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Metabolomics Research with Tandem Mass Spectrometry

Guo-Fang Zhang[1], Qingling Li[1], Ling Li[2] and Takhar Kasumov[2]
[1]*Department of Nutrition, Case Western Reserve University, Cleveland, Ohio;*
[2]*Department of Gastroenterology and Hepatology, Cleveland Clinic, Cleveland, Ohio, USA*

1. Introduction

Metabolomics, the large-scale analysis of metabolites generated in metabolic pathways, has recently joined with "omics" integration studies (Tan *et al.*, 2008;Martinez-Pinna *et al.*, 2010;Gehlenborg *et al.*, 2010;Kint *et al.*, 2010;Vlaanderen *et al.*, 2010). In addition to metabolite profiling characteristic to metabolomics, metabonomics describes the wide spectrum of metabolites changing under certain conditions, such as disease, interventions and functional gene changes. Since these two terms (metabolomics and metabonomics) used interchangeably in literature (Mishur and Rea, 2011), in future discussion we will use only metabolomics for describing both quantitative metabolic profiling and changes in response to stimulus. Biochemically, metabolomics is the ultimate endpoint measurement of biological events and capturing the influences of nutrition, environmental influences, responses to pharmaceuticals, and many more. The history of metabolomics starts with the pioneering work of Horning who in the early 1960s applied gas chromatography (GC) for the metabolites profiling in urine (SWEELEY and Horning, 1960;Horning and VANDENHEUVEL, 1963). A few years later Pauling et al. expanded application of GC for metabolites analysis in both urine and breath (Pauling *et al.*, 1971). Current advancements in bioanalytical instrumentations in combination with sample preparation techniques enables metabolomics studies virtually in all kinds of biological tissues and fluids. Ultimately clinical application of metabolic profiling became an important clinical tool in diagnosis and screening for inherited metabolic disorders (IMD), a group of over 200 single gene disorders.

The vital progress of metabolomics usually occurs when new instrumentation or technique appears. Various state of the art techniques have been applied to metabolomics studies, including: nuclear magnetic resonance (NMR) spectroscopy (Reo, 2002;Ward *et al.*, 2007;Jordan and Cheng, 2007;Serkova *et al.*, 2007;Duarte *et al.*, 2009;Maher *et al.*, 2009;Sofia *et al.*, 2011), magnetic resonance imaging (MRI) (Elias *et al.*, 2008;De Leon-Rodriguez *et al.*, 2009), infrared spectroscopy (IR) (Singh and Sinclair, 2007;Shaw *et al.*, 2009;Corte *et al.*, 2010), gas chromatography mass spectrometry (GC-MS) (Styczynski *et al.*, 2007;Xu *et al.*, 2009;Lin *et al.*, 2010;Dehaven *et al.*, 2010;Carroll *et al.*, 2010;Tsugawa *et al.*, 2011), capillary electrophoresis-MS (Lee *et al.*, 2007;Soga, 2007;Lapainis *et al.*, 2009;Barbas *et al.*, 2011;Britz-McKibbin, 2011) and HPLC-MS (Chen *et al.*, 2007;Lu *et al.*, 2008;Llorach *et al.*, 2009;Gika and Theodoridis, 2011;Bajad and Shulaev, 2011) . Current metabolomics and metabolic profiling

studies rely almost exclusively on ^{1}H NMR, GC-MS and LC-MS due to the technological maturity of these instrumentations.

Although the concept of the application of NMR to metabolomics studies dates back to early nineteen eighties (Nicholson *et al.*, 1983a;Nicholson *et al.*, 1983b;Bales *et al.*, 1984), Nicholson and colleagues laid the foundation of the contemporary NMR based metabolomics in 1999 (Nicholson *et al.*, 1999). In this elegant study, a wide range of biochemical, clinical and toxicological problems were addressed using a high resolution NMR based matabolomics approach. Nowadays NMR (mainly ^{1}H NMR) is widely used for metabolomics studies. NMR has the following advantages in the metabolic studies: (i) sample preparation for NMR is much simplified; (ii) NMR is non-invasive and non-destructive so that the sample could be used in subsequent analysis by an alternative technique; (iii) the data is easily quantified and highly reproducible (Zhang *et al.*, 2010); and (iv) various NMR spectral libraries are available for metabolites identifications, such as Human Metabolome Database (HMDB), the Madison Metabolomics Consortium Database (MMCD), the Biological Magnetic Resonance Data Bank (BMRB), the Magnetic Resonance Metabolomics Database or the Chenomx Database and either open source or publicly unavailable software tools (e.g. Analysis of MIXtures (AMIX) program, MetaboMiner, and rNMR which also permits to quantify the metabolites) (Malet-Martino *et al.*, 2011). However, the low sensitivity of NMR analysis limits its application in metabolomics research. Recent effort has been put on improving the sensitivity of NMR by improving Larmor frequency, decreasing probe diameter and reducing noise (Malet-Martino *et al.*, 2011).

GC-MS technique has been seen as a traditional and standard approach for metabolomics studies because of its high separation ability, high sensitivity and easy analyte identification. Typical GC column has a length of 10 to 100 meters with the theoretical plate number of 1,000 to 1000,000. This enables the GC-MS to analyze very complex biological samples. The high sensitivity of the GC-MS allows metabolomics profiling of a complex mixture with a small volume of sample injection (1 µl) into GC-MS. Most compounds can be detected at 1 pmol or lower levels by GC-MS. Many public spectral libraries are available for electron-impact (EI) GC-MS. The available libraries can save huge amount of work and time to identify the metabolites. The national institute of standardization and technology mass spectral library (NIST 11) contains 243,893 carefully evaluated spectra of 212,961 unique compounds, with identifications, nearly all with chemical structures. Other available GC-MS libraries are Fiehn libraries for GC-quadrupole mass spectrometers from Agilent, and for GC-TOF mass spectrometers from Leco. These libraries are collected using EI mass spectrometers that combined with GC. Since only volatile compounds can be analyzed by GC-MS, therefore GC-MS data only covers part of metabolites from biological samples although some nonvolatile metabolites could become more volatile after derivatization.

Notwithstanding the specific strengths of those different methodologies, the unique role of tandem mass spectrometry in metabolomics research is indisputable. Tandem mass spectrometry for metabolomics study has the following merits: (i) the high sensitivity of tandem mass spectrometry provides more information on trace amounts of metabolites; (ii) specific mass scan features of tandem mass spectrometry, such as precursor ion scan, neutral loss scan and multiple reaction monitoring scan, can simplify ion chromatograms. The application of liquid chromatography-coupled tandem mass spectrometry has revolutionized not only metabolic profiling of inherited metabolic disorders (IMD) but also the entire metabolic research, particularly the allied field of metabolomics.

In turn, recent advances in metabolomics have aided major discoveries made in several areas, including the identification of new metabolites and biomarkers of different diseases in both animal models and humans. In addition, metabolomics has been utilized in the investigation of metabolic pathways, biomarker identifications and molecular interactions and regulations. More recently, in combination with stable isotope technology, metabolomics has been successfully used for the identification of new metabolic pathways and the quantification of metabolic fluxes. Because of the objective to profile thousands or more metabolites in complex biological samples, untargeted metabolomics research requires a very elegant methodology or multiple combined methodologies. In this chapter we will focus on our recent metabolomics studies by LC-MS/MS as well as some seminal literature as an introduction to the current applications of tandem mass spectrometry in metabolomics research.

2. Targeted and untargeted metabolite profiling with tandem mass spectrometry

The tandem mass spectrometry is a vital technique in identifying and quantifying different metabolites. We will use our recent work on lipid and folate metabolism as examples to highlight the unique role of tandem mass spectrometry in both targeted and untargeted metabolite profiling. Briefly, the targeted metabolomics experiment with tandem mass spectrometry measures defined ion transitions from known metabolites. The untargeted metabolomics experiment records all ions within a certain mass range, including the ions belonging to structurally novel metabolites (Vinayavekhin and Saghatelian, 2010).

As a part of our research on lipid metabolism we applied tandem mass spectrometry to the analysis of different acyl-CoA derivatives. Acyl-CoAs are intermediates in both fatty acid oxidation and synthesis. Acyl-CoAs are also formed during metabolism of some drugs containing carboxyl groups and therefore they can be used to track the metabolic pathways of several drugs. Structurally acyl-CoAs belong to a class of a large number of diverse compounds that have coenzyme A part and different acyl moieties. Despite the difference in acyl groups, all acyl-CoAs are catabolized through two distinct oxidation pathways in mitochondria and peroxisomes. Mitochondrial beta oxidation is the primary pathway of fatty acid oxidation that produces energy. Each cycle of mitochondrial beta oxidation generates enoyl-CoA, 3-hydroxyacyl-CoA and 3-ketoacyl-CoA intermediates before losing one acetyl-CoA. In contrast to mitochondrial oxidation, peroxisomal fatty acid oxidation can operate relatively independent of the cellular energy demand. This characterizes peroxisomal fatty acid oxidation as the pathway that eliminates poorly metabolized compounds, including the spillover of fatty acids and drug metabolites. Peroxisomal oxidation is primarily responsible for chain shortenings of very-long-chain fatty acids, methyl-branched fatty acids and dicarboxylic acids. Except the first step, all other steps in peroxisomal oxidation are very similar. The first and rate limiting step in peroxisomal beta oxidation is catalyzed by acyl-CoA oxidase utilizing molecular oxygen with the production of hydrogen peroxide. In this step, instead of ATP formation, the energy produced is dissipated as heat. The acetyl-CoA generated in peroxisomes is transferred into mitochondria for complete oxidation to CO_2. The concentrations of acyl-CoAs formed in these metabolic pathways are quite different, some acyl-CoAs can reach 50-100 nmol/g, some of them are only 0.1-1 nmol/g (Gu *et al.*, 2010;Harris *et al.*, 2011). Therefore, acyl-CoA analysis is a challenge to the method development. Tandem mass spectrometry offers

several unique capacities for the acyl-CoA analysis. First of all, multiple reaction monitoring (MRM) has high sensitivity to acyl-CoA, because acyl-CoA containing multiple nitrogen atoms has very good ionization yield in positive electrospray ionization (ESI) mass spectrometry. Second, the collision induced dissociation (CID) of all acyl-CoA derivatives in tandem mass spectrometry generate the the common fragments at m/z 428 and 261 derived from coenzyme A moiety. Third, all acyl-CoA molecules in tandem mass spectrometry has the same neutral loss of 507. Most importantly, the neutral loss of 507 is the most abundant daughter ion for most of acyl-CoAs.

2.1 Untargeted metabolites profile by tandem mass spectrometry

The tandem mass spectrometer is designed for sensitive quantification of analytes. To quantify an analyte by tandem mass spectrometer, some compound information and parameters including precursor ion, daughter ion, declustering potential, and collision energy etc., should be known and optimized first. All these seem to determine that tandem mass spectrometer is only used for targeted metabolomics analysis. However, tandem mass spectrometry provides additional features that can be applied to untargeted metabolomics studies. Two types of tandem mass spectrometric untargeted metabolic profiling approaches are introduced here. The first one is the precursor ion scan that profiles all the metabolites having the same fragment. The second approach is MRM scan that is basically similar to neutral loss scan.

2.1.1 Precursor ion scan

Tandem mass spectrometric characteristics of the acyl-CoA molecules can be used for the comprehensive analysis of fatty acid oxidation intermediates. The typical fragmentation of acyl-CoA is shown in Fig 1. As a survey analysis of all CoA derivatives, we developed acyl-CoA precursor ion scan method using fragment at either 428 or 261 (Dalluge *et al.*, 2002). Application of tandem mass spectrometry for discovery of new metabolites, including phospho-hydroxyacyl-CoA derivatives will be discussed in detail in section 3. The precursor ion scan is extremely helpful for this type of untargeted metabolite profiling because it profoundly filters out interferences by focusing on the metabolites that gives specific daughter ion(s).

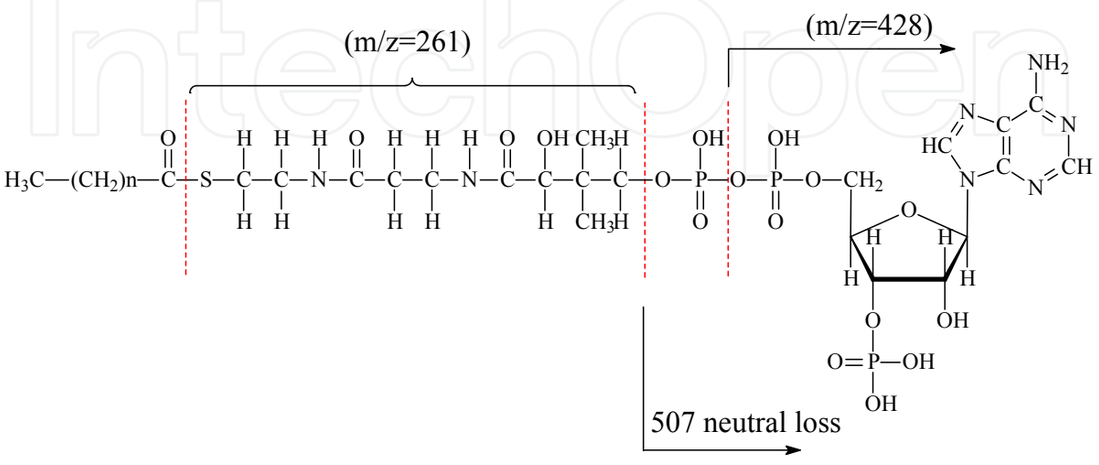


Fig. 1. The acyl-CoA structure and typical fragmentation pattern in positive ESI.

The applications of precursor ion scan to profile metabolites have been reported by others (Millington *et al.*, 1989;Wen *et al.*, 2008;Millington and Stevens, 2011). Acylcarnitines that are from their corresponding acyl-CoAs are used for diagnostic test for inherited disorders of fatty acid and branched-chain amino acid catabolism. Millington et al. used precursor ion scan mode to profile acylcarnitines in plasma and whole blood using acylcarnitine methyl esters typical fragment ion at m/z 99 (CH_2-CH=CH-$COOCH_3$, positive ion) (Millington *et al.*, 1989;Millington *et al.*, 2011). In addition to qualitative profiling, precursor ion scan was also used for acylcarnitines quantiation by adding deuterium labeled acyl-carnitines in their work. Another fragment (m/z 85, CH_2-CH=CH-COOH, positive ion) of acylcarnitine butyl esters was chosen for precursor ion scan to profile acylcarnitines in blood. Ten common acylcarnitines and 40 rare acylcarnitines were detected in defined mutant mice (Rolinski *et al.*, 2000). Wen et al. screened unknown glutathione conjugate by negative precursor ion scan using fragment m/z 272 that corresponds to deprotonated γ-glutamyl-dehydroalanyl-glycine originating from the glutathione moiety. The new metabolite, 2-hydroxy-3-(glutathione-S-yl)-deschloro-meclofenamic acid, from meclofenamic acid was found by this method (Wen *et al.*, 2008).

2.1.2 MRM scan

The advantage of MRM scan is its high sensitivity suitable for targeted quantification purposes. To establish a method in MRM mode one needs to obtain both the precursor and daughter ions information. This requires direct infusion of standard (precursor) compound into mass spectrometer and optimization of ionization and fragmentation parameters. Here we present our strategy to perform non-targeted analysis of acyl-CoA metabolites by MRM. As it is mentioned earlier, all acyl-CoAs have a neutral loss of 507 in tandem mass spectrometry (Fig 1). In addition, the mass of most endogenous acyl-CoA molecules ranges from 767 (non-esterified free CoA) to 1117 (C24 acyl-CoA, the largest physiological CoA derivative). For the comprehensive analysis of all acyl-CoA one can set the precursor ion scan from 768 ($[M+H]^+$ for free CoA) to 1118 ($[M+H]^+$ for C24 acyl-CoA), and their corresponding daughter ions' m/z values are their precursor ions' m/z data minus 507 at positive ESI. Therefore, MRM scan is programmed based on the neutral loss. However, MRM mode is more sensitive than neutral loss scan mode.

After validation this MRM method with known acyl-CoAs, we performed a non-targeted acyl-CoA profiling from the isolated rat liver perfused with levulinic acid (4-ketopentanoic acid) (Harris *et al.*, 2011). "Head-to-head" comparison of acyl-CoA profiles with the sham liver tissue enabled identification of the following acyl-CoA metabolites in the rat livers perfused with levulinic acid: levulinyl-CoA, 4-hydroxypentanoyl-CoA; 4-phosphopentanoyl-CoA, 3-hydroxypentanaoyl-CoA, 3-ketopentanoyl-CoA, 4-hydroxy-2-pentenoyl-CoA, 4-keto-2-pentenoyl-CoA, 3,4-dihydroxypentanoyl-CoA, 4-keto-3-hydroxy-pentanoyl-CoA, 3-keto-4-hydroxypentanoyl-CoA and 3,4-diketopentanoyl-CoA. With such detailed acyl-CoA metabolites profile information, we can deduce three parallel catabolic pathways of levulinic acid in rat livers (Harris *et al.*, 2011). Pathway A is the phosphorylation of 4-hydroxypentanoyl-CoA to form 4-phosphopentanoyl-CoA followed by isomerization to form 3-hyroxypentanoyl-CoA. The latter compound is a regular beta oxidation intermediate that can be further oxidized completely to form acetyl-CoA and propionyl-CoA. In pathway B, levulinic acid is reduced to 4-hydroxypentanoic acid and activated to 4-hydroxypentanoyl-CoA. The latter intermediate is cleaved to form lactyl-CoA

and acetyl-CoA via one cycle of beta oxidation. In pathway C, levulinic acid can be directly activated to form levulinyl-CoA that is further metabolized to 3,4-diketopentanoyl-CoA. Partial enzymatic reduction of this metabolite generates 3-keto-4-hydroxypentanoyl-CoA. Further degradation of 3-Keto-4-hydroxypentanoyl-CoA forms lactyl-CoA and acetyl-CoA (Harris *et al.*, 2011). Some of these intermediates are in small amount. For example, 4-Keto-2-petanoyl-CoA is approximately 500 fold less than acetyl-CoA. Therefore, only a sensitive method utilizing the state-of-the-art technique like tandem mass spectrometry can identify such small quantities of these intermediates.

2.2 Targeted metabolic profiling of ceramide species by tandem mass spectrometry

Recently we utilized LC-MS/MS for the simultaneous profiling and quantification of different ceramide species in biological samples. Ceramides represent a class of compounds characterized with acyl group attached to amino group of sphingosine backbone. The carbon number before the name, such as C14-ceramide for N-myrystyl-sphyngosine, specifies fatty acyl chain length. The fatty acids with chain length of C14-C26 are most common ceramide species existing in nature, although ceramides with shorter (C10, C12) and longer (C30) chains are also found.

Ceramides play an important role in insulin resistance, cell signaling, cell differentiation, proliferation and apoptosis and serve as a precursor for many other sphingolipids (Hannun and Obeid, 2008;Kewalramani *et al.*, 2010). Interestingly, ceramide and distinct ceramide metabolites have different and sometimes opposing functions in cell survival and apoptosis, glucose uptake and insulin resistance (Fig.2). Particularly, it is known that ceramide causes apoptosis and inhibits insulin signaling while sphingosine-1-phosphate increases intracellular glucose uptake and inhibits apoptic pathways through the activation of Akt. Because of these opposing effects ceramide/sphigosine-1-phosphate rheostat notion was formulated (Hannun *et al.*, 2008).

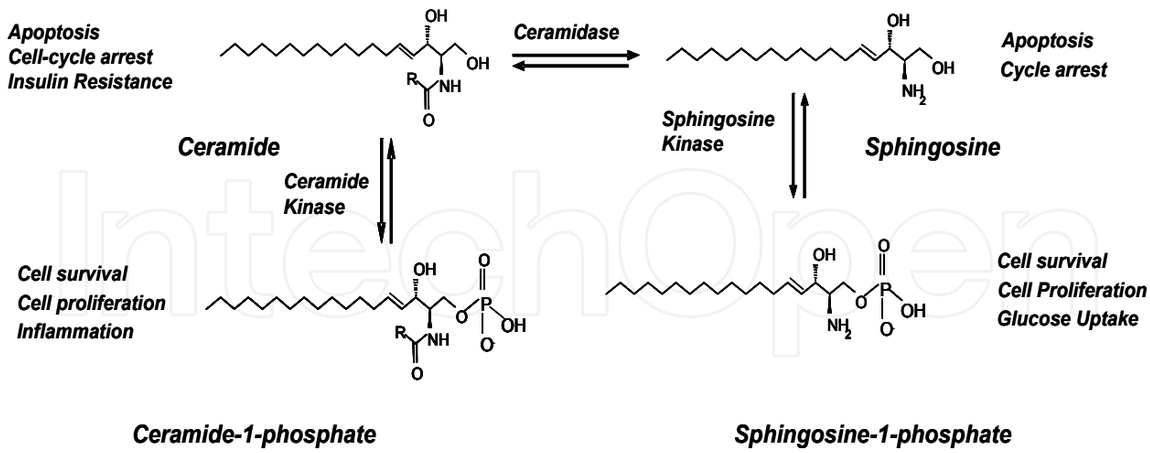


Fig. 2. Role of ceramide and its metabolites in cell signaling.

Although it is not known how the structure of individual ceramide species defines their physiological functions, it has been shown that ceramide containing specific fatty acids are generated in response to certain stimuli, underscoring the structure/function relationship for different ceramide species (Pewzner-Jung *et al.*, 2006). For example, it is known that C16 and C24 ceramide species are involved in cell death (Osawa *et al.*, 2005), while C18 ceramide

inhibits cell growth (Koybasi *et al.*, 2004). Investigation of the physiological function of distinct ceramides and their metabolites requires an accurate and sensitive method for their quantification in biological samples. Ceramides were analyzed by an enzymatic diacylglycerol kinase assay (Preiss *et al.*, 1987), by high performance liquid chromatography (HPLC) (Yano *et al.*, 1998), and GC-MS analysis after derivatization (Tserng and Griffin, 2003). However, these methods are labor intensive and time consuming. Although, some of these approaches may be used to analyze total ceramide or fatty acid moiety of ceramide, they do not provide information on individual ceramide species. In addition, these methods generate discrepant results on ceramide levels in different biological samples (Turinsky *et al.*, 1990;Yamaguchi *et al.*, 2004). The tandem mass spectrometry offers several advantages for the analysis of ceramides and their metabolites. The MS/MS mode enables high specificity of identification of the structurally related isomeric and isobaric complex compounds through the precursor/product relationship of ionized molecular and fragment ions. Monitoring of selected precursor and daughter ions in MRM mode improves the sensitivity of the analysis. A wide dynamic range of tandem mass spectrometry allows analysis of both low and high abundant ceramides and their metabolites in a single run. However, structural similarities, wide range biological levels of related sphingolipids and different physico-chemical properties (solubility, ionization) of ceramides and metabolites requires very careful selection of extraction methods, internal standards, instrumentation and mass spectrometric parameters.

Sphingolipids, including ceramides, are easily ionized and they produce several product ions characteristic for the backbone and fatty acids attached to the backbone. Most of the basic and complex sphingolipids form $(M+H)^+$ and $(M+H-H_2O)^+$ positive ions, while phosphorylated sphinglipids also form negative $(M-H)^-$ ions in negative ESI mode. Recently lipidomic studies have used ESI-MS/MS technologies to analyze ceramides and related sphingolipids in biological samples (Schmelzer *et al.*, 2007;Sullards *et al.*, 2007).

We have optimized and validated a reverse-phase liquid chromatography coupled ESI-MS/MS technique for the simultaneous measurement of multiple ceramide species in different biological matrices (Kasumov *et al.*, 2010). The method of analysis of tissue samples is based on Bligh and Dyer extraction (BLIGH and DYER, 1959), reverse-phase HPLC separation and MRM of ceramides. Preparation of plasma samples also requires isolation of sphingolipids by silica gel column chromatography prior to LC-ESI-MS/MS analysis. The limits of detection and quantification are in a range of 5-50 pg/ml for distinct ceramides. The method is reliable for inter-assay and intra-assay precision, accuracy and linearity. The separation and quantification of several endogenous long-chain and very-long-chain ceramides using two non-physiological odd chain ceramide (C17 and C25) as internal standards is achieved on a C8 reverse-phase column in less than 5 min during a single 21 min chromatographic run. This method took advantage of the formation of m/z 264.3 daughter ion representing the sphingosine backbone of ceramide (Fig. 3).

To perform the survey analysis of different subspecies a scan of precursor ions was performed over a wide range of collision energies (10-65 eV) in a triple quadrupole tandem mass spectrometry. The MRM method was composed based on existing species in biological samples. The following transitions were selected for sensitive and selective analysis of biological ceramide species: 482.3/264.3 (C12:0), 510.3/264.3 (C14:0), 538.3/264.3 (C16:0), 552.3/264.3 (C17:0), 564.3/264.3 (C18:1), 566.3/264.3 (C18:0), 592.6/264.3 (C20:1), 594.6/264.3 (C20:0), 620.6/264.3 (C22:1), 622.6/264.3 (C22:0), 648.6/264.3 (C24:1), 650.6/264.3 (C24:0), 676.6/264.3 (C26:1) and 678.6/264.3 (C26:0). In addition, the following

transitions were used for non-physiological internal standards: 552.3/264.3 (C17:0) and 664.6/264.3 (C25:0).

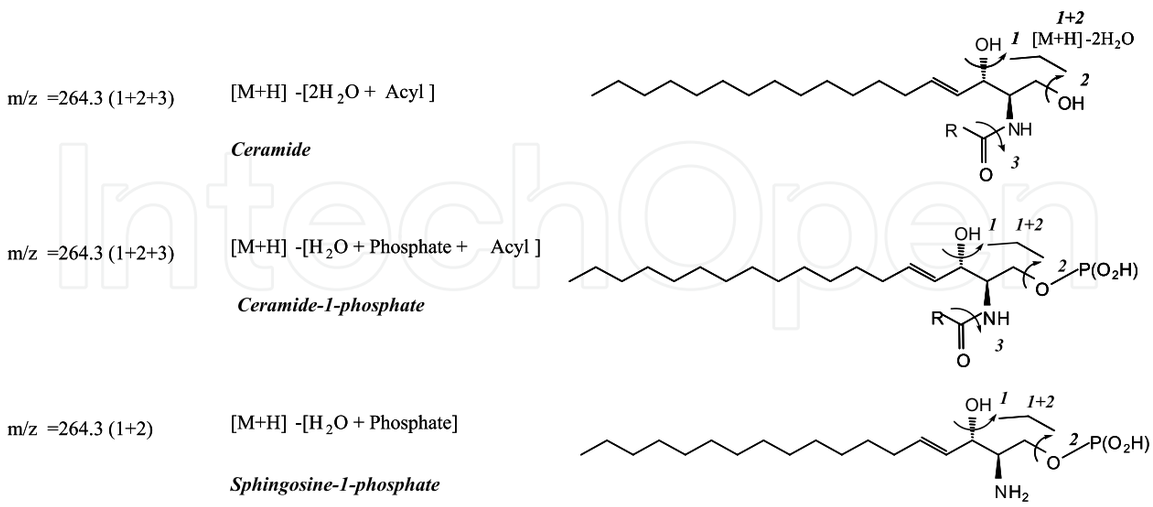
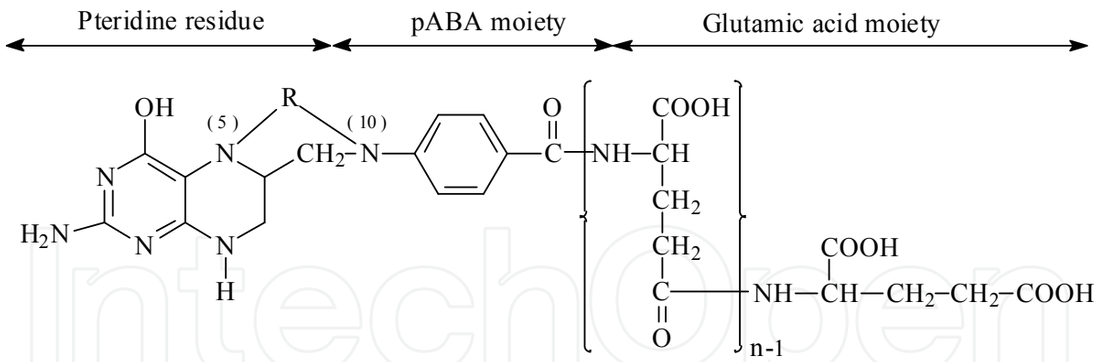


Fig. 3. Structure and fragmentation of ceramide, ceramide-1-phosphate and sphingosine-1-phosphate with m/z 264.3 as the most abundant product ion during collision induced ionization.

The technique was applied to quantify distinct ceramide species and metabolites in different rat tissues and in human plasma. Using this analytical technique we demonstrated that plasma and muscle ceramides are increased in obese subjects with type 2 diabetes and are associated with reduced insulin sensitivity (Haus *et al.*, 2009). This technique was extended for the quantification of ceramide metabolites ceramide-1-phosphate and sphingosine-1-phosphate which utilized 380.3/264.3, 618.7/264.3 and 366.3/250.3 transitions for the sphingosine-1-phosphate and ceramide-1-phosphate and C17-sphingosine-1-phosphate (internal standard), respectively (Fig. 3). This method also could be applied to the analysis of other sphingolipids without significant modification.

2.3 Targeted folate derivatives quantitation by tandem mass spectrometry

Another good example of the application of tandem mass spectrometry for metabolite profiling is folate analysis. Folate is a water-soluble vitamin (B9), which plays a key role in the methylation cycle and in DNA biosynthesis. Folate deficiency has been implicated in hyperhomocysteinemia, which results in an increased risk of cardiovascular disease and dementia, and in neural tube defects. The folate analysis is challenging because of the complexity of folate derivatives in biological samples (see Fig 4) (Zhang *et al.*, 2003;Zhang *et al.*, 2005). Different substitutes (R, Fig 4)) and various glutamate units account for the most of the structural diversity of folate derivatives. It is especially challenging for the most methods to differentiate such large number of folate derivatives. The classical gold method for folate analysis is a microbiological method that analyzes bacterial growth dependent on the available amount of folate (Koontz *et al.*, 2005). Although microbiological method for folate analysis is very sensitive, it suffers from being not reproducible. In addition, only the total amount of folate (but not individual species) is measured by microbiological method.



Substituent (R)	Position
—CH_3 (methyl)	5
—CHO (formyl)	5 or 10
—CH_2— (methylene)	5 and 10
═CH— (methenyl)	5 and 10

Tetrahydropteroylpolyglutamates

Fig. 4. The structure of folate derivatives with different substituent and different number of glutamate.

Tandem mass spectrometry has the unique capacity to differentiate the compounds as long as they have different masses of precursor ions and/or product ions. Mono-glutamate folates have different precursor ion and product ion masses, poly-glutamate folates have the same product ion as their corresponding mono-glutamate folate product ion with different precursor ion masses. For example: precursor/product transitions for 5-methyltetrahydrofolate mono-glutamate, 5-methyltetrahydrofolate di-glutamate, 5-methyltetrahydrofolate tri-glutamate, 5-methyltetrahydrofolate tetra-glutamate, 5-methyltetrahydrofolate penta-glutamate and 5-methyltetrahydrofolate hexa-glutamate are 460/313, 589/313, 718/313, 847/313, 976/313 and 1105/313, respectively. Folate molecules with different number of glutamate have the following tandem mass spectrometric characteristics: (i) each additional glutamate has an additional 129 mass unit increase at the m/z of the precursor ion, (ii) they all have the same product ion.

Antifolates are among the first anti-microbial agents invented. Clinical resistance to anti-folates has been mainly attributed to mutations that alter the structure or the expression of enzymes involved in *de novo* folate synthesis. Our recent study showed that the deficiency of 5,10-methenyltetrahydrofolate synthase was found to be hyper susceptible to anti-folate agents. We applied the tandem mass spectrometry technique to measure folates in the *Mycobacterium smegmatis* with the mutation of 5,10-methenyltetrahydrofolate synthase. The folate tandem mass spectrometry method is briefly summarized here. A Thermo Scientific Hypersil GOLD C18 column (150 × 2.1 mm), protected by a guard column (Hypersil GOLD C18 5 µm, 10 × 2.1 mm) was used to separate folates by applying gradient elution of two

mobile phases. Mobile phase A is 98% H_2O with 2% acetonitrile containing 0.1% formic acid and mobile phase B is 98% acetonitrile with 2% H_2O containing 0.1% formic acid. The starting eluent was 98% A / 2% B. Mobile phase B was linearly increased to 30% in 15 minutes, then further increased to 90% in 2 minutes. The mobile B was kept at 90% for 4 minutes and then adjusted to initial condition. The column was re-equilibrated at initial condition for 10 minutes before the next injection. The column oven and auto sampler were kept at 35 and 4°C, respectively. The 4000 Qtrap mass spectrometer (AB Sciex, Foster City, CA) source parameters are set as follows: Turbo ion-spray source at 600 °C, gas 1: 60 psi, gas 2: 70 psi, curtain gas at 30 psi. The mono-glutamate folate mass transitions and compound parameters are shown in Table 1. Poly-glutamate folate parent m/z is 129 more for one additional glutamate but with the same product ion m/z as the one corresponding to mono-glutamate folate. Profiling of the folate derivatives showed that mono-glutamate folates had no change compared to the wild type *Mycobacterium smegmatis*, but dramatically decreased poly-glutamate 5-formyltetrahydrofolate, which indicates the important physiological role of poly-glutamate 5-formyltetrahydrofolate in the folate metabolism (Ogwang *et al.*, 2011).

Folates	Parent *m/z*	Product *m/z*	CE (V)	DP (V)	EP (V)	CXP (V)
Folate	442.1	295.0	27	75	10	10
Dihydrofolate	444.1	178.0	27	70	10	10
Tetrahydrofolate	446.1	299.1	20	76	10	10
5,10-methenyltetrahydroflate	456.1	412.1	31	60	10	10
5,10-methylenetetrahydrofolate	458.1	311.1	23	70	10	10
5-methyltetrahydrofolate	460.1	313.1	25	60	10	10
5/10-formyltetrahydrofolate	474.1	327.1	20	71	10	10

Table 1. Mono-glutamate folate compound parameters and mass transitions in tandem mass spectrometry.

3. New metabolite discovery by tandem mass spectrometry

Identification of new metabolites is a challenge due to (i) the complexity of biological samples, and (ii) the fact that novel metabolites usually have no reference compound or existing library data. In addition, it is difficult to characterize compound identities based on molecular mass alone. In this section we will show how tandem mass spectrometry can provide more fragmentation information to help elucidating the structure of molecules. In addition we will examine how high resolution tandem mass spectrometry confirms the hypothesized molecule and fragment formula.

Tandem mass spectrometry provides information on the fragmentation, which is critical in the identification of new unknown metabolites. For example, the identity of 4-phosphopentanoyl-CoA, a metabolite of 4-hydroxypentanoic acid, is deduced by careful analyzing its fragmentation pattern in positive ionization mode of tandem mass spectrometry (Zhang *et al.*, 2009;Sadhukhan *et al.*, 2010). 4-Phosphopentanoyl-CoA (m/z 948) is 80 mass unit heavier than 4-hydroxypentanoyl-CoA (m/z=868). This suggests a possible phosphorylation. In addition, 4-phosphopentanoyl-CoA has fragments with m/z of

441, 428, 343, 312, 261, 214 and 160. The m/z at 428 and 261 confirms that this unknown compound is an acyl-CoA. The neutral loss of 507 from precursor ion (m/z=948) yields a fragment ion at m/z=441. As we discussed earlier, neutral loss of 507 fragmentation pattern is one of the acyl-CoA characteristics in positive ionization tandem mass spectrometry. Two steps of further fragmentation with a loss of 98 (one phosphoric acid) from fragments of 441 and 312 to form daughter ions 343 and 214. This fragmentation pattern confirms the phosphorylation at 4-hydroxy group of 4-hydroxypentanoyl-CoA. The detailed scheme of fragmentation and chemical structures of fragment ions are shown in Fig 5.

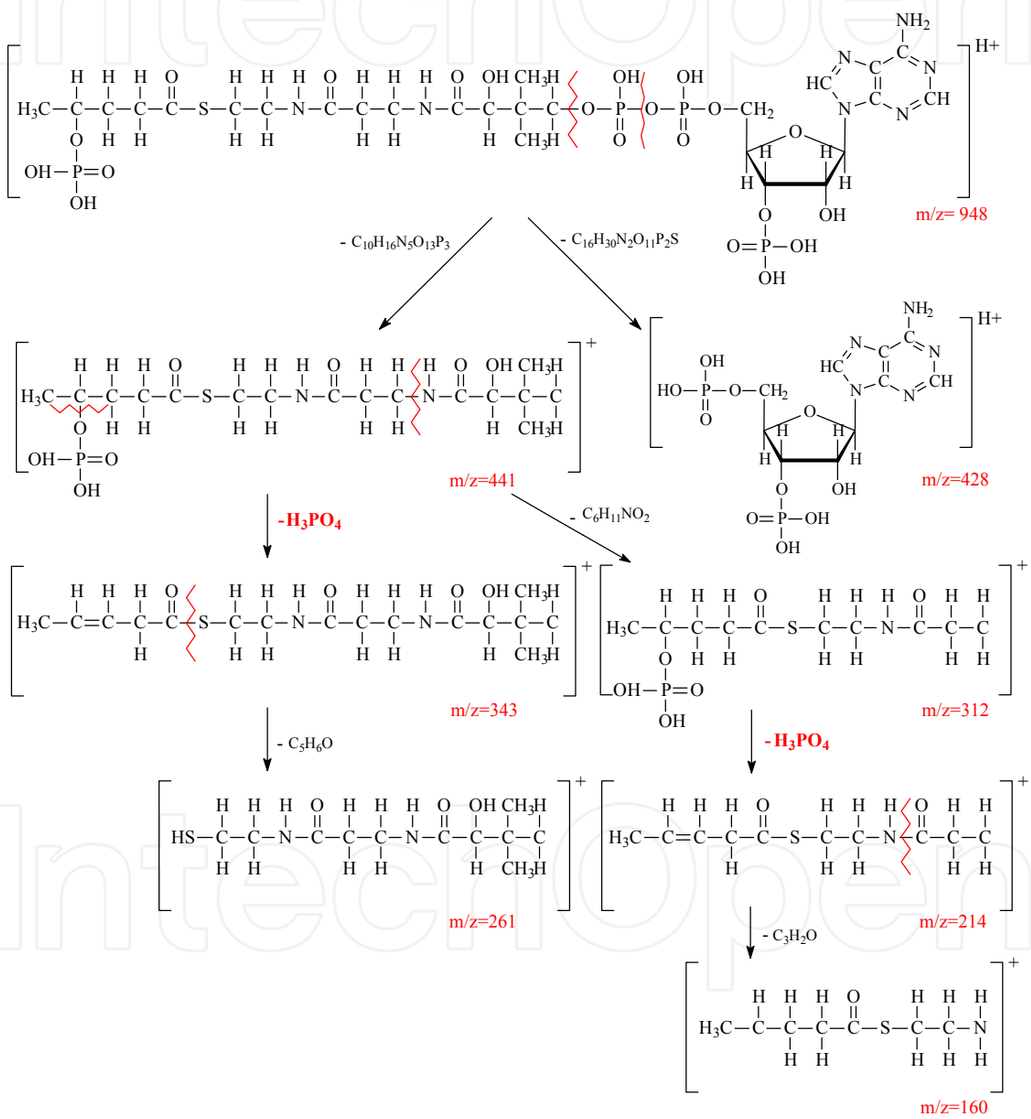


Fig. 5. Fragmentation pathway of 4-phosphopentanoyl-CoA in tandem mass spectrometry.

This example demonstrates the utility of product ion scan for identification of new metabolites. However, the success rate of determining metabolite structures based on MRM fragmentation data will ultimately depend on the experience of the individual investigator, the availability of in house or public fragmentation libraries of the same or similar class of

compounds, and the chemical nature of the metabolite itself, in terms of fragmentation information produced. Compared to EI NIST library data, LC-tandem mass spectrometry library is still at initial stage.

Single or triple quadrupole mass spectrometers usually have relatively low mass resolution (unit mass resolution). However, instruments such as time of flight (TOF), Orbitrap™, and Fourier transform ion cyclotron resonance mass spectrometry (FTICR) have high mass resolution of up to 100,000 (at m/z 400). The high mass resolution and mass accuracy can ensure mass error less than 3 ppm (Metz *et al.*, 2007). From such accurate mass measurements, candidate empirical formula of detected species may be determined. One or more empirical formula may be generated for each metabolite. The high resolution tandem mass spectrometry provides additional information on the identity of unknown metabolites. The excellent example of using accurate mass spectrometer is the identification of the unknown metabolite (4-phosphopentanoyl-CoA) from 4-hydroxypentanoic acid catabolism (see section 2.1). The exact mass of 4-phosphopentanoyl-CoA and its three fragments (A, B and C) were measured by high resolution mass spectrometry to obtain their exact masses. Table 2 represents both measured exact mass and the theoretical mass, calculated from chemical formula of hypothesized metabolite (4-phosphopentanoyl-CoA), and its three fragments (m/z values of fragments are 441, 428 and 343). The differences between measured exact mass and theoretical values are all lower than 0.5 ppm. Thus, the exact mass measurements by high resolution tandem mass spectrometry confirmed the hypothesized chemical formula.

	Hypothesized formula	Theoretical mass	Measured mass	Difference (ppm)
[4-Phospho-pentanoyl-CoA-H]$^+$	$C_{26}H_{46}N_7O_{21}P_4S$	948.141803	948.14141	0.41449
Fragment A	$C_{16}H_{30}N_2O_8PS$	441.146051	441.14612	0.15641
Fragment B	$C_{10}H_{16}N_5O_{10}P_2$	428.037244	428.03732	0.17755
Fragment C	$C_{16}H_{27}N_2O_4S$	343.169154	343.16905	0.30306

Table 2. The exact mass of 4-phosphopentanoyl-CoA and its three fragments.

4. Identification of biomarkers for specific diseases

A biomarker is a substance that is objectively measured for indication of the presence of an abnormal condition in a patient and allows disease progression and/or therapeutic response to be monitored. In this section we will review selected examples of the applications of tandem mass spectrometry to the discoveries of metabolic biomarkers characteristic of several pathological conditions.

Recently Sreekumar et al. found that sarcosine was significantly increased in the invasive prostate cancer cell line (Sreekumar *et al.*, 2009). If this finding is true, the sarcosine analysis in urine sample has significance in prostate cancer diagnosis. However, the relation between sarcosine and prostate cancer are still subject to dispute (Pavlou and Diamandis, 2009;Jentzmik *et al.*, 2010;Struys *et al.*, 2010). Soga et al. identified ophthalmic acid as a new oxidative stress biomarker in mice treated with acetaminophen (Soga *et al.*, 2006). Ophthalmic acid, L-γ-glutamyl-L-α-aminobutyrylglycine, is a tripeptide analogue of glutathione in which the cysteine group is replaced by L-2-aminobutyrate. Glutathione is a

major cellular anti-oxidant and is involved in the second phase detoxification of several drugs, including acetaminophen. As expected, acetaminophen treated mice had a sudden drop in hepatic glutathione levels which was paralleled with the appearance of an unknown compound with m/z of 290 in positive ionization mass spectrometry. The product ion spectrum of this unknown metabolite was comparable with that of the product ion spectra of glutathione but with 17.957 differences in mass. Based on these results authors deduced that the SH group of the cysteine residue of glutathione was replaced by a methyl (CH_3) group. This non polar side chain corresponds to 2-aminobutyrate, the side product of *de novo* cysteine synthesis in transsulfuration pathway. The replacement of cysteine residue by 2-aminobutyrate in glutathione molecule forms ophthalmic acid. The reduced glutathione and cysteine are decreased in conditions associated with oxidative stress. Sequestration of glutathione and cysteine induces both cysteine and glutathione synthesis. Stimulated cysteine production generates 2-aminobutyrate while increased demand for glutathione induces glycine cysteine synthase that utilizes 2-aminobutyric acid when cysteine levels are below the Km of this enzyme. The mechanism of ophthalmate formation in conditions associated with oxidative stress is outlined in Fig 6. Abbas et al. found increased ophthalmate formation in the rabbit liver with implanted tumor which was paralleled with the reduced glutathione synthesis (Abbas *et al.*, 2011).

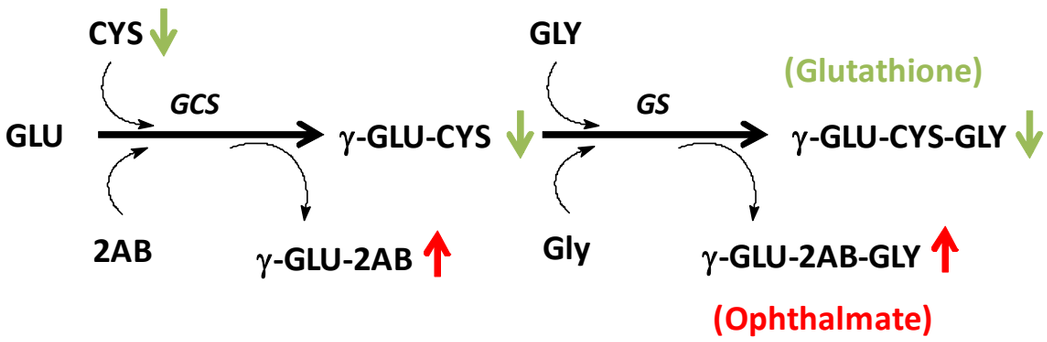


Fig. 6. The relationship between ophthalmate and glutathione concentration changes in the oxidative stress.

Recently the tandem mass spectrometry assisted biomarker discovery was applied to predict the development of diabetes (Wang *et al.*, 2011). Among 2422 normoglycemic individuals followed by 12 years, 201 developed diabetes. In these cases, 5 branched-chain and aromatic amino acids, i.e. isoleucine, leucine, valine, tyrosine and phenylalanine, were found to have significant associations with future development of diabetes. The risk of future diabetes was elevated at least 4-fold in subjects with high plasma amino acid concentrations.

5. Tandem mass spectrometry combined with the mass isotopomer analysis in metabolomics research

Because of the natural existence of low proportions of heavy ^{13}C (1.10%), ^{2}H (0.015%), ^{15}N (0.37%), and ^{18}O (0.20%) isotopes, biological molecules are identified by the number and position(s) of these isotopes. Mass isotopomers differ by the number of heavy atoms in their molecules, resulting in different molecular weights. In mass spectrometric jargon, they are

referred to as M, M1, M2, …, Mn (Brunengraber *et al.*, 1997). The application of stable isotope labeled compounds in metabolomics research is not only useful for the identification and the characterization of metabolic pathways but also provides a powerful strategy to measure metabolite fluxes, i.e. the synthesis and degradation rates of those metabolites. In this section, we will briefly discuss the application of ^{13}C and ^{2}H isotope containing compounds to the quantification of metabolic fluxes through fatty acid oxidation and protein synthesis.

First we consider $^{2}H_2O$-metabolic labeling based approach utilizing tandem mass spectrometry for protein turnover studies (Kasumov *et al.*, 2011). Proteins exist in a dynamic state of equilibrium with their surrounding environment. The net static protein expression is determined by changes in a protein turnover, i.e. both synthesis and degradation. It is difficult to detect small changes in protein abundance, whereas the changes in the turnover rates are easily measurable. Advances in isotopic tracer methods and improvements in subcellular isolation methods have enabled studies of various protein fractions, i.e. total membrane, mitochondrial, sarcoplasmic or cytosolic proteins. However, understanding the pathologies related to the regulations of protein metabolism requires methods for studying the synthesis of an individual protein. Classical protein turnover studies relied on precursor-product relationships and involved the administration of a labeled amino acid. These methods require a long-term oral consumption or intravenous infusion of labeled amino acids, isolation mixed or individual proteins at different time points and degradation of proteins to individual amino acids. After column purification amino acids are derivatized and their labeling measured by GC-MS. This is a labor-intensive protocol that requires extensive purification of proteins and amino acids and often associated with contaminations. Recently we used a high resolution ion-trap tandem mass spectrometry, i.e. FTICR MS, to study the protein synthesis. Predictable fragmentation pattern of peptides in an ion-trap as a result of CID allows *de novo* sequencing of a protein. Particularly, the cleavage at the C-N amide (along with Cα-C and N-Cα bonds) in an ion-trap MS results in the mass difference between consecutive ions within a series corresponding to the specific amino acid and allows deduction of the peptide sequence. In addition, the high resolution of the ICR detector allows an accurate mass isotopomer analysis of a selected peptide. We took advantage of these capacities of the high resolution ion-trap tandem mass spectrometry for the measurement of ^{2}H-enrichment of protein-bound amino acids in our $^{2}H_2O$-based protein turnover studies. Oral administration of $^{2}H_2O$ (a safe, non-radioactive isotope) in drinking water results in a rapid steady state labeling of body water in free living organisms. $^{2}H_2O$ rapidly labels proteogenic amino acids, indicating that amino acids transfer to protein chain is a rate liming step in a protein biosynthesis (Rachdaoui *et al.*, 2009). Incorporation of multiple copies of ^{2}H from $^{2}H_2O$ to non essential amino acids results in the amplification of the isotopic enrichment in a product and enhances the measurements of their labeling. A time-domain stepwise fragmentation of a peptide in a selective reaction monitoring mode with the zoom scans of the precursor peptide and two of its consecutive fragments allows measurement of the ^{2}H-labeling of a protein bound amino acids. Thus, instead of purification of an individual protein and following degradation to amino acids for labeling measurement, this technique allows the measurement of an individual protein-bound amino acid even in a complex mixture of proteins. This approach was applied to measure the turnover rate constant of albumin in rats with the total body water enrichment of ~2.65% (Kasumov *et al.*, 2011). Fig 7 demonstrates the time dependent

labeling observed in daughter ions derived from tryptic albumin peptide LVQEVTDFAK. The difference between the labeling of two consecutive fragment ions, i.e. QEVTDFAK *vs* EVTDFAK represents the labeling of peptide-bound glutamine. As expected, glutamine with several exchangeable hydrogen atoms incorporates a substantial quantity of deuterium. Thus, measurement of a peptide-bound amino acid labeling allows a reliable estimation of albumin kinetic parameters, i.e. the fractional synthesis rate (k= 0.37±0.05 day^{-1}) and the half-life ($t_{1/2}$ = 1.71 day) when compared with data obtained using the precursor ion (k= 0.36±0.06 day^{-1} and $t_{1/2}$ = 1.76 day). Assessment of the labeling of individual protein-bound amino acids via tandem mass spectrometry is especially important when calculating the kinetics of a protein with shorter half-life. The major assumption for a protein turnover study is that amino acids precursor labeling reaches the steady state much faster than that of their incorporation into proteins. Therefore, simultaneous measurements of labeling of proteolytic peptides and a peptide-bound amino acid allow both the validation of this assumption and the calculation the kinetic parameters.

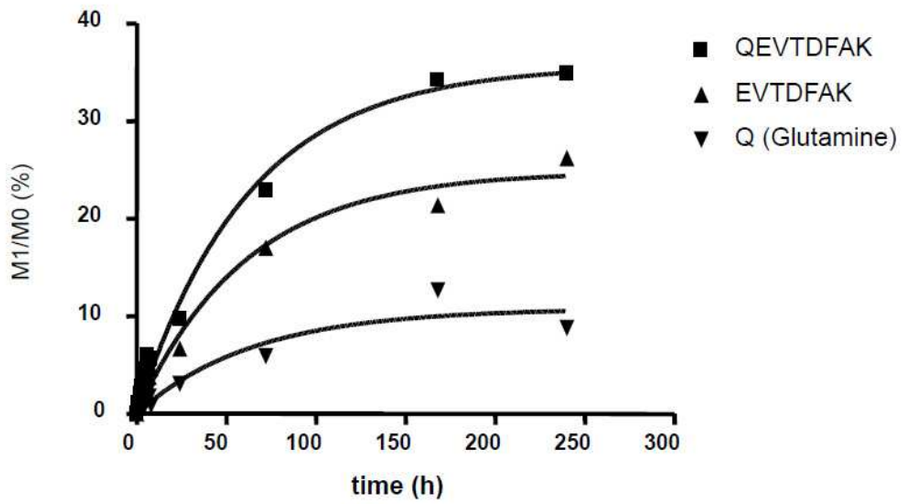


Fig. 7. Time course labeling of measured tryptic peptides QEVTDFAK and EVTDFAK and calculated labeling of peptide-bound glutamine (Q).

The second example highlights the utilization of the mass isotopomer analysis coupled metabolomics in the metabolic pathway discovery. In this case metabolomics in combination with stable isotopes is used to dissect 4-hydroxy-2-trans-nonenal (HNE) catabolism. HNE is a reactive unsaturated aldehyde derived from lipid peroxidation. HNE modified proteins, lipids and DNAs are the pathogenic factors. The detoxification of HNE is mainly via glutathione conjugation or reduction to 1,4-dihydroxynonene. HNE oxidation to 4-hydroxynonanoic acid is another major detoxification pathway. We identified two parallel catabolic pathways of 4-hydroxynonanoic acid in rat liver (Zhang *et al.*, 2009;Sadhukhan *et al.*, 2010). Two pathways are shown in Fig 8. The first round beta oxidation is the same for both pathways. The difference between pathway A and B starts from carbon 3 and 4 of 4-hydroxynonanoic acid. With a specifically isotope positional labeled 4-hydroxynonanoic

acid, i.e. [3,4-$^{13}C_2$]-4-hydroxynonanoic acid, one can track the catabolic pathways of 4-hydroxynonante. In addition, quantification of the isotopically labeled products also allows calculating the relative rate of two pathways. [3,4-$^{13}C_2$]-4-Hydroxynonanoic acid is activated to M2 4-hydroxynonanoyl-CoA in the rat liver. In pathway A, M2 4-hydroxynonanoyl-CoA is isomerized to M2 3-hydroxynonanoyl-CoA that undergoes regular beta oxidations. Continuous beta oxidations of 3-hydroxynonanoyl-CoA complete HNE degradation in the pathway A and generate one M2 acetyl-CoA. In the pathway B, 4-hydroxynonanoyl-CoA splits one acetyl-CoA through first round beta oxidation to form M2 2-hydroxyheptanoyl-CoA. M2 2-hydroxyheptanoyl-CoA is alpha oxidized to form M1 formic acid and M1 hexanoyl-CoA. M1 hexanoyl-CoA is further beta oxidized to form one M1 acetyl-CoA. Therefore, M1 and M2 acetyl-CoA are generated through pathway B and A, respectively. One can analyze mass isotopomer enrichment of acetyl-CoA and determine the rate of two pathways based on M2 acetyl-CoA/M1 acetyl-CoA ratio if M1 and M2 acetyl-CoA are exclusively from pathway B and A.

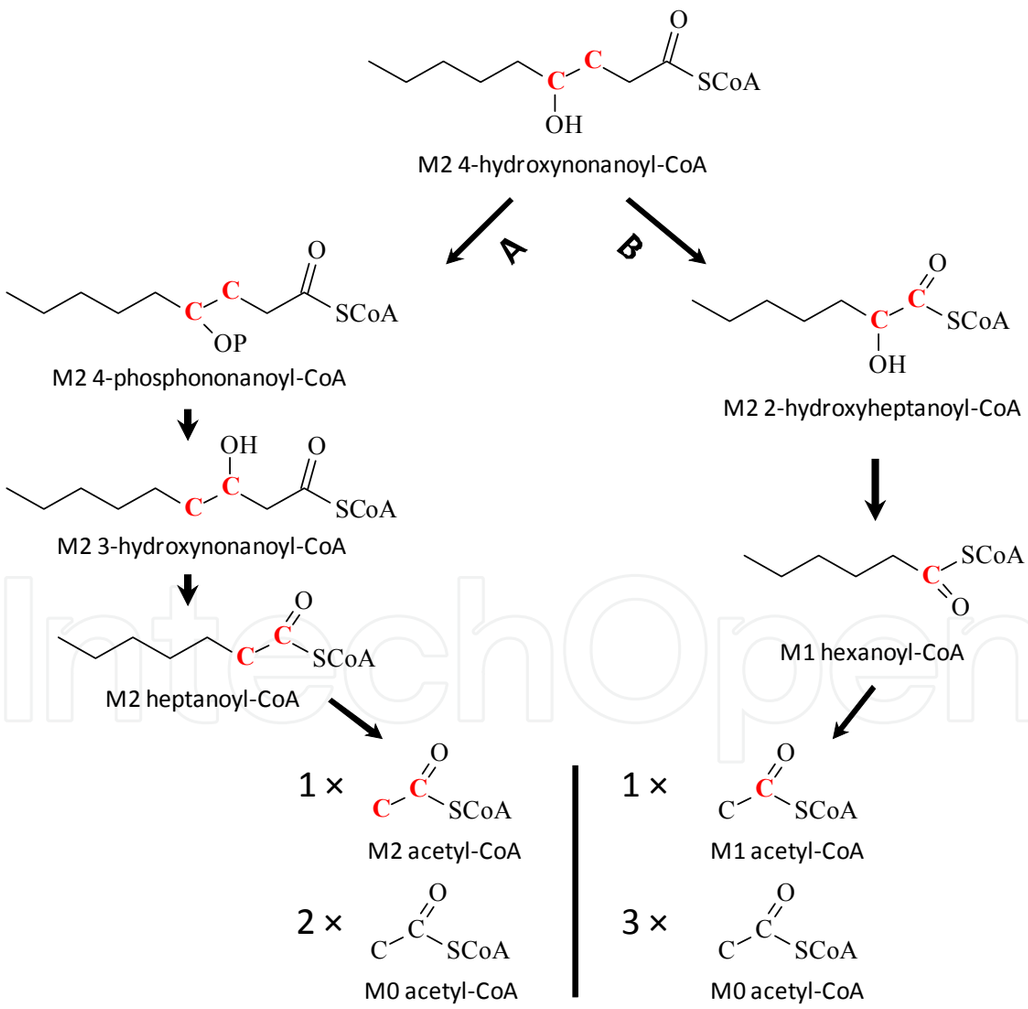


Fig. 8. The formation of M2 and M1 acetyl-CoA from two parallel catabolic pathways (A and B) of [3,4-$^{13}C_2$]-4-hydroxynonanoyl-CoA (M2 4-hydroxynonanoyl-CoA).

In both examples discussed above tandem mass spectrometry was used to measure the labeling of the fragment ions of the analytes. The mass isotopomer analysis of fragment ions, rather than whole molecule has following advantages: (i) the lower natural mass isotopomer distribution background of fragment improves the accuracy of mass isotopomer measurement, and (ii) the fragment mass isotopomer analysis provides positional labeling information. The positional mass isotope labeling measured by tandem mass spectrometry provides additional information on metabolic pathways and enables flux calculation that enriches metabolomics studies (Jeffrey *et al.*, 2002;Antoniewicz *et al.*, 2007;Kiefer *et al.*, 2007;Choi and Antoniewicz, 2011).

6. Conclusions

Tandem mass spectrometry is a sensitive and accurate technique that has been applied for targeted metabolites quantification. Traditional metabolomics investigation was limited by the sensitivity of analytical methods. Therefore the application of tandem mass spectrometry in metaboliomics study has emerged recently. This chapter mainly discussed the major applications of tandem mass spectrometry in recent metabolic studies: targeted metabolites quantification, untargeted analysis of a class of metabolites, biomarker assay, unknown metabolites identification and mass isotopomer analysis coupled tandem mass spectrometry. More insightful metabolic findings have been achieved with the help of sensitive tandem mass spectrometric technique. Current tandem mass spectrometry is limited to the targeted or semi un-targeted metabolic profiling. The single cell metabolism study is a challenging task because of the limitations related to the sensitivity of all existing methods. Achieving these goals in metabolomics studies requires the following improvements: (i) a more general un-targeted metabolomics methodology using tandem mass spectrometry is needed, (ii) a standardized tandem mass spectrometric condition is needed so that a public tandem mass spectrometric library could be generated, and (iii) further improvement of sensitivity. The future advancement of tandem mass spectrometry and bioinformatics tools should address these and other related issues in the metabolomics field.

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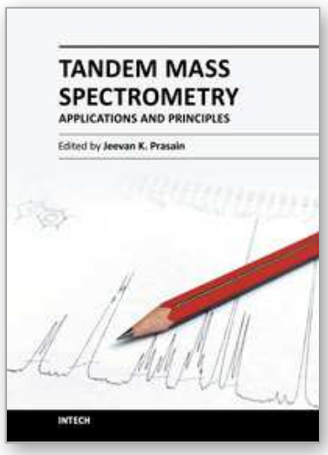

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Tandem Mass Spectrometry - Applications and Principles presents comprehensive coverage of theory, instrumentation and major applications of tandem mass spectrometry. The areas covered range from the analysis of drug metabolites, proteins and complex lipids to clinical diagnosis. This book serves multiple groups of audiences; professional (academic and industry), graduate students and general readers interested in the use of modern mass spectrometry in solving critical questions of chemical and biological sciences.

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University Campus STeP Ri
Slavka Krautzeka 83/A
51000 Rijeka, Croatia
Phone: +385 (51) 770 447
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中国上海市延安西路65号上海国际贵都大饭店办公楼405单元
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