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Technological advances in current metabolomics and its application in tradition Chinese medicine

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During the last few years, many metabolomics technologies have been established in biomedical research for analyzing the changes of metabolite levels. They have also become important tools in the field of life sciences and are widely applied to discover innovative drugs, providing valuable methods to explore the function and essence of formulas in traditional Chinese medicine (TCM) research. Metabolomics shows major potential to provide a unique perspective for disease diagnosis and elucidate the action mechanism of TCM by measuring endogenous metabolites in biofluids. With the gradual expansion of the application fields of metabolomics, the related number of techniques is increasing day by day. The techniques of choice for the study of various low-molecular-weight metabolic pathways, and for the analysis of metabolites in search of the changes of biomarkers in TCM treatment of disease in biological samples include nuclear magnetic resonance (NMR) spectroscopy, chromatography coupled with mass spectrometry, and so forth. These techniques have been proven to be powerful tools for the detection of metabolites and biomarkers. Here, we will introduce the newly used analytical techniques for metabolomics, and metabolomics in TCM is also summarized.

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1 Introduction

Metabolomics aims to characterize and quantify all small molecular metabolites in complex biological specimens including serum, plasma, urine, *etc.*[1] Metabolomics is applied for rapid metabolite identification in global profiling of different living systems, because of improvements in the detection capabilities of current instrumentation techniques used in biomedical research. Since then, the technique has been greatly improved by coupling mass spectrometers to chromatography, such as liquid chromatography (LC), gas chromatography (GC), and capillary electrophoresis (CE), and various types of mass analyzers including magnetic or electric sector, time-of-flight (TOF), quadrupole (Q), ion trap (IT), and Fourier transform ion cyclotron resonance (FTICR) mass spectrometers.[2,3] Due to the diverse properties of metabolites and the complexity of the metabolome, there is no single analytical platform that can be used for the detection of all metabolites in biological samples. A variety of techniques such as chromatography coupled with mass spectrometry[4–6] and NMR spectroscopy are available in metabolomics at present.[7,8] This is because these techniques can provide excellent resolution to determine individual molecular species. These are valuable tools for the screening of metabolites and biomarkers, and they are extremely beneficial in terms of the identification and analysis of interest.

Based on the compatibility theory of Chinese medical science, traditional Chinese medicine (TCM) is usually prescribed in combination of several medicinal herbs at a certain mass ratio, playing a key role in treatment of disease and the clinical prevention for centuries in China.[9,10] TCM treats diseases with multi-component acting on multi-target simultaneously through multiple pathways, generating a range of actions that manifest as a comprehensive overall effect. The sources of these active constituents are complicated, ranging from original compounds of the prescription to their metabolites of the drugs *in vivo* after administration[11,12] (Fig. 1). The identification of metabolites and absorbed constituents plays a critical role in elucidating mechanism of action and the therapeutic material basis. Thus, technique is important for reliable and practical analysis of the chemical constituents of TCM and the detection of potential biomarkers associated with TCM treatment. The findings demonstrate that the efficiency of ultra performance liquid chromatography (UPLC), the accuracy of Q-TOF-MS, and the sensitivity and quantitation ability of Qtrap-MS provide a method for the efficient and comprehensive chemical characterization and quality control of complex TCM.[13] It is necessary and valuable to develop a reliability sensitivity analysis approach for the detection and identification of metabolites and absorbed constituents of TCM. It might pave the way for further investigations into the mechanisms of action of TCM.

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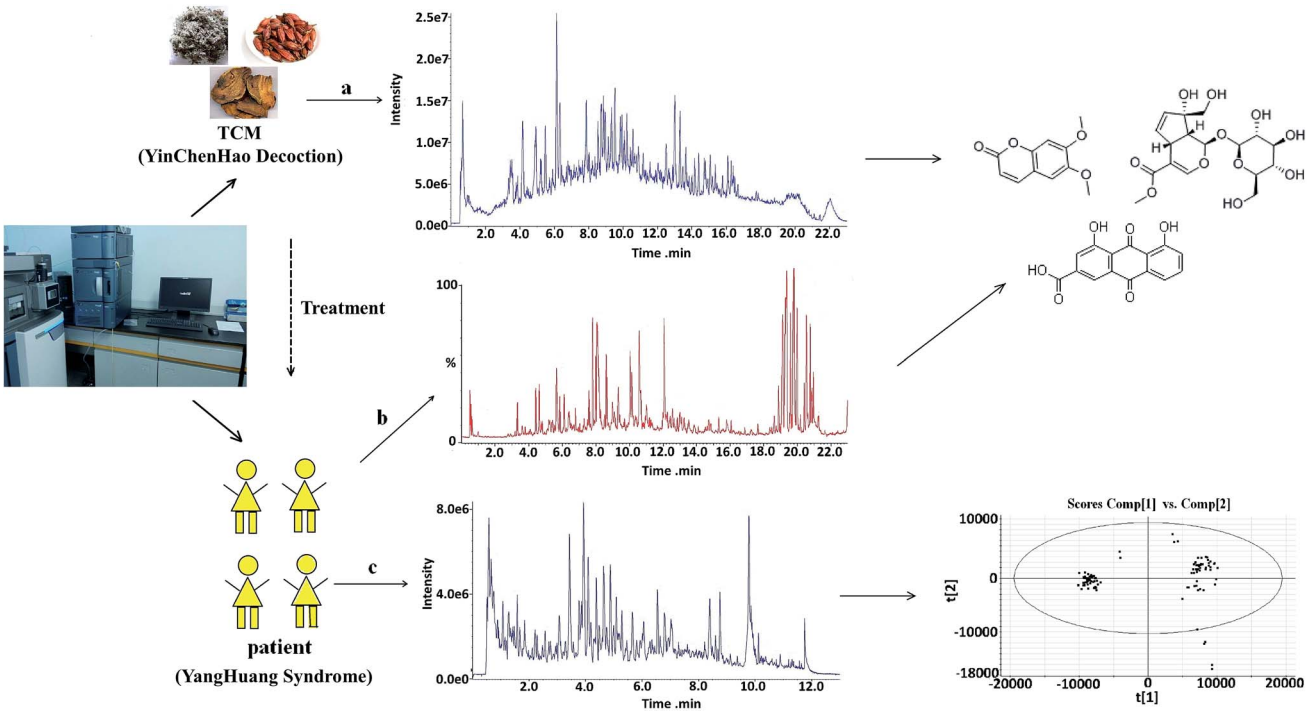

Fig. 1 The application of metabolomics technology in TCM. YinChenHao Decoction (YCHD), a well-known TCM formula, is used for the treatment of YangHuang syndrome. A metabolomics approach and the multivariate statistical methods have been developed for identification and screening of original compounds and their metabolites of YCHD. Based on the same instrumental conditions, potential biomarkers associated with YangHuang syndrome and with YCHD treatment were detected and identified. (a) Original compounds of YCHD *in vitro*. (b) Original compounds and their metabolites of YCHD *in vivo* after administration. (c) Metabolomics-based studies on YangHuang syndrome.

2 Analytical technology

Over the past twenty years, an explosion of technological developments has been inspired by renewed interest in metabolic research to study metabolism. Chromatography-mass spectrometry and nuclear magnetic resonance (NMR) spectroscopy are the two most commonly used analytical technologies in metabolomics.[14]

2.1 NMR spectroscopy

NMR spectroscopy, a powerful technique, can be applied to elucidate the structures of organic compounds. NMR-based metabolomics analysis of biofluids accurately and quickly, without the need for initial separation or processing, has found wide applications, for example, in diagnosis of disease,[15] physiological evaluation,[16] characterization of animal models of disease,[17] drug safety assessment,[18,19] and drug therapy monitoring.[20] The major strengths of NMR spectroscopy are its efficiency and ability to measure analytes. Recently, improvements have included stop-flow chromatography samples, lower instrument cost, and higher spectral resolution.[21] This extremely important experimental technique is based on magnetic nuclear spin of ^{1}H, ^{13}C, ^{15}N, ^{119}Sn, and so on, and among them ^{1}H and ^{13}C-NMR spectroscopy are the common analytical technologies used for metabolomics primarily.[22] A large amount of metabolic data can be generated by simple ^{1}H-NMR experiments, which provides a surprising detailed to explore the biochemical events throughout the organism and study species differences based on toxicological biomarkers.[23] The application of ^{13}C-NMR raises the possibility of better separation between experimental groups and easier identification of metabolites using multivariate analyses, owing to better spectral dispersion.[24] In the study of small molecule mixtures, two dimensional NMR (2D-NMR) spectra provides the benefit of more detailed structural information, which is particularly relevant to detect novel chemotypes.[25] There are a series of 2D-NMR experiments commonly used in metabolomics, such as 2D *J*-resolved NMR, 2D and 3D diffusion ordered spectroscopy (DOSY), 2D ^{1}H–^{1}H correlation spectroscopy (COSY), 2D ^{1}H–^{13}C heteronuclear NMR, *etc.*[26] Although, 2D-NMR experiments are relatively less sensitive compared to 1D experiments, the additional information available from 1H–13C cross peaks are invaluable for spectral annotation/quantitation, avoiding the peak overlaps that usually clutter the 1D spectrum.[27] These technologies have commonality and own their features (Fig. 2), and thus it is essential to choose an appropriate analytical technology for the specific class of analyte of interest in the biomatrix.

2.2 Chromatography-mass spectrometry

Mass spectrometry is a recommended approach for metabolomics research at present because it can directly and simultaneously analyze many compounds.[28,29] The efficacy of MS in the study of endogenous metabolites stems from its proven

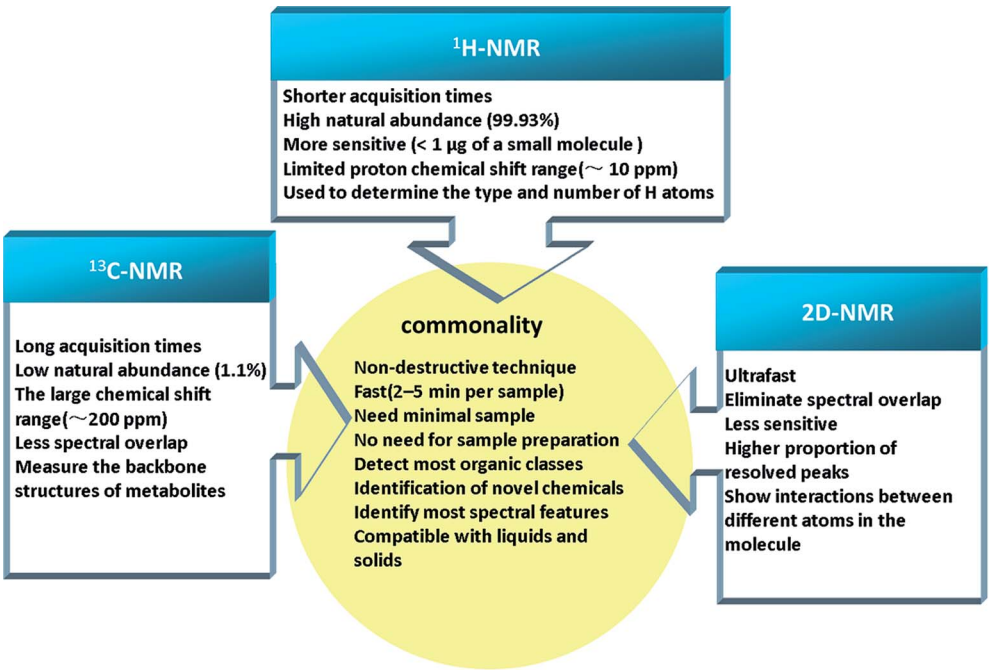

Fig. 2 The commonality and features between ^{1}H-NMR, ^{13}C-NMR and 2D-NMR.

success in disease studies,[30–32] pharmacokinetics[33,34] and drug-metabolite analysis.[35] MS (especially Q-TOF) also helps to identify previously uncharacterized metabolites.[13,36,37] MS in quantitative analysis of small molecules has been established, unlike its application to proteomics. Due to the complexity of metabolites and hundreds of metabolites therein, high-resolution MS has combined with advanced and high-throughput separation techniques, but not exclusively, to perform these measurements. MS provides an additional and completely independent method for the identification of compounds. By coupling chromatography to MS, the sensitivity of mass spectrometry has been widely used, and among them liquid chromatography-mass spectrometry (LC-MS) has already been applied to metabolomics studies of seminal plasma.[38] Although these technologies each have their own strengths and weaknesses (Table 1), numerous studies have shown how they may be used to complement each other. Gas chromatography coupled with mass spectrometry (GC-MS) has been recognized as a primary tool for small molecule analysis,[39] and is still used to detect various metabolic disorders today.[40] Recent interest in metabolomics stems from the ability to carry out more comprehensive metabolome analysis using new liquid chromatography combined with mass spectrometry (LC-MS), which has the potential to discover and identify both disease and pharmaceutical biomarkers, exploring the hidden secrets of the biochemical processes of tissues and cells.[41,42] Advances in LC-MS are very impressive, with recent developments in UPLC, the speed of analysis is further improved. Recent developments in analytical separation sciences have led to the emergence of technologies based on MS and UPLC, demonstrating ever improving sensitivity of mass measurements and resolution of metabolite species.[43] In the field of metabolomics, today capillary electrophoresis-mass spectrometry (CE-MS), as a particularly useful complementary analytical technique, has been unitized to separate and detect ionic compounds based on the different migration rates of charged metabolites.[6,44] In view of these advances, this current metabolomics technology can allow the extent of capability to become better diagnostic and therapeutic tools.

3 Technological advances in metabolomics

Metabolomics, a new emerging discipline, evaluates the concentration of different metabolites in complex biological samples to understand the ongoing metabolism. Metabolites are the end products of a variety of metabolic pathways, which are involved in the occurrence of disease, and can be used as biomarkers for the evaluation of diagnosis, treatment, and prognosis of disease.[45] At the forefront of methodological innovation is an approach called ''non-targeted'' or ''discovery'' metabolomics. In order to comprehensive analysis of the metabolome at the systems level, non-targeted metabolomics attempts to measure a largely undefined set of metabolites in a given biological specimen. In view of its potential comprehensive coverage, non-targeted metabolomics is often the first choice of experiments for investigators to pursue a metabolic research question.[46]

A new opportunity to discover biomarkers in complex diseases has been provided by metabolomics, which may improve the clinical course and provide pathological understanding of the disease, beyond the traditional technology.[47] The potential of this approach for clinical diagnostics is enormous, since only minimal biological preparation is required.

Table 1 The strengths and weaknesses of hyphenated MS techniques

	Strengths	Weaknesses	Ref.
GC-MS	Good sensitivity (LOD = 0.5 μM) Suitable sample volume (0.1–0.2 mL) Superb separation reproducibility wide dynamic range Detects some inorganic and most organic molecules Can be mostly automated	Sample not recoverable Sample derivatization is required Separation is required Long analysis time (20–40 min per sample) Not compatible with solids Novel compound identification is difficult	81–85
LC-MS	Great sensitivity (LOD = 0.5 nM) Very flexible technology Detects some inorganic and most organic molecules Small sample volume (10–100 μL) Direct infusion Has the potential to detect the largest portion of metabolome Can be mostly automated	Sample not recoverable Not very quantitative Time-consuming sample preparation procedures Long analysis time (15–40 min per sample) Lower reproducibility and poor separation resolution Less-robust instrumentation Not compatible with gases Novel compound identification is difficult	60 and 85–89
CE-MS	Good sensitivity (LOD = 0.5 fM) Smaller sample volume (1–50 nL) High resolution Polar ionogenic metabolites Short analysis time (1–20 min per sample) Can be mostly automated Detects most organic, inorganic molecules, and biological macromolecules Low solvent consumption Can be done without separation (direct injection)	Sample not recoverable Relatively low sample throughputs Low separation reproducibility Not compatible with gases and solids Relatively less-robust instrumentation Poor migration time reproducibility	43, 90 and 91

Recently, metabolomics-based studies on disease such as cancer have been used to screen plasma, urine, and tumor tissue from control populations and cancer patients.[48–52] The first discovery of metabolic changes in cancer occurred almost a century ago. Recently altered metabolism has been identified as a key marker of cancer and metabolism-focused research has received renewed attention.[50] Dereplication based on hyphenated techniques has been extensively applied in metabolomics, thereby recognizing disease with the metabolic relationship. Over the past decade, metabolomics research has provided the necessary advanced methods to identify changing metabolite levels, leading to rapid progress in disease biomarker discovery.[53–55] Diagnostic cancer biomarkers detected by metabolomics have gained much attention in the field of clinical cancer research, to further understand its complex heterogeneity, to indicate changes in metabolic biomarkers during therapeutic intervention and to explore pathways involving cancer that can be used for new targets.[56] We take the colon cancer as an example to demonstrate the metabolomics used within the field of colorectal cancer (CRC).

Denkert *et al.* reported a cluster of paired colorectal cancer and normal mucosa samples were studied by GC-TOF/MS, which detected 206 metabolites. Compared to normal colon mucosa, intermediates of lipids and the TCA cycle were decreased in colorectal cancer, while pyrimidines, purines, amino acids, and urea cycle metabolites were generally upregulated.[57] Different types of samples were discriminated by the NMR spectrum, in this case between serum samples from patients with cancer and those from healthy volunteers. Identification of metabolites indicated upregulated levels of pyruvate, lactate and ketone bodies in patients with cancer.[50] Qiu *et al.* used GC-MS coupled with a multivariate statistics technique to profile urine metabolites from healthy controls and patients with CRC. Discovery of abnormal glutamate metabolism and histamine metabolism only in the patient urine, and discovery of abnormal polyamine metabolism only in the urine samples of rats. This study demonstrated the metabolomic variations associated with CRC, and thus providing supplementary information to tissue and serum metabonomics to elucidate fully the underlying metabolic mechanisms of CRC.[58]

Biomarkers were found to be performed using FTICR-MS. Comprehensive metabolomic analyses showed that all three independent sets of patients with CRC had significantly reduced levels of hydroxylated, polyunsaturated ultra long-chain fatty acids compared to healthy subjects.[59] Multivariate analysis of the GC peak areas was performed to visualise clusters within cases, discriminating the volatile metabolites between healthy individuals and cancer patients. It was well achieved to discriminate within cancer groups and between control and cancer groups.[60] Cheng *et al.* investigated a second urinary metabonomic of a larger set of CRC patients and healthy controls studied by GC-TOF/MS and UPLC-qTOF-MS. Their findings confirmed different urinary metabolic footprints of patients with CRC, which were characterized by the changes in the levels of metabolites obtained from gut microbial-host co-metabolism. A set of metabolite markers composed of 2-aminobutyrate, myristate, citrate, putrescine, *p*-cresol, kynurenate, and hippurate was selected to distinguish between CRC patients and healthy subjects.[61] We explored

a variety of analytical technologies applied in the study of CRC metabolomics or in specific metabolites associated with CRC research. Early studies using NMR and GC-MS showed that characterization of tumor cells with aerobic glycolysis, up-regulation of purine metabolism for DNA synthesis, and protein synthesis. LC, CE, and GC, each in conjunction with MS, promise to advance the field and allow exploration of metabolic pathways using cancer cells. Technology improvement is required for conducting studies to determine better biomarkers and potential therapeutic targets to treat or prevent CRC.[62]

4 Application in TCM

Metabolomics technology platforms for disease research can offer more concise, direct, effective and rapid methods. In particular, metabolomics can serve as powerful tools for insight into the essence of Chinese medicine syndromes (CMS) disease, promoting personalized TCM. Metabolomics is a powerful technology that provides holistic metabolic profiles which is the assemblage of individuals and the dynamic change of an individual at a particular time point similar to the holistic and dynamic nature of TCM.[63] Robust metabolomic approaches have played a key role in traditional herbal medicine; particular focusing on the past successes in metabonomics applications will help biomarkers discovery in TCM research. In light of the advantages of metabolomics, many studies have applied this approach to explore the effect of Chinese herbal medicines and the mechanisms of CMS disease.[63,64]

Up to now, there are several analytical technologies for the determination of metabolites in biological samples after oral administration of TCM. Blood plasma after oral administration of TCM contains hundreds even thousands of low-molecular-weight compounds that vary widely in stabilities and concentrations and are typically noncovalently bound to proteins.[65] However, it is quite difficult to find the metabolites for TCMs by mass spectrometric data acquired automatically, due to the high background noise of biological samples, the absorbed compounds at low concentration and the shortage of reference standards.[66–68] Therefore, the development of better technologies to find a way out of the difficulty is challenging.

4.1 Application of NMR and GC-MS in TCM

In order to explore the field of medicine especially Tradition Chinese Medicine (TCM) we should make the best of technology to develop metabonomics. As a case study, Jiang *et al.* explained the protective mechanisms of TCM, Xue–Sai–Tong injection, against myocardial ischemia/reperfusion (I/R) injury by the combination of ^{1}H-NMR metabolomics and biochemical factors analysis. By comparing the metabolic characteristics of serum samples, it was found that 9 metabolites altered by I/R injury were restored to normal status (sham operation) after XST treatment. Pathway analysis showed the metabolic changes were mainly involved in citrate cycle, glycolysis, and pyruvate metabolism.[69] Xiaoyaosan (XYS) which is one of the most famous TCM formulas in China has been prescribed to treat mental disorders. Tian *et al.* monitored the changes of metabolites in patients treated with XYS and determined changes in those metabolites concentrations respectively with GC-MS. In this study, 33 healthy volunteers and 25 depressed patients were recruited. By analyzing the urine metabolites of the healthy controls compared with depressed patients before and after treatment, five metabolites have been identified as therapeutic and potential disease biomarkers of Xiaoyaosan and depression.[70] An empirical TCM prescription of Quzhuo-tongbi decoction (QZTBD) had no serious adverse effects in the treatment of hyperuricemia. Chen *et al.* developed the serum metabolic profiling of rats by a GC-MS approach, and further, explored the different mechanism of urate-lowering therapy by evaluating the effects of allopurinol and QZTBD on metabolic profiles.[71]

4.2 Application of UPLC-MS in TCM

Recently, the rapid and efficient technique of ultra-performance liquid chromatography-electrospray ionization quadrupole time-of-flight tandem mass spectrometry (UPLC-Q-TOF-MS) has been applied to analyze and identify the chemical constituents in TCM.[13,72–74] This technique has particular advantages over conventional analytical methods in showing sensitivity or low concentration of metabolites and rapid extraction of target metabolites.[75] Among the well-known TCM formulas, one named Zhi-zi-chi decoction (ZZCD). Characterization of the potential metabolites and bioactive constituents of ZZCD was performed by an UPLC-triple TOF-MS/MS approach comprehensively, which detected 109 potential bioactive compounds. After oral administration of ZZCD, 100 compounds were found in the rat biofluids, including 61 original ZZCD compounds and 39 metabolites under the same instrumental conditions.[75] Senkyunolide I (SEI), a bioactive phthalides of Ligusticum chuanxiong Hort, was effectively analyzed and detected the metabolites in rats after oral administration and their chemical constituents with 1D and 2D-NMR and UPLC/Q-TOF-MS. They determined structures and obtained the major pathways of SEI metabolism *in vivo* by this method, which helped to identify unknown compounds and elucidated the mechanism of action of SEI.[76] Traditional Chinese formula of Fangji Huangqi Tang (FHT) was screened and characterized the multiple constituents *in vitro*, and the potential bioactive components in the serum of rats *in vivo* with UHPLC-ESI-Q-TOF-MS.[77] Zi Shen Wan (ZSW), a TCM formula, has been widely used in treatment of prostatitis and infection diseases. Li *et al.* indicated that UHPLC-MS in conjunction with MassLynx software and multivariate data processing approach could be used to rapidly screen and comprehensive analyze chemical constituents *in vitro* and prototype components or metabolites *in vivo* of TCM.[78]

5 Conclusion and perspective

Metabolomics applications as a methodology for insight into human disease continue to expand rapidly. Metabolomics has the potential to improve understanding disease, diagnosing disease, personalized medicine, risk stratification, monitoring the success of drug discovery and disease treatments. In

particular, the technology platform of metabolomics is playing a key role in studying in-depth research of the constituents of TCM *in vivo* and *in vitro*. Over the years, chromatography-mass spectrometry and NMR have demonstrated themselves as powerful techniques for detecting and annotating diverse metabolite classes and have become necessary tools for metabolomics analysis in numerous organisms. Nowadays, a variety of conventional MS-based multiclass analyses are replaced by metabolomics approaches, providing excellent combinations of analytical and bioinformatics tools, and can offer comprehensive information on a large number of metabolites in any particular system. NMR allows a much better resolution of peaks since they are spread along an additional dimension.[26,79] The current databases commonly store hundreds of metabolites, while a single organism contains several thousand diverse metabolites. Thus, a database-dependent approach has a significant limitation when it comes to determining of the entire metabolome of a complex biological system. A new approach, named SUMMIT MS/NMR, is well suited for high-throughput applications for the discovery of new metabolites in biological and biomedical mixtures, overcoming the need of experimental MS and NMR metabolite databases.[80] Although the rapid development of chromatography-mass spectrometry and NMR has great prospects, there are many challenges in metabolomics applications, including complexity, high upfront costs, lack of user-friendliness, and the complexity of the science and the associated regulatory processes. For chemist/biochemists, it is important to open up new avenues to identify potentially unknown metabolites and overcome the obstacle and bottleneck in finding novel metabolites in complex biological mixtures.

Conflicts of interest

The authors declare no competing financial interests.

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