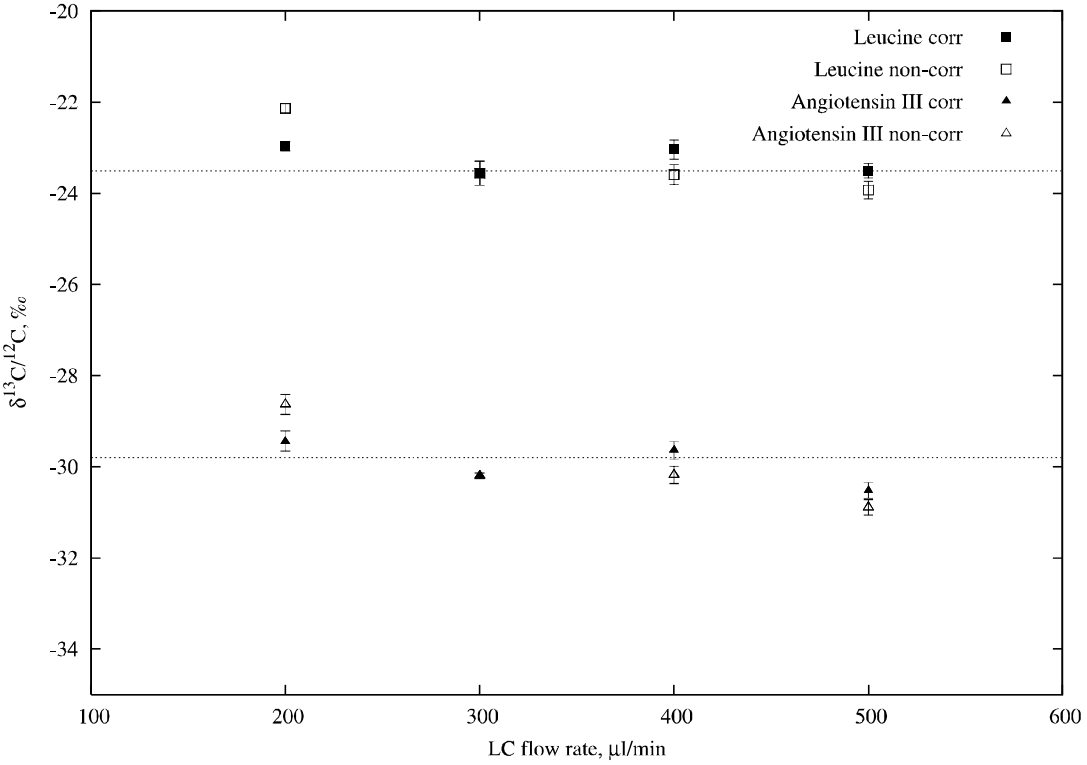


Figure 25: Variations of $\delta^{13}C$ values of Leu and angiotensin III as a function of LC flow rate. Injected were 750ng of Leu and 800ng of angiotensin III

In Figure 25, each data point represents triplicate injections with a standard deviation $\delta^{13}C$ values better than 0.3‰. Reagent flow was kept constant at 50µLmin^{-1}. The two dotted lines represent the $\delta^{13}C$ values of the same analyte assessed by EA-IRMS.

3.4.2 LINEAR RANGE AND LIMIT OF QUANTIFICATION BY FIA-LC/MS

Krummen *et al.* studied linearity, i.e. peak area versus amount of carbon injected for benzoic acid in the range of 300 to 2907ng of carbon (Krummen et al., 2004). Here, we investigated the linearity for Leu and angiotensin III at a lower range and used the values to assess their limit of quantification. All determinations were carried out in triplicate. For both Leu and angiotensin III, a linear relationship (not shown) was observed between peak area and the amount of carbon injected. For Leu, six data points were obtained corresponding to 293ng to 4.3ng of carbon (4.1 to 0.06nmol Leu); the slope of the regression line was 0.196 and a regression coefficient r^2 of 0.988. For angiotensin III, five data points from 327 to 20ng of carbon were obtained (0.6 to 0.04nmol); the slope of the regression line =0.221 and r^2 =1.000. The standard deviation of the $\delta^{13}C$ values in the range given was 0.20‰ for Leu and 1.00‰ for angiotensin III. This shows that the FIA-LC/MS instrument can be used for quantifying of low amounts of both low molecular weight (lmw) compounds and peptides, at least in the range from 20 to 300ng of carbon injected, with a precision of $\delta^{13}C$ values better than 1‰.

Besides linearity, the precision of $\delta^{13}C$ values is an important parameter. According to Schmitt's work in GC-C-IRMS (Schmitt et al., 2003), a "correction factor" expressed as a linear or logarithmic regression can be used to describe the changes of $\delta^{13}C$ values according to the range of compound injected. The results obtained for Leu and for angiotensin III confirm this (see Figure 26).

To assess the limit of quantification with the IRMS instrument, Brenna *et al.* (Brenna et al., 1997) recommended expressing the limit of quantification as the amount of (elemental) analyte, such as carbon, that is required to produce a specified precision (in ‰) of $\delta^{13}C$ values. It is also possible to use a higher gain of the amplifier of the *m/z* 45 cup to increase the limit of quantification for a labeled compound (Brenna et al., 1997; Mottram & Evershed, 2003). In our study, samples of non-labeled Leu and angiotensin III were used to assess the limit of quantification in flow injection analysis mode (FIA).

To assess the contribution of water injected with the bulk of sample to the $CO_2^{+\cdot}$ signal, water was injected separately, yielding a peak height and area of 143mV and 3Vs for *m/z* 44. Since this corresponds to the contribution of the blank, we can estimate that, in our conditions and for less than 0.5ng of carbon injected, the

standard deviation of the $\delta^{13}C$ will exceed 2‰ and therefore the results will be unreliable, even if identification and integration of the peak are still feasible. Therefore, we report as limit of quantification the value that yields a peak area of twice that of the water signal and for which the standard deviation of $\delta^{13}C$ value was better than 0.4‰. Using these considerations and under the specific conditions used here (e.g. a $CO_2^{+\cdot}$ baseline of 80mV with a noise of 5mV peak to peak), the limit of quantification was 7.3ng of carbon for Leu (100pmol of compound), and 19.7ng of carbon for angiotensin III (38pmol of compound). At the LOQ, the peak area was around 6V.s and the peak amplitude was around 330mV for both compounds. This is of the same order of magnitude as the limit of quantification for lmw compounds (such amino acids) in GC-C-IRMS, i.e. around 10ng of carbon.

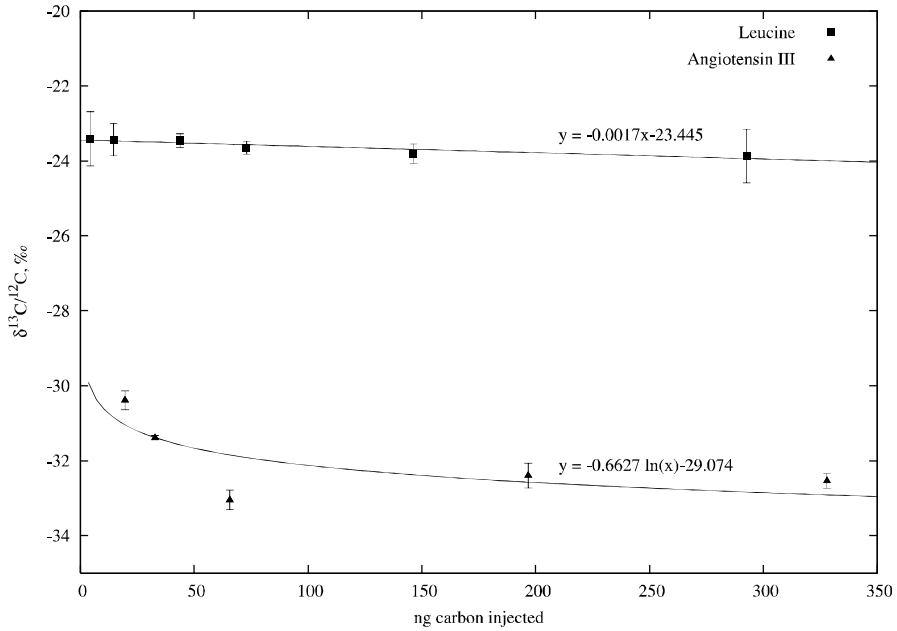


Figure 26: $\delta^{13}C$ values as a function of the amount of carbon injected in FIA-LC/MS.

In Figure 26, the $CO_2^{+\cdot}$ signal on m/z 44 ranged from 300mV at 7ng of carbon to 3V at 300ng of carbon injected (Leu). Each data point is the average of three injections with a standard deviation better than 0.4‰. The slope of the line for Leu is statistically significantly different from zero (p:0.01, n=6, 95% upper/lower slope confidence level at -0.0007 and -0.0027, respectively). For angiotensin III, an isotopic bias is observed with amounts below 70ng.

3.4.3 ACCURACY AND PRECISION OF $\delta^{13}C$ VALUES OF MACROMOLECULES

Several publications have reported isotopic analyses of macromolecules such as proteins using EA-IRMS (Apostol et al., 2001), LC-CRI-IRMS (Abramson et al., 1996) or LC-C-IRMS (Caimi & Brenna, 1996) instruments. Nevertheless, to date LC-IRMS was only used for lmw compounds, and no reports have appeared on the analysis of $\delta^{13}C$ values of peptides and proteins. We report here the isotopic analysis of such compounds, validated by comparing the results from EA-IRMS and FIA-LC/MS (Table 16).

For lmw compounds such as beet sugar, cane sugar and Leu, the precision of $\delta^{13}C$ value was better than 0.3‰ and the accuracy of $\delta^{13}C$ value was better than 0.2‰. For compounds with higher molecular weights, such as angiotensin III, bovine insulin and bovine albumin (897, 5733 and 66390Da, respectively), the precision was still better than 0.3‰. The accuracy of $\delta^{13}C$ value, expressed as the difference of the value obtained with EA-IRMS and FIA-LC/MS, was around 0.08‰ for peptides and around 0.7‰ for proteins. The observed bias for insulin and albumin (0.66 and 0.73‰, respectively) needs further investigation. Eventually, the bias could be correlated to the high molecular weight of the compound, and/or a combination of lower purity and less homogeneity of the sample.

Table 16: Accuracy and precision of $\delta^{13}C$ values obtained by EA-IRMS and FIA-LC/MS

		EA-IRMS	FIA-LC/MS	Amount inj. FIA		Δ (EA-FIA)
Compounds	MW	$\delta^{13}C \pm SD$, ‰	$\delta^{13}C \pm SD$, ‰	nmol	ng C	Δ (‰)
Beet sugar	180	-25,55 ± 0.08	-25.38 ± 0.23	2.8	201.6	0.17
Cane sugar	180	-11,71 ± 0.25	-11.78 ± 0.03	5.6	403.2	0.07
Leu	131	- 23.51 ± 0.39	-23.66 ± 0.04	6.8	489.6	0.15
Angiotensin III	897	- 29.81 ± 0.06	-29.89 ± 0.24	0.21	109.9	0.08
Bovine insulin	5733	- 14.20 ± 0.04	-14.86 ± 0.04	0.26	647.6	0.66
Bovine albumin	66390	- 12.88 ± 0.39	-13.61 ± 0.07	0.01	381.8	0.73

Data represent three replicates each. The number of carbon atoms is 254 and 2934 for bovine insulin and bovine albumin, respectively. The Δ (EA-FIA) is calculated as ($\delta^{13}C$ of EA-IRMS) – ($\delta^{13}C$ of FIA-LC/MS). SD is the standard deviation.

3.4.4 CHROMATOGRAPHIC CONSIDERATIONS

When using GC-C-IRMS, Meier-Augenstein (Meier-Augenstein, 1999) pointed out that baseline separation of analyte peaks is mandatory for a precise determinations of $\delta^{13}C$ values. Therefore, a pivotal analytical challenge for coupling LC to IRMS is to develop a LC method that allows baseline separation of analytes within a given mixture. Figure 27 is a schematic summary of different analytical approaches, which can be useful in isotope ratio monitoring to deconvolute biological samples. Besides column chemistry, different operational parameters can be used (within certain practical limits) to decrease the peak width in an on-line approach, such as column diameter, column packing particle size, column temperature, LC flow rate, and the helium flow rate in the interface. If satisfactory resolution between two components cannot be reached, Goodman and Brenna suggested that curve fitting could be employed to correct $\delta^{13}C$ values (Goodman & Brenna, 1994).

Due to its design, the LC interface used here presents several practical and analytical constraints that need to be considered. The main constraint is that no organic solvent or organic buffer can be used, as any organic components of the buffer will produce a $CO_2^{+\cdot}$ background, which would introduce a significant offset and thus reduce the dynamic range. Experiments performed using the flow injection analysis mode with "classical" organic modifiers, such as acetonitrile and methanol, showed that with only 0.01% v/v of such organic modifiers, the $CO_2^{+\cdot}$ signal was about 40% full scale, which covers almost all of the dynamic range of the instrument. Since LC separations employing organic modifiers usually require at least two orders of magnitude higher concentrations, the IRMS would become saturated with the $CO_2^{+\cdot}$ signal from these compounds. In addition, due to mechanical constraints, the LC flow rate entering the interface must not exceed $500\mu L min^{-1}$, and the total flow rate of LC plus oxidant plus acid must be below $700\mu L min^{-1}$. Furthermore, the pH of the mobile phase must be acidic to ensure CO_2 extraction from the eluent (Krummen et al., 2004).

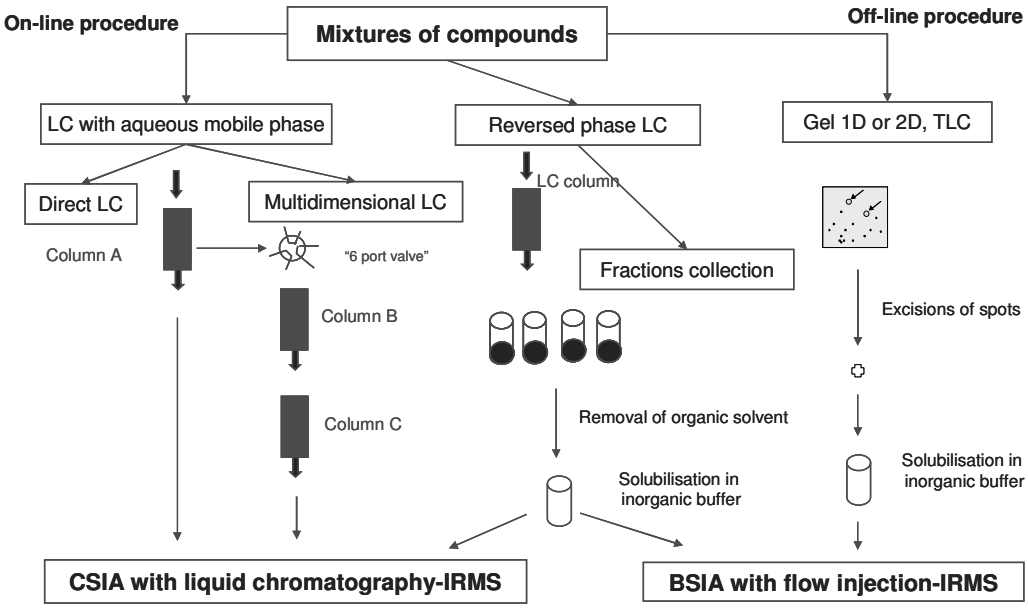


Figure 27: Analytical approaches to deconvolute biological samples for CSIA or BSIA

3.4.5 IRM-LC/MS OF UNDERIVATISED AMINO ACIDS

To date, the analysis of free amino acids by reversed-phase LC is usually performed after derivatisation with non-polar reagents such as fluorescent 9-fluorenylmethyl carbonate. For underivatised amino acid analysis, most of the methods involve an anion exchange chromatography or ion-pair reversed-phase chromatography (Chaimbault et al., 2000; Petritis et al., 2001). The addition of organic modifiers, the derivatisation steps and the basic pH used are inappropriate for accurate isotopic analysis by irm-LC/MS. The following protocol was developed for the LC separation of underivatised amino acids that is fully compatible with IRMS.

Our approach involves the coupling of a strong cation exchange column, directly to a reversed phase (RP-C30) column. The advantage of this combination is their compatibility with aqueous buffers (Majors & Przybyciel, 2003). The RP-C30 phase avoids the "phase collapse" phenomenon that is commonly observed due to the highly aqueous nature of the mobile phase in combination with the very long alkyl chains which are probably in a solid state at room temperature (which is not the case for RP-C8 or RP-C18 columns) (Majors & Przybyciel, 2002). The long alkyl chains of the RP-C30 column is also known to resolve polar compounds by increasing their retention time due to the increased of the hydrophobicity of the stationary phase.

Thus, the mobile phase used here was an aqueous buffer with a gradient in concentration and pH of phosphate buffer.

A representative irm-LC/MS chromatogram of underivatised amino acids is presented in Figure 28. 5 flat top peaks in the beginning and during the run are the CO_2 reference gas pulses. Assignment of the 15 underivatised amino acids was based on their retention times compared to standard compounds. The overall mechanism of retention of these compounds is probably based on a mixed mode of separation, combining ion exchange and reversed-phase behaviour. The influence of this mode of separation is demonstrated (Fig. 29) by injecting a commercial mixture of 17 amino acids separately onto each column. The chromatogram in Figure 28 shows that using this 2D-LC baseline separation can be achieved for 11 of the 15 amino acids. The basic amino acids such as Arg, Lys, His as well as Phe, Ser, Thr, Gln, Gly, Asn were well resolved, while Ala, Val, Pro, Leu and Tyr coelute. To further separate these components, an additional step, such as column switching, may be necessary.

3.4.6 ISOTOPE SWING OF AMINO ACIDS ISOTOPOMERS

In GC, it is known that ^{13}C labeled compounds have a slightly shorter chromatographic retention time compared to their ^{12}C-labeled counterparts (ca. 100-150 ms) (Brand, 1996)·(Meier-Augenstein, 1999). If the ratio of the signals at *m/z* 45 and *m/z* 44 is plotted, this yields a characteristic signal with a bimodal oscillation, knows as the isotope swing. Under the irm-LC/MS operating conditions described above with a $CO_2^{+\cdot}$ background level of 210mV, the chromatogram of the fifteen amino acids clearly showed that this isotope swing is opposite to that observed in GC-C-IRMS (see Fig. 28). The isotope swing is disappearing over time, which may be due to modification of the chromatographic isotope effect on these conditions.

The separation of ^{13}C and ^{12}C isotopomers shows that the ^{12}C isomers have a slightly shorter retention times than their ^{13}C counterparts. This may be explained by the two mixed modes of separation used in this method: According to Filer (Filer, 1999), ^{14}C labeled amino acids can migrate slower than their corresponding ^{12}C counterparts using ion exchange chromatography with a pH gradient. On the other hand, in reversed-phase LC the elution order of the isotopomers depends on the nature of the compounds and on the exact chromatographic conditions (Caimi & Brenna, 1997): For Leu with ion-pairing agents, the ^{12}C isotopomer elutes first (Baumann et al., 1992).

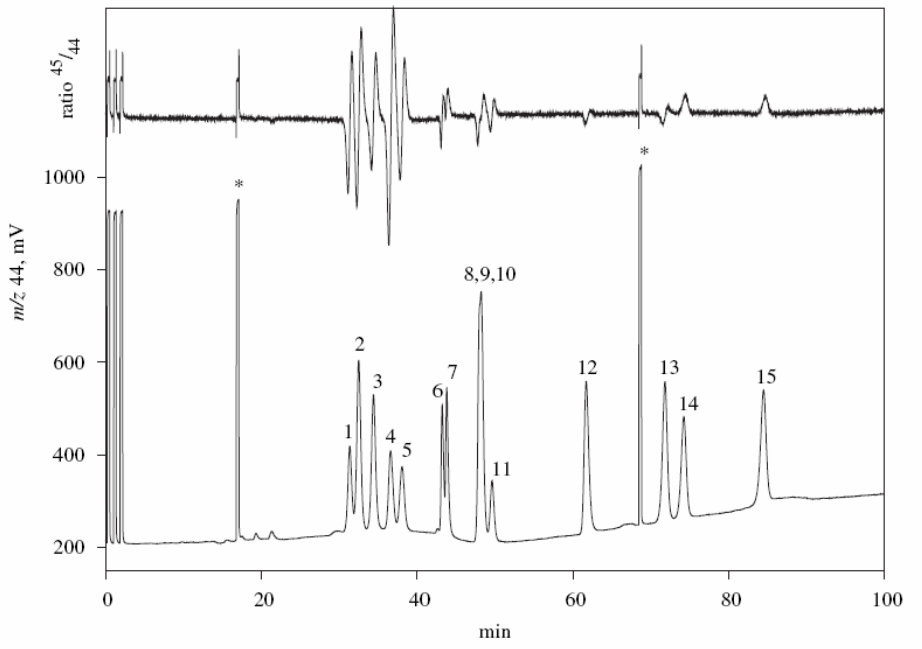


Figure 28: Chromatogram of 15 underivatised amino acids, obtained by irm-LC/MS.

In Figure 28, the amount injected was (12.8 ± 3.7) nmol per amino acid. The lower trace shows the signal at m/z 44, the upper trace demonstrates the bimodal oscillation (isotope swing) of the ratio m/z (45/44). Amino acids elute in the following order: 1, Ser; 2, Thr; 3, Gln; 4, Gly; 5, Asn; 6, Pro; 7, Val; 8, Ala; 9, Leu; 10, Tyr; 11, Ile; 12, Phe; 13, Lys; 14, His; 15, Arg. The asterisks denote reference gas peaks

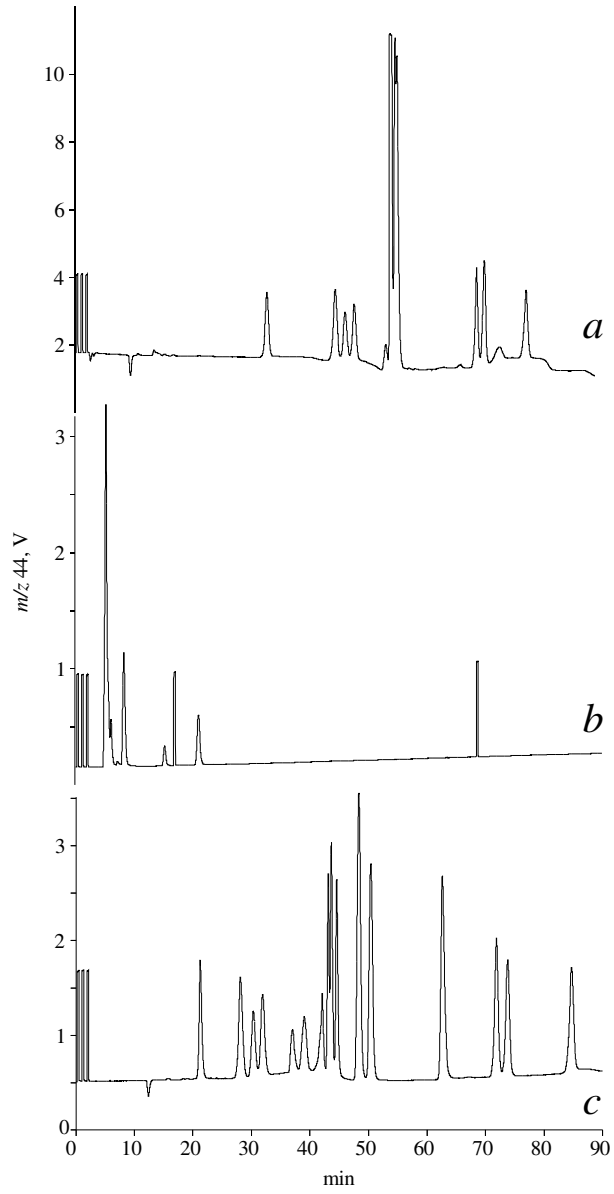


Figure 29: Separation of 17 underivatised amino acids using different LC columns but with the same LC flow rate and the same gradient. a) SCX column only; b) RP-C30 column only; c) mixture injected on serial combination of SCX and RP-C30 columns connected in series

3.4.7 PRECISION OF ISOTOPIC RATIOS OF NON-LABELED AND LABELED UNDERIVATISED AMINO ACIDS

Another problem that influences irm-LC/MS is column bleeding. To perform accurate measurements of $\delta^{13}C$ values, it is mandatory to evaluate the stability of the $CO_2^{+\cdot}$ background, since any imprecision will increase the uncertainty in the calculated $\delta^{13}C$ values and decrease the dynamic range. The $CO_2^{+\cdot}$ background obtained with our 2D-LC columns was typically 500 ± 250 mV. The variation of 250mV, observed over a period of several days, was probably due to residual organic solvent present in the columns, which was completely eliminated only after several days of use. The increase of the background during a gradient run (99min) was around 100mV. To determine the influence of this variation on the precision of $\delta^{13}C$ values during a run a standard "on-off" procedure was performed, normally used to assess signal stability, during the LC run. A set of three reference gas pulses ($\delta^{13}C$= -7.47‰) is admitted to the ion source every 1000sec. Although this procedure does not take into account the increasing peak width through the LC gradient run, an offset of the $\delta^{13}C$ values of 0.5 and 0.8‰ between the first set of CO_2 pulses and the subsequent of pulses at 3000 and 6000sec was observed, corresponding to $\delta^{13}C$ values of (-7.53 ± 0.05)‰, (-7.23 ± 0.1)‰ and (-6.75 ± 0.10)‰, respectively. This means that the corresponding drift rate was around 0.010‰min^{-1} (n=2). This drift is a typical drawback of the gradient elution, but pulses of reference CO_2 gas during the run may be used to compensate for it.

We investigated the precision of the $\delta^{13}C$ values through the LC run with four underivatised amino acids, Thr, Val, Ala and Phe, eluting between 30 and 60min. These amino acids were injected both at natural abundance and at low isotopic enrichment, and the results are reported in Table 14. At natural abundance, the precision of the $\delta^{13}C$ value was better than 0.2‰ (or <0.0001atom%), and the intra-assay coefficient of variation (CV) was 0.01%, which is in agreement with the data for derivatised Leu at natural abundance reported by Yarasheski *et al.* using GC-C-IRMS (Yarasheski et al., 1992).

For the labeled amino acids, the precision of the $\delta^{13}C$ values shown in Table 14 varied according to the amino acid and its enrichment. For Thr and Ala, the precision of isotopic ratios was 0.6 and 1‰ or 0.0006 and 0.001atom%, respectively, giving an intra-assay coefficient of variation of 0.9 and 1.3% for 0.07atom% excess (APE). For Val and Phe, the precision was 0.1 and 0.6‰ or 0.0001 and 0.0006 atom%,

respectively, giving an intra-assay coefficient of variation of 10.7 and 7.5% for 0.03APE. This is also in the same range as the values reported by Yarasheski *et al.* by GC-C-IRMS (CV of 3% for 0.08APE, 15% for 0.02APE) (Yarasheski et al., 1992).

Table 17: Intra-assay precision of unlabeled (natural abundance) and ^{13}C-labeled standard amino acids, analysed by irm-LC/MS

Non-labelled amino acids				^{13}C-Labelled amino acids			
Amino acid	$\delta^{13}C \pm SD$	atom% ± SD	CV in %	APE cal.	$\delta^{13}C \pm SD$	APE meas. ± SD	CV in %
Thr	-31.00 ± 0.12	1.0772 ± 0.0001	0.011	0.1254	33.12 ± 0.55	0.0704 ± 0.0006	0.9
Ala	-22.88 ± 0.12	1.0861± 0.0001	0.011	0.1328	49.52 ± 0.94	0.0795 ± 0.0010	1.3
Val	-10.75 ± 0.15	1.0994 ± 0.0002	0.015	0.0268	16.44 ± 0.10	0.0281 ± 0.0030	10.7
Phe	-26.88 ± 0.12	1.0817 ± 0.0001	0.011	0.0261	5.73 ± 0.64	0.0343 ± 0.0026	7.5

Data represent triplicate injections of 10µL each. The labeled mixtures were prepared using known amounts of natural (5 to 7nmol) and ^{13}C-labeled amino acids (0.01 to 0.03nmol).

3.4.8 BIOLOGICAL APPLICATIONS

One of the many applications of ^{13}C tracers is in the study of protein and amino acid metabolism. Different tracer methods have been used to investigate protein synthesis, catabolism and protein oxidation (Wagenmakers, 1999). In humans, the determination of the muscle protein fractional synthesis rate is based on the use of labeled amino acids administrated intravenously or orally. Demonstrated here is the applicability of the irm-LC/MS coupling to ^{13}C tracer analysis of mucoproteins from rat colon.

The mucus gel protects the mucosa against stomach acidity and proteolytic enzyme activities. Its main role is to provide a physical barrier against bacteria and pathogens. Its capacity to protect epithelial surfaces is due to glycoproteins called mucins or mucoproteins (Faure et al., 2002). However, many gastrointestinal diseases such as inflammatory bowel diseases, ulcerative colitis, and Crohn's disease have been associated with disorders of mucoprotein expression (Corfield et al., 2000). In many of these pathologies, the mucus gel thickness and the mucoprotein production were altered. To assess the *in vivo* protein and mucoprotein synthesis rate using the flooding dose method, determinations of the ^{13}C enrichment of free ^{13}C labeled amino acids (intracellular) and protein (or mucoprotein) bound amino acid need to be performed. Currently, the standard technique to determine the ^{13}C-labelling of tracer

amino acids in the free pool is GC-MS. This is an adequate technique for determining high enrichment levels. However, for amino acids bound to proteins (with low enrichment), the GC-C-IRMS approach offers enhancement of precision and sensitivity. However, as mentioned above, the amino acids need to be derivatised (Glaser & Amelung, 2002; Metges & Daenzer, 2000; Zaideh et al., 2001), which alters the $\delta^{13}C$ values of the investigated amino acids derivative introducing an additional source of errors into the isotopic determinations, especially at natural abundance (Docherty et al., 2001). Therefore, irm-LC/MS would be an ideal alternative to GC-C-IRMS since it allows for a simple and straightforward preparation (dilution, filtration and injection) and potentially reduced error.

The 2D-LC separation described above was applied to a mixture of mucoproteins. To check the specificity of the develop 2D-LC method, a mixture of fifteen underivatised amino acids (Fig. 30) that comprise mucoproteins purified from rat jejunum mucosa was injected (Faure et al., 2002) The peak for Thr (eluting at 33 min; peak 2 in Fig. 28) was readily detected and well separated from the other underivatised amino acids. A representative irm-LC/MS chromatogram of mucin originating from the rat colon is shown in Figure 30. The average $\delta^{13}C$ value for the labeled Thr (n=3) was 44.08 ± 0.54‰ (or 1.160 ± 0.001atom%), which shows that irm-LC/MS can be used to perform isotopic analyses of biological sample achieving a precision comparable to GC-C-IRMS, and avoiding derivatisation. A detailed biological study is ongoing, and details will be reported at a later date.

It should be noted, however, that verifying the peak purity of the compound of interest is essential in the stable isotopic analysis of components of complex mixtures. One way to achieve this is to use other detectors, such as UV or electrospray-MS, either on-line or off-line. However, such detectors present problems of compatibility due to the high content of inorganic salt in the mobile phase and the poor response of underivatised amino acids to a UV detector.

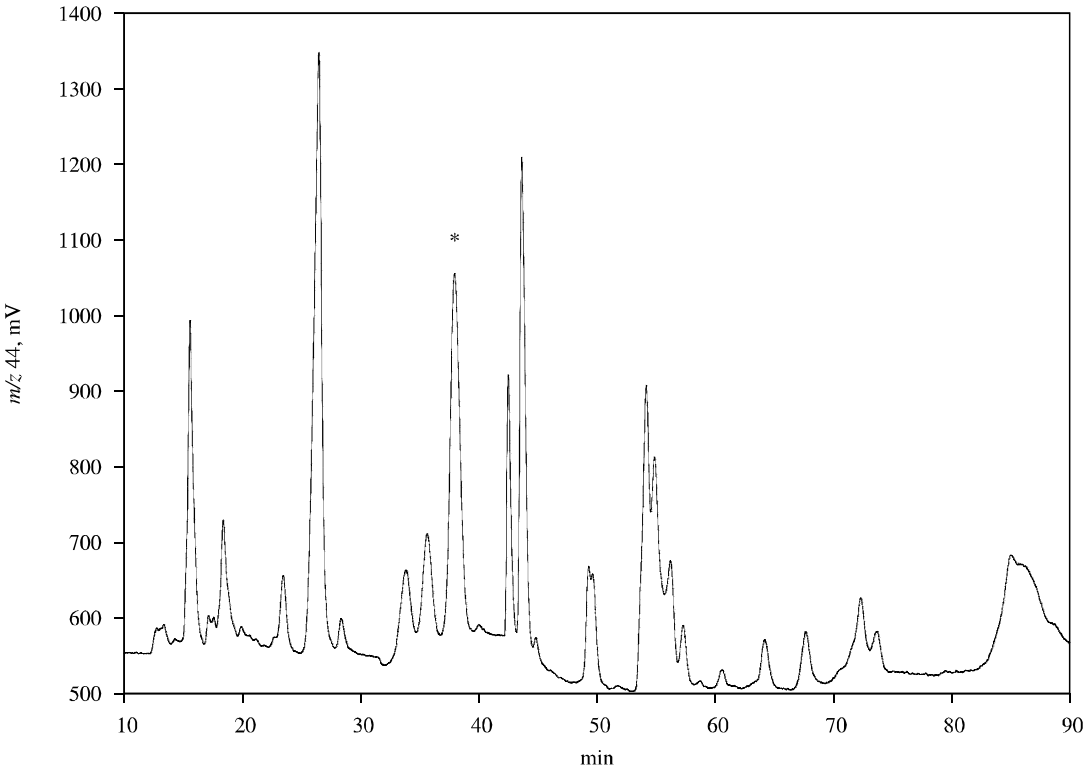


Figure 30: LC-IRMS chromatogram of undervivatised amino acids from mucin extract from rat colon.

In Figure 30, the average of the isotopic enrichment of ^{13}C-labeled Thr, marked by the asterisk, was $\delta^{13}C$ value = 44.08 ± 0.54‰, equivalent to 1.160 ± 0.001atom% ($\delta^{13}C$ values obtained were 43.55, 44.05 and 44.63‰).

3.5 CONCLUSIONS

The irm-LC/MS instrument has been shown to be applicable to the analysis of organic compounds of very different molecular mass. One of principle advantage is that isotopic determinations can be performed both for small and macromolecules avoiding the complex sample preparation procedures needed for GC-C-IRMS analysis (e.g. derivatisation, hydrolysis).

Flow injection isotopic analyses of low and high molecular weight compounds shows for the first time that the analysis of compounds ranging from 130 to 66390Da can be performed with low amounts of material and with the precision of the $\delta^{13}C$ values better than 0.3‰.

In a flow range between 200 and 500µLmin^{-1}, the interface is directly compatible with the use of narrow-bore LC columns, and the $CO_2^{+\cdot}$ signal is nearly independent of the LC flow rate.

The 2D-LC separation for the fifteen underivatised amino acids can be applied to assess the carbon isotope composition of underivatised amino acids at natural abundance and at tracer levels. Nevertheless, for some amino acids with very close retention times, the method still needs to be fully optimised to assess the isotopic compositions of all components in complex mixtures.

CHAPTER 4

^{13}C isotopic analyses of valine by GC-C-IRMS and LC-IRMS

Keywords: valine, GC-C-IRMS, LC-IRMS, MPE, APE

4 LIQUID AND GAS CHROMATOGRAPHY COUPLED TO IRMS FOR THE DETERMINATION OF ^{13}C VALINE ISOTOPIC RATIOS IN COMPLEX BIOLOGICAL SAMPLES

In press ***Journal of Mass Spectrometry,*** *2008, by Jean-Philippe Godin, Denis Breuillé, Christiane Obled, Isabelle Papet, Henk Schierbeek, Gérard Hopfgartner and Laurent-Bernard Fay*

4.1 CONTEXT

Three years after its first commercialisation, only a limited number of papers were published using the LC-IRMS device showing reliable data for biological ^{13}C isotopic ratio measurements. Schierbeek et al. (Schierbeek et al., 2007) published a work focused on the measurement of the FSR of glutathione in newborn after administration of ^{13}C glycine as precursor. The underivatised glutathione (GSH) is separated (especially its native and oxidised forms) from amino acids in erythrocyte using a mixed mode column (see Figure 31 for the LC-IRMS separation). McCullagh showed also an elegant LC-IRMS separation of 15 underivatised amino acids. However, to the best of our knowledge, no papers have been published about the comparison of ^{13}C isotopic enrichments carried out by the GC-C-IRMS and the LC-IRMS devices.

In the next chapter, we proposed to perform ^{13}C isotopic measurements of native and derivative valine by LC-IRMS and GC-C-IRMS, respectively. The work will:

- Report a new LC-IRMS method for measuring isotopic ratio of valine
- Compare isotopic enrichments between LC-IRMS and GC-C-IRMS for relative and absolute isotopic enrichments
- Study a possible isotopic fractionation for the N(O,S)-ethoxycarbonly ethyl ester derivative of valine.

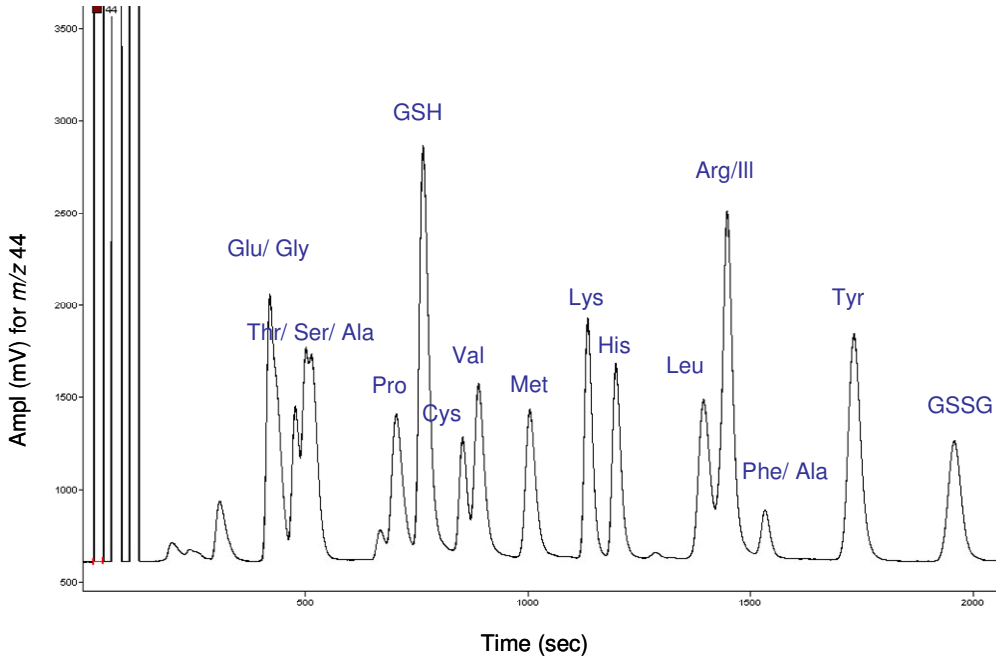


Figure 31: LC-IRMS chromatogram of standard underivatised amino acids with a spiked of glutathione using a mixed mode column with sulphuric acid as LC buffer (Schierbeek et al.)

4.2 INTRODUCTION

In 2004, the commercialization of an interface allowing to couple Liquid Chromatography (LC) with Isotope Ratio Mass Spectrometry (IRMS) added a novel analytical tool (LC-IRMS) for high-precision ^{13}C isotopic analysis in the domain of continuous-flow techniques. With LC-IRMS, isotopic analyses of polar and thermo-labile compounds such as amino acids (Krummen et al., 2004; McCullagh et al., 2006), sugars (Cabanero et al., 2006), organic acids (Heuer et al., 2006), standard macromolecules (Godin et al., 2005) and gluthatione (Schierbeek et al., 2007) are now straightforward and give reliable results. If compared to a gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS) or to an elemental analyzer coupled to IRMS (EA-IRMS) instruments, the LC-IRMS technique presents very few specific requirements in terms of method development and amount of compound needed (Godin et al., 2007). Briefly, with the LC-IRMS instrument, only inorganic buffers can be used as the LC eluents entering in the interface and the LC

flow rate is limited to 700µLmin^{-1}. Moreover, by LC-IRMS, the amount of material needed is 3 order of magnitude less than what is needed by EA-IRMS (a few hundred nanograms of carbon by LC-IRMS versus a few hundred micrograms by EA-IRMS). When comparing the LC-IRMS to GC-C-IRMS, which is still the state of the art for CSIA (compound specific isotopic analysis) in various areas (Aguilera et al., 2002; Ando & Tanaka, 2005; Lichtfouse, 2000; Meier-Augenstein, 1999; Schmidt et al., 2004), the effects of the derivatisation needed in the latter must also be considered. This step affects the physico-chemical properties of the compounds, but also its $^{13}C/^{12}C$ ratio by adding extra carbons brought in by the derivative. This chemical modification of the targeted compound can lead to an isotopic fractionation due to a kinetic isotopic effect and an isotopic dilution which might consequently produce an isotopic error (Docherty et al., 2001; Rieley, 1994). For studies performed with compounds at natural abundance by GC-C-IRMS (reported with relative isotopic value with $\delta^{13}C$ ‰ as units), a mass balance equation (see equation 1) must be used to remove the carbon contribution added by the derivative (Gross & Glaser, 2004; Silfer et al., 1991). On the other hand, using LC-IRMS, the derivatisation step is not needed as the targeted compound is analyzed directly. By contrast in the tracer studies where the interest is not in the knowledge of the relative isotopic ratio ($\delta^{13}C$) but rather on the absolute isotopic enrichment of the sample (expressed as Atom% or Atom Percent Excess or Molar Percent Excess, APE and MPE respectively), the mass balance equation is rarely used whatever the devices (LC-IRMS and GC-C-IRMS). Additionally, GC and LC features have other key parameters which need to be optimized in order to obtain precise and accurate isotopic ratio (Godin et al., 2007; Meier-Augenstein et al., 1996; Sessions, 2006).

Only a few papers have been published concerning the comparison of the isotopic precision and accuracy at natural abundance and above performed by both GC-C-IRMS and other isotopic methods (Demmelmair & Schmidt, 1993; Guo et al., 1993; Wong et al., 1995). To the best of our knowledge, comparison of isotopic precision/accuracy between LC-IRMS and GC-C-IRMS as well as the correlation for relative/absolute isotopic enrichments has never been reported in a peer-reviewed publication. It would be important to obtain this information, allowing the building of “bridges” between the two techniques thus preventing misinterpretation of the results and other sources of error.

The main objective of this chapter was to obtain a comprehensive view of ^{13}C isotopic data generated by GC-C-IRMS and LC-IRMS. For this purpose we developed a new LC-IRMS method enabling the measurement of the isotopic ratio of several amino acids (Asp, Thr-Ser, Glu, Pro, Gly, Ala, Cys and Val). Subsequently, ^{13}C isotopic enrichments were acquired for Val by LC-IRMS and by GC-C-IRMS devices to assess the isotopic precision and accuracy of both devices. Accordingly, we also derived the mathematical equations and we built the corresponding curves linking the relative and absolute isotopic enrichments acquired both by GC-C-IRMS and LC-IRMS. Finally, both systems (GC-C-IRMS and LC-IRMS) were used for measuring ^{13}C Val in bone samples within the frame of a tracer study.

4.3 EXPERIMENTAL SECTION

4.3.1 CHEMICALS

Phosphoric acid, sodium peroxodisulfate (p.A.), potassium hydrogen phosphate (p.A.) and hydrochloric acid (p.A) were purchased from Merck. The mixture of 17 amino acids was purchased from Sigma (St Louis, USA). The ^{13}C-labeled Val was purchased from Cambridge Isotope Laboratories (Innerberg, Switzerland). Deionised water was prepared using a Milli-Q system (with a resistance level equal to 18.2 MΩ.cm).

Phosphate buffers were prepared with Milli-Q grade water; pH was adjusted with hydrochloric acid (Wolfe, 1992). To decrease the ubiquitous CO_2^+ background signal *(m/z* 44, measured with a preamplifier feedback resistor of 300 MΩ), all reagents and LC eluents were thoroughly degassed in an ultrasonic bath (Digitana AG, Switzerland) at (30 ± 5℃ for 15 ± 5min using a water vacuum). The concentration of the acidic reagent was approx. 1.0molL^{-1} phosphoric acid in water, and the concentration of sodium peroxodisulfate was approx. 0.5molL^{-1} in water.

For the standard solutions, approximately 2.1molL^{-1} of natural Val and 0.9molL^{-1} of ^{13}C-Val were prepared. Then by adequate dilution, standard solutions between 0.0 and 2.3 MPE with a final concentration at approximately 0.5molL^{-1} were performed.

4.3.2 INSTRUMENTS (LC-IRMS AND GC-C-IRMS)

Experiments were carried out on a MAT252 IRMS (Finnigan MAT, Bremen, Germany). The IRMS was operated at an accelerating voltage of 8kV. The ion source

was held at a pressure of 1 x 10^{-6}Torr, and ions were generated by electron impact at 70eV. Three faraday cup detectors monitored simultaneously and continuously the CO_2^+ signals for the three major ions at *m/z* 44 ($^{12}CO_2$), *m/z* 45 ($^{13}CO_2$ and $^{12}C^{17}O^{16}O$) and *m/z* 46 ($^{12}C^{18}O^{16}O$). To obtain comparable signal outputs, the preamplifier feedback resistors were selected with 300MΩ, 30GΩ and 100GΩ for *m/z* 44, *m/z* 45 and *m/z* 46, respectively (on this IRMS instrument, the maximum signal level is 10V). Carbon dioxide from Carbogas (quality 57) was used as working reference gas, and was itself calibrated against Iso Top CO_2 (Messer Griesheim, Krefeld, Germany) using the dual inlet of MAT252.

An LC Isolink® interface (Thermo Electron, Bremen, Germany) was coupled to the MAT252 without any modification. The temperature of the interface reactor was set at 99.9°C. The helium (99.9998 %) flow rate of the separation unit was set at 1mLmin^{-1}. The liquid chromatographic eluents were delivered with two Rheos 2000 (Flux, Basel, Switzerland) liquid chromatography pumps. The injection was performed with a CTC auto sampler equipped with a 10µL loop. This instrument was also used to perform flow injection isotopic analysis (FIA-IRMS) meaning injection without any LC column.

A Hewlett Packard 5890 series II gas chromatograph interfaced with the same MAT 252 IRMS was used to perform the GC-C-IRMS experiments. The combustion interface consisting of a NiO/CuO/Pt oxidation furnace reactor set at 940°C, and a copper reduction furnace heated at 600°C. A water trap Nafion® was placed between the reduction furnace and the isotope ratio mass spectrometer. The ^{13}C isotopic enrichment of derivatised Val was determined by monitoring ions of masses *m/z* 44, *m/z* 45 and *m/z* 46 of CO_2 gas. A CTC Pal auto sampler (Switzerland) was used to inject 1µL of each sample.

Data acquired with LC, FIA and GC-C-IRMS were processed using the Finnigan Isodat NT software.

4.3.3 UNITS OF ISOTOPIC ABUNDANCE AND CALCULATIONS

The mass balance equation (Equation 1) is generally used to take into account the exogenous carbon added during the GC-C-IRMS derivatisation to obtain the "corrected" isotopic ratio (Rieley, 1994). The concept of this equation is that a heavy isotope in a product is the sum of the heavy isotopes from precursors (Hayes, 1983).

$$m_T F_T = m_1 F_1 + m_2 F_2 \qquad (\textit{Equation 1})$$

where the 'm' terms represent the molar quantities of the element and the 'F' terms represent the fractional isotopic abundances.
The fractional isotopic abundance is defined in Equation 2 as:

$$F_{^{13}C} = \frac{[^{13}C]}{[^{12}C]+[^{13}C]} \qquad (Equation\ 2)$$

Due to isotopic differences between samples at natural abundance, which may occur in the third or fourth digit after the decimal point, the isotopic ratio is also expressed with a delta unit (Slater et al., 2001b). Thus, the $^{13}C/^{12}C$ isotopic ratio is expressed as a $\delta^{13}C$ value calibrated against the international standard (Vienna Pee Dee Belemnite, VPDB). The delta notation which is a measure of the relative isotopic concentration, is defined as:

$$\delta^{13}C_{sample}\ , ‰ = [(R_s / R_{std}) - 1] \times 1000, \qquad (Equation\ 3)$$

where R_s is the ratio of $^{13}C/^{12}C$ in the sample and R_{std} is the ratio of ($^{13}C/^{12}C$) of the International Standard of Pee Dee Belemnite, R_{std}=0.0112372. For tracer studies absolute isotopic ratio is preferred with Atom%, APE (Atom Percent Excess) and MPE (Molar Percent Excess) as possible units. Atom% value refers to the number of heavy atoms compared to the total number of atoms of the element (Equation 4).

$$\text{Atom \% } ^{13}C = \frac{[^{13}C]}{[^{12}C]+[^{13}C]} \times 100 \qquad (Equation\ 4)$$

To transform $\delta^{13}C$ to Atom%, equations 3 and 4 are rearranged to obtain Equation 5.

$$\text{Atom\%} = \left[\frac{100 \times R \times \left(\left(\delta^{13}C/1000\right)+1\right)}{1+R \times \left(\left(\delta^{13}C/1000\right)+1\right)}\right] \qquad (Equation\ 5)$$

Subsequently, Atom% can also be used to determine APE value using Equation 6 and then MPE (see Equation 7) where n_{total} and $n_{labeled}$ are respectively the total number of carbons in the molecule and the number of labeled carbons in the

molecule. APE and MPE refer respectively to the excess of Atom fraction or Mole fraction of heavy species.

$$APE = Atom\ \%_{\ Sample} - Atom\ \%_{\ Basal} \qquad (Equation\ 6)$$

$$MPE = APE \times \frac{(n_{total})}{(n_{labelled})} \qquad (Equation\ 7)$$

In practice, by weighing the heavy molecule (^{13}C-labeled compound) and the non-labeled compound (at natural abundance), and by taking into account the isotopic purity of compounds (Xa and Xb), the absolute theoretical mole ratio (MPE_{theo}) of heavy molecule can be determined using Equation 8.

$$MPE_{theo} = \frac{[X_a N_a]}{[X_a N_a + X_b N_b]} \qquad (Equation\ 8)$$

where N_a and N_b are respectively the number of moles of heavy and light molecules.
A paired t-test was used to test the significant of data acquired with GC-C-IRMS and LC-IRMS devices.

In order to easily compare isotopic enrichments acquired by the GC-C-IRMS, FIA-IRMS and LC-IRMS devices, we also reported the root-mean-square (rms) of SD ($\delta^{13}C$) and the root-mean-square of the differences between the measured and the theoretical APE or MPE (ΔAPE or ΔMPE) (see Equation 9).

$$rms = \sqrt{\frac{\sum_{i=1}^{i=n} Xi}{n}} \qquad (Equation\ 9)$$

where *Xi* represents either SD ($\delta^{13}C$) or Δ (APE or MPE) and n is the number of points involved in the evaluation.

4.3.4 ANIMAL STUDY

All procedures were performed according to the French legislation on animal experimentation. Rats aged 12 weeks (M&B A/S Bomholtgärd, Ry, Denmark and Iffa

Credo, Saint Germain sur l'Arbresle, France) were housed in a temperature-controlled room (22-23°C) in individual wire-bottom cages with a 12h-12h light-dark cycle. At the end of the experimental period, animals received a bolus injection of ^{13}C Val (150µmol100g^{-1} body wt, 80% MPE, 0.5ml100g^{-1} body wt) in a lateral tail vein. Animals were anaesthetised with sodium pentobarbital (6mg100g^{-1} body weight) and killed by blood puncture in the abdominal aorta. Blood was collected on heparin, centrifuged and the plasma was collected for analysis of ^{13}C Val enrichment by GC-MS. After sacrifice, tibial epiphysis were quickly dissected, weighed and frozen in liquid nitrogen.

Protein synthesis rate was measured as described previously (Breuille et al., 1998). Briefly, frozen samples were powdered in liquid nitrogen with a ball mill (Dangoumeau, Prolabo, Paris, France) and homogenised in eight volumes of a 10% trichloroacetic acid solution (TCA). Tissue homogenates were centrifuged to separate the acid-soluble fraction. The remaining pellet containing proteins was washed twice with 10% TCA, twice with a 0.2 M perchloric acid solution (PCA) and solubilised in 0.3N NaOH an hour at 37°C. An aliquot was then collected for protein assay and the pellet was hydrolysed in 6N HCl at 110°C for 48hours. HCl was evaporated, the pellet was diluted in 0.1N HCl and desalted by cation-exchange chromatography. Amino acids were eluted with 4M NH_4OH, dried by evaporation and re-suspended in 0.1N HCl. Finally, each sample was divided into two sets of samples: approx. half of the sample was analyzed by GC-C-IRMS and the second half of the sample was analyzed by LC-IRMS according to the method described in this paper.

4.4 LC-IRMS METHOD

For LC-IRMS analyses, Rheos 2000 pump (Flux, Switzerland) was used directly connected to LC Isolink interface. An in-line filter of 0.2µm (Vici, Schmidlin Labor, Switzerland) was placed after the LC column to avoid any particles passing into the interface. Samples were filtered through 0.2µm Nylon membrane filters (Nalgene, Waters, Switzerland). The LC separation of underivatised amino acids was achieved using a strong sodium cation exchange chromatography column (sodium-SCX, 250x3.0mm, 8µm, Pickering Laboratories). The column was held at 48°C. The LC flow rate was 300µLmin^{-1}, and the flow rate of the acid and oxidant reagents in the LC interface was 50µLmin^{-1} in both cases. The LC separation was achieved under isocratic condition with degassed phosphate buffer at 200mM (pH 3.16 ± 0.05). After

the elution of Val, the column was washed with a commercial buffer (RG011® from Pickering Laboratories) for 12min. This commercial buffer contains water (99%), sodium hydroxide (0.6%) and sodium chloride (0.4%). However, the introduction of this buffer in the interface increases dramatically the CO_2 background (higher than 10V measured on *m/z* 44). Therefore, in each run, the open split is set in position "OFF" after the elution of Val, thus preventing CO_2 gas entering the IRMS source. An alternative solution might be to use a switching valve system.

For FIA-IRMS analyses, the phosphate buffer eluent was degassed. The flow rates were 300µLmin^{-1}, and 50µLmin^{-1} respectively for the acid and oxidant reagents in the interface. For each run, 4 injections of sample were performed followed by two other injections of working standard with a known isotopic ratio (cane sugar with $\delta^{13}C$ =-11.3‰).

4.5 CONVENTIONAL GC-C-IRMS METHOD

For the GC-C-IRMS analyses, the injector temperature was set at 250°C. The separation was achieved on a DB 5-MS (30m x 0.32mm I.D., 0.25µm film thickness) fused silica capillary column (J&W Scientific, Koeniz, Switzerland). Helium was used as carrier gas and the column head pressure was set at 15psi. Splitless injection was used with a purge delay of 0.8min. The initial temperature gradient was 60°C, 1min held, and then increased to 150°C with a rate of 15°Cmin^{-1}; 1.1°Cmin^{-1} to 155°C; and finally to 35°Cmin^{-1} to 300°C, with a held of 1min. Ions with *m/z* of 44, 45 and 46 were measured for the [1-^{13}C] Val enrichment determinations. Before each sequence, O_2 was flushed for approximatively 30min in back-flush mode to regenerate the reduction furnace.

Sample preparation

The dried residue obtained after the sample preparation was derivatized using an N(O,S)-ethoxycarbonyl ethyl ester derivative according to protocols already published (Faure et al., 2002; Godin et al., 2007; Montigon et al., 2001). Using the GC-C-IRMS instrument, isotopic precision, SD ($\delta^{13}C$), measured for Val was 0.3‰ that was in the specification of the system. The intra-assay repeatability measured on 4 days for Val at natural abundance was 2.4% (with $\delta^{13}C$ = -26.13 ± 0.62‰, n=12).

Typical retention time of Val was approximately 520sec and the total run time of the GC-C-IRMS method was approx. 700sec (see Figure 32).

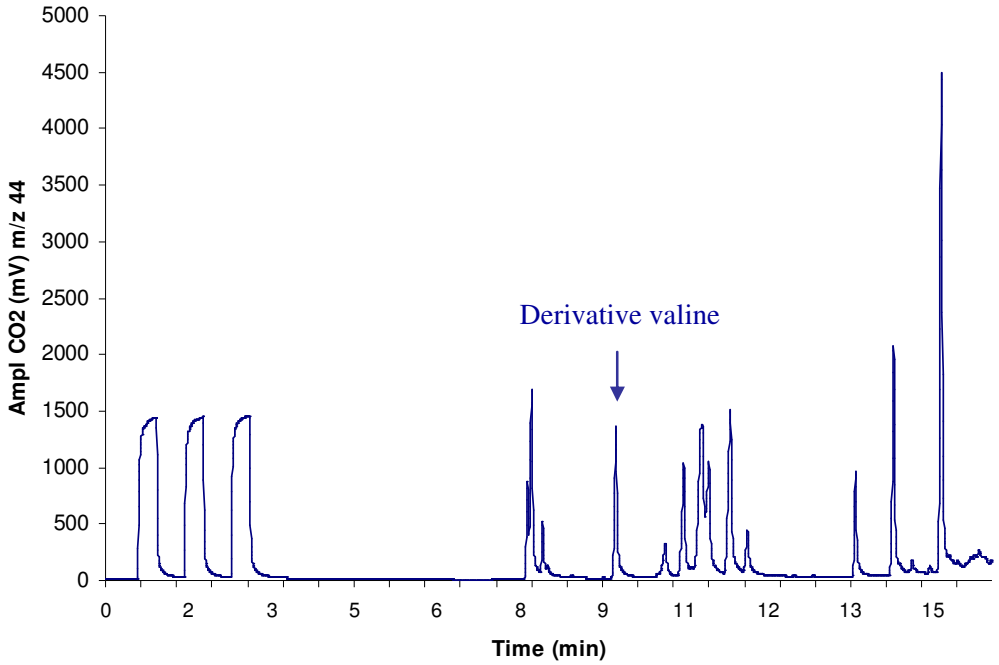


Figure 32: Typical GC-C-IRMS chromatogram ($^{12}CO_2$ trace measured on m/z 44) developed for measuring ^{13}C isotopic enrichment of Val in metabolic study using an apolar GC column.

The three peaks in the beginning of the chromatogram correspond to calibrated reference CO_2 standard peaks.

4.6 MATHEMATICAL TRANSFORMATION TO OBTAIN THE SAME ISOTOPIC ENRICHMENTS BETWEEN GC-C-IRMS AND LC-IRMS

Experimentally, the LC-IRMS and the GC-C-IRMS results were acquired on different days with different sets of standard mother solutions. It means that a slight difference of theoretical isotopic enrichment for standard solutions was used for the GC and LC

instruments. In order to correlate isotopic data measured by LC-IRMS and GC-C-IRMS with the same theoretical isotopic enrichment, mathematical transformations were performed. The approach to operate this transformation was based on experimental data acquired by the GC-C-IRMS instrument. A standard curve was built with the GC-C-IRMS data, where theoretical MPE was plotted in the x-axis against measured Atom% values in the y-axis. A linear equation was found: $y= 0.104x + 1.082$, $r^2=0.999$. Thus, the theoretical MPE used in LC was subsequently transformed in Atom% measured by GC-C-IRMS using the previous equation. And then, APE and MPE values were also calculated for the GC-C-IRMS using the previous equations (see Table 18).

Table 18: Theoretical and experimental isotopic enrichment of ^{13}C Val (from 0 to 2.3MPE) measured by LC-IRMS and then mathematically transformed into GC-C-IRMS data using a curve (Atom% = f(MPE)) acquired by GC-C-IRMS

Theoretical values		Measured LC-IRMS data				GC-IRMS data generated using experimental data, Atom% = f(MPE)			LC-IRMS data calculated with mass balance eq. and $\Delta^{13}C$ ($Atom\%_{Predi.}$ - $Atom\%_{Meas.}$)			
MPE	APE	$\delta^{13}C$‰	Atom%	APE	MPE	Atom%	APE	MPE	Atom%	ΔAtom%	MPE
0	0	-12.33	1.098	0	0	1.082	0	0	1.1	0.0022	0
0.012	0.0023	-9.73	1.101	0.003	0.014	1.083	0.0012	0.012	1.102	0.0018	0.012
0.023	0.0045	-8.41	1.102	0.004	0.022	1.084	0.0023	0.023	1.105	0.0024	0.023
0.046	0.0091	-3.21	1.108	0.01	0.05	1.086	0.0048	0.048	1.109	0.0017	0.048
0.117	0.0234	10.88	1.123	0.026	0.128	1.094	0.0122	0.122	1.124	0.0011	0.122
0.448	0.0895	72.26	1.191	0.093	0.464	1.128	0.0467	0.467	1.193	0.0028	0.467
0.9	0.18	150.76	1.278	0.18	0.901	1.176	0.094	0.94	1.288	0.0099	0.94
2.29	0.458	433.66	1.588	0.49	2.451	1.321	0.2391	2.391	1.578	-0.0098	2.391

4.7 ISOTOPIC FRACTIONATION DURING DERIVATISATION

The variations of magnitude in kinetic isotope effect, KIE (Rieley, 1994) may appear between different amino acids due to their difference of reactivity and also vary according to the derivatisation method chosen. To the best of our knowledge, no KIE studies using the N(O,S)-ethoxycarbonyl ethyl ester derivative for different amino acids were reported in the literature. To predict the LC-IRMS Atom% value of underivatized Val, we used the mass balance equation (Equation 1) with on one hand the GC-C-IRMS isotopic value and on the other hand the isotopic value of the derivative reagents used for the GC derivatisation of Val (ethyl chloroformate and ethanol). The derivative $\delta^{13}C$ ($\delta^{13}C_d$) was calculated at - 43.68 ± 0.33‰ (or 1.0633 Atom%) by analysing Ala and Gly successively by GC-C-IRMS and by FIA-IRMS. Thus, using Equation 1 with Atom% value of derivative Val measured by GC-C-IRMS and Atom% of the derivative ($\delta^{13}C_d$), the theoretical LC-IRMS Atom% data was calculated (see Table 18). Then, by plotting predicted and measured Atom% values obtained by LC-IRMS between 0.00 and 2.3MPE (or -12.3 to 433.7‰, or 1.098 to 1.588Atom%), an excellent correlation was reported: $y=0.98x + 0.025$ with $r^2=0.999$. At natural abundance, an isotopic discrepancy was observed with a ΔAtom% at 0.0022 (or a $\delta^{13}C=2.2$‰) between the measured and the predicted value. Moreover this bias was constant ($\delta^{13}C=2.0 \pm 0.6$‰) between 0 and 0.47MPE (see Table 18). The origin of this isotopic discrepancy is not apparent at present. It may originate from the derivatisation reaction (KIE associated to the derivatisation) or from one of the parts of the system used (isotopic bias due to furnaces). For the N(O,S)-ethoxycarbonyl ethyl ester derivative, reproducible isotopic measurement was reported (Montigon et al., 2001) showing that the bias reported here is suitable for isotopic analysis. Moreover, in GC-C-IRMS, previous papers have reported that the Val derivative if compared to other amino acids has higher isotopic fractionation. The isotopic discrepancy reported was - 2.46‰ for N-TFA isopropyl ester of Val (Silfer et al., 1991) against off-line IRMS measurement of the same compound, and 5.6‰ for the N-acetyl propyl (NAP) Val between predicted and measured values (Demmelmair & Schmidt, 1993).

4.8 RESULTS AND DISCUSSION

Ion exchange chromatography with sulfonated polystyrene resins operating in sodium or lithium forms with buffers (in which organic buffers are present) and temperature gradient followed by post column ninhydrin derivatisation has been the method of choice for amino acid analysis since the development of automated amino acids analysis by Moore & Stein in the 1950's (Ettre & Gehrke, 2006). However, the buffers do not match the analytical constraints of the LC-IRMS due to the presence of organic salts. Here we present a new method for underivatised amino acids using the sodium ion exchange chromatography column with aqueous buffer. The key benefit of this method is the regeneration of the column after each run, which allows using it for a long period of time and for complex biological samples.

4.8.1 LC-IRMS SEPARATION OF VAL

The chromatographic conditions used here (with an ion exchange chromatography column with phosphate buffer in isocratic mode) allow separating Val from several other amino acids as illustrated in Figure 33. Baseline separation was obtained for Asp, Thr, Glu, Pro, Gly, Ala, Cys and Val (peak assignment was performed based on the relative retention time assessed by injection of standard mixtures). In order to get rid of the amino acids eluted after Val with the same column, a gradient of temperature and buffer is required (Moore & Stein, 1951). We have, however used the washing buffer recommended to store the LC column as LC buffer to remove the rest of amino acids. This presents a significant advantage compared to the existing LC-IRMS methods because no organic solvent (acetonitrile or methanol) is introduced in the LC column, thus avoiding the time consuming process of the organic buffer removal. With the new method, Ser coelutes with Thr and Val is located between Cys and Ala. However, to master reproducibility of the separation, the pH of the mobile phase must be strictly controlled. Cys is in particular very sensitive to the pH. At pH higher than 3.3, Cys coelutes with Ala whereas at pH lower than 3.1 Cys coelutes with Val. In our condition the retention factor (k) for Val is approx 3.6 ± 0.2 (corresponding to a retention time of about 27min with a t_o=350sec).

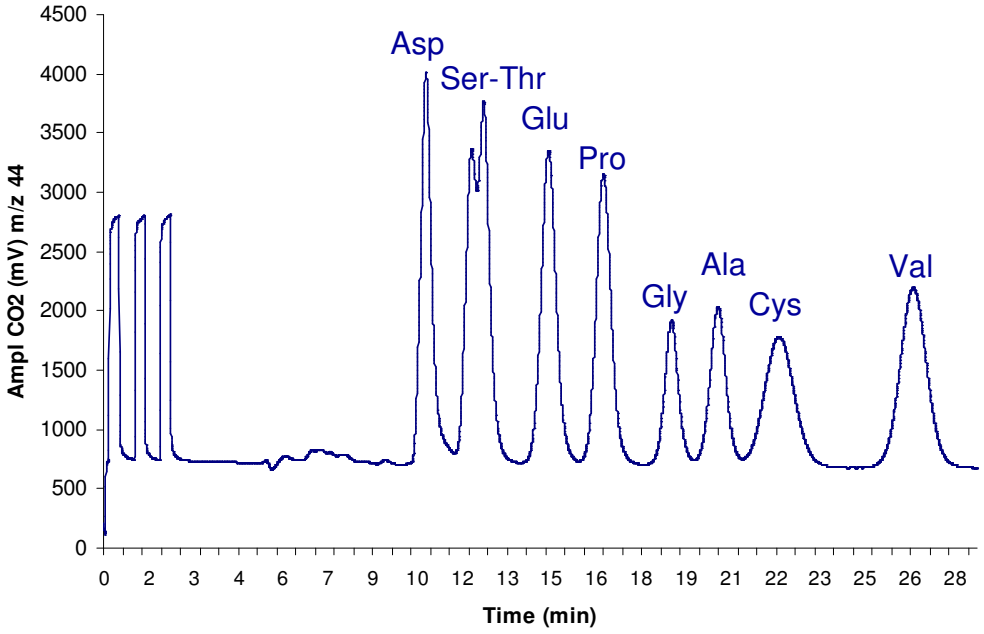


Figure 33: Typical LC-IRMS chromatogram of 8 underivatised amino acids eluted with a phosphate buffer (200mM at pH 3.2) in isocratic mode through a strong cation exchange column heated at 48°C

In Figure 33, the solution injected corresponds to a mixture of 17 pure amino acids (approximately 5nmol injected). The isocratic elution mode allows separation of several amino acids with baseline resolution. The order of elution is Asp, Thr-Ser, Glu, Pro, Gly, Ala, Cys and Val. Ser is coeluted with Thr. The three peaks in the beginning of the chromatogram correspond to calibrated reference CO_2 standard peaks.

4.8.1.1 Peak width and band broadening in LC-IRMS

In LC, the band broadening observed is generally expressed as the addition of the variances of the different contributions (Snyder et al., 1997). The contributions can originate from the separation, the transport within the column itself and from the extra-contributions due to the connected apparatus such as the injector, the fittings, the tubings and the detector itself. With the LC-IRMS in FIA mode (without any LC column), a peak width lower or equal to 60sec is within the specification (for 300ng carbon). Additionally, by injecting directly and manually in the interface 10µL of

diluted sparkling water, a peak width of 90sec was observed (measured on cup used for *m/z* 44). This experiment showed that the main extra contribution to the peak width in our system comes from the interface itself (excluding the chemical reactor). Thus, the commercialized LC-IRMS interface may cause a significant extra-column broadening which may affect peak width as well as the sensitivity (due to the decrease of the signal). Additionally, the increase of peak width with the retention factor is typically associated to the isocratic and/or gradient elution modes in an ion exchange chromatography with a polymeric column (Knox, 1977; Neue, 2005). Here, with this LC-IRMS method, the peak width increases with the retention factor. The corresponding peak widths (measured at the baseline) varied from 99sec for Asp to 177sec for Val (SD=20sec), for approximately 36sec by LC-UV (determined with a gradient elution mode from the test chromatogram of the supplier).

4.8.1.2 Quantitative oxidation conversion with FIA and LC coupled to IRMS

Based on the observation made on the peak width, it was also important to study if the band broadening can affect the efficacy of the oxidation of organic molecules within the interface. Moreover, to obtain accurate and precise isotopic ratio using LC-IRMS and in addition to the baseline separation of the analytes, it is also necessary to quantitatively convert the molecule of interest into CO_2 and subsequently transfer it efficiently into the IRMS**.** To assess this oxidation efficacy, a response curve was established using both the LC separation and the flow injection analysis (FIA-IRMS) over carbon range from 101 to 1605ng and 153 to 1532ng, for FIA-IRMS and LC-IRMS respectively. Linear curves were found in both cases: for FIA-IRMS, equation was y=0.0714 x + 0.244, r^2=0.999 and for LC-IRMS, the equation was y=0.0732 x + 0.442, r^2=0.999. The values (raw data) were not statistically different (p>0.05). Thus, these results showed that the same amount of carbon was transformed in CO_2 and then transferred into the counter-current of helium of the separation unit using both FIA and LC-IRMS. Additionally, these results also demonstrate that the LC conditions do not affect the oxidation process and the CO_2 transfer even if the peak width for Val is broader than the one of the first amino acid eluted.

4.8.2 ISOTOPIC PRECISION AND ACCURACY MEASURED BY LC-IRMS

Since fast eluting compounds give sharper peaks than compounds eluting later on, in an isocratic mode, the SD (δ^{13}C) varies through the LC run. We observed that the SD

($\delta^{13}C$) assessed through 10 consecutive injections at natural abundance of amino acids standard solutions was 0.1‰ for Asp (k=0.7), 0.2‰ for Thr (injected alone) (k=1.2), 0.3‰ for Pro (k=1.9), 0.7‰ for Ala (k=2.6) and 0.6‰ for Val (k=3.6). Therefore, in an isocratic elution mode, when the LC peak width increases, the SD ($\delta^{13}C$) of the corresponding peak increases too. This observation was previously reported for LC-C-IRMS (Goodman & Brenna, 1992). The inter-assay repeatability over three different days measured for Val at natural abundance by LC-IRMS showed an SD ($\delta^{13}C$) at 0.4‰ ($\delta^{13}C$ was measured at -11.57, -11.96 and -12.33‰ with SD lower than 0.6‰ for each determination).

In order to ascertain the accuracy of isotopic enrichment measured by LC-IRMS over a wide dynamic range, known amounts of ^{13}C Val were added to a known amount of unlabeled Val to cover a theoretical isotopic enrichment range from 0.00 to 2.29MPE ($\delta^{13}C$ between -12.3 to 433.7‰). The values (Atom% and APE measured with their SD) are presented in Table 19. Subsequently, accuracy curve (using the measured MPE values in the y-axis *versus* the theoretical MPE values in x-axis) were built. A linear relation was then obtained with slope of 1.05. Additionally, the difference between theoretical MPE and measured MPE (ΔMPE) was found to be a function of the level of isotopic enrichment: ΔMPE ranges from 0.0002 to 0.15 in LC-IRMS (for a range of theoretical MPE varying from 0.00 to 2.29MPE). Moreover, low level of isotopic enrichment (0.002APE) was also measured accurately.

The new developed LC-IRMS method for Val can measure very low ^{13}C isotopic enrichment and higher isotopic enrichment with very good accuracy and with an SD ($\delta^{13}C$) lower than 1‰ at natural abundance which is suitable for a tracer study performed through clinical and animal studies.

Table 19: Theoretical and experimental isotopic enrichment of ^{13}C Val measured by GC-C-IRMS, FIA-IRMS and LC-IRMS with different standard mixtures of natural Val mixed with known amount of ^{13}C tracer.

	GC-C-IRMS				LC-IRMS				FIA-IRMS			
Theor.	Atom%	APE	MPE	Theor	Atom%	APE	MPE	Theor.	Atom%	APE	MPE	
MPE	mean ± SD	mean ± SD	mean ± SD	MPE	mean ± SD	mean ± SD	mean ± SD	MPE	mean ± SD	mean ± SD	mean ± SD	
		Δ APE	Δ MPE			Δ APE	Δ MPE			Δ APE	Δ MPE	
0.000	1.079 ± 0.0007			0	1.098 ± 0.0007			0	1.099 ± 0.0001			
0.021	1.081 ± 0.0005	0.0025 ± 0.0005	0.025 ± 0.005	0.011	1.101± 0.0001	0.0029 ± 0.0003	0.014 ± 0.002	0.035	1.106 ± 0.0001	0.0071 ± 0.0001	0.035 ± 0.001	
		-0.0004	0.004			0.0000	0.003			0.0001	0.001	
0.053	1.087± 0.0002	0.0078 ± 0.0002	0.077 ± 0.002	0.023	1.102 ± 0.0001	0.0045 ± 0.0004	0.023 ± 0.002	0.053	1.110 ± 0.0001	0.0112 ± 0.0001	0.056 ± 0.0001	
		0.0025	0.024			0.0000	0.0002			0.000	0.003	
0.106	1.091 ± 0.0001	0.0125 ± 0.0001	0.125 ± 0.001	0.046	1.108 ± 0.0001	0.0100 ± 0.0003	0.05 ± 0.002	0.095	1.117 ± 0.0001	0.0189± 0.0001	0.095 ± 0.001	
		0.0019	0.019			0.001	0.005			0.0000	0.001	
0.523	1.139 ± 0.0002	0.0596 ± 0.0002	0.596 ± 0.002	0.117	1.123 ± 0.0001	0.0255 ± 0.0006	0.128 ± 0.003	0.190	1.138 ± 0.0001	0.0393 ± 0.0001	0.196 ± 0.0007	
		0.0073	0.073			0.0021	0.010			0.0012	0.006	
1.046	1.189 ± 0.0015	0.1099 ± 0.0015	1.099 ± 0.015	0.448	1.191 ± 0.0001	0.0929 ± 0.0006	0.464 ± 0.003	0.383	1.170 ± 0.0001	0.0719 ± 0.0001	0.358 ± 0.002	
		0.0053	0.053			0.003	0.017			-0.0048	0.025	
3.103	1.422 ± 0.0057	0.3429 ± 0.0057	3.429 ± 0.057	0.900	1.278 ± 0.0001	0.1789 ± 0.0018	0.895 ± 0.009	0.983	1.303 ± 0.0003	0.2044 ± 0.0003	1.021 ± 0.004	
		0.0299	0.299			0.001	-0.005			0.0079	0.039	
5.536	1.652 ± 0.0047	0.5732 ± 0.0047	5.732 ± 0.047	2.290	1.588 ± 0.0001	0.4902 ± 0.0033	2.439 ± 0.024					
		0.0196	0.196			0.032	0.149					

4.8.3 THEORETICAL AND EXPERIMENTAL CORRELATIONS BETWEEN GC AND LC-IRMS FOR ABSOLUTE ISOTOPIC ENRICHMENTS (ATOM%, APE AND MPE)

In essence, for all the correlations between LC and GC coupled to IRMS the calculation of the isotopic values is performed using stochiometric mass balance relationships (Equation 1). After rearrangement of equation 1, a correlation between the $F^{13}C$ measured by LC (without derivatisation) and by GC (with derivatisation) is presented in equation 10. The slope of this curve depends on the number of carbons added by the derivative (n_d) and the intercept corresponds also to a dilution of the isotopic value of the derivative. n_{LC} and n_{GC} are the number of carbons present in the intact molecule and in the derivative molecule, respectively. n' corresponds to the number of labeled carbon in the molecule.

$$F_{GC} = \frac{n_{LC}}{n_{LC} + n_d} \times F_{LC} + \frac{n_d}{n_{LC} + n_d} \times F_d \qquad (\textit{Equation 10})$$

In order to link the absolute isotopic enrichment measured by LC-IRMS and GC-C-IRMS, equations 6 and 10 are used to obtain equations 11 and 12. Consecutively, for APE measurement, the slope of the curve is also expressed by $[n_{LC}/(n_{LC}+n_d)]$.

$$APE_{GC} = \left(n_{LC}\ F_{LC1} - n_{LC}\ F_{LC2}\right) \times \frac{1}{n_{LC} + n_d} \qquad (\textit{Equation 11})$$

$$APE_{GC} = \left(APE_{LC}\right) \times \frac{n_{LC}}{n_{LC} + n_d} \qquad (\textit{Equation 12})$$

Finally, to obtain the equivalence between MPE values measured by LC-IRMS and GC-C-IRMS systems, a correction factor corresponding to the transformation of APE into MPE is added to equation 12. Equations 13, 14 and 15 are thus obtained:

$$APE_{GC} \times \left(\frac{n_{LC} + n_d}{n'}\right) = \left(APE_{LC}\right) \times \left(\frac{n_{LC}}{n_{LC} + n_d}\right) \times \left(\frac{n_{LC} + n_d}{n'}\right) \qquad (\textit{Equation 13})$$

$$MPE_{GC} = \left(APE_{LC}\right) \times \left(\frac{n_{LC}}{n_{LC} + n_d}\right) \times \left(\frac{n_{LC} + n_d}{n'}\right) \qquad (\textit{Equation 14})$$

$$MPE_{GC} = (APE_{LC}) \times \left(\frac{n_{LC}}{n'} \right) = MPE_{LC} \qquad (\textit{Equation 15})$$

Using GC-C-IRMS device with the N(O,S)-ethoxycarbonyl ethyl ester derivative of Val (with $n_{LC}=n_d=5$), fifty percent of carbon in the final molecule comes from the derivative reagents ($n_{LC}=n_d$ are the number of carbon atoms in the molecule and brought by the derivative, respectively). The theoretical slopes of the curves representing $\delta^{13}C$, Atom% and APE values of GC versus LC can be expressed according to equations 10 and 12 by the isotopic dilution or $[n_{LC}/(n_{LC}+n_d)]$ which presently equals to 0.50. The experimental slope (as illustrated in Figure 34) is 0.51 in all cases. Theoretically, according to equation 15, a slope of 1 is obtained between MPE measured by LC-IRMS and GC-C-IRMS which is also the case for the experimental measurements performed (measured value is 0.98, see Figure 35).

In summary, the series of Equations (10, 12 and 15) describe the isotopic dilution existing between the isotopic data of labeled Val measured by GC-C-IRMS after derivatisation and the LC-IRMS data acquired directly with the underivatised analyte. The theoretical and measured slopes and intercepts are both consistent. Finally, if the GC-C-IRMS and LC-IRMS are used in case of tracer studies, the comparison of absolute isotopic enrichments is not straightforward when determining the APE values since isotopic dilution needs to be taken into account. Typically, using this derivative and whatever the number of ^{13}C labeled carbon, for Leu (containing 6 carbons, $n_{LC}=6$), the slope between the GC and the LC should be 0.54. And for phenylalanine ($n_{LC}=9$), the slope should be 0.64. In contrast, the MPE values acquired by the LC-IRMS and GC-C-IRMS are directly comparable and similar whatever the amino acids.

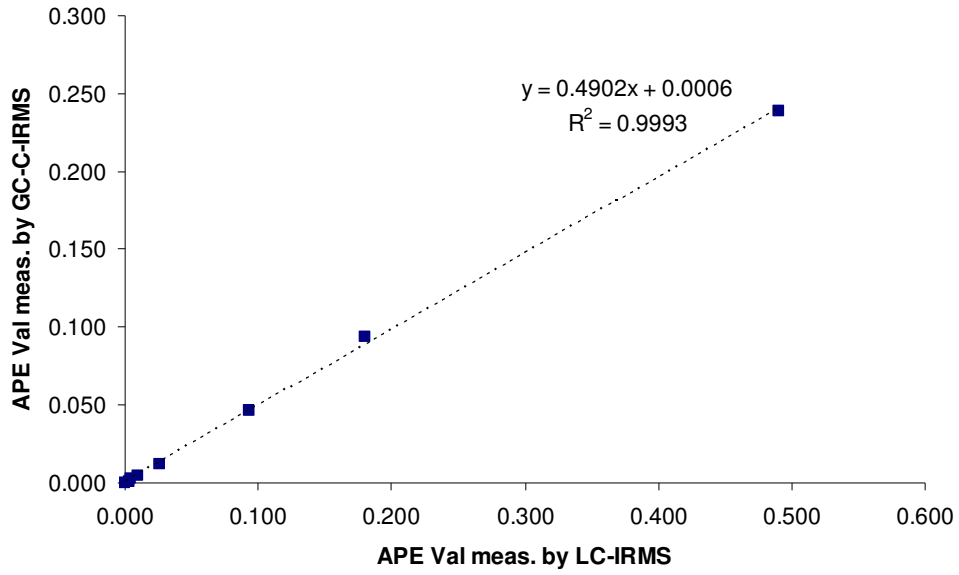


Figure 34: Experimental correlation between Atom% of Val acquired by GC-C-IRMS after derivatisation with N(O,S)-ethoxycarbonyl ethyl ester derivative and by LC-IRMS without any derivatisation

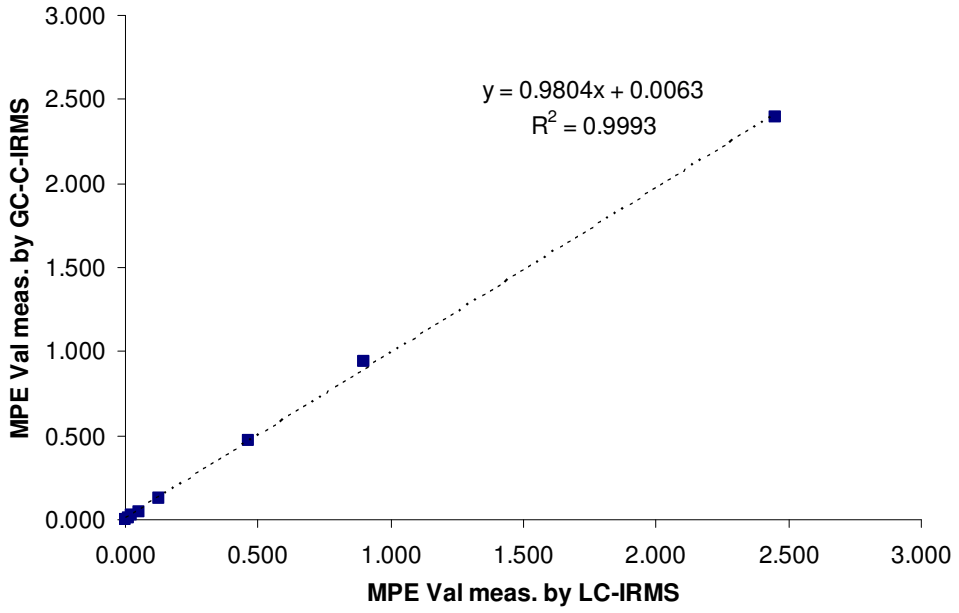


Figure 35: Experimental correlation between the MPE of Val acquired by the GC-C-IRMS after derivatisation with N(O,S)-ethoxycarbonyl ethyl ester and by the LC-IRMS without any derivatisation.

Based on the same principle and using equations described in the text, the correlation between experimental GC-C-IRMS and LC-IRMS obtained for APE was $y=0.49x + 0.0006$, $r^2=0.999$.

4.8.4 COMPARISON BETWEEN GC-C-IRMS AND LC-IRMS IN TERMS OF ISOTOPIC PRECISION AND ACCURACY

In order to perform a comparison between two separation methods coupled to the same IRMS, standard curves between 0.0 to 1.0MPE (-29.3 to 70.7‰) for GC-C-IRMS, 0.00 to 0.9MPE (-12.3 to 150.8‰) for LC-IRMS were processed. Additionally, a standard curve between 0 to 1.0MPE were also processed by FIA-IRMS (see Table 19). The results reported in Table 20 show that the calculated rms for precision was 0.004 and 0.003MPE respectively for GC and LC coupled to IRMS (corresponding respectively to a $\delta^{13}C$ at 0.46 and 0.38‰). A similar result between GC and LC-IRMS devices indicating identical isotopic precision for amino acids at natural abundance was reported by McCullagh (personal talk given at the Stable Isotope Meeting User Group, 2007). Additionally, in the same range of isotopic enrichment (0 to 1MPE), the calculated rms for accuracy was 0.023 and 0.005MPE respectively for GC and LC-IRMS. By summing the rms of precision and accuracy, the total error of each system using the methods described in this work and for Val, is 0.027MPE and 0.007MPE respectively for GC and LC-IRMS (see Table 20). Our results show that both systems give similar precision between 0 to 1MPE. Nevertheless, the accuracy within the isotopic ranges investigated was affected by the isotopic bias. In our case, the use of CO_2 pulses didn't correct this isotopic fractionation. This result also supports the use of the LC-IRMS in which the derivatisation step and its associated error are not present. Additionally, the results (see Table 20) also demonstrate that the FIA-IRMS mode is the most precise with a calculated rms of precision at 0.0008MPE (or a $\delta^{13}C$ = 0.11‰). Nevertheless, the accuracy between 0 to 1MPE is 0.008MPE for FIA-IRMS (compared to 0.005 in LC-IRMS). This result might be explained by the fact that possible impurities are present in the sample and may affect the isotopic ratio.

Table 20: Summary of isotopic precision and isotopic accuracy measured by GC-C-IRMS, LC-IRMS and FIA-IRMS for isotopic enrichment of ^{13}C Val between 0 to 1MPE. The total error reported corresponds to the sum of the isotopic precision and isotopic accuracy

	LC-IRMS (0-0.9MPE), n=18			GC-C-IRMS (0-1MPE), n=18			FIA-IRMS (0-1MPE), n=35		
	δ^{13}C,‰	APE	MPE	δ^{13}C,‰	APE	MPE	δ^{13}C,‰	APE	MPE
rms precision	0.46	0.0005	0.002	0.38	0.0004	0.004	0.11	0.0001	0.0008
rms accuracy		0.001	0.005		0.0023	0.023		0.0016	0.008
Total error		0.0015	0.007		0.0027	0.027		0.0017	0.009

Δ APE corresponds to the measured APE minus theoretical APE.

rms precision means root-mean-square of SD(δ^{13}C)

rms accuracy means root-mean-square of ΔAPE and ΔMPE. The corresponding formula is described in the text

4.8.5 IN VIVO TRACER STUDY MEASURED BY LC-IRMS AND GC-C-IRMS

Bone loss (osteopenia) is a common complication of inflammatory bowel disease (IBD). To measure the bone formation or resorption rates different biochemical methods are available (Robins & New, 1997). Moreover, recently, bone collagen synthesis or skin protein breakdown were also measured directly using the incorporation of labeled amino acids (Babraj et al., 2005; Volpi et al., 2000). Within this frame, we performed an animal study to compare the results of bone formation in the tibial epiphysis given by the determination of osteocalcin and the measurement of protein synthesis using ^{13}C Val. The isotopic enrichments of protein-bound Val were measured both by GC-C-IRMS and by LC-IRMS at the end of the incorporation period of the tracer amino acid. Figure 36 shows a typical section of a LC-IRMS chromatogram with the elution of Val in bone sample.

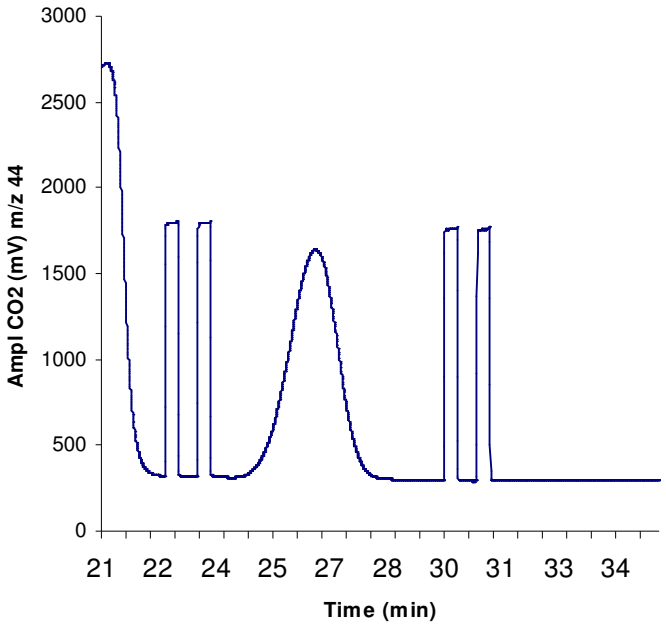


Figure 36: Section of an LC-IRMS chromatogram of bone extract after acidic hydrolysis and re-suspension of free amino acids in initial LC mobile phase of phosphate buffer at pH 3.16.

In Figure 36, the time window corresponds to the Val elution with 4 pulses of CO_2 calibrated reference gas.

Table 21 summarizes the results for seven rats: two rats were used to assess the isotopic ratio at natural abundance and the other five rats the different time points of the ^{13}C-Val incorporation into the bone. For the two rats at natural abundance, they showed $\delta^{13}C$= -32.03 ± 0.41‰ (or 1.0760 ± 0.0004Atom%) and -21.71±1.54‰ (1.087 ± 0.0020 Atom%) measured by GC and by LC respectively. Using these data, the predicted isotopic ratio measured using the GC-C-IRMS result (as described before) is in agreement with LC-IRMS data, with a difference of 0.0016Atom% (equivalent to 1.6‰). Additionally, to compare the absolute isotopic enrichment acquired by the LC and GC coupled to IRMS, a ratio (S) of the isotopic enrichments (IE) measured by the GC-C-IRMS over LC-IRMS using APE and MPE values were calculated using equation 16:

$$S = \frac{IE_{GC}}{IE_{LC}} \qquad (\textit{Equation 16})$$

The results showed that the "S" ratio was 0.53 ± 0.1APE and 1.07 ± 0.2MPE. These results are in agreement with the previous theoretical calculation between the GC-C-IRMS and LC-IRMS (0.5 and 1 for APE and MPE respectively). In summary, for the tracer study the isotopic enrichment (MPE) measured by LC-IRMS and GC-C-IRMS are not statistically different ($p>0.05$). This result demonstrates that both methods give equivalent isotopic enrichments when isotopic dilution (added by the derivatisation step) in GC-C-IRMS is taken into account. The possibility to analyze amino acids without any derivatisation with results in agreement with the conventional GC-C-IRMS shows that the LC-IRMS is the method of choice for analyzing such compounds in metabolic studies.

Table 21: Isotopic enrichments of ^{13}C Val bound proteins in rats tibial epiphysis after administration of ^{13}C Val measured by LC-IRMS and GC-C-IRMS

	GC-C-IRMS				LC-IRMS				MPE
	$\delta^{13}C$‰	SD	MPE	SD	$\delta^{13}C$‰	SD	MPE	SD	
Rat 1	-32.08	0.53	-	-	-20.48	0.52	-	-	
Rat 2	-31.39	0.38	-	-	-22.94	1.06	-	-	
Rat 5	-16.71	1.01	0.184	0.011	12.18	0.13	0.181	0.001	1.02
Rat 6	-3.72	0.11	0.324	0.001	52.05	0.97	0.387	0.005	0.84
Rat 7	-15.56	0.02	0.197	0.001	11.96	0.2	0.18	0.001	1.09
Rat 8	-19.48	0.21	0.155	0.002	2.64	0.31	0.132	0.002	1.17
Rat 9	-17.37	0.13	0.177	0.001	5.05	0.33	0.144	0.002	1.23

4.9 CONCLUSIONS

The data presented in this work indicate that ^{13}C isotopic analysis by LC-IRMS for amino acids overcomes the GC derivatisation step that leads to isotopic dilution and isotopic error. Compared to the conventional GC-C-IRMS technique, by LC-IRMS, the measured limit of detection was 0.002APE. In the range of isotopic enrichment investigated (-12 to 434‰ by LC-IRMS equivalent to 0 to 1MPE), the isotopic precision between the GC-C-IRMS and the LC-IRMS was in agreement (with SD at 0.4-0.5‰). The measured accuracy between the GC-C-IRMS and the LC-IRMS illustrates the potential isotopic bias that may occur within the GC-C-IRMS method. Moreover, by taking into account the carbon added by the derivative, it is possible to correlate relative and absolute isotopic enrichments acquired by both tools. Finally, as the LC-IRMS gives similar isotopic enrichment to GC-C-IRMS in tracer study, we consider it as the method of choice in metabolic studies, particularly for the measurement of underivatised amino acids in nutritional and medical research.

CHAPTER 5

Hyphenation of high temperature LC with IRMS (HTLC-IRMS)

Keywords: high temperature liquid chromatography, IRMS, organic acids, phenolic acids, porous carbon graphite column

5 HYPHENATION OF HIGH TEMPERATURE LIQUID CHROMATOGRAPHY AND IRMS (HTLC-IRMS)

5.1 STATIONARY PHASES MATERIALS WITH 100% AQUEOUS MOBILE PHASE

With reversed stationary phase columns, water is highly polar, a weak solvent and a poor solvent for most of organic analytes. When separating very polar, water-soluble compounds, it is unusual to use mobile phases that contain less than 10% organic modifier (such as methanol or acetontrile) to achieve sufficient retention. LC separation under 100% aqueous mobile phase conditions leads generally to poor chromatographic reproducibility. It means that the retention time will be shorter and shorter and the chromatographic resolution will deteriorate. The modification of the stationary phase material is explained by a phenomenum called “phase collapse”. Thus, under aqueous mobile phase, there are less and less interaction between the alkyl phase and the analytes and the surface area accessible to the analytes is reduced.

Column manufacturers have addressed the problem of LC separation with a 100% aqueous mobile phase by using polar embedded phase (with amide and carbamate groups) and hydrophilic end-capped stationary phases. Thus, the alkyl phases are extended leading to a better reproducibility under aqueous mobile phase (Majors & Przybylski, 2002).

Thus, for LC separation of water-soluble compounds with a 100% aqueous mobile phase by LC-IRMS, in addition to ion exchange and mixed mode stationary phases, specific reversed stationary phases can be also used.

To achieve better performance in liquid chromatography, different parameters are classically optimized such as the composition and the ionic strength of the mobile phase, the pH, the type, the particle size, the stationary phase and the dimension of

the column and the temperature. There are many advantages of considering the temperature in method developments:

- Superheated water is cheap, safe, non-toxic, non-inflammable and recyclable.
- The analysis speed is increased due to higher linear velocities (explained by the decrease of the viscosity with the temperature which also decreases the back-pressure); by reducing the back-pressure, a smaller particles size and longer column could be used which also increased the efficiency (N) (Vanhoenacker & Sandra, 2006) (Guillarme et al., 2004) (Dolan, 2002).
- The reduction of backpressure enables replacement of methanol and acetonitrile by ethanol. As a consequence, these modifications lead to a "green mobile phase" which respects the environment by reducing the chemical waste (Smith, 2006).
- Temperature is also known to play a role on the chromatographic resolution (through efficiency and selectivity, especially for polar and ionisable compounds, by changing the pKa of the molecules) (Heinisch et al., 2006).
- Temperature can also improve peak capacity by reducing the peak width, and peak asymmetry (Kondo & Yang, 2003).
- Temperature is also an easier parameter for controlling the selectivity compared to the adjustment of the pH.
- Temperature is also a straightforward parameter to change and can be easily transferred between different laboratories.
- Additionally, the amount of organic modifier can be decreased or eliminated in LC by increasing the temperature which also reduces the retention time.

In the next chapter, the use of heated water (between 40 and 200°C) will be evaluated as chromatographic eluent for LC-IRMS. The main advantage foreseen is that by increasing the temperature, the dielectic constant of water is reduced. In effect, a 3.5°C rise in water temperature is equivalent to a 1% methanol increase for a methanol-water mixture.

To illustrate the usefulness of the high temperature liquid chromotagraphy approach with IRMS, the phenolic and organic acids will be used as model compounds for HTLC-IRMS studies.

5.2 HTLC-IRMS: A NEW APPROACH FOR MEASURING ^{13}C ISOTOPIC RATIOS OF ORGANIC AND PHENOLIC ACIDS

Submitted March 2008, by Jean-Philippe Godin, Gérard Hopfgartner and Laurent-Bernard Fay

5.2.1 INTRODUCTION

Although the idea of combining liquid chromatography (LC) to isotope ratio mass spectrometry (IRMS) was proposed over 10 years ago (see Godin et al., 2007), the interface allowing the LC-IRMS coupling was only commercialized in 2004 (Krummen et al., 2004). The LC-IRMS coupling is based on a wet chemical oxidation of organic molecules into CO_2 gas. The main constraint of the LC method development for an LC-IRMS run is the imperative absence of organic buffers in the mobile phase entering in the interface. This limitation makes of the most widespread LC stationary phase, the reversed phase, a phase which is not suitable for the LC-IRMS technique. Several reports showed how to overcome all these constraints for various classes of compounds (e.g. as amino acids (Godin et al., 2005; McCullagh et al., 2006), small peptide (Schierbeek et al., 2007), sugars (Cabanero et al., 2006), or volatile organic acids (Heuer et al., 2006) by using a specific and restrictive chromatography mode of elution. This mode of elution is based on ion exchange or mixed mode interactions with gradients of inorganic salts such as phosphate buffers, diluted sulphuric acid or water. However, high-precision isotopic analyses of vitamins, other natural products (i.e. phenolic acid), basic or neutral drugs compound classically analyzed by reversed stationary phases with organic solvents currently remain a challenge for the LC-IRMS device. At present, the measurement of isotopic ratios at natural abundance of these compounds requires an isolation of the molecules prior to the isotopic analyses (Jamin et al., 2005) (Jasper et al., 2004; Moreno Rojas et al., 2007) or a derivatisation followed by gas chromatography-combustion-IRMS (GC-C-IRMS) (Meier-Augenstein, 2002), which is time consuming and tedious in terms of sample preparation. A new strategy needs, therefore, to be developed in order to allow LC-IRMS analyses of such compounds.

Currently, LC analysis carried out at high temperature is a valuable approach to the use of the Ultra Performance Liquid Chromatography (UPLC) (Mazzeo et al., 2005) (Guillarme et al., 2007) and the monolith separation (Guiochon, 2007) for increasing the throughput. Although the concept of high temperature is not new (Miller & Hawthorne, 1997), the high temperature liquid chromatography (HTLC) is also seen as a powerful optimization variable for many LC separations (de Boer et al., 2005; Plumb et al., 2006; Riddle & Guiochon, 2006; Teutenberg et al., 2001; Vanhoenacker & Sandra, 2005) with many advantages (Dolan, 2002; Guillarme et al., 2004; Heinisch et al., 2006; Smith, 2006; Vanhoenacker & Sandra, 2006). The interest in this direction is also driven by the commercialization of LC columns resisting to temperatures higher than 120°C (Vanhoenacker & Sandra, 2006) and instruments allowing heating of the LC columns and the mobile phases up to 200°C (McNeff et al., 2007). Last but not least, HTLC is hyphenated with UV, MS, NMR and ICP-MS devices (Guillarme & Heinisch, 2005). Using high temperature condition associated with a 100% aqueous mobile phase, uncommon hyphenation is also reported between the HTLC with the FID detector (Guillarme & Rocca, 2005). Thus, the HTLC-FID coupling presents similarities with the IRMS since the organic eluent increased the background and for both detectors the LC flow rate introduced is limited.

This novel HTLC-IRMS approach expands for the first time the on-line high-precision ^{13}C isotopic analysis of underivatised phenolic acids (*p*-coumaric acid and its sulfate and glucuronide conjugates) at high temperature. In this condition, elevated column and eluent temperatures (i.e. above room temperature) replace organic solvents which are “forbidden”. Moreover, performing ramps of temperatures in LC may mimic the modification of the mobile phase strength, as when modifying the organic buffer in reversed phase mode, or the ramp of temperature in gas chromatography (GC). Here, ^{13}C isotopic organic acids analyses are also reported with isothermal and temperature gradient with different LC conditions. Moreover, other investigation is reported to increase the range of compounds which were excluded beforehand from the range of analysable compounds. At high temperature, assays are reported to study the range of methanol which might be added in the injected solution for measuring reliable isotopic ratio of compounds not completely soluble in a 100% aqueous mobile phase. All these results clearly improve the attractiveness of the LC separation for IRMS analyses for high-precision isotopic analysis.

5.2.2 EXPERIMENTAL

5.2.2.1 Materials and reagents

Phosphoric acid, sodium peroxodisulfate (p.A.), potassium hydrogen phosphate (p.A.) were purchased from Merck. De-ionised water was prepared using a Milli-Q system (with a resistance level equal to 18.2MΩ.cm). Organic acids were purchased from Sigma (St Louis, USA). *p*-coumaric acid was purchased from Sigma (France). Sulfate and glucuronide conjugate of *p*-coumaric acid were chemically synthesized in house. Each solution was prepared by dissolving the compounds in the aqueous eluent and finally passed by ultrason for obtaining a complete dissolution.

Phosphate buffers were prepared with Milli-Q grade water. To decrease the ubiquitous CO_2^+ background signal *(m/z* 44, measured with a pre-amplifier feedback resistor of 300MΩ), all reagents and LC eluents were thoroughly degassed in an ultrasonic bath (Digitana AG, Switzerland) at (30 ± 5℃ for 15 ± 5min using a water vacuum). The concentration of the acidic reagent was approx. 1.0molL^{-1} phosphoric acid in water, and the concentration of sodium peroxodisulfate was approx. 0.5molL^{-1} in water.

5.2.2.2 Instrumentations

Experiments were carried out on a MAT252 IRMS (Finnigan MAT, Bremen, Germany). The IRMS operated at an accelerating voltage of 8kV. The ion source was held at a pressure of 1 x 10^{-6} Torr, and ions were generated by electron impact at 70eV. Three faraday cup detectors monitored simultaneously and continuously the $CO_2^{+\cdot}$ signals for the three major ions at *m/z* 44 ($^{12}CO_2$), *m/z* 45 ($^{13}CO_2$ and $^{12}C^{17}O^{16}O$) and *m/z* 46 ($^{12}C^{18}O^{16}O$). To obtain comparable signal outputs, the preamplifier feedback resistors were selected with 300MΩ, 30GΩ and 100GΩ for *m/z* 44, *m/z* 45 and *m/z* 46, respectively (on this IRMS instrument, the maximum signal level is 10V).

The liquid chromatographic eluents were delivered with one Rheos 2000 (Flux, Basel, Switzerland) liquid chromatography pump. The injection was performed with a CTC autosampler equipped with a 10µL loop. The pump was connected to the heating module via a switching valve. One on-line filter (0.2µm, Vicci) was placed between the pumps and the heating module. The heating module used was a PatSelerity Polaratherm® 9000 series (RIC, Belgium) and was connected to an LC Isolink® interface (Thermo Electron, Bremen, Germany) with peek tubing. At the entrance of

the LC-IRMS interface, a second on-line filter (0.2µm) was placed. Finally, the LC Isolink® interface was coupled to the MAT252. A plexiglass window was installed between the heater and the LC-IRMS interfaces as a preventive measure to avoid any potential physical disturbance. However, no technical problems occurred during our extensive work to justify this installation. Within the Polaratherm 9000, the LC mobile phase was heated at the same temperature as the column and the LC effluent (after the column) was set at 30°C. Inside the LC-IRMS interface, the temperature of the reactor was set at 99.9°C. The helium (99.9998 %) flow rate of the separation unit was set at 1mLmin^{-1}. The LC Isolink® flow rates were set at 50µL/min for both acid and oxidant reagents (respectively phosphoric acid and sodium peroxodisulfate). This configuration was used to perform flow injection isotopic analysis (FIA-IRMS), i.e. injection without any LC column, and LC-IRMS analyses with an LC column. Here, in the whole set of analyses, the backpressure measured was higher than 40bar whatever the temperature used (the boiling point of water at 200°C is 20bar. Therefore, only a small back-pressure is needed to prevent boiling of water). The scheme of the installation is described in Figure 37.

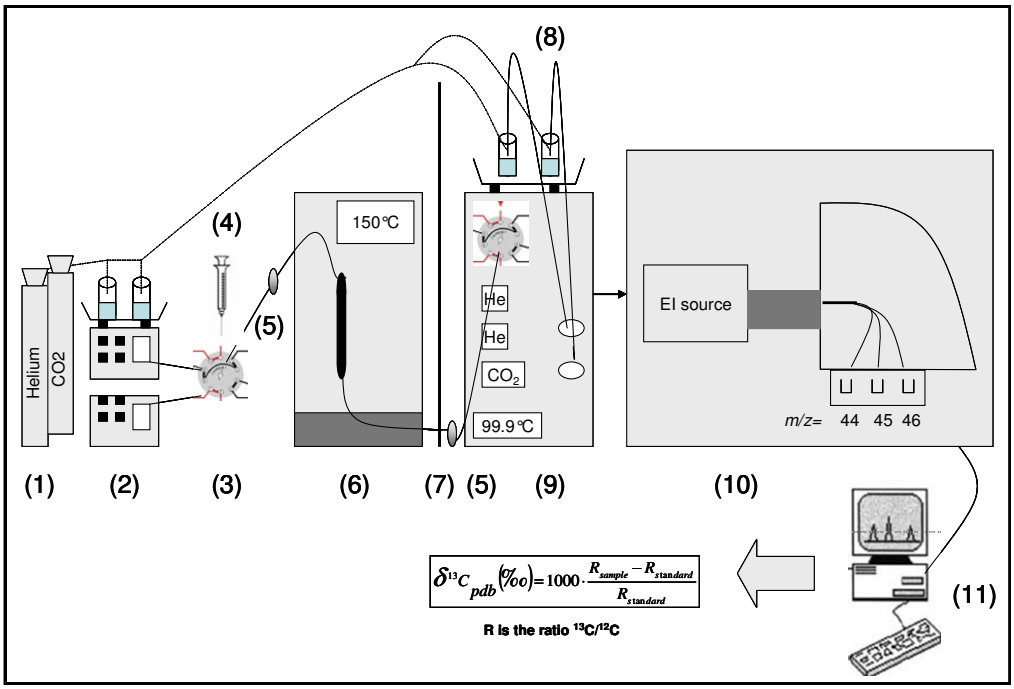


Figure 37: Schematic representation of the High Temperature Liquid Chromatography hyphenated with an Isotope Ratio Mass Spectrometer (HTLC-IRMS).

In Figure 37, (1) is the helium and CO_2 tanks used to degas the eluent buffer and as the reference gas, respectively; (2) are the LC pumps and the degasser; (3) the injection valve with a 10µL loop; (4) is the autosampler; (5) are the 0.2 µm on-line filters; (6) is the Polaratherm® 9000 heater; (7) is a plexiglass window; (8) are the oxidant and acid reagents used in the LC-IRMS interface; (9) is the LC Isolink® interface; (10) is the magnetic sector instrument; and (11) represents the data treatment.

5.2.2.3 Choice of the chromatographic conditions for HTLC-IRMS separations

In the work presented here, two different columns were used. Firstly, a polymeric column packed with sulfonated polystyrene-divinylbenzene (PS-DVB) copolymers stable up to 90°C (and 0 ≤pH ≤14). The mechanism of separation is based on the ion exclusion principle. The negatively charged species are repelled from the membrane and the uncharged ones are absorbed on the beads. Thus, the equilibrium is regulated through the acidic dissociation (pKa) of the organic acid. Secondly, for LC run at higher temperatures, LC separation was carried out with a porous graphitized carbon (PGC) column (Hypercarb 100 x 2.1mm, 5µm, Thermo). The rigid and planar surface of the PGC column is composed of hexagonally arranged and covalently bonded carbon atoms. Therefore, this column is particularly resistant to high temperature (up to 200°C) and to 1≤pH ≤14. As pointed out by West (West et al., 2004), the retention mechanism is governed by three phenomena: the hydrophobic effect, the dispersive interactions, and the interactions of polarized and polarisable functional groups of the solute with the graphite surface. Several work reported the use of the PGC column for the separation of polar compounds such as carbohydrates, polar phenolic acids (amongst them coumaric acid using organic buffer such as methanol and THF), steroidal drugs and lipids (Roy et al., 2006). Additionally, in drug metabolism, the lipophilic parent drug is generally well retained on a reversed phase silica stationary phase whereas glucuronide or sulphate metabolites are less retained under the same chromatographic elution. One approach used was to separate drug metabolites with a PGC column (Ayrton et al., 1995).

For the LC separation of organic acids (see Figure 38), the isotopic analyses were carried out with a Coregel 87H3 column (300 x 7.8mm, Transgenomic). Here, the

column works in isothermal mode at 35°C and 60°C, with 0.008N sulphuric acid as eluent and a flow rate of 500µL/min.

For high-precision isotopic analyses of organic acids at higher temperature (lower than 120°C), the PGC column used phosphoric acid (20mM) in isocratic and isothermal mode (LC flow rate at 220µL/min). Additionally, other experiments were performed with a gradient of temperature started at 35°C held for 5min, followed by a ramp of temperature of 20°C/min until 110°C held for 1 or 5min.

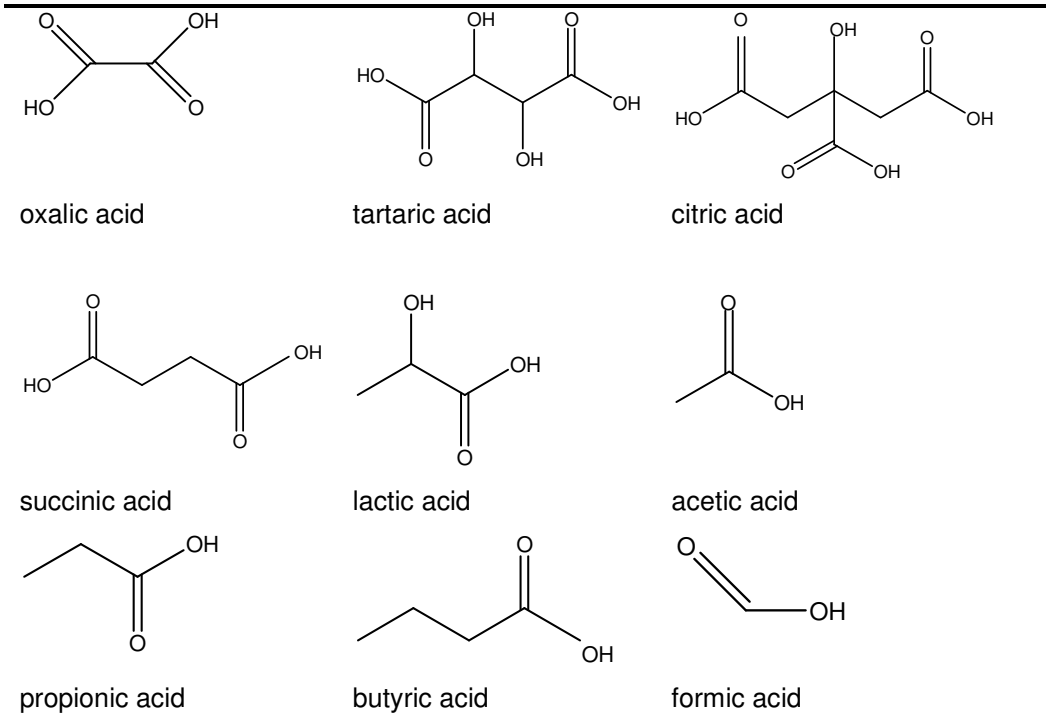
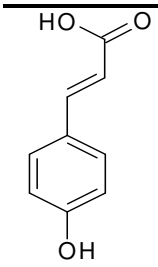


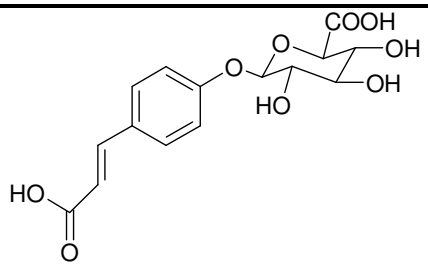
Figure 38: Chemical structure of organic acids used in this work

For high-precision ^{13}C isotopic analyses of phenolic acids (see Figure 39 for their chemical structure), we tested different analytical conditions. Non-published data showed that by using 100% aqueous mobile phase (with binary buffers containing phosphoric acid, sodium fluoride and sodium hydroxide, sodium fluoride) with a Zirchrom-PDB column (2.1 x 150mm, 3µm), the baseline separation of *p*-coumaric acid, and its sulfate and glucuronide conjugates was difficult to achieve at temperature lower than 80°C. Then, the LC separation was carried out with the PGC column at 150°C, 160°C and 170°C under isothermal and isocratic conditions (phosphate buffer at 20mM, pH 7.2, LC flow rate at 500µL/min). In these HTLC-IRMS

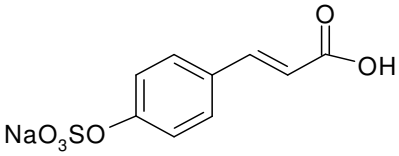
conditions (with temperature above 150°C), other phenolic acids such as dihydrocaffeic acid, protocatechuic acid and dihydrobenzoic acid were eluted in the dead volume of the column. Caffeic acid was nearly coeluted with *p*-coumaric acid (data not reported).



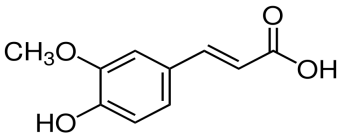
p-coumaric acid



p-coumaric acid 4'-O-β-D-glucuronide



p-coumaric acid 4'-O-sulfate sodium salt



ferulic acid

Figure 39: Chemical structure of phenolic acids used in this work

Injection of standard CO_2 pulses (20sec of width) every 30sec was also performed using the LC-IRMS interface through different LC runs (with gradient of temperature and isothermal conditions). This allows us to mimic elution of compounds and to study the effect of the LC conditions on isotopic precision and accuracy. In this case, each reported $\delta^{13}C$ values are a mean of a duplicate run.

5.2.2.4 Isotopic measurements and calculation

Data were acquired with Isodat NT software. The $^{13}C/^{12}C$ isotopic ratio is expressed as a $\delta^{13}C$ value calibrated against the international standard (Vienna Pee Dee Belemnite, VPDB). The delta notation which measures the relative isotopic concentration is defined as:

$$\delta^{13}C_{sample}\ ,\ ‰ = [(R_s / R_{std}) - 1] \times 1000, \qquad (\textit{Equation 1})$$

where R_s is the ratio of ^{13}C in the sample and R_{std} is the ratio of ($^{13}C/^{12}C$) of International Standard of Pee Dee Belemnite, R_{std}=0.0112372.
All the $\delta^{13}C$ values are reported relative to the reference CO_2 of a known carbon isotopic composition, introduced directly into the ion source in three pulses at the beginning of each run. The CO_2 reference gas was calibrated against VPDB international standard (IAEA-CH6 through the LC Isolink® interface).

5.2.3 RESULTS AND DISCUSSION

5.2.3.1 HTLC-IRMS, a counter-nature coupling?

In order to use the high temperature LC with the IRMS device, the choice of the size i.e. internal diameter (i.d.) of the column is important. Typically, with the LC-IRMS, the LC flow rate is limited to 700µL/min due to the geometry of the interface. However with high temperature liquid chromatography (HTLC) hyphenated to the UV or MS devices, based on van Deemter curve (which is flattened when the temperature is increased), the chromatography efficiency is not affected if the chromatographic separation is carried out at a higher flow rate (Lestremau et al., 2007). Therefore HTLC is generally associated with a high LC flow rate for narrow bore column if compared to separations carried out at room temperature (Guillarme et al., 2005). Typically, between 30°C and 200°C, for water as LC eluent, for 5µm of particle size the optimum linear velocity increase is 10 fold for 4.6, 2.1 or 1.0mm i.d. columns. With a 2.1mm i.d. column, the optimum flow rate is ~145µL/min at ambiant temperature and reached 600 and 1500µL/min at 100 and 200°C, respectively. For a 4.6mm i.d. column, already at 30°C the optimum flow rate is at 700µL/min which is already the maximum of the flow rate typically allowed when the LC-IRMS runs under standard temperature conditions. Therefore, the miniaturization of the LC column using 1mm i.d. column might be one solution for running LC separation at temperature higher than 150°C. At 200°C, the optimum LC flow rate is ~ 340µL/min which matches the requirement of the LC-IRMS interface. However, the loading capacity is four times less with a 1mm i.d column versus a 2mm i.d. column (Abian J., 1999) which might be a problem as the IRMS is sensitive in terms of amount of material injected (the required amount is 1nmol). Thus with a 1mm i.d. column, the volume required to obtain good sensitivity would be increased which would affect the peak shape. In order to demonstrate the usefulness of the high temperature for

isotopic analysis carried out by the LC-IRMS, the chromatographic efficiency is compromised here by working with non-optimal velocity (according to the van Deemter curve) for the LC eluent to respect the constraints of the LC-IRMS interface.

5.2.3.2 Effect of the temperature on the retention factor of citric acid

Currently, it is time consuming to measure high-precision isotopic ratios (^{13}C and ^{2}H) of exogenous citric acid (used as food additive) in fruit products. The sample preparation involves a preparative LC followed by the crystallisation of citric acid into calcium citrate (Jamin et al., 2005). Here, standard solutions of a mixture of organic acids (containing formic, acetic, propionic, tartaric, butyric, succinic and citric acids) were injected though the PGC (Hypercarb column), and eluted in isothermal mode between 50 to 110°C with a phosphate buffer at constant flow rate. Figure 40 shows that by increasing the temperature of the mobile phase and of the LC column, the retention factor (k) of organic acids is decreased. For instance, the retention factor of citric acid was 5.5 at 50°C and decreased to 1.9 at 110°C. This corresponds to a 2.8 fold gain in speed of analysis. The modification of the retention factor with the temperature can be evaluated using the van't Hoff equation (equation 2).

$$\ln K = -\frac{\Delta H}{RT} + \frac{\Delta S}{R} + \ln \Phi \qquad (\textit{Equation 2})$$

where k is the retention factor, ΔH and ΔS are respectively the enthalpy and the entropy change, associated with the transfer of solute from one phase to another, R is the gas constant, T is the temperature (in Kelvin) and Φ is the volume phase ratio corresponding to volume of the stationary phases divided by the volume of the mobile phase.

Experimentally, by plotting 1/T (in the x-axis) and lnk (in the y-axis), a quadratic correlation is found (r^2 at 1.000) for citric acid. This curve demonstrates that the retention factor of this compound may be estimated by varying the temperature. It also means that the retention mechanism is constant in the range of temperatures studied as already observed for other compounds with the PGC column (Pereira et al., 2007).

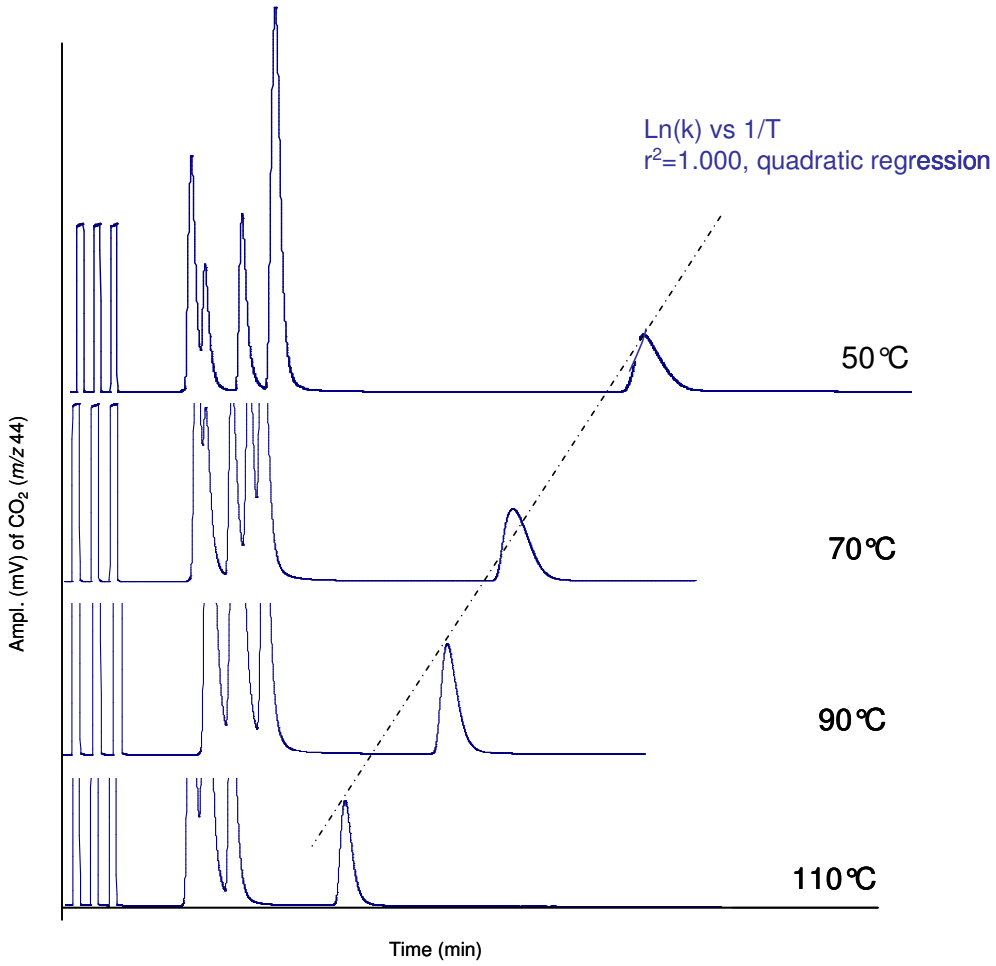


Figure 40: HTLC-IRMS chromatograms of a mixture of organic acids carried out in isothermal conditions (from 50 to 110°C) on a PGC column (Hypercarb column)

In Figure 40, HTLC-IRMS conditions are: a phosphoric acid 20mM as eluent; flow rate 220µL/min. The three square peaks in the beginning of each chromatogram are reference CO_2 peaks. To illustrate the influence of the temperature on chromatographic parameters, the evolution of the retention factor (k) of the citric acid is plotted versus the temperatures. The slope of 1 was assessed using a quadratic regression model.

The second effect related to the variation of the temperature of the mobile phase and of the LC column are the modifications of the peak width and peak height. Typically here, the peak width of citric acid is reduced from 200sec to 120sec and the peak

height is increased by a factor of 2.2 in the range of temperature studied (the peak area is nearly constant with SD at 3 V.s). This result is explained by the contribution of water viscosity on the mass transfer (C term of the van Deemter curve). Thus, the band broadening is decreased. Nevertheless, Dugo et al. (Dugo et al., 2007) showed that this phenomenon is not constant between 110-190°C for butylparaben (analyzed with water and 5% of THF) and presents an inflection of the slope of the curve around 170°C (above this temperature the tailing factor is increased).

All these results highlighted that the temperature with isothermal mode gives a powerful tool to improve the retention factor, peak width and peak height as well as the chromatographic selectivity in LC separation. Thus, compared to the conventional parameters to optimize for LC-IRMS method development (which are the columns, the ionic strength of the buffer and the pH), the temperature can play a major role by changing the eluent strength of aqueous mobile phase and by improving the peak shape.

5.2.3.3 Effect of temperature gradient on ^{13}C isotopic ratios measured by LC-IRMS

We tested the temperature gradient of the high temperature on the high-precision LC-IRMS ^{13}C measurements. The typical precision of $\delta^{13}C$ values by LC-IRMS is 0.1-0.3‰ (Krummen et al., 2004). The influence of a temperature gradient on the isotopic precision and accuracy were tested by comparing the isotopic ratios of organic acids measured at ambient temperature with a PS-DVB column to the isotopic ratios acquired with a gradient of temperature (below 120°C) with the PGC column (Hypercarb). As reported in Table 22, for the compounds injected separately, the precision, SD($\delta^{13}C$) is good (below 0.3‰) and the accuracy ($\Delta^{13}C$, ‰), assessed as the difference between the two LC conditions, was also below 0.3‰. However, as illustrated in Figure 41, for succinic and citric acids, SD($\delta^{13}C$) was higher than 0.7‰ and $\Delta^{13}C$ higher than 0.8‰. For other organic acids reported in these conditions (Table 23), the accuracy of isotopic ratio is affected by the poor resolution with neighbouring peaks.

Table 22: ^{13}C isotopic ratios at natural abundance of acetic, propionic, butyric and citric acid measured with the Hypercarb with temperature gradient and with Coregel 87H3 columns in isothermal condition.

	Gradient of temperature (Hypercarb) $\delta^{13}C \pm SD$ ‰ (n=3)	Isothermal elution (Coregel at 35°C) $\delta^{13}C \pm SD$ ‰ (n=14)	$\delta^{13}C$ (Hypercarb – Coregel at 35°C) Δ ‰
Acetic acid	-32.11 ± 0.25	-32.17 ± 0.17	0.06
Propionic acid	-28.99 ± 0.11	-29.23 ± 0.25	0.24
Butyric acid	-22.39 ± 0.37	-22.66 ± 0.26	-0.27
Citric acid	-16.28 ± 1.50	-18.60 ± 0.14	2.32

In Table 22, the temperature gradient started at 35°C, held for 5min and then ramped at 20°C/min until 110°C, held for 1min column; flow rate 220µL/min; and with the PS-DVB column; flow rate 500µL/min in isothermal conditions at 35°C and at 60°C.

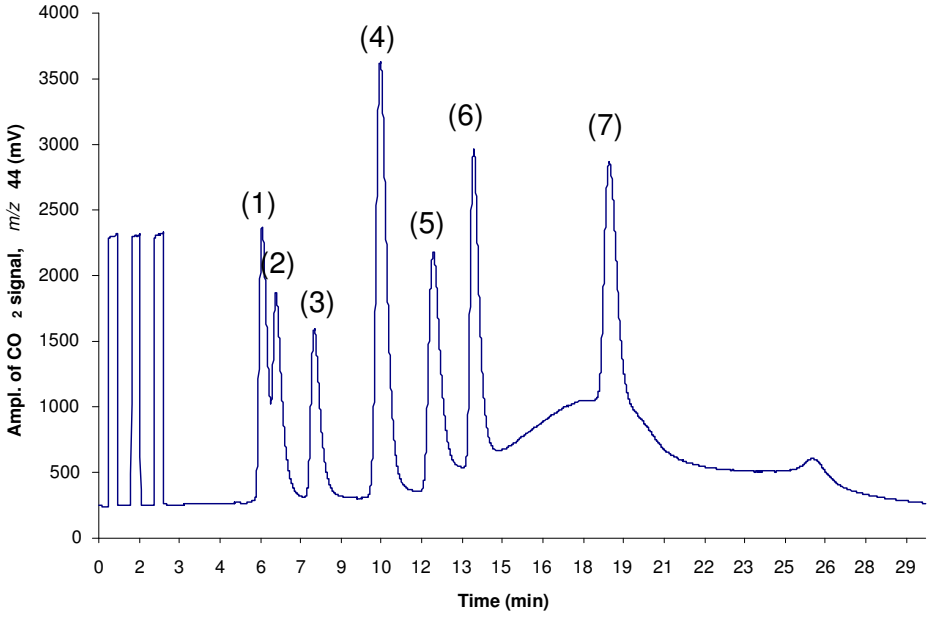


Figure 41: HTLC-IRMS chromatogram of organic acids (standard mixtures) injected on a PGC column with a temperature gradient

The conditions used in Figure 41 are: Hypercarb column; phosphoric acid; flow rate 220µL/min; temperature gradient started at 35°C, held for 5min and then rampled at 20°C/min until 110°C, held for 1min.

The list of compounds is (1) formic acid; (2) acetic acid; (3) propionic acid; (4) tartaric acid; (5) butyric acid; (6) succinic acid; (7) citric acid..

Table 23: isotopic ratios at natural abundance of (1) formic acid; (2) acetic acid; (3) propionic aicd; (4) tartaric acid; (5) butyric acid; (6) succinic acid; (7) citric acid; carried out with the PS-DVB column in isothermal conditions at 40°C and with Hypercarb column with gradient of temperature

	Isothermal elution (with Coregel at 40°C)	Gradient of temperature (with Hypercarb)	$\Delta^{13}C$ (Isothermal – gradient)
	$\delta^{13}C \pm SD$ ‰ (n=5)	$\delta^{13}C \pm SD$ ‰ (n=3)	Δ, ‰
Citric acid (7)	-18.32 ± 0.39	-16.22 ± 1.53	2.10 (‡)
Tartaric acid (4)	-21.77 ± 0.67	-21.49 ± 0.06	0.28
Succinic acid (6)	-22.40 ± 0.11	-21.56 ± 0.74	0.84 (‡)
Acetic acid (2)	-31.98 ± 0.35	-29.23 ± 0.17	2.75 (†)
Propionic acid (3)	-29.27 ± 0.24	-28.46 ± 0.32	0.81(†)
Butyric acid (5)	-22.57 ± 0.17	-21.95 ± 0.36	0.62 (†)

(‡) represents the isotopic values that are affected by the temperature gradient leading to Δ ‰ (accuracy) higher than 1‰ which is not suitable for studies at natural abundance.
(†) is the isotopic values affected by the low chromatographic resolution obtained with adjacent compound.

As pointed out by Teutenberg (Teutenberg et al., 2006) who used UV or CAD (charged aerosol detector) devices with various LC columns (used for high temperature LC), the bleeding of the stationary phase varies according to the stationary phases of the LC columns, the temperature and the instruments used for measuring. The column bleed can be easily overcome in LC-UV by subtracting the background using appropriate LC software. However, for isotopic work using an IRMS device, the CO_2 background subtraction is currently impossible to perform with an Isodat® software. An alternative solution might be to use a mathematical software program to remove the drift of the baseline (assuming that this is reproducible) followed by the re-calculation of the isotopic ratio.
In order to avoid non-reliable isotopic values in this condition, an alternative solution would be to elute the compound after the temperature gradient in a plateau of

temperature to obtain a stable CO_2 background. This might help to obtain a meaningful improvement of isotopic precision. In Figure 42, using successive pulses of the CO_2 reference gas (arbitrary set at 0‰), the precision of CO_2 pulses was assessed at 0.04‰ at the beginning of the run and in the plateau (after the temperature gradient and in isothermal condition at 110°C) the SD was measured at 0.06‰ for 4 successive CO_2 pulses. Additionally, one advantage foreseen when using a temperature gradient in LC-IRMS is the elution of compounds which are stacked when using isothermal conditions. Thus, by applying temperature gradient, the cross contamination through successive injections due to compounds potentially stacking on the column can be avoided.

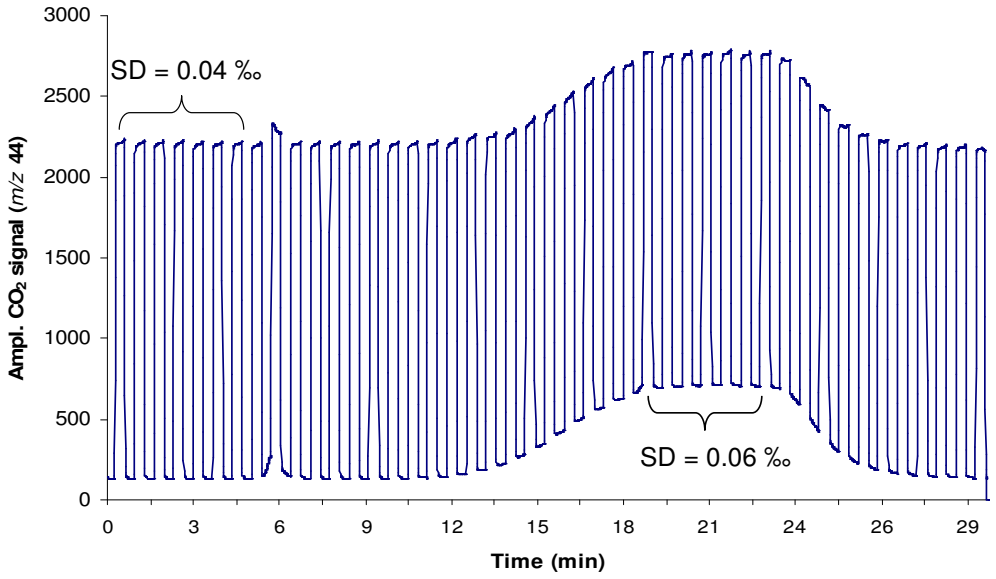


Figure 42: HTLC-IRMS chromatogram of CO_2 pulses injected every 30sec on Hypercarb column with a temperature gradient

In Figure 42, the LC flow rate is 220µL/min; phosphoric acid 20mM; temperature gradient started at 35°C hold for 5min and then a ramp of temperature of 20°C/min until 110°C, held for 5min. This chromatogram illustrates the LC bleed of the column during a temperature gradient and shows that after a temperature gradient, an isothermal temperature is needed to retrieve the isotopic precision (SD) in the same order of range as in the beginning of the run.

5.2.3.4 Isothermal LC separations of phenolic acids using HTLC-IRMS

Phenolic compounds are found in substantial levels in commonly consumed fruits, vegetables, herbal products, and beverages. There are numerous epidemiological studies that suggest that consumption of fruits and vegetables are correlated with reduced risk of chronic diseases associated with oxidative stress. The precise role that phenolics have in human health is not yet well understood because these compounds may be present either in the free aglycone form or conjugated forms. To study the bioavailability of phenolic acids, it is important to discriminate between endogenous and exogenous phenolic acids. Recent availability of stable-isotope-labeled analogues of daidzein and genistein (isoflavone compounds) show that their pharmacokinetics in humans was measured independently from the presence of naturally occurring isoflavones from the diet (Setchell et al., 2003). These labeled tracers circumvent the need to use radioisotopic tracers, and importantly are synthesized with ^{13}C atoms that are both chemically and metabolically inert. Our interest was to measure ^{13}C isotopic ratio of non-labeled phenolic compounds such as *p*-coumaric acid, its sulfate and glucuronide conjugates and ferulic acid, using the LC-IRMS device (with 100% aqueous mobile phase). Current LC methods with ODS-silica packing materials required the use of an organic modifier such as methanol to elute the phenolic molecules with addition of organic acid to improve the peak shape at ambient temperature (Robbins & Bean, 2004).

Using the porous carbon graphite column in isothermal conditions (at 170°C) with a phosphate buffer, baseline separation was achieved for *p*-coumaric acid, its glucuronide conjugate and ferulic acid (Figure 43). In these conditions, the sulfate conjugate of *p*-coumaric acid has a poor chromatographic resolution with *p*-coumaric acid. The comparison of $\delta^{13}C$ values acquired by flow injection-IRMS (measured at ambient temperature) and by HTLC-IRMS at 170°C (Table 24) showed that excellent isotopic precision (SD of $\delta^{13}C$ lower than 0.3‰) and accuracy were obtained for *p*-coumaric acid and its glucuronide conjugate. Due to the moderate chromatographic resolution of the sulfate conjugate with the *p*-coumaric acid, poor accuracy (with a difference ~1.0‰) and good precision were measured. For ferulic acid, the $\delta^{13}C$ values were not consistent between FIA-IRMS and HTLC-IRMS analyses.

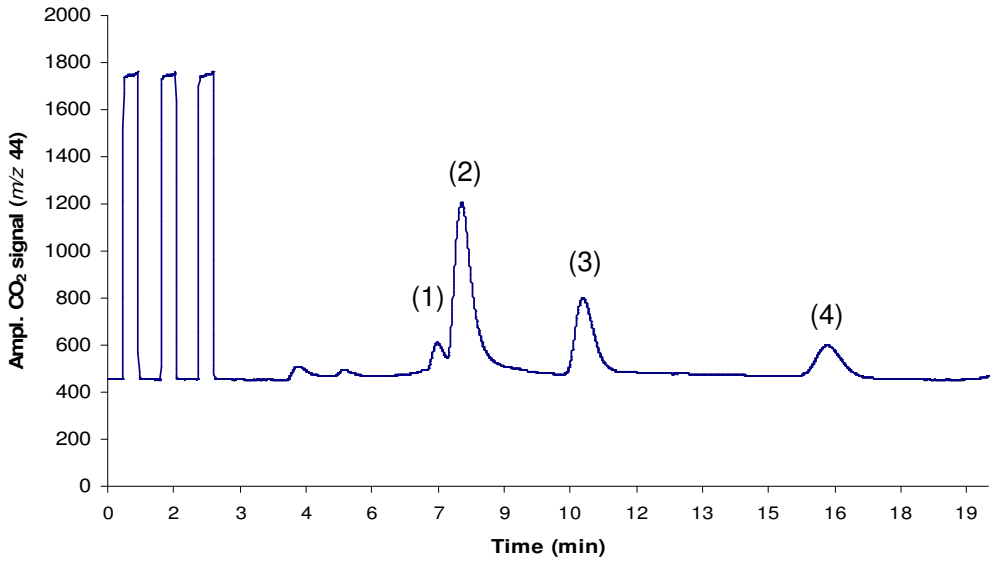


Figure 43: HTLC-IRMS chromatogram of phenolic acids eluted with the Hypercarb column in isothermal conditions at 170°C

In Figure 43, the conditions are: flow rate 500µL/min; phosphate buffer 20mM, pH 7.2; isothermal temperature at 170°C. The compounds are (1) sulfate conjugate of p-coumaric acid; (2) p-coumaric acid; (3) glucuronide conjugate of p-coumaric acid; (4) ferulic acid

Table 24: ^{13}C isotopic ratios of phenolic acids carried out with the Hypercarb column in isothermal conditions at 170°C and at ambient temperature

Compounds	FIA-IRMS $\delta^{13}C \pm SD$ ‰	HTLC-IRMS (170°C) $\delta^{13}C \pm SD$ ‰, (n=5)	HTLC-FIA Δ ‰
sulfate *p*-coumaric acid	-30.95 ± 0.71	-32.04 ± 1.60	1.09 (†)
p-coumaric acid	-28.80 ± 0.14	-28.88 ± 0.47	0.08
glucuronide *p*-coumaric acid	-22.87 ± 0.20	-22.70 ± 0.28	0.17
ferulic acid	-26.23 ± 0.13	-22.79 ± 1.48	3.44

In Table 24, individual phenolic acid was analyzed by flow injection mode-IRMS using the same eluent and flow rate at ambient temperature. Δ (‰) represents the difference of $\delta^{13}C$ values measured between the Hypercarb column at 170°C and the

FIA-IRMS analyses at ambient temperature. Typically, ferulic acid in this condition is degraded due to its long residence time at high temperature.
(†) is the isotopic values affected by the low chromatographic resolution obtained with adjacent compound.

Additionally, by decreasing the temperature to 160°C and to 150°C, isothermal analyses of *p*-coumaric acid by HTLC-IRMS showed that the $\delta^{13}C$ of *p*-coumaric acid was -28.50 ± 0.2‰ (with k=1.4) and -25.45 ± 0.04‰ (with k=3.1), respectively. The inaccuracy of the isotopic values of ferulic acid at 170°C as well as for *p*-coumaric at 150°C showed that the "long" residence time of the compounds in the column at this temperature degrades the ferulic acid at 170°C and the *p*-coumaric acid at 150°C. As already pointed out for the separation of vitamins for instance (stable LC chromatography can be performed for pyridoxine and riboflavin at 180 and 160°C, respectively (Chienthavorn et al., 2004), the degradation of the compounds at high temperature depends on the analytical conditions (the column, temperature and flow rate affecting the residence time of the compound at high temperature).
This experiment on conjugated metabolites of *p*-coumaric acid highlights that using judicious separation conditions, the HTLC-IRMS separation allows a rapid separation of the parent compound (*p*-coumaric acid) and its metabolites (sulfate and glucuronide) in a single isocratic and isothermal run as native forms avoiding the step of enzymatic reduction of the conjugates prior to their analyses. Such a tool could have its relevance within the frame of metabolism studies using ^{13}C labeled drugs as already reported by many works of Abramson through the use of the chemical reaction interface hyphenated to mass spectrometry (CRI-MS) (Abramson et al., 1996; Abramson, 2001).

5.2.3.5 Quantitative measurements by HTLC-IRMS of *p*-coumaric acid

With the era of LC-IRMS, the use of the IRMS device to perform quantitative measurements becomes an appropriate and complementary tool to the high-precision ^{13}C isotopic ratio measurements. As reported by several authors, the signal response is proportional to the number of carbon injected for a specific compound. Schierbeek et al. used this approach to measure the glutathione concentration in human erythrocyte blood using an internal standard and were able to make the measurement in the range of 0.2 to 2mg/mL (Schierbeek et al., 2007). Here, we

report such an approach to quantitate *p*-coumaric acid with the PGC column (Hypercarb) at 160°C with phosphate buffer using benzoic acid as internal standard. In the range measured (between 95ng and 2375ng of carbon corresponding to 0.9nmol to 22.5nmol injected or 0.01 to 0.4mg/mL), a linear correlation was found with r^2=0.999. In the range studied, for *p*-coumaric acid and benzoic acid, $\delta^{13}C$ was -28.50 ± 0.20‰ (n=15) and -29.95 ± 0.31‰ (n=15), respectively. Additionally, in this range the response factor was 0.106V.s/ng carbon for *p*-coumaric acid. However, the comparison to the response factor measured by FIA-IRMS (assessed to 0.173V.s/ng C between 126 and 504ng C), shows that a correction factor is needed for taking into account the potential modification of the ionization and the flow rate (FIA-IRMS and HTLC-IRMS experiments were run at 300 and 500µL/min, respectively). By normalizing the response factor in the FIA and HTLC modes versus the response factor of the benzoic acid, and by taking into account the known decrease of the area of the peaks between 300 and 500µL/min, it is possible to measure a response factor. The response factor between the FIA and HTLC-IRMS are then in agreement (0.106 measured versus 0.102 calculated), and are affected neither by the choice of the LC column nor by the analytical conditions used. This experiment highlights that an internal standard, which is sometimes difficult to separate from other compounds in LC-IRMS, might easily be replaced by an external calibration with FIA-IRMS. Further work is in progress extending this approach to study the response factor of LC-IRMS for different classes of compounds.

5.2.3.6 Injection of methanol by HTLC-IRMS

In order to increase the solubility of compounds not completely soluble in a 100% aqueous buffer, we investigate the effect of the addition of a small percentage of methanol in the injected solution on the $\delta^{13}C$ values of compounds which would be eluted after the methanol peak in HTLC-IRMS. Thus, we performed different experiments at 35, 85 and 170°C. By injecting methanol within a solution of phosphate buffer using the PGC column (Hypercarb) at 35°C and 85°C in isothermal run, it appears (data not shown) that with a spike of 10% v/v of methanol (~25nmol injected), the CO_2 peak is huge (amplitude higher than 10V) and the peak tailing is very broad (higher than 800sec). However, by increasing the temperature to 170°C and by reducing the amount of methanol to 5% and 1% (v/v), the CO_2 peak width is decreased to ~200sec (with an amplitude higher than 10V for both conditions). As

reported in Figure 44, we performed a series of standard "ON-OFF" pulses of CO_2 reference gas (arbitrary set at 0‰).

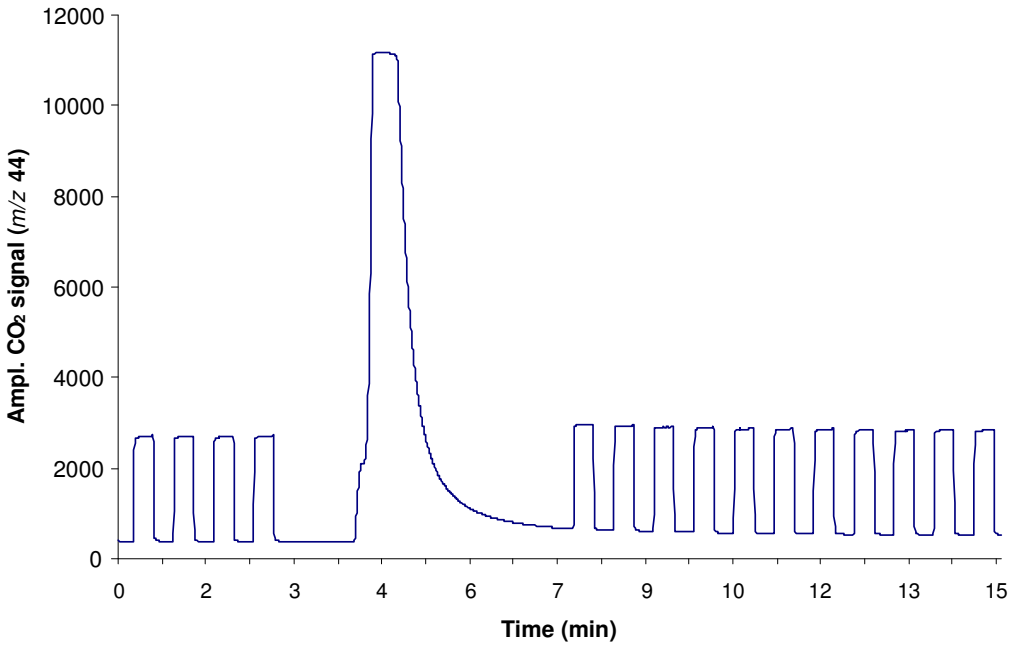


Figure 44: HTLC-IRMS chromatogram of methanol (12.5nmol or 5% v/v) carried out with the Hypercarb column at 170°C

In Figure 44, the conditions are: flow rate 500µL/min; phosphate buffer 20mM, pH 7.2; isothermal temperature at 170°C. Before and after the CO_2 peak of methanol, CO_2 pulses are injected every 30sec to mimic the elution of other compounds.

By injecting 5% of methanol (12.5nmol) and grouping 4 successive CO_2 pulses together for each determination (corresponding to a width of 150sec) before and after the elution of methanol, the isotopic precision and accuracy of the CO_2 pulses are affected 4min after the elution of methanol (Figure 45). Thus the isotopic precision is 10 fold lower than for the first group of pulses in the beginning of the run (SD $\delta^{13}C$ is 0.29 versus 0.04‰). After 10min, SD ($\delta^{13}C$) returns to 0.04‰. Additionally, with the injection of 1% of methanol (2.5nmol), the isotopic precision of the CO_2 pulses is slightly affected 3min after the elution of the CO_2 peak of methanol when compared to pulses before its injection, SD ($\delta^{13}C$) is 0.06 versus 0.04‰.

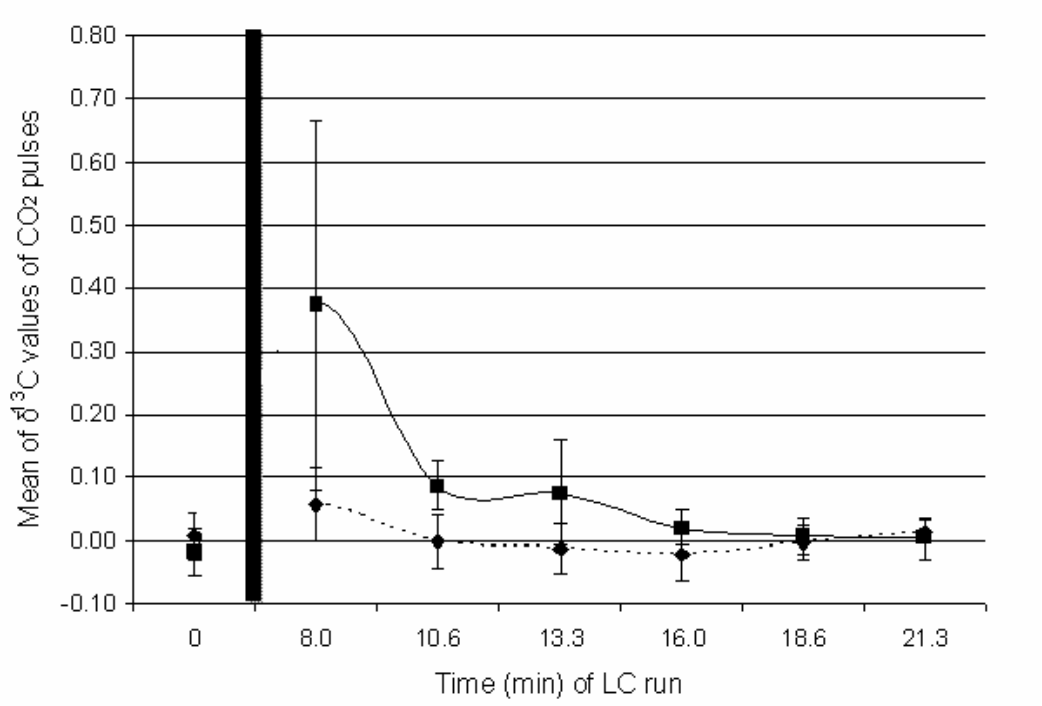


Figure 45: Schematic representation of the variation of the ^{13}C isotopic ratios of CO_2 pulses before and after the elution of methanol (represented by the black bar).

In Figure 45, arbitrarily the reference CO_2 gas was set at 0‰. Each point represents the grouping of 4 successive pulses within a duplicate run (n=8). The dotted line is the evolution for the injection of 1% methanol (2.5nmol). The bold line represents the injection of 5% methanol.

The experiments showed that the HTLC-IRMS might also be a powerful tool to measure isotopic ratio of molecules not completely soluble in an aqueous mobile phase by adding a small percentage of methanol to the solution.

5.2.3.7 Perspective for HTLC-IRMS

With the new stationary phase packing materials available for HTLC-IRMS compatible with a 100% aqueous mobile phase, it may also be relevant to carry out isotopic analysis with a column-switching approach using orthogonal mode of separation. Ion exchange column as first dimension followed by HTLC column as second dimension would have several benefits. i) to act as a pre-concentration step

before the isotopic analysis; ii) to decrease the peak width and increase the peak height for compounds eluted at the end of gradient with ion exchange column and iii) to help improving baseline resolution for difficult LC separation.

5.2.4 CONCLUSIONS

The new analytical strategy based on the coupling of the high temperature liquid chromatography to IRMS present interested insights for high-precision ^{13}C isotopic analyses. LC separation as high at 170°C can be used without changing the ^{13}C isotopic ratio of compounds. With these new temperature conditions, the peak height and peak width can be improved. However at this temperature, degradation is observed for compounds with long residence time giving unreliable ^{13}C isotopic ratio. Moreover, at 170°C, addition of 10µL of methanol (1-5%) in the injected solution showed that isotopic precision of CO_2 peaks eluted after the CO_2 peak of methanol is affected but remains acceptable for isotopic anylyses. Thus, this possibility exists to slightly increase the range of analyzable compounds by HTLC-IRMS which are not fully soluble in a 100% aqueous buffer and to which small percentage of methanol need to be added for solubilisation purpose. Despite the column bleeding which affects the isotopic precision and accuracy, HTLC adds new and orthogonal stationary phase packing materials to the field of high-precision ^{13}C isotopic analyses. While this work demonstrated the effective use of the high temperature for high-precision ^{13}C isotopic measurement, this work also lays the groundwork for future isotopic applications with a new tool for deciphering complex samples.

CHAPTER 6

^{13}C isotopic analyses of glucose in plasma by LC-IRMS

Keywords: glucose, plasma, LC-IRMS

6 ^{13}C ISOTOPIC ANALYSES OF GLUCOSE IN PLASMA BY LC-IRMS

In this chapter, a new approach for measuring ^{13}C isotopic enrichment of underivatised glucose in plasma, after infusion of [^{13}C-U] glucose tracer, is reported (data unpublished).

6.1 *IN VIVO* GLUCOSE PRODUCTION

There are two components related to glucose production *in vivo*: glycogenolysis and gluconeogenesis. Following the ingestion of a meal or during a glucose infusion, a significant portion of the glucose is stored as glycogen in liver. Glycogen is released into the blood circulation during fasting. The glycogenolysis is under hormonal control (such as glucagon as the main hormone). The gluconeogenesis is the new formation of glucose from noncarbohydrate precusors (such as lactate and alanine). To measure the kinetics of glucose production (rate of appearance and rate of disappearance) in human, different approaches have been described using stable isotope tracers (Landau, 1999). The physiological information obtained from tracer studies is important to understand the role of the gluconeogenesis during exercice (Jeukendrup et al., 1999) and to study the effect of glucose ingestion on gluconeogenesis (Wolfe, 1992).

6.2 CONVENTIONAL GC-C-IRMS APPROACH

In order to measure ^{13}C isotopic enrichment of glucose in plasma, glucose is routinely converted into alditol acetates with acetic anhydride prior to GC-C-IRMS isotopic analysis. This procedure introduces 10moles of derivative C per mole of glucose by adding an ethyl group to each free hydroxyl group. Therefore, due to the isotopic dilution, the sensitivity for high-precision ^{13}C isotopic analysis of glucose derivative is reduced by 60% in GC-C-IRMS when compared to LC-IRMS analysis using native form of glucose (6 carbons for underivatised glucose versus 16 for the derivatized one). Moreover, it is known that the alditol derivativatization produces isotopic

fractionation. The isotopic bias observed is due to the formation of a bond between the oxygen of the monosaccharide and the carbon of the acetic anhydride. Hence, carbon atoms derived from the acetic anhydride are fractionated. The best way to avoid this, would be to consume quantitatively the acetic anhydride which is not possible since it is present in an excess amount. Another alternative is to used methyl boronic acid with sylilating reagent for the remaining hydroxyl group (van Dongen et al., 2001).

6.3 LC-IRMS METHOD AND RESULTS

In the frame of a clinical study, after administration of tracers (among them U-^{13}C glucose), ^{13}C isotopic enrichment of plasma was measured by LC-IRMS on 100µL of plasma.

6.3.1 SAMPLE PREPARATION

Before the infusion of the tracer, five blood samples (5mL) were drawn every 25-30 min. Subsequently during the continuous infusion of the tracer, eight other blood samples were drawn every 25-30min.

After deproteinisation with 150µL of barium hydroxide (0.3N from Sigma) and 150µL of zinc sulfate (0.3N from Sigma), the upper layer was transferred to resins (cationic and anionic resins, AG50W-X8 and AG1-X8, respectively). The elution of glucose was then performed with 2 mL of water. An aliquot of 30µL is used and then diluted with H_2SO_4 (0.008N) to obtain 100µL of solution. Finally, 10µL is injected into the LC-IRMS.

Isotopic analysis is carried out with a Coregel 87H3 column (300 x 7.8 mm) heated at 50°C in isothermal conditions with sulphuric acid as eluent. The instruments used (LC and IRMS) was already described in the previous chapters.

6.3.2 RESULTS & DISCUSSIONS

Using the LC-IRMS method briefly presented here, the retention time of native glucose is approximately 14min and an unknown peak elutes at 18min (Figure 46). Based on the work published by Jackson et al. (Jackson et al., 2007) by GC-C-IRMS using methyl boronic and BSTFA as sylilating reagent for the last hydroxyl group, the

retention time of derivative glucose was lower than 10min with standard compounds. Here, using our LC-IRMS method, an improvement of the retention time is still envisageable using a shorter LC column (work in progress).

Using the LC-IRMS method described here, ^{13}C isotopic values of glucose ($\delta^{13}C$) in plasma, at natural abundance, were measured at -25.54 ± 0.42‰ (n=5). Additionally, the unknown peak eluting at the very end of the run showed a reproducible $\delta^{13}C$ with SD at 0.50‰ ($\delta^{13}C$= - 26.93‰, n=13) over all the kinetic study (over 5hours) Morover, by spiking control plasma with various amount of U-^{13}C glucose, linear regression is reported to be r^2=0.999. The difference of isotopic enrichment between two spiked solutions was measured at 1.1‰. Thus, using standard solution of known amount of glucose spiked with a known amount of ^{13}C glucose, the slope of the accuracy curves obtained were 0.919 ± 0.007 (r^2=0.999). It showed that a potential isotopic bias occurred during the analysis (sample preparation, or analysis). Further work is in progress to understand this result as well as to identify the unknown peak.

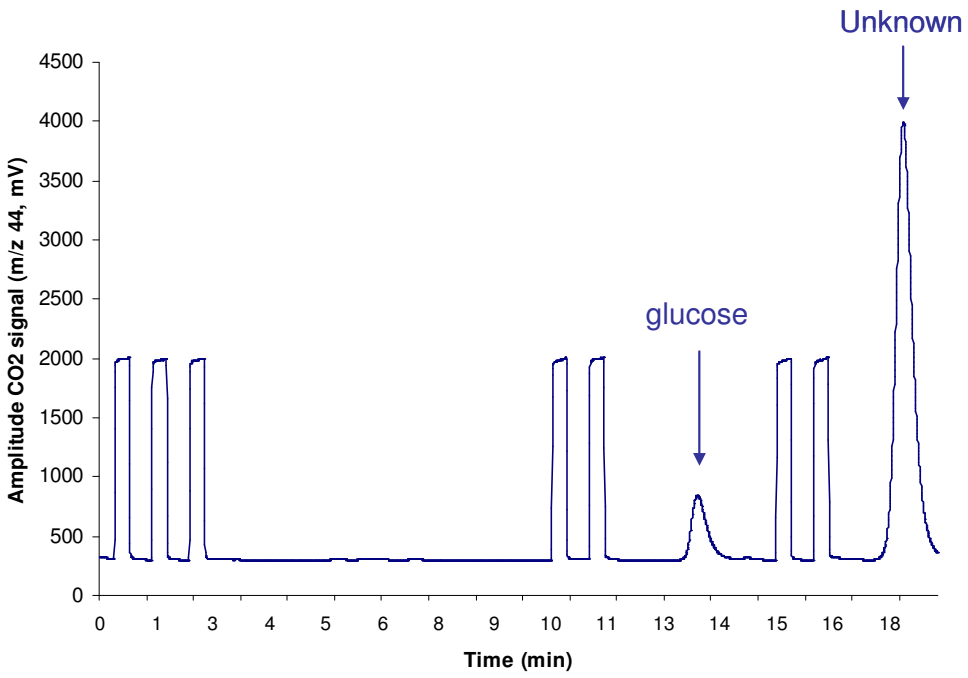


Figure 46: LC-IRMS chromatogram of glucose extracted from 100µL of plasma

6.4 CONCLUSIONS

The method described allows the precise, reliable, rapid and convenient determination of ^{13}C glucose isotopic enrichment in plasma with a small volume of analyte (as 100µL). SD ($\delta^{13}C$) measured at natural abundance is 0.5‰ and is suitable for tracer study.

CHAPTER 7

Technology highlights for LC-IRMS

Keywords: water, nitrogen atoms and oxygen

7 TECHNOLOGY HIGHLIGHTS

This section summarizes remarks concerning the LC-IRMS interface if compared to GC-C-IRMS. It includes the level of gases (argon and water), and the presence of nitrogen oxides species in the ion source. Moreover, the latest LC-IRMS development to remove oxygen in the ion source is also reported.

The LC-IRMS is less sensitive to air leaks than GC-C-IRMS since the connections within the interface between the fused silica capillaries and stainless steel pieces are fewer. Typically, the argon level measured in the ion source is higher than in GC-C-IRMS (higher than 1V versus lower than 100mV, respectively for LC and GC-C-IRMS). In LC-IRMS the degassing of the eluent is crucial to obtain reliable isotopic ratios. The eluent is degassed off-line under vacuum at 40°C in an ultrasonic bath, followed successively by on-line helium stripping and by a degasser module.

Experimentally, further studies would be needed on the LC-IRMS device to understand the influence of various parameters such as:

- The influence of the water content on the isotopic precision and accuracy: Typically, in our case, the water level measured is ~200mV in the cup used for measuring *m/z* 44. The water level thus measured is more intense than in GC-C-IRMS. As pointed out by Leckrone (Leckrone & Hayes, 1998), water in the ion source might produce interfering species such as HCO_2^+ (*m/z* 45) which produce a systematic bias on the isotopic ratio.

- The nitrogen atoms present in compounds analyzed by LC-IRMS. Under oxidation conditions, nitrogen oxides can produced N_2O, NO and NO_2 gases resulting of the chemical oxidation of N atoms. Thus, these species could contribute to the *m/z* 44 with $^{14}N^{14}N^{16}O$, *m/z* 45 with $^{15}N^{14}N^{16}O$ and *m/z* 46 with $^{14}N^{16}O^{16}O$ and $^{14}N^{16}O^{18}O$ gases. These contributions could deteriorate isotopic accuracy. No peer-reviewed article has reported in detail such studies. Nevertheless, during this work, the oxidation process inside the LC-IRMS interface has changed, based on the recommendation of Thermo (Bremen). $Na_2S_2O_8$ has replaced $(NH_4)_2S_2O_8$ as oxidation reagent. This cation modification (sodium versus the ammonium) was carried out because the ammonium cation can be oxidized into NO_2 and can increase *m/z* 46 (leading to isotopic bias). Furthermore, with the sodium oxidation reagent, isotopic

analysis is performed without $AgNO_3$ as catalyst, because of the "higher oxidation potential" of the sodium reagent. Therefore, the precipitation of AgCl is no longer a problem.

- The production of O_2 in large excess during the oxidation process reduced the throuput of the system. Typically, in our ion source (at Nestlé), the level of O_2 is ~2V measured on *m/z* 44. In this condition, the filament lifetime is severely decreased and generates some electric problems in the ion source. After discussions with Thermo (Bremen), the accelerating potential of the ion source (MAT252) was set at 8kV instead of 10kV. This issue has also been considered recently by Brand's team (Hettmann et al., 2007). The solution for the O_2 removal has a direct and considerable advantage of improving the filament lifetime by 600%. Thus, inside the LC-IRMS interface, a new O_2 scrubber consisting of two parallel hot copper reduction reactors (0.8mm i.d., active length 120mm) is installed together with a switch-over valve in between (Figure 47). One reactor is regenerated using He/H_2 while the other is actively scavenging O_2 from the gas stream. These reactors have a lifetime of 200 reduction/ oxidation cycles according to the flow rate and the concentration of oxidant reagent used. With this new modification of the LC Isolink interface, for example, the precision of the mean of $\delta^{13}C$ values for long-term measurement (1 month) of sucrose was 0.11 and 0.08‰, without and with the system, respectively.

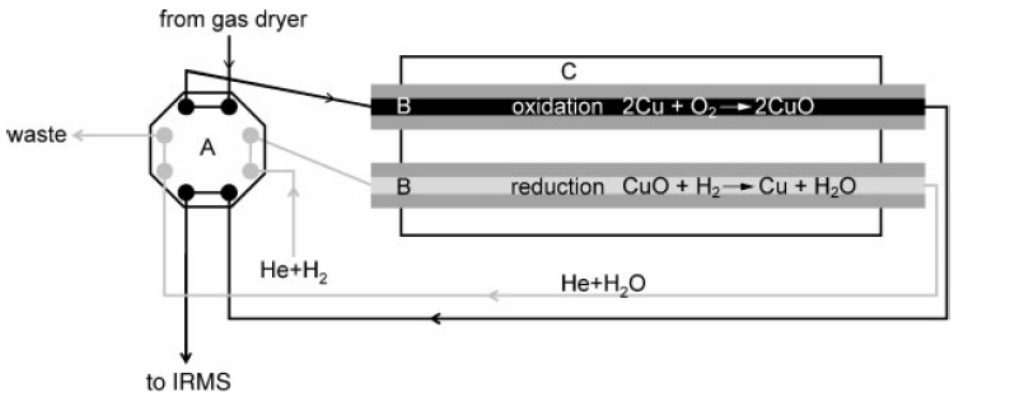


Figure 47: Schematic of the reduction reactor and heating reactor implemented within the LC-IRMS interface (Hettmann et al., 2007)

CHAPTER 8

Perspectives and conclusions

Keywords: DNA, arginine, functional metabolomics

8 PERSPECTIVES AND CONCLUSIONS

8.1 PERSPECTIVES

With the combination of LC to IRMS, the "isotopic-world" has made a major step forward. This technology allows the direct isotopic analyses of a wide range of molecules (low molecular weight compounds and macromolecules) at natural abundance or within tracer studies to be carried out.

- The LC-IRMS improves the reliability of isotopic measurements by a straightforward reading of $\delta^{13}C$ value for underivatised compounds at natural abundance.

- To measure isotopic ratios of a maximum number of amino acids, the LC-IRMS device is more suitable than the GC-C-IRMS. Moreover, LC avoids the issues related to the derivatisation typically present in GC. Thus, amino acid such as arginine, which plays an important role in cell division, immune function, muscle recovery and release of hormones, is preferably analyzed by LC-IRMS as shown by the LC-IRMS separation developed by McCullagh (McCullagh et al., 2006).

- DNA and RNA are also two major targets in biology due their central role for cell proliferation. DNA turnover can be assessed as described in the literature using GC-MS (Nissim et al., 2000) or by LC-CRIMS (Black & Abramson, 2003) with tracer. Isotopic analyses by LC-IRMS seem also particularly interesting as a tool to tackle isotopic analysis of nucleotides (Du et al., 2008) with the advantage to use lower amount of tracer if compared to GC-MS.

- LC-IRMS can measure small variation of isotopic enrichments (or variation of natural abundance) for a set of compounds where other mass spectrometer devices cannot detect any change. Over and above the insights provided by proteomic and genomic studies (Goodacre, 2007), functional metabolomic concerned with the measurement and interpretation of the flux of small

molecules with tracer administration. Thus, to scale up functional metabolomics within human, although, the IRMS device cannot measure isotopomers, LC-IRMS would have a role to play in these analyses.

- As recently illustrated by the paper of Hettmann (Hettmann et al., 2007) for oxygen removal, further developments on LC-IRMS system are still needed to improve its robustness, to optimize the parameters and to develop $^{15}N/^{14}N$ isotopic ratio measurements.

8.2 GENERAL SUMMARY

During recent decades, the use of carbon stable isotopes as tracers or in natural abundance, in different fields of life sciences, has been increasing. The development of high-precision ^{13}C isotopic instruments with GC-C-IRMS has extended the working range of molecules amenable to high-precision ^{13}C isotopic analyses in many fields (nutrition, forensic sciences and medicine) when compared to off-line methods. With the recent introduction of the LC-IRMS instrument (2004), a complementary tool is available for high-precision ^{13}C isotopic analyses of water-soluble non-volatile and thermolabile compounds. Due to the design of the interface currently commercialized, the analytical LC-IRMS methods are restricted to a 100% aqueous mobile phase.

The aim of the present work was to i) extend new GC-C-IRMS applications; ii) develop new LC-IRMS applications and iii) correlate isotopic data acquired between the GC-C-IRMS and LC-IRMS.

In this framework, we extended the use of N(O,S)-ethoxycarbonyl ethyl ester derivative for valine and threonine by GC-C-IRMS in the frame of a tracer study. The isotopic reproducibility of the method, assessed by measuring ^{13}C isotopic ratio at natural abundance of alanine, valine, leucine, isoleucine, glycine, proline, aspartic acid, threonine and glutamine of rats, was lower than 0.5% (except for aspartic acid measured at 2%). Additionally, the work performed using data acquired by LC-IRMS and GC-C-IRMS showed that N(O,S)-ethoxyethylcarbonyl ethyl ester derivative of valine introduced an isotopic bias of 2‰ between 0 and 0.47MPE. To avoid the isotopic bias and the isotopic dilution classically associated to GC-C-IRMS isotopic measurements, we presented a new LC-IRMS method for measuring ^{13}C isotopic ratio of underivatised amino acids, in particular the valine. The reliable method

developed allows comparing isotopic data between GC-C-IRMS and LC-IRMS. The total error (estimated by summing the rms of precision and accuracy) was calculated at 0.007 and 0.027MPE between 0 and 1MPE, for LC-IRMS and GC-C-IRMS respectively. Moreover, this method was used for measuring the incorporation of ^{13}C valine in bone protein over the time. Another molecule of great interest in life science is glucose, which was analyzed in the framework of a tracer study. Results showed that the LC-IRMS method developed can be routinely used and gives reliable data. SD ($\delta^{13}C$) of glucose at natural abundance from plasma was assessed at 0.5‰ over a few hours. On top of that study, LC-IRMS presents interesting insights compared to conventional continuous flow isotopic methods. Flow injection isotopic analyses (FIA-IRMS) of macromolecules were also carried out with LC-IRMS. When isotopic values are compared with data acquired with EA-IRMS device, the results showed that the difference of $\delta^{13}C$ was lower than 0.8‰ for 1000 fold less amount of carbon. In view of the limited choice of eluents and stationary phases available for LC-IRMS methods, high temperature liquid chromatography coupled to IRMS (HTLC-IRMS) was implemented. By increasing the temperature of LC eluent and columns, we mimic the addition of organic solvent or temperature ramp in GC. Isotopic analyses of phenolic acids and organic acids were performed using isothermal and temperature gradient. Isotopic analysis of standard phenolic acid at high temperature (170°C) gave accurate and precise $\delta^{13}C$ values compared to FIA-IRMS analysis at ambient temperature. Thus, with this new HTLC-IRMS approach, the selectivity and efficacy of LC columns available for LC-IRMS are increased. Furthermore, at high temperature, 1-5% of methanol added in the injected solution showed that ^{13}C isotopic ratio could still be measured.

So far, the research carried out in the frame of this PhD thesis highlights the potential of this new LC-IRMS technique. However, it is our feeling that this work represents only the tip of an iceberg. We sincerely hope that further work will be carried out for confirming of our initial results.

CHAPTER 9

References list

9 REFERENCES LIST

Abian J. 1999. The coupling of Gas and Liquid Chromatography with Mass Spectrometry. J Mass Spectrom 34:157-168.

Abramson FP. 1994. CRIMS: Chemical reaction interface mass spectrometry. Mass Spectrom Rev 13:341-356.

Abramson FP. 2001. The use of stable isotopes in drug metabolism studies. Seminars in Perinatology 25:133-138.

Abramson FP., Black GE., Lecchi P. 2001. Application of high-performance liquid chromatography with isotope-ratio mass spectrometry for measuring low levels of enrichment of underivatized materials. J Chromatograph A 913:269-273.

Abramson FP., Markey SP. 1986. Mass spectrometric analysis of sulfur in microgram quantities of biological macromolecules using a reaction interface. Biomed Environ Mass Spectrom 13:411-415.

Abramson FP., Osborn BL., Teffera Y. 1996. Isotopic differences in human growth hormone preparations. Anal Chem 68:1971-1972.

Abramson FP., Teffera Y., Kusmierz J., Steenwyk RC., Pearson PG. 1996. Replacing ^{14}C with stable isotopes in drug metabolism studies. Drug Metab Dispos 24:697-701.

Aguilera R., Hatton CK., Catlin DH. 2002. Detection of epitestosterone doping by isotope ratio mass spectrometry. Clin Chem 48:629-636.

Ando S.,Tanaka Y. 2005. Mass spectrometric studies on brain metabolism, using stable isotopes. Mass Spectrom Rev 24:865-886.

Apostol I., Brooks PD., Mathews AJ. 2001. Application of high-precision isotope ratio monitoring mass spectrometry to identify the biosynthetic origins of proteins. Protein Sc 10:1466-1469.

Asche S., Michaud AL., Brenna JT. 2003. Sourcing organic compounds based on natural isotopic variations measured by high precision isotope ratio mass spectrometry. Current Organic Chem 7:1527-1543.

Ayotte C., Levesque JF., Cleroux M., Lajeunesse A., Goudreault D., Fakirian A. 2001. Sport nutritional supplements: quality and doping controls. Canadian journal of applied physiology = Revue canadienne de physiologie appliquée 26 Suppl:S120-S129.

Ayrton J., Evans MB., Harris AJ., Plumb RS. 1995. Porous graphitic carbon shows promise for the rapid chromatographic analysis of polar drug metabolites. J Chromatogr B 667:173-178.

Babraj JA., Smith K., Cuthbertson DJ., Rickhuss P., Dorling JS., Rennie MJ. 2005. Human bone collagen synthesis is a rapid, nutritionally modulated process. J Bon Miner Res 20:930-937.

Badoud R., Fay L-B., Richli U., Husek P. 1991. Gas chromatography determination of N-carboxymethyl amin acids, the periodate oxidation products of Amadori compounds. J Chromatogr 552:345-351.

Bates CJ., Jones KS., Bluck LJ. 2004. Stable isotope-labeled vitamin C as a probe for vitamin C absorption by human subjects. Br J Nutr 91:699-705.

Baumann PQ., Ebenstein DB., O'Rourke BD., Nair KS. 1992. High-performance liquid chromatographic technique for non-derivatized leucine purification: evidence for carbon isotope fractionation. J Chromatogr 573:11-16.

Beauchemin D. 2006. Inductively coupled plasma mass spectrometry. Anal Chem 78:4111-4136.

Bederman IR., Reszko AE., Kasumov T., David F., Wasserman DH., Kelleher JK., Brunengraber H. 2004. Zonation of labeling of lipogenic acetyl-CoA across the liver: implications for studies of lipogenesis by mass isotopomer analysis. J Biol Chem 279:43207-43216.

Begley IS., Scrimgeour CM. 1997. High precision $\delta^{2}H$ and $\delta^{18}O$ measurement for water and volatile organic compounds by continuous-flow pyrolysis isotope ratio mass spectrometry. Anal Biochem 69:1530-1535.

Benson S., Lennard C., Maynard P., Rox C. 2006. Forensic applications of isotope ratio mass spectrometry - a review. Forensic Science International 157:1-22.

Berthold HK., Hachey DL., Reeds P., Thomas OP., Hoeksema S., Klein P. 1991. Uniformly ^{13}C-labeled algal protein used to determine amino acid essentiality in vivo. Proc Nat Acad Sci 88:8091-8095.

Bisutti I., Hilke I., Raessler M. 2004. Determination of total organic carbon - an overview of current methods. Trac-Trends Anal Chem 23:716-726.

Black GE., Abramson FP. 2003. Measuring DNA synthesis rates with stable isotopes. Anal Chem 75:56A-63A.

Blessing M., Jochmann MA., Schmidt TC. 2007. Pitfalls in compound-specific isotope analysis of environmental samples. Anal Bioanal Chem, DOI 10-1007/s00126-007-1588-1.

Blomstrand E. 2006. A role for branched-chain amino acids in reducing central fatigue. J Nutr 136:544S-547S.

Brand WA, Dobberstein P. 1996. Isotope-Ratio-Monitoring Liquid chromatography mass spectrometry (IRMS-LCMS): First results from a moving wire interface system. Isotopes Environ Health Stud 32:275-283.

Brand WA. 1996. High precision isotope ratio monitoring techniques in mass spectrometry. J Mass Spectrom 31:225-235.

Brenna JT. 2001. Natural intramolecular isotope measurements in physiology: elements of the case for an effort toward high-precision position-specific isotope analysis. Rapid Commun Mass Spectrom 15:1252-1262.

Brenna JT., Corso TN., Tobias HJ., Caimi RJ. 1997. High-precision continuous-flow isotope ratio mass spectrometry. Mass Spectrom Rev 16:227-258.

Breuille D., Arnal M., Rambourdin F., Bayle G., Levieux D., Obled C. 1998. Sustained modifications of protein metabolism in various tissues in a rat model of long-lasting sepsis. Clin Sci (Lond) 94:413-423.

Browne TR., Szabo GK., Ajami A., Browne DG. 1998. Performance of human mass balance studies with stable isotope-labeled drug and continuous flow-isotope ratio mass spectrometry: a progress report. J Clin Pharmacol 38:309-314.

Browne TR., Szabo GK., Ajami A., Wagner D. 1993. Performance of human mass balance/metabolite identification studies using stable isotope (^{13}C, ^{15}N) labeling and continuous-flow isotope-ratio mass spectrometry as an alternative to radioactive labeling methods. J Clin Pharmacol 33:246-252.

Cabanero AI., Recio JL., Ruperez M. 2006. Liquid chromatography coupled to isotope ratio mass spectrometry: a new perspective on honey adulteration detection. J Agric Food Chem 54:9719-9727.

Caimi RJ., Brenna JT. 1995. High-sensitivity Liquid Chromatography-Combustion Isotope Ratio Mass Spectrometry of Fat-soluble vitamins. J Mass Spectrom 30:466-472.

Caimi RJ., Brenna JT. 1993. High-precision liquid chromatography-combustion isotope ratio mass spectrometry. Anal Chem 65:3497-3500.

Caimi RJ., Brenna JT. 1996. Direct Analysis of Carbon Isotope variability in Albumins by Liquid Flow-injection Isotope Ratio Mass Spectrometry. J Am Soc Mass Spectrom 7:605-610.

Caimi RJ., Brenna JT. 1997. Quantitative evaluation of carbon isotopic fractionation during reversed-phase high-performance liquid chromatography. J Chromatogr A 757:307-310.

Calder AG., Garden KE., Anderson SE., Lobley GE. 1999. Quantitation of blood and plasma amino acids using isotope dilution electron impact gas chromatography/mass spectrometry with U-^{13}C amino acids as internal standards. Rapid Commun Mass Spectrom 13:2080-2083.

Capitan P., Malmezat T., Breuille D., Obled C. 1999. Gas chromatographic-mass spectrometric analysis of stable isotopes of cysteine and glutathione in biological samples. J Chromatogr B 732:127-135.

Chaimbault P., Petritis K., Elfakir C., Dreux M. 2000. Ion-pair chromatography on a porous graphitic carbon stationary phase for the analysis of twenty underivatized protein amino acids. J Chromatograph A 870:245-254.

Chan DC., Barrett PH., Watts GF. 2004. Lipoprotein transport in the metabolic syndrome: methodological aspects of stable isotope kinetic studies. Clin Sci (Lond) 107:221-232.

Chen P., Abramson FP. 1998. Measuring DNA synthesis rates with [1-^{13}C]glycine. Anal Chem 70:1664-1669.

Chen P., Teffera Y., Black GE., Abramson FP. 1999. Flow injection with chemical reaction interface-isotope ratio mass spectrometry: An alternative to off-line combustion for detecting low levels of enriched ^{13}C in mass balance studies. J Am Soc Mass Spectrom 10:153-158.

Chienthavorn O., Smith RM., Saha S., Wilson ID., Wright B., Taylor SD., Lenz EM. 2004. Superheated water chromatography-nuclear magnetic resonance spectroscopy and mass spectrometry of vitamins. J Pharm Biomed Anal 36:477-482.

Clapperton AT., Coward WA., Bluck LJ. 2002. Measurement of insulin sensitivity indices using ^{13}C-glucose and gas chromatography/combustion/isotope ratio mass spectrometry. Rapid Commun Mass Spectrom 16:2009-2014.

Collins ML., Eng S., Hoh R., Hellerstein MK. 2003. Measurement of mitochondrial DNA synthesis in vivo using a stable isotope-mass spectrometric technique. J Appl Physiol 94:2203-2211.

Coplen TB., Brand WA., Gehre M., Groning M., Meijer HA., Toman B., Verkouteren RM. 2006. New guidelines for δ^{13}C measurements. Anal Chem 78:2439-2441.

Corfield AP., Myerscough N., Longman R., Sylvester P., Arul S., Pignatelli M. 2000. Mucins and mucosal protection in the gastrointestinal tract: new prospects for mucins in the pathology of gastrointestinal disease. Gut 47:589-594.

Corso TN., Lewis BA., Brenna JT. 1998. Reduction of fatty acid methyl esters to fatty alcohols to improve volatility for isotopic analysis without extraneous carbon. Anal Chem 70:3752-3756.

Courtois D., Kastenmayer P., Clough J., Vigo M., Sabatier M., Arnaud MJ. 2003. Magnesium enrichment and distribution in plants. Isotopes Environ Health Stud 39:273-279.

Couzy F., Kastenmayer P., Vigo M., Clough J., Munoz-Box R., Barclay DV. 1995. Calcium bioavailability from a calcium- and sulfate-rich mineral water, compared with milk, in young adult women. Am J Clin Nutr 62:1239-1244.

Coym JW,.Dorsey JG. 2004. Superheated water chromatography: a brief review of an emerging technique. Anal Letters 37:1013-1023.

Craig H. 1953. The geochemistry of the stable carbon isotopes. Geochim Cosmochim Acta 3:53-92.

De Boer AR., Alcaide-Hidalgo JM., Krabbe JG., Kolkman J., Emde Boas CN., Niessen WM., Lingeman H., Irth H. 2005. High-temperature liquid chromatography coupled on-line to a continuous-flow biochemical screening assay with electrospray ionization mass spectrometric detection. Anal Chem 77:7894-7900.

De Laeter J., Kurz MD. 2006. Alfred Nier and the sector field mass spectrometer. J Mass Spectrom 41:847-854.

De Villiers A., Lestremau F., Szucs R., Gelebart S., David F., Sandra P. 2006. Evaluation of ultra performance liquid chromatography. Part I. Possibilities and limitations. J Chromatogr A 1127:60-69.

Demmelmair H., Sauerwald T., Koletzko B., Richter T. 1997. New insights into lipid and fatty acid metabolism via stable isotopes. Eur J Pediatr 156 Suppl 1:S70-S74.

Demmelmair H., Schmidt H-L. 1993. Precise $\delta^{13}C$ determination in the range of natural abundance on amino acids from protein hydrolysates by gas chromatography-isotope ratio mass spectrometry. Isotopes Environ Health Stud 29:237-250.

Dettmer K., Aronov PA., Hammock BD. 2007. Mass spectrometry-based metabolomics. Mass Spectrom Rev 26:51-78.

Docherty G., Jones V., Evershed RP. 2001. Practical and theoretical considerations in the gas chromatography/combustion/isotope ratio mass spectrometry $\delta^{13}C$ analysis of small polyfunctional compounds. Rapid Commun Mass Spectrom 15:730-738.

Doherty MK., Beynon RJ. 2006. Protein turnover on the scale of the proteome. Expert Rev Proteomics 3:97-110.

Doherty MK., Whitehead C., McCormack H., Gaskell SJ., Beynon RJ. 2005. Proteome dynamics in complex organisms: using stable isotopes to monitor individual protein turnover rates. Proteomics 5:522-533.

Dolan JW. 2002. Temperature selectivity in reversed-phase high performance liquid chromatography. J Chromatogr A 965:195-205.

Dolnikowski GG., Marsh JB., Das SK., Welty FK. 2005. Stable isotopes in obesity research. Mass Spectrom Rev 24:311-327.

Du X., Ferguson K., Gregory R., Sprang SR. 2008. A method to determine ^{18}O kinetic isotope effects in the hydrolysis of nucleotide triphosphates. Anal Biochem 372:213-221.

Dugo P., Buonasera K., Crupi ML., Cacciola F., Dugo G., Mondello L. 2007. Superheated water as chromatographic eluent for parabens separation on octadecyl coated zirconia stationary phase. J Sep Sci 30:1125-1130.

Engel MH.,.Macko SA. 1997. Isotopic evidence for extraterrestrial non-racemic amino acids in the Murchison meteorite. Nature 389:265-268.

Esteban NV., Liberato DJ., Sidbury JB., Yergey AL. 1987. Stable isotope dilution thermospray liquid chromatography/mass spectrometry method for determination of sugars and sugar alcohols in humans. Anal Chem 59:1674-1677.

Ettre LS., Gehrke CW. 2006. The development of the amino acid analyzer. LC GC North Am

Faure M., Moennoz D., Montigon F., Fay LB., Breuille D., Finot PA., Ballevre O., Boza J. 2002. Development of a rapid and convenient method to purify mucins and determine their in vivo synthesis rate in rats. Anal Biochem 307:244-251.

Ferchaud-Roucher V., Albert C., Champ M., Krempf M. 2006. Solid-phase microextraction method for carbon isotopic analysis of volatile carboxylic acids in human plasma by gas chromatography/combustion/isotope ratio mass spectrometry. Rapid Commun Mass Spectrom 20:3573-3578.

Fields SM., Ye CQ., Zhang DD., Branch BR., Zhang XJ., Okafo N. 2001. Superheated water as eluent in high-temperature high-performance liquid chromatographic separations of steroids on a polymer-coated zirconia column. J Chromatogr A 913:197-204.

Filer CN. 1999. Isotopic fractionation of organic compounds in chromatography. J Labeled Compounds & Radiopharmaceuticals 42:169-197.

Fortunato G., Ritter A., Fabian D. 2005. Old Masters' lead white pigments: investigations of paintings from the 16th to the 17th century using high precision lead isotope abundance ratios. Analyst 130:898-906.

Fuller BT., Fuller JL., Sage NE., Harris DA., O'Connell TC., Hedges RE. 2005. Nitrogen balance and $\delta^{15}N$: why you're not what you eat during nutritional stress. Rapid Commun Mass Spectrom 19:2497-2506.

Garlick PJ., McNurlan MA., Preedy VR. 1980. A rapid and convenient technique for measuring the rate of protein synthesis in tissues by injection of [^{3}H]phenylalanine. Biochem J 192:719-723.

Ghosh P., Brand WA. 2003. stable isotope ratio mass spectrometry in global climate change research. Int J Mass Spectrom 228:1-33.

Glaser B., Amelung W. 2002. Determination of $\delta^{13}C$ natural abundance of amino acid enantiomers in soil: methodological considerations and first results. Rapid Commun Mass Spectrom 16:891-898.

Godin JP., Faure M., Breuille D., Hopfgartner G., Fay LB. 2007. Determination of ^{13}C isotopic enrichment of valine and threonine by GC-C-IRMS after formation of the N(O,S)-ethoxycarbonyl ethyl ester derivatives of the amino acids. Anal Bioanal Chem 388:909-918.

Godin JP., Fay LB., Hopfgartner G. 2007. Liquid chromatography combined with Mass Spectrometry for ^{13}C isotopic analysis in life science research. Mass Spectrom Rev 26:751-774.

Godin JP., Hau J., Fay LB., Hopfgartner G. 2005. Isotope ratio monitoring of small molecules and macromolecules by liquid chromatography coupled to isotope ratio mass spectrometry. Rapid Commun Mass Spectrom 19:2689-2698.

Goodacre R. 2007. Metabolomics of a superorganism. J Nutr 137:259S-266S.

Goodman KJ., Brenna JT. 1992. High sensitivity tracer detection using high-precision gas chromatography-combustion isotope ratio mass spectrometry and highly enriched [U-^{13}C]-labeled precursors. Anal Chem 64:1088-1095.

Goodman KJ., Brenna JT. 1994. Curve fitting for restoration of accuracy for overlapping peaks in gas chromatography/combustion isotope ratio mass spectrometry. Anal Chem 66:1294-1301.

Goupry S, Rochut N, Robins RJ, Gentil E. 2000. Evaluation of Solid Phase Microextraction for the Isotopic Analyses of volatiles compounds produced during fermentation by lactic acid bacteria. J Agr Food Chem 48: 2222-2227

Grimble GK., Malik SB., Boza JJ. 2000. Methods for measuring tissue RNA turnover. Curr Opin Clin Nutr Metab Care 3:399-408.

Gross S., Glaser B. 2004. Minimization of carbon addition during derivatization of monosaccharides for compound-specific $\delta^{13}C$ analysis in environmental research. Rapid Commun Mass Spectrom 18:2753-2764.

Guillarme D., Heinisch S. 2005. Detection modes with High Tempearature Liquid Chromatography-A review. Separation & Purification Reviews 34:181-216.

Guillarme D., Heinisch S., Gauvrit JY., Lanteri P., Rocca JL. 2005. Optimization of the coupling of high-temperature liquid chromatography and flame ionization detection application to the separations of alcohols. J Chromatogr A 1078:22-27.

Guillarme D., Heinisch S., Rocca JL. 2004. Effect of temperature in reversed phase liquid chromatography. J Chromatogr A 1052:39-51.

Guillarme D., Nguyen DT., Rudaz S., Veuthey JL. 2007. Recent developments in liquid chromatography-impact on qualitative and quantitative performance. J Chromatogr A 1149:20-29.

Guiochon G. 2007. Monolithic columns in high-performance liquid chromatography. J Chromatogr A 1168:101-168.

Guo Z., Yarasheski K., Jensen MD. 2006. High-precision isotopic analysis of palmitoylcarnitine by liquid chromatography/electrospray ionization ion-trap tandem mass spectrometry. Rapid Commun Mass Spectrom 20:3361-3366.

Guo ZK., Luke AH., Lee WP., Schoeller D. 1993. Compound-specific carbon isotope ratio determination of enriched cholesterol. Anal Chem 65:1954-1959.

Halket JM., Waterman D., Przyborowska AM., Patel RK., Fraser PD., Bramley PM. 2005. Chemical derivatization and mass spectral libraries in metabolic profiling by GC/MS and LC/MS/MS. J Exp Bot 56:219-243.

Hatch KA., Crawford MA., Kunz AW., Thomsen SR., Eggett DL., Nelson ST., Roeder BL. 2006. An objective means of diagnosing anorexia nervosa and bulimia nervosa using $^{15}N/^{14}N$ and $^{13}C/^{12}C$ ratios in hair. Rapid Commun Mass Spectrom 20:3367-3373.

Hayes JM. 1983. Practice and Principles of isotopic measurement in organic Geochemistry.5.1-5.31.

Hayes JM., Freeman KH., Popp BN., Hoham CH. 1990. Compound-specific isotopic analyses: a novel tool for reconstruction of ancient biogeochemical processes. Org Geochem 16:1115-1128.

Heinisch S., Puy G., Barrioulet MP., Rocca JL. 2006. Effect of temperature on the retention of ionizable compounds in reversed-phase liquid chromatography: application to method development. J Chromatogr A 1118:234-243.

Hellerstein MK. 2004. New stable isotope-mass spectrometric techniques for measuring fluxes through intact metabolic pathways in mammalian systems:

introduction of moving pictures into functional genomics and biochemical phenotyping. Metab Eng 6:85-100.

Hellerstein MK., Murphy E. 2004. Stable isotope-mass spectrometric measurements of molecular fluxes in vivo: emerging applications in drug development. Curr Opin Mol Ther 6:249-264.

Hellerstein MK., Neese RA. 2000. Mass isotopomer distribution analysis at eight years: Theoretical, analytic, and experimental considerations. Am J Physiol 276:E1146-E1170.

Hernandez-Perez JM., Cabre E., Fluvia L., Motos A., Pastor C., Corominas A., Gassull MA. 2002. Improved method for gas chromatographic-mass spectrometric analysis of ^{13}C-labeled long-chain fatty acids in plasma samples. Clin Chem 48:906-912.

Hettmann E., Brand WA., Gleixner G. 2007. Improved isotope ratio measurement performance in liquid chromatography/isotope ratio mass spectrometry by removing excess oxygen. Rapid Commun Mass Spectrom 21:4135-4141.

Heuer V., Elvert M., Tille S., Krummen M., Mollar XP., Hmelo LR., Hinrichs KU. 2006. Online δ^{13}C analysis of fatty acids in sediment/ porewater systems by liquid chromatography isotope ratio mass spectrometry. Limnology and oceanography: methods 4:346-357.

Heuer V., Tille S., Finke N., Elvert M., Hinrichs KV. 2006. Carbon isotopic compositions of volatile fatty acids in sediment/pore-water systems measured by isotope-ratio-monitoring liquid chromatography/mass spectrometry. Geopghysical Research Abstracts 8:

Hilkert AW., Douthitt CB., Schlueter HJ., Brand WA. 1999. Isotope ratio monitoring gas chromatography/mass spectrometry of D/H by high temperature conversion isotope ratio mass spectrometry. Rapid Commun Mass Spectrom 13:1226-1230.

Hopfgartner G., Bourgogne E. 2003. Quantitative high-throughput analysis of drugs in biological matrices by mass spectrometry. Mass Spectrom Rev 22:195-214.

Hopfgartner G., Varesio E., Tschappat V., Grivet C., Bourgogne E., Leuthold LA. 2004. Triple quadrupole linear ion trap mass spectrometer for the analysis of small molecules and macromolecules. J Mass Spectrom 39:845-855.

Hor K., Ruff C., Weckerle B., Konig T., Schreier P. 2001. Flavor authenticity studies by H-2/H-1 ratio determination using on-line gas chromatography pyrolysis isotope ratio mass spectrometry. J Agr Food Chem 49:21-25.

Hu Q.,Noll RJ., Li H., Makarov A., Hardman M., Graham Cooks R. 2005. The Orbitrap: a new mass spectrometer. J Mass Spectrom 40:430-443

Husek P. 1998. Chloroformates in gas chromatography as general purpose derivatizing agents. J Chromatogr B 717:57-91.

Husek P. 1975. Gas chromatography of amino acids. J Chromatogr 113:139-230.

Husek P., Simek P. 2001. Advances in amino acid analysis. LC GC North America 19:986.

Jackson SJ., Waterhouse JS., Bluck LJ. 2007. A single glucose derivative suitable for gas chromatography/ mass spectrometry and gas chromatography/combustion/isotope ratio mass spectrometry. Rapid Commun Mass Spectrom 21:3123-3128.

Jamin E., Martin F., Santamaria-Fernandez R., Lees M. 2005. Detection of exogenous citric acid in fruit juices by stable isotope ratio analysis. J Agric Food Chem 53:5130-5133.

Jasper JP. 1999. The increasing use of stable isotopes in the pharmaceutical industry. Pharmaceutical technology October:106-114.

Jasper JP., Westenberger BJ., Spencer JA., Buhse LF., Nasr M. 2004. Stable isotopic characterization of active pharmaceutical ingredients. J Pharm and Biomedical Analysis 35:21-30.

Jeukendrup AE., Raben A., Gijsen A., Stegen JH., Brouns F., Saris WH., Wagenmakers AJ. 1999. Glucose kinetics during prolonged exercise in highly trained human subjects: effect of glucose ingestion. J Physiol 515 (Pt 2):579-589.

Jim S., Jones V., Copley MS., Ambrose SH., Evershed RP. 2003. Effects of hydrolysis on the $\delta^{13}C$ values of individual amino acids derived from polypeptides and proteins. Rapid Commun Mass Spectrom 17:2283-2289.

Jones KS., Bluck LJ., Coward WA. 2006. Analysis of isotope ratios in vitamin K1 (phylloquinone) from human plasma by gas chromatography/mass spectrometry. Rapid Commun Mass Spectrom 20:1894-1898.

Jorabchi K., Kahen K., Lecchi P., Montaser A. 2005. Chemical reaction interface mass spectrometry with high efficiency nebulization. Anal Chem 77:5402-5406.

Kalderon B., Sheena V., Shachrur S., Hertz R., Bar-Tana J. 2002. Modulation by nutrients and drugs of liver acyl-CoAs analyzed by mass spectrometry. J Lipid Res 43:1125-1132.

Kastenmayer P., Czarnecki-Maulden GL., King W. 2002. Mineral and trace element absorption from dry dog food by dogs, determined using stable isotopes. J Nutr 132:1670S-1672S.

Kastenmayer P., Davidsson L., Galan P., Cherouvrier F., Hercberg S., Hurrell RF. 1994. A double stable isotope technique for measuring iron absorption in infants. Br J Nutr 71:411-424.

Kasumov T., Martini WZ., Reszko AE., Bian F., Pierce BA., David F., Roe CR., Brunengraber H. 2002. Assay of the concentration and ^{13}C isotopic

enrichment of propionyl-CoA, methylmalonyl-CoA, and succinyl-CoA by gas chromatography-mass spectrometry. Anal Biochem 305:90-96.

Knox JH. 1977. Practical aspect of LC theory. J Chrom Sci 15:352-64.

Koletzko B., Sauerwald T., Demmelmair H. 1997. Safety of stable isotope use. Eur J Pediatr 156 Suppl 1:S12-S17.

Kondo T.,.Yang Yu. 2003. Comparison of elution strengh, column efficiency, and peak symmetry in subcritical water chromaography and traditional reversed-phase liquid chromatography. Analytica Chimica Acta 494:157-166.

Krummen M., Hilkert AW., Juchelka D., Duhr A., Schluter HJ., Pesch R. 2004. A new concept for isotope ratio monitoring liquid chromatography/mass spectrometry. Rapid Commun Mass Spectrom 18:2260-2266.

Kulik W., Meesterburrie JA., Jakobs C., de Meer K. 1998. Determination of $\delta^{13}C$ values of valine in protein hydrolysate by gas chromatography-combustion isotope ratio mass spectrometry. J Chromatogr B 710:37-47.

Kussmann M. 2007. How to comprehensively analyse proteins and how this influences nutritional research. Clin Chem Lab Med 45:288-300.

Landau BR. 1999. Quantifying the contribution of gluconeogenesis to glucose production in fasted human subjects using stable isotopes. Proc Nutr Soc 58:963-972.

Lecchi P., Abramson FP. 1998. Analysis of biopolymers by size-exclusion chromatography mass spectrometry. J Chromatograph A 828:509-513.

Lecchi P., Abramson FP. 1999. Size exclusion chromatography-chemical reaction interface mass spectrometry: "a perfect match". Anal Chem 71:2951-2955.

Leckrone KJ., Hayes JM. 1998. Water-induced errors in continuous-flow carbon isotope ratio mass spectrometry. Anal Chem 70:2737-2744.

Lees M., Kelly SD. 2003. Using stable isotope ratio mass spectrometry (IRMS) in food authentification and traceability. Woodhead Publishing:156-183.

Lestremau F., de Villiers A., Lynen F., Cooper A., Szucs R., Sandra P. 2007. High efficiency liquid chromatography on conventional columns and instrumentation by using temperature as a variable. Kinetic plots and experimental verification. J Chromatogr A 1138:120-131.

Lichtfouse E. 2000. Compound-specific isotope analysis. Application to archaelogy, biomedical sciences, biosynthesis, environment, extraterrestrial chemistry, food science, forensic science, humic substances, microbiology, organic geochemistry, soil science and sport. Rapid Commun Mass Spectrom 14:1337-1344.

Lobinski R., Shaumloffel D., Szpunar J. 2006. Mass spectrometry in bioinorganic analytical chemistry. Mass Spectrom Rev 25:255-289.

Luong ET., Houk RS. 2003. Determination of carbon isotope ratios in amino acids, proteins, and oligosaccharides by inductively coupled plasma-mass spectrometry. J Am Soc Mass Spectrom 14:295-301.

Lyon TD., Baxter MS. 1978. Stable carbon isotopes in human tissues. Nature 273:750-751.

MacCoss MJ., Matthews DE. 2005. Quantitative MS for proteomics: teaching a new dog old tricks. Anal Chem 77:294A-302A.

MacCoss MJ., Wu CC., Matthews DE., Yates JR., III. 2005. Measurement of the isotope enrichment of stable isotope-labeled proteins using high-resolution mass spectra of peptides. Anal Chem 77:7646-7653.

Mahmoud M., Gentil E., Robins RJ. 2004. Natural-abundance isotope ratio mass spectrometry as a means of evaluating carbon redistribution during glucose-citrate cofermentation by Lactococcus lactis. Eur J Biochem 271:4392-4400.

Majors R., Przybylski M. 2002. Columns for reversed-phase LC separations in Highly Aqueous Mobile Phases. LC GC Europe 2-7.

Majors RE., Przybyciel M. 2002. Columns for reversed-phase LC separations in highly aqueous mobile phases. LC GC Europe 15:780-.

Majors RE., Przybyciel M. 2003. Columns for reversed-phase LC separations in highly aqueous mobile phases (vol 15, pg 780, 2002). LC GC Europe 16:81-81.

Marin SJ., Jones BA., Felix WD., Clark J. 2004. Effect of high-temperature on high-performance liquid chromatography column stability and performance under temperature-programmed conditions. J Chromatogr A 1030:255-262.

Matthews DE., Hayes JM. 1978. Isotope-ratio-monitoring gas chromatography-mass spectrometry. Anal Chem 50:1465-1473.

Mazzeo JR., Neue UD., Kele M., Plumb RS. 2005. A new separation technique takes advantage of sub-2 um porous particle. Anal Chem 461A-467A.

McCullagh JS., Juchelka D., Hedges RE. 2006. Analysis of amino acid ^{13}C abundance from human and faunal bone collagen using liquid chromatography/isotope ratio mass spectrometry. Rapid Commun Mass Spectrom 20:2761-2768.

McLean M., Vestal ML., Teffera Y., Abramson FP. 1996. Element- and isotope-specific detection for high-performance liquid chromatography using chemical reaction interface mass spectrometry. J Chromatogr A 732:189-199.

McNeff CV., Yan B., Stoll DR., Henry RA. 2007. Practice and theory of high temperature liquid chromatography. J Sep Sci 30:1672-1685.

McRae C., Sun CG., McMillan CF., Snape CE., Fallick AE. 2000. Sourcing of fossil fuel-derived PAH in the environment. Polycyclic Aromatic Compounds 20:97-109.

Meier-Augenstein W. 1999. Applied gas chromatography coupled to isotope ratio mass spectrometry. J Chromatogr A 842:351-371.

Meier-Augenstein W. 1999. Use of gas chromatography-combustion-isotope ratio mass spectrometry in nutrition and metabolic research. Curr Opin Clin Nutr Metab Care 2:465-470.

Meier-Augenstein W. 2002. Stable isotope analysis of fatty acids by gas chromatography-isotope ratio mass spectrometry. Analytica Chimica Acta 465:63-79.

Meier-Augenstein W. 2004. GC and IRMS technology for ^{13}C and ^{15}N analysis on Organic compounds and related gases.153-176. Chapter 8, Handbook of stable isotopes analytical techniques. Ed. de Groot PA.

Meier-Augenstein W., Watt PW., Langhans CD. 1996. Influence of gas chromatographic parameters on measurement of $^{13}C/^{12}C$ isotope ratios by gas-liquid chromatography-combustion isotope ratio mass spectrometry. J of Chromatogr A 752:233-241.

Menand C., Pouteau E., Marchini S., Maugere P., Krempf M., Darmaun D. 1997. Determination of low ^{13}C-glutamine enrichments using gas chromatography-combustion-isotope ratio mass spectrometry. J Mass Spectrom 32:1094-1099.

Merritt DA., Brand WA., Hayes JM. 1994. Isotope-ratio-monitoring gas chromatography-mass spectrometry: methods for isotopic calibration. Org Geochem 21:573-583.

Merritt DA., Hayes JM. 1994. Factors controlling precision and accuracy in isotope-ratio-monitoring mass spectrometry. Anal Chem 66:2336-2347.

Metges CC., Daenzer M. 2000. ^{13}C gas chromatography-combustion isotope ratio mass spectrometry analysis of N-pivaloyl amino acid esters of tissue and plasma samples. Anal Biochem 278:156-164.

Miller DJ., Hawthorne SB. 1997. Subcritical water chromatography with flame ionization detection. Anal Chem 69:623-627.

Moco S., Bino R., de Vos R., Vervoort J. 2007. Metabolomics technologies and metabolite identification. Trends in Analytical Chemistry doi: 10.1016/j.trac.2007.08.003-

Moens T., Bouillon S., Gallucci F.. 2005. Dual stable isotope abundances unravel trophic position of estuarine nematodes. Journal of the Marine Biological Association of the United Kingdom 85:1401-1407.

Moini M., Abramson FP. 1991. A moving belt device to couple high-performance liquid chromatography and chemical reaction interface mass spectrometry. Biol Mass Spectrom 20:308-312.

Montigon F., Boza JJ., Fay LB. 2001. Determination of ^{13}C- and ^{15}N-enrichment of glutamine by gas chromatography/mass spectrometry and gas chromatography/combustion/isotope ratio mass spectrometry after N(O,S)-ethoxycarbonyl ethyl ester derivatization. Rapid Comm Mass Spectrom 15:116-123.

Moore S., Stein WH. 1951. Chromatography of amino acids on sulfonated polystyrene resins. J Biol Chem 192:663-681.

Moreno Rojas JM., Cosofret S., Reniero F., Guillou C., Serra F. 2007. Control of oenological products: discrimination between different botanical sources of L-tartaric acid by isotope ratio mass spectrometry. Rapid Commun Mass Spectrom 21:2447-2450.

Mottram HR., Evershed RP. 2003. Practical considerations in the gas chromatography/combustion/isotope ratio monitoring mass spectrometry of ^{13}C-enriched compounds: detection limits and carryover effects. Rapid Commun Mass Spectrom 17:2669-2674.

Namera A., Yashiki M., Nishida M., Kojima T. 2002. Direct extract derivatization for determination of amino acids in human urine by gas chromatography and mass spectrometry. J Chromatogr B 776:49-55.

Neue UD. 2005. Theory of peak capacity in gradient elution. J Chromatogr A 1079:153-161.

Nguyen DT., Guillarme D., Rudaz S., Veuthey JL. 2006. Fast analysis in liquid chromatography using small particle size and high pressure. J Sep Sci 29:1836-1848.

Niessen WM. 2003. Progress in liquid chromatography-mass spectrometry instrumentation and its impact on high-throughput screening. J Chromatogr A 1000:413-436.

Nissim I., Starr SE., Sullivan KE., Campbell DE., Douglas SD., Daikhin Y., Yudkoff M. 2000. Rapid method for determining the rate of DNA synthesis and cellular proliferation. Anal Biochem 278:198-205.

O'Brien DM., Fogel ML., Boggs CL. 2002. Renewable and nonrenewable resources: amino acid turnover and allocation to reproduction in Lepidoptera. Proc Natl Acad Sci U S A 99:4413-4418.

Osborn BL., Abramson FP. 1998. Pharmacokinetic and metabolism studies using uniformly stable isotope labeled proteins with HPLC/CRIMS detection. Biopharm Drug Dispos 19:439-444.

Palau J., Soler A., Teixidor P., Aravena R. 2007. Compound-specific carbon isotope analysis of volatile organic compounds in water using solid-phase microextraction. J Chromatogr A 1163:260-268.

Patterson BW. 1997. Use of stable isotopically labeled tracers for studies of metabolic kinetics: an overview. Metabolism 46:322-329.

Penning H., Conrad R. 2006. Effect of inhibition of acetoclastic methanogenesis on growth of archaeal populations in an anoxic model environment. Appl Environ Microbiol 72:178-184.

Pereira L., Aspey S., Ritchie H. 2007. High temperature to increase throughput in liquid chromatography and liquid chromatography-mass spectrometry with a porous graphitic carbon stationary phase. J Sep Sci 30:1115-1124.

Petritis K., Chaimbault P., Elfakir C., Dreux M. 2001. Ion-pair reversed phase liquid chromatography-indirect conductivity detection and response deviations of underivatised amino acids. J Sep Sci 24:397-405.

Petritis K., Elfakir C., Dreux M. 2002. A comparative study of commercial liquid chromatographic detectors for the analysis of underivatized amino acids. J. Chromatogr A 961:9-21.

Petzke KJ., Feist T., Fleig WE., Metges CC. 2006. Nitrogen isotopic composition in hair protein is different in liver cirrhotic patients. Rapid Commun Mass Spectrom 20:2973-2978.

Piraud M., Vianey-Saban C., Petritis K., Elfakir C., Steghens JP., Morla A., Bouchu D. 2003. ESI-MS/MS analysis of underivatised amino acids: a new tool for the diagnosis of inherited disorders of amino acid metabolism. Fragmentation study of 79 molecules of biological interest in positive and negative ionisation mode. Rapid Commun Mass Spectrom 17:1297-1311.

Plumb RS., Rainville P., Smith BW., Johnson KA., Castro-Perez J., Wilson ID., Nicholson JK. 2006. Generation of ultrahigh peak capacity LC separations via elevated temperatures and high linear mobile-phase velocities. Anal Chem 78:7278-7283.

Reeds PJ., Berthold HK., Boza JJ., Burrin DG., Jahoor F., Jaksic T., Klein PD., Keshen T., Miller R., Stoll B., Wykes LJ. 1997. Integration of amino acid and carbon intermediary metabolism: studies with uniformly labeled tracers and mass isotopomer analysis. Eur J Pediatr 156 Suppl 1:S50-S58.

Reijngoud DJ., Hellstern G., Elzinga H., de Sain-van der Velden MG, Okken A., Stellaard F. 1998. Determination of low isotopic enrichment of L-[1-^{13}C] valine by gas chromatography/combustion/isotope ratio mass spectrometry: a robust method for measuring protein fractional synthetic rates in vivo. J Mass Spectrom 33:621-626.

Rennie MJ. 1999. An introduction to the use of tracers in nutrition and metabolism. Proceedings of the Nutrition Society 58:935-944.

Rezzi S., Ramadan Z., Fay L., Kochhar S. 2007. Nutritional metabolomics: applications and perspectives. J Proteome Res 6:513-525.

Riddle LA., Guiochon G. 2006. Separation of free sterols by high temperature liquid chromatography. J Chromatogr A 1137:173-179.

Rieley G. 1994. Derivatization of Organic compounds prior to gas chroamtographic-combustion-isotope ratio mass spectrometric analysis: identification of isotope fractionation processes. Analyst 119:915-919.

Robbins RJ., Bean SR. 2004. Development of a quantitative high-performance liquid chromatography-photodiode array detection measurement system for phenolic acids. J Chromatogr A 1038:97-105.

Robins SP., New SA. 1997. Markers of bone turnover in relation to bone health. Proc Nutr Soc 56:903-914.

Roy S., Delobel A., Gaudin K., Touboul D., Germain DP., Baillet A., Prognon P., Laprevote O., Chaminade P. 2006. Liquid chromatography on porous graphitic carbon with atmospheric pressure photoionization mass spectrometry and tandem mass spectrometry for the analysis of glycosphingolipids. J Chromatogr A 1117:154-162.

Sacks GL., Brenna JT. 2005. ^{15}N/^{14}N position-specific isotopic analyses of polynitrogenous amino acids. Anal Chem 77:1013-1019.

Sacks GL., Wolyniak CJ., Brenna JT. 2003. Analysis of quantization error in high-precision continuous-flow isotope ratio mass spectrometry. J Chromatogr A 1020:273-282.

Sano M., Yotsui Y., Abe H., Sasaki S. 1976. A new technique for the detection of metabolites labeled by the isotope ^{13}C using mass fragmentography. Biomed Mass Spectrom 3:1-3.

Saudan C., Augsburger M., Kintz P., Saugy M., Mangin P. 2005. Detection of exogenous GHB in blood by gas chromatography-combustion-isotope ratio mass spectrometry: implications in postmortem toxicology. J Anal Toxicol 29:777-781.

Schierbeek H., Te Braake F., Godin JP., Fay LB., van Goudoever JB. 2007. Novel method for measurement of glutathione kinetics in neonates using liquid chromatography coupled to isotope ratio mass spectrometry. Rapid Commun Mass Spectrom 21:2805-2812.

Schmidt TC., Zwank L., Elsner M., Berg M., Meckenstock RU., Haderlein SB. 2004. Compound-specific stable isotope analysis of organic contaminants in natural environments: a critical review of the state of the art, prospects, and future challenges. Anal Bioanal Chem 378:283-300.

Schmitt J., Glaser B., Zech W. 2003. Amount-dependent isotopic fractionation during compound-specific isotope analysis. Rapid Commun Mass Spectrom 17:970-977.

Sessions AL. 2006. Isotope-ratio detection for gas chromatography. J Sep Sci 29:1946-1961.

Sessions AL., Sylva SP., Hayes JM. 2005. Moving-wire device for carbon isotopic analyses of nanogram quantities of nonvolatile organic carbon. Anal Chem 77:6519-6527.

Setchell KD., Faughnan MS., Avades T., Zimmer-Nechemias L., Brown NM., Wolfe BE., Brashear WT., Desai P., Oldfield MF., Botting NP., Cassidy A.

2003. Comparing the pharmacokinetics of daidzein and genistein with the use of ^{13}C-labeled tracers in premenopausal women. Am J Clin Nutr 77:411-419.

Sidossis LS., Magkos F., Mittendorfer B., Wolfe RR. 2004. Stable isotope tracer dilution for quantifying very low-density lipoprotein-triacylglycerol kinetics in man. Clin Nutr 23:457-466.

Silfer JA., Engel MH., Macko SA., Jumeau EJ. 1991. Stable isotope analysis of amino acid enantioners by conventional isotope ratio MS and combined GC isotope ratio MS. Anal Chem 63:370-374.

Siuzdak G. 2006. The expanding role of Mass Spectrometry in Biotechnology. Second: MCC Press, San Diego.

Slater C., Ling SC., Preston T., Weaver LT. 2002. Analysis of ^{13}C-mixed triacylglycerol in stool by bulk (EA-IRMS) and compound specific (GC/MS) methods. Isotopes in Environ and Health Stud 38:79-86.

Slater C., Preston T., Weaver TL. 2001. Stables isotopes and the international system of units. Rapid Commun Mass Spectrom 15:1270-1273.

Smith C., Jensen BP., Wilson ID., Abou-Shakra F., Crowther D. 2004. High-performance liquid chromatography/inductively coupled plasma mass spectrometry and tandem mass spectrometry for the detection of carbon-containing compounds. Rapid Commun Mass Spectrom 18:1487-1492.

Smith K., Rennie MJ. 1996. The measurement of tissue protein turnover. Baillieres Clin Endocrinol Metab 10:469-495.

Smith RM. 2006. Superheated water: the ultimate green solvent for separation science. Anal Bioanal Chem 385:419-421.

Snyder LR, Kirkland JJ., Glajch JL. 1997. Practical HPLC method development. Second Ed.: Wiley intersciences, New York.

Sobolevsky TG., Chernetsova ES., Revelsky A I., Revelsky IA., Starostin AB., Miller B., Oriedo V. 2003. Electron ionization mass spectra and their reproducibility for trialkylsilylated derivatives of organic acids, sugars and alcohols. Eur J Mass Spectrom (Chichester, Eng) 9:487-495.

St Jean G. 2003. Automated quantitative and isotopic ^{13}C analysis of dissolved inorganic carbon and dissolved organic carbon in continuous-flow using a total organic carbon analyser. Rapid Comm in Mass Spectrom 17:419-428.

Stellaard F.,.Elzinga H. 2005. Analytical techniques in biomedical stable isotope applications: (isotope ratio) mass spectrometry or infrared spectrometry? Isotopes Environ Health Stud 41:345-361.

Stott AW., Evershed RP. 1996. $\delta^{13}C$ analysis of cholesterol preserved in archaeological bones and teeth. Anal Chem 68:4402-4408.

Sun D., Cree MG., Wolfe RR. 2006. Quantification of the concentration and ^{13}C tracer enrichment of long-chain fatty acyl-coenzyme A in muscle by liquid chromatography/mass spectrometry. Anal Biochem 349:87-95.

Tanumihardjo SA. 2000. Vitamin A status assessment in rats with $^{13}C_4$ retinyl acetate and gas chromatography/combustion/isotope ratio mass spectrometry. J Nutr 130:2844-2849.

Tea I., Ferchaud-Roucher V., Kuster A., Darmaun D., Robins RJ. 2007. Determination of ^{13}C isotopic enrichment of glutathione and glycine by gas chromatography/combustion/isotope ratio mass spectrometry after formation of the N- or (N,S)-ethoxycarbonyl methyl ester derivatives. Rapid Commun Mass Spectrom 21:3245-3252.

Teffera Y., Abramson FP. 1994. Application of high-performance liquid chromatography/chemical reaction interface mass spectrometry for the analysis of conjugated metabolites: a demonstration using deuterated acetaminophen. Biol Mass Spectrom 23:776-783.

Teffera Y., Abramson FP., McLean M., Vestal M. 1993. Development of an isotope-selective high-performance liquid chromatography detector using chemical-reaction-interface mass spectrometry: application to deuterated cortisol metabolites in urine. J Chromatogr 620:89-96.

Teffera Y., Kusmierz JJ., Abramson FP. 1996. Continuous-flow isotope ratio mass spectrometry using the chemical reaction interface with either gas or liquid chromatographic introduction. Anal Chem 68:1888-1894.

Teutenberg T., Lerch O., Gotze HJ., Zinn P. 2001. Separation of selected anticancer drugs using superheated water as the mobile phase. Anal Chem 73:3896-3899.

Teutenberg T., Tuerk J., Holzhauser M., Kiffmeyer TK. 2006. Evaluation of column bleed by using an ultraviolet and a charged aerosol detector coupled to a high-temperature liquid chromatographic system. J Chromatogr A 1119:197-201.

Tripp JA., McCullagh JS., Hedges RE. 2006. Preparative separation of underivatized amino acids for compound-specific stable isotope analysis and radiocarbon dating of hydrolyzed bone collagen. J Sep Sci 29:41-48.

Turner SM. 2006. Stable isotopes, mass spectrometry, and molecular fluxes: applications to toxicology. J Pharmacol Toxicol Methods 53:75-85.

Van Dongen BE., Schouten S., Damste JS. 2001. Gas chromatography/combustion/isotope-ratio-monitoring mass spectrometric analysis of methylboronic derivatives of monosaccharides: a new method for determining natural ^{13}C abundances of carbohydrates. Rapid Commun Mass Spectrom 15:496-500.

Van Eijk Hans MH, Rooyakkers DR, Wagen MaKersa JM, Soeters PB., Deutz NEP. 1997. Isolation and quantification of isotopically labeled amino acids from biological samples. J Chromatogr B 691:287-296.

Van Eijk HM., Rooyakkers DR., Soeters PB., Deutz NE. 1999. Determination of amino acid isotope enrichment using liquid chromatography-mass spectrometry. Anal Biochem 271:8-17.

Van Lieshout M., West CE., van Breemen RB. 2003. Isotopic tracer techniques for studying the bioavailability and bioefficacy of dietary carotenoids, particularly beta-carotene, in humans: a review. Am J Clin Nutr 77:12-28.

Vanhoenacker G., Sandra P. 2005. High temperature liquid chromatography and liquid chromatography-mass spectroscopy analysis of octylphenol ethoxylates on different stationary phases. J Chromatogr A 1082:193-202.

Vanhoenacker G., Sandra P. 2006. Elevated temperature and temperature programming in conventional liquid chromatography-fundamentals and applications. J Sep Sci 29:1822-1835.

Vogt J. 1997. Availability of stable isotope tracers for human use. Eur J Pediatr 156 Suppl 1:S9-11.

Volpi E., Jeschke MG., Herndon DN., Wolfe RR. 2000. Measurement of skin protein breakdown in a rat model. Am J Physiol Endocrinol Metab 279:E900-E906.

Wagenmakers AJM. 1999. Tracers to investigate protein and amino acid metabolism in human subjects. Proceedings of the Nutrition Society 58:987-1000.

Werner RA, Brand WA. 2001. Referencing strategies and techniques in stable isotope ratio analysis. Rapid Commun Mass Spectrom 15:501-519.

West C., Lesellier E., Tchapla A. 2004. Retention characteristics of porous graphitic carbon in subcritical fluid chromatography with carbon dioxide-methanol mobile phases. J Chromatogr A 1048:99-109.

Wolfe RR. 1992. Radioactive and Stable Isotope Tracers in Biomedicine : Principles and Practice of Kinetic Analysis.

Wong WW., Hachey DL., Zhang S., Clarke LL. 1995. Accuracy and precision of gas chromatography/combustion isotope ratio mass spectrometry for stable carbon isotope ratio measurement. Rapid Commun Mass Spectrom 9:1007-1011.

Wu CC., MacCoss MJ., Howell KE., Matthews DE., Yates JR. 2004. Metabolic labeling of mammalian organisms with stable isotopes for quantitative proteomic analysis. Anal Chem 76:4951-4959.

Wu Z., Zhang XJ., Cody RB., Wolfe RR. 2004. Determination of phenylalanine isotope ratio enrichment by liquid chromatography/time- of-flight mass spectrometry. Eur J Mass Spectrom (Chichester, Eng) 10:619-623.

Yarasheski KE., Smith K., Rennie MJ., Bier DM. 1992. Measurement of muscle protein fractional synthetic rate by capillary gas chromatography/combustion isotope ratio mass spectrometry. Biol Mass Spectrom 21:486-490.

Young VR., Ajami AM. 1999. Jonathan E. Rhoads lecture. Isotopic metaprobes, nutrition, and the roads ahead. JPEN J Parenter Enteral Nutr 23:175-194.

Zaideh BI., Saad NM., Lewis BA., Brenna JT. 2001. Reduction of nonpolar amino acids to amino alcohols to enhance volatility for high-precision isotopic analysis. Anal Chem 73:799-802.

Zwank L., Berg M., Schmidt TC., Haderlein SB. 2003. Compound-specific carbon isotope analysis of volatile organic compounds in the low-microgram per liter range. Anal Chem 75:5575-5583.