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Review

Gas chromatography/mass spectrometry in metabolic profiling of biological fluids☆

Kishore K. Pasikanti, P.C. Ho, E.C.Y. Chan*

Department of Pharmacy, Faculty of Science, National University of Singapore, 18 Science Drive 4, Singapore 117543, Singapore

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ABSTRACT

One of the objectives of metabonomics is to identify subtle changes in metabolite profiles between biological systems of different physiological or pathological states. Gas chromatography mass spectrometry (GC/MS) is a widely used analytical tool for metabolic profiling in various biofluids, such as urine and blood due to its high sensitivity, peak resolution and reproducibility. The availability of the GC/MS electron impact (EI) spectral library further facilitates the identification of diagnostic biomarkers and aids the subsequent mechanistic elucidation of the biological or pathological variations. With the advent of new comprehensive two dimensional GC (GC × GC) coupled to time-of-flight mass spectrometry (TOFMS), it is possible to detect more than 1200 compounds in a single analytical run. In this review, we discuss the applications of GC/MS in the metabolic profiling of urine and blood, and discuss its advances in methodologies and technologies.

Contents

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* Corresponding author. Tel.: +65 65166137; fax: +65 67791554.
E-mail address: phaccye@nus.edu.sg (E.C.Y. Chan).

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1. Introduction

Metabolites are the end products and by-products of the many complex biosynthetic and catabolism pathways that exist in humans and other living systems. Recently, global metabolite profiling techniques are finding increasing applications in the diagnosis of a number of pathologies and in the assessment of the exposure of biological systems to xenobiotics. Metabonomics or metabolomics are terms commonly used to describe the non-targeted global analysis of tissues and biofluids for endogenous metabolites. More precisely, metabonomics is defined as the quantitative measurement of the dynamic multiparametric response of a living system to pathophysiological stimuli or genetic modification [1]. On the other hand, the comprehensive and quantitative analysis of the whole metabolome under a given set of conditions is termed metabolomics [2]. However, in some cases, these terms were found to be used interchangeably. In genomics and proteomics, the analytes of interest are biological macromolecules which are polymeric in nature and the chemistries of the building blocks are relatively well defined. On the other hand, in metabonomics, the chemical space associated with the endogenous metabolites is large and highly diversified. This great diversity in chemical properties of the metabolites and their wide concentration ranges pose a significant challenge in developing a generic, robust and reproducible global profiling assay [3]. The endogenous metabolites that are typically profiled include organic acids, amino acids, amines, sugars, steroids, nucleic acid bases, and other substances that are intermediates in cellular metabolism. As these small molecular metabolites vary greatly in terms of their physicochemical properties and acid/base characteristics, several separation techniques have been investigated to resolve these analytes prior to mass spectrometry (MS) detection. Among the tandem techniques investigated, the coupling of capillary GC to MS (GC/MS) proved to be a potentially useful method based on its high sensitivity, peak resolution and reproducibility. Availability of GC/MS electron impact (EI) spectral library further facilitates the identification of diagnostic biomarkers and aids the subsequent mechanistic elucidation of the biological or pathological variations [4]. However, a major prerequisite for GC/MS analysis is that the compound should be volatile and thermally stable. As most of the metabolites are polar and non-volatile in nature, they cannot be readily analyzed by GC/MS. Therefore, metabolic profiling via GC/MS usually requires chemical derivatization at the polar functional groups to reduce the polarity, increase the thermal stability and volatility of the analytes. Derivatization, extensive sample preparation and long chromatographic analysis render GC/MS a relatively low throughput technique for metabonomic applications. With the advent of new separation and detection techniques such as comprehensive two-dimensional GC ($GC \times GC$) coupled to time-of-flight mass spectrometry (TOFMS), the analysis time can be significantly reduced and a large number of metabolites can be detected in a single analysis. NMR has traditionally been the preferred analytical platform for the reproducible detection of low molecular weight compounds in biological fluids in toxicological metabonomic studies [5–7]. Although NMR spectroscopy provides detailed structural information of a large number of biofluid constituents with minimal sample preparation, its sensitivity is lower compared to most of the MS techniques. Liquid chromatography (LC), particularly ultra high pressure LC (UPLC), coupled to MS is being used increasingly in metabonomic research [4,8,9]. Numerous review papers had been published that discussed the use of NMR and LC technologies in metabonomic profiling and it is not the aim of this paper to reiterate these concepts. Use of LC/MS for metabonomic analysis had been extensively reviewed by Wilson et al. [8]. Comparison was also made between the use of NMR spectroscopy and LC/MS in metabonomic analysis including their advantages and limitations [8]. An overview of various analytical technologies utilized in metabonomic studies and their strengths and weakness were reviewed by Dunn et al. [10]. Reviews on current trends in NMR application in metabonomics, challenges of data processing and data interpretation can be found elsewhere [10,11]. In this review paper, we focus our discussion on the laboratory techniques adopted in the metabolic profiling of urine and blood matrices using GC/MS and highlight a few examples of GC/MS metabonomic applications performed to date.

2. GC/MS in urinary metabolic profiling

Urine and blood are the most frequently profiled biological matrices for exploring the systematic modification of the metabolome [12–16]. Compared to the analysis of blood samples, advantages of urinalysis are numerous; especially urine sample collection is noninvasive. The use of GC/MS in urinary metabolic profiling is a long-standing practice. As early as 1971, Horning et al. used GC/MS to study metabolic profiles of steroids and organic acids in human urine [17]. Largely, GC/MS was initially utilized in identifying metabolites rather than profiling in human urine [18–20]. By 1980, Tanaka et al. developed a GC/MS method to identify 155 metabolically important compounds in urine samples and proved the potential of GC/MS applications in disease diagnosis [21,22]. However, sample preparation for the analysis of urinary metabolites using GC/MS was extensive and tedious and the quality of MS chromatograms was not optimized. These challenges were addressed in the benchmark paper by Shoemaker and Elliott [23]. Shoemaker and his co-workers used urease enzyme to deplete urea that caused major chromatographic interference and masked many of the low intensity metabolite peaks. The pretreatment of urine with urease has enabled the simultaneous analysis of several categories of compounds in a single analytical run. A practical and simplified protocol for urease pretreatment, stable-isotope dilution and GC/MS detection of marker metabolites in urine was developed and successfully utilized to screen a number of inborn errors of metabolism (IEM) [24–27]. The sample preparation required up to 200 μL of urine. Urine samples were incubated with 30 U of urease enzyme for 10 min, then urease and other proteins were precipitated with 0.9 mL of ethanol and the supernatant is dried under nitrogen and derivatized using 100 μL of BSTFA [*N*,*O*-bis-(trimethylsilyl)trifluoroacetamide)] containing 1% TMCS (trimethylchlorosilane) at 80 °C for 30 min [28]. This procedure, clinically applicable yet comprehensive from the metabolic point of view, is a valuable tool for screening and diagnosis of many IEMs. However, chromatographic resolution achieved using the method is not sufficient to be used as a standard method for global metabolic profiling.

2.1. Collection and storage of urine samples

First-void urine or spot urine samples are used commonly for metabonomic analyses. First-void urine samples are preferred compared to spot urine samples because the influence of lifestyle factors (such as diet, physical exertion, stress, etc.) on the metabolic urinary profiles is relatively minimized and normalized in the case of first-void urine [29]. However, collection of first-void urine samples may be more challenging due to poor patient compliance especially for outpatients in the hospitals. Recently, Slupsky et al. observed that the effect of diurnal variation on healthy human urine samples is related to diet [30]. Based on the NMR profiling of morning and afternoon urine samples, the authors suggested that concentrations of most metabolites between the two groups of samples are not significantly different. As some of the metabolites profiled by

GC/MS are different from those of NMR, the effect of urine collection on urinary metabolic profiles has to be investigated using GC/MS specifically. While the variations between first-void and spot urine samples may be minimal, the effects of diet and other lifestyle factors are clear [30]. Hence, first-void urine samples should be used for metabonomic experiments as far as possible.

It is well established that storage conditions exert significant impact on the stability of few urine metabolites over time. If the stability of urinary metabolites is not evaluated, it may lead to confounding factors in biomarker identification as the variation of the measured metabolites may be due to degradation rather than a biological response. By outlining proper sample handling and storage conditions for urine samples, the integrity of the sample is ensured and the original state of the biological system can be reflected accurately in the metabonomic study. Different methods of sample preparation (centrifugation, filtration, or addition of the preservative such as sodium azide), effect of urine pH, as well as sample storage conditions (room temperature (25 °C), refrigerator (4 °C, or −80 °C)) need to be systematically evaluated using GC/MS. Effects of urine sample storage conditions in metabonomic studies had been investigated using NMR [31–33], LC/MS and UPLC/MS to a certain extent [34]. However, no study till date has systematically evaluated urine sample storage conditions for metabonomic analysis using GC/MS. NMR studies revealed that storage of the urine sample in a refrigerator (4 °C) produced a slight reduction in the degree of metabolite change, but storage at −80 °C provided urine with a metabolic profile that best reflected the original metabolite concentrations. Recently, evaluation of urinary metabolic profiles for long term stability using LC and UPLC/MS showed that urine samples were stable up to 6 months when stored at −20 °C or below and repeated freeze thaw cycles had little effect on overall urinary metabolic profiles [34]. Since influences of storage conditions on GC/MS metabolic profiles is not clearly understood till date. Urine samples should be preferably stored at −80 °C and repeated freeze thaw cycles should be minimized.

2.2. Derivatization of urine samples

Many derivatizing agents were explored in metabolic profiling including BSTFA and MSTFA (*N*-methyl-trimethylsilyltrifluoroacetamide), which are being used predominantly in metabonomic investigations [35–38]. However, it has been noted that silylation can cause conversion reactions, for example, arginine is converted into ornithine by reaction with BSTFA or MSTFA [39]. The most commonly used derivatization procedure, following extraction, is where the dried extract is dissolved in pyridine, while oximation is carried out using methoxamine hydrochloride (28–37 °C, up to 120 min or 16 h) followed by trimethylsilyl (TMS) derivatization using MSTFA (37 °C for 30 min to 1 h) [40]. Due to the cyclic and open chain structures of sugars, silylation of monosaccharides without oximation step leads to multiple peaks belonging to each individual sugar compound. By introducing an oximation step prior to silylation, cyclization is inhibited, resulting in fewer peaks per sugar. Moreover, α-ketoacids are protected against decarboxylation, and enolizable keto groups are fixed by oximation. A sample GC/MS chromatogram of a human urine sample analyzed as TMS derivatives in our laboratory is shown in Fig. 1.

Derivatization using ethyl chloroformate is now gaining popularity especially in the analysis of metabolites in urine samples. Unlike TMS derivatizing agents, which only work in nonaqueous phase, ethyl chloroformate (ECF) is reactive in aqueous medium. A method for metabonomic analysis of urine samples using ECF derivatization has been extensively optimized and validated over a broad range of different compounds and urine samples [41].

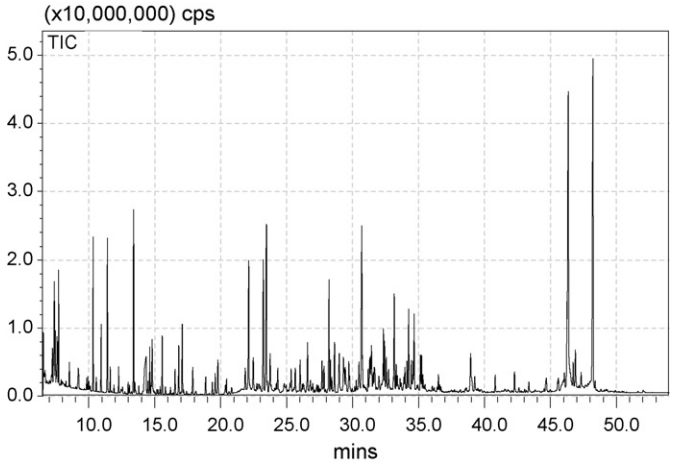

Fig. 1. GC/MS total ion chromatogram showing the metabolic profile of healthy male urine sample using BSTFA derivatization.

Recently, a number of important metabolites related to aristolochic acid-induced nephrotoxicity were identified as ECF derivatives by Ni et al. in rat urine using GC/MS [14]. Likewise, Li et al. utilized ECF derivatization to study pre-dose urinary metabolic profiles of two classical experimental models, the Streptozotocin-induced diabetic model of Wistar rats and the high-energy, diet-induced obesity model of Sprague–Dawley rats [42]. ECF derivatization is carried out in two steps. Initially, anhydrous ethanol and pyridine are added to urine samples followed by 50 μL of ECF. The mixture is then sonicated for 1 min. Subsequently, extraction is performed using chloroform, with the aqueous layer pH adjusted to 9–10 using NaOH. The derivatization procedure is repeated with the addition of 50 μL ECF into the mixture. After the two successive derivatization steps, the aqueous layer is aspirated off, while the remaining chloroform layer containing the derivatives is isolated and dried with anhydrous sodium sulfate and subsequently subjected to GC/MS analysis [41].

Finally, *tert*.-butyldimethylsilylation (TBDMS) had also been utilized in the metabolic profiling of urine samples [43–46]. TBDMS derivatives are less sensitive to hydrolytic effects of moisture than the corresponding TMS derivatives [47]. However, TBDMS derivatization can significantly increase the molecular mass, particularly where multiple derivatizable groups are present. Moreover, steric hindrances in the molecule can lead to a mixture of fully and partially derivatized analytes.

2.3. Applications of GC/MS in urinary metabolic profiling

The majority of metabolic profiling studies using combined GC/MS and chemometric techniques reside in the field of plant metabolomics [2,48,49]. At the same time, GC/MS has also been used in toxicological evaluation [14,36,50], disease mechanism elucidation [51] and biomarker discovery [37,52,53]. Few examples of urinary metabolic profiling applications in toxicological research and disease biomarker profiling are briefly discussed in this paper.

The application of GC/MS metabolic profiling in the area of toxicology is relatively underdeveloped as compared to NMR and LC/MS. However, recent studies demonstrate the potential of urinary metabolic profiling by GC/MS as a complementary tool in toxicological evaluations, providing a comprehensive understanding of the response of biological system to xenobiotic intervention. Recently, Chen et al. utilized GC/MS and LC/MS

based urinary metabolic profiling to elucidate the toxicity induced by orally administered multiglycosides of *Tripterygium wilfordii* Hook. f. (GTW) in rats [50]. Urine samples were collected at various time points before and after the dosing of GTW. The work indicated that GTW caused a time-dependent toxic effect at a high dose as revealed by the perturbed metabolic regulatory network [50]. This integrated MS-based metabolic profiling approach successfully captured metabolic alterations associated with the onset and progression of multi organ toxicity induced by GTW. Similarly, urine samples were analyzed by GC/MS and LC/MS to evaluate aristolochic acid-induced nephrotoxicity in rat [14]. Alteration of metabolic networks involving free fatty acids generation, energy and amino acids metabolism, and alteration in the structure of gut microbiota were observed. Collectively, these studies demonstrated the complementariness of GC/MS and LC/MS techniques in urine metabolic profiling for toxicological evaluation.

Urinary metabolic profiling has also shown much success in disease diagnosis. A recent study which evaluated urine profile by using both GC/MS and NMR suggested that GC is a valuable and complementary tool to NMR for the metabonomic analysis of urine samples [13]. Similarly, hydrophilic interaction chromatography (HILIC), UPLC/MS, and gas chromatography time-of-flight mass spectrometry (GC/TOFMS) were all used as complementary technologies to investigate the suitability of analytical techniques for profiling of urine samples obtained from renal cell carcinoma (RCC) patients. The combination of these techniques is best suited to cover a very large part of the urine metabolome by enabling the detection of both lipophilic and hydrophilic metabolites present in urine [54]. Apart from the global metabolite profiling, GC/MS has been successfully utilized in analysis of specific class of metabolites in urine [55–58]. In one study, GC/MS based metabonomics has been applied to identify differences in urinary metabolic profiles between healthy subjects and type 2 diabetes patients [12]. Another similar study was also performed to differentiate urinary metabolic profiles of uterine myoma and cervical cancer patients [46]. Both these studies focused on the profiling of endogenous organic acids.

2.4. Sources of variability and recommendations

Although GC/MS has been successfully used in a large number of urinary metabolic profiling studies, there are still minor but important aspects that have been largely overlooked. Firstly, urine sample storage conditions for metabonomic analysis are not systematically investigated using GC/MS (discussed in detail in Section 2.2).

Secondly, the urinary metabolic profile is influenced by many factors including demographic, environmental and pathological conditions. Before any metabolic fluxes can be attributed to the etiology of a disease, metabolic fluctuations due to general demographic and environmental factors need to be elucidated and understood [59]. Some of the key demographic factors that may influence urinary metabolic profiles include gender (sex), age and ethnic grouping (race). Therefore when designing a GC/MS metabonomic experiment, it is necessary to control all the factors that affect the baseline metabolic profiles. If some of the factors cannot to be controlled, at least metabolic differences in different pathophysiological states need to be understood. This can help in identifying biomarkers specific to disease condition and to eliminate confounding factors due to differences in baseline characteristics. Until now, only limited work has been done to explore the inherent changes in metabolic profiles in various pathophysiological conditions of which majority of work has been done using NMR [30,60–64]. Therefore, there is a need for establishing differences in metabolic profiles in various pathological and physiological conditions using GC/MS.

Thirdly, GC/MS metabonomic studies typically involve extensive sample preparation and long analysis time. Therefore, it is important to use quality control (QC) samples for monitoring the performance of the method and to increase the credibility of data obtained. Most of the studies performing metabolic profiling seldom use QC samples because there are no clear guidelines on what should be used as QC samples in metabonomic studies. Recently, Sangster et al., used pooled biological fluid samples as QC samples in metabonomic analysis using LC/MS or GC/MS [65]. Pooled samples were split to form multiple QC samples which were analyzed at the beginning, randomly in the middle and at the end of a series of analyses [65]. These QC samples were then subjected to similar data pre-processing and principle component analysis (PCA) along with other test samples. QC samples are expected to cluster closely together, and show no time related trends if the analysis is satisfactory [65,66]. Further, identified biomarker compounds were observed for any time-related changes in their intensity or peak area using the QC samples. As the variation in peak intensity of biomarkers is minimal in the QC samples, it suggests that the analysis is satisfactory. Therefore, QC data were also successfully utilized to validate the results of identified biomarkers. However, it remains unclear if these QC samples are suitable to be used to monitor inter-day variation.

Lastly, with the increasing production of metabonomic data, there is a need for standardized description of this data to aid assessment, exchange, storage and curation of information from different metabonomic studies. Recently, metabonomic researchers are investigating the reporting needs to make recommendations for standardizing reports for metabonomic studies [67,68]. More recently, the metabolomics standards initiative-mammalian context working sub-group (MSI-MCWSG) published guidelines for the reporting of the biological materials and processes examined in a metabonomic study involving mammalian subjects [69]. For example, reporting of urine sample collection in preclinical metabonomic studies should include details of how samples are collected (metabolic cage, cystocentesis, catheterisation), frequency of collection, duration of collection, time of collection relative to dose and light cycle (if less than 24 h collection), use of bacteriostatic agent or any other additive (final concentration), urine volume (for 24 h collection), and temperature of urine collection tube (on ice or room temperature). Similarly, reporting requirements for metabonomic studies involving collection of urine and blood samples in preclinical and clinical studies were also presented. Following a standard reporting format for all the metabonomic experiments, the guidelines will allow users to collate and cross-compare their data between diverse sets of experiments.

3. GC/MS in analyzing plasma metabolic profiles

Sample preparation for metabolic profiling of plasma sample is relatively similar to that of urinary metabolic profiling. A major difference to urine metabolic profiling is that plasma samples are not incubated with urease enzyme. Comparison of sample preparation protocols for urine, plasma and tissue samples is shown in Fig. 2. Plasma samples are extracted either with acetonitrile [70,71], ethanol [72,73] or methanol [74,75] and supernatant is dried, methoximated, derivatized and injected for GC/MS analysis. Jiye et al. adopted the design of experiments procedures to investigate the effects of extraction solvent, derivatization protocol and extraction condition on the analysis of human blood plasma metabolome by GC/MS [75]. A D-optimal design was used to investigate how five commonly used solvents (methanol, ethanol,

acetonitrile, acetone, chloroform) for protein precipitation affect the efficiency of metabolite extraction. Methanol was found to be the best extraction solvent as maximum numbers of metabolites were detected. In addition, peak areas of most of the detected metabolites were maximum when methanol was used compared to other solvents. Fractional factorial design was then constructed for optimizing the extraction and derivatization conditions. The experimental factors investigated included methanol volume, extraction duration, temperature and duration of the incubation before and after extraction, and temperature and duration of the methoxymation and silylation. Results from the study shows that 100 μL of plasma extracted with 800 μL of methanol, vortex mixed, centrifuged, supernatant collected (200 μL), dried, methoxymated at room temperature for 16 h and derivatized with MSTFA for 1 h is the optimized protocol for the metabolic profiling of plasma samples [75].

3.1. Derivatization of plasma samples

MSTFA is used predominantly as the derivatizing agent in metabolic profiling of plasma samples [71,76]. Many other derivatizing agents had also been investigated. An analytical method was developed by Yoon et al. to quantify organic acids, amino acids, and glycines simultaneously in a two-step derivatization procedure [77]. Use of other derivatizing reagents such as ethylchloroformate [72,78–81], methylchlorofomate [82] and isobutyl chloroformate [83] were also reported in studies involving plasma metabolic profiling.

3.2. Applications of GC/MS in plasma metabolic profiling

In order to relate therapeutic or toxic effect to normality or to understand biochemical alterations caused by disease, it is nec-

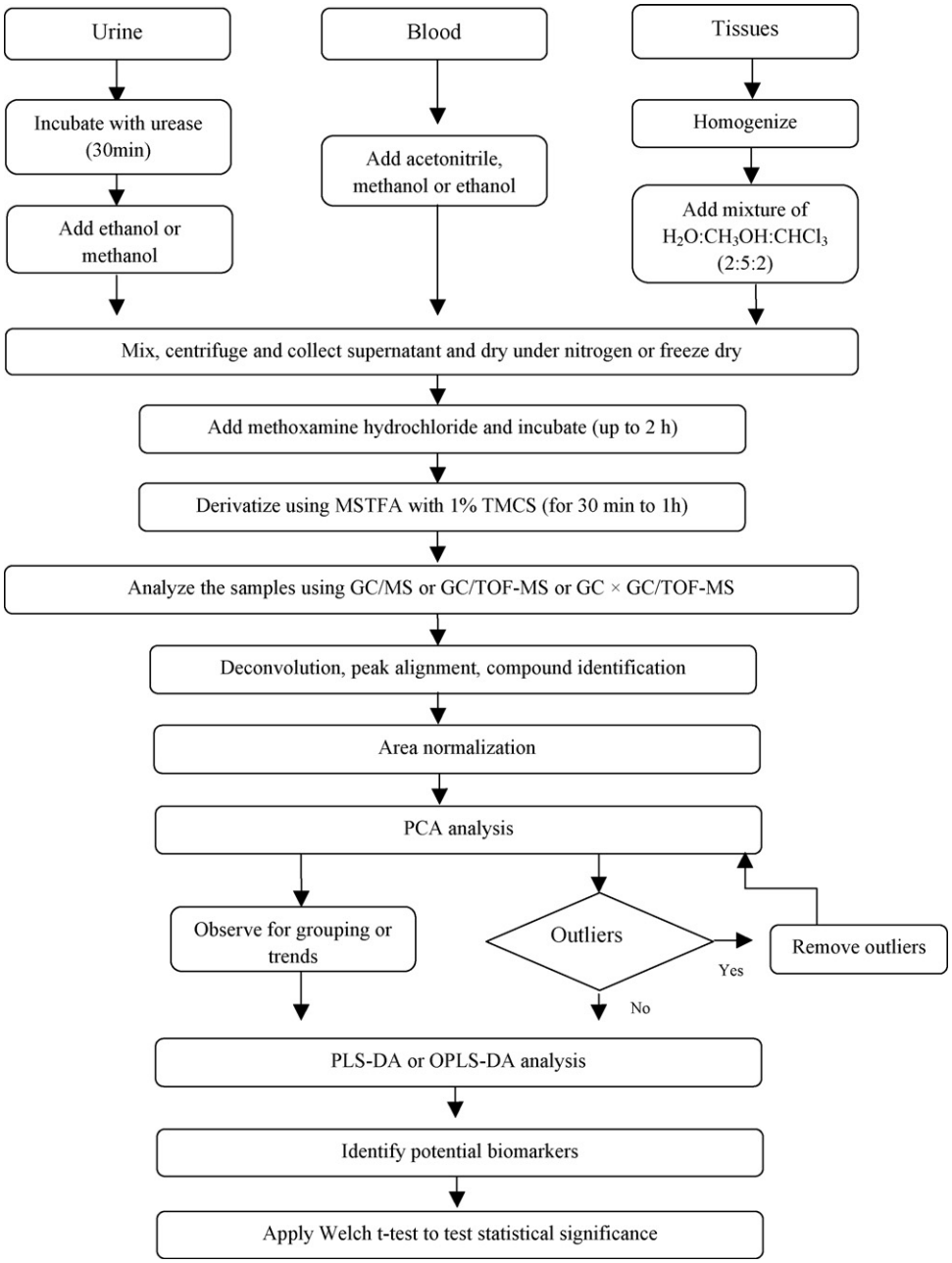

Fig. 2. Metabonomic approaches for biomarker screening in urine, blood and tissue samples using GC/MS.

essary to have good understanding of what constitutes a normal biochemical profile. Recently, a number of studies adopted plasma metabolic profiling to identify metabolic differences in experimental animals. In one such study, plasma samples obtained from three strains of Zucker rats were analyzed using capillary GC/MS to obtain the global metabolite profiles [70]. Plasma samples were analyzed following protein precipitation with acetonitrile, derivatization with MSTFA, and GC/MS analysis with electron ionization (EI) and chemical ionization (CI) modes. Subsequent data analysis using PCA and orthogonal projection to latent structures (OPLS [84,85]) revealed differences in metabolite profiles of the three strains. Metabolic profiles of Zucker lean and the lean/(fa) strains were found to be similar to each other whilst differing from the (fa/fa) obese strain. In another separate study, NMR, UPLC/MS and GC/MS were used for metabonomic analysis of plasma obtained from normal and zucker (fa/fa) obese rats to identify biomarkers related to strain difference [61]. The objective of the study was to establish biomarkers due to strain difference in order to facilitate the profiling of other disease biomarkers [61]. It was also observed that whilst there was some overlap in metabolites identified between GC/MS and NMR spectroscopy, this was not so apparent with UPLC/MS. Recently, changes in metabolic profiles in human serum in relation to strenuous physical exercise were evaluated using metabolic profiling where a specialized multivariate data analysis tool, hierarchical multivariate curve resolution (H-MCR) [86], was employed to identify metabolites *via* spectral database comparisons [87].

Recently, GC/TOFMS is increasingly used for metabolic profiling of plasma or serum samples [71,88]. Fast acquisition rates and the absence of spectral skewing render TOFMS the ideal detector for metabonomic analysis when GC is selected as the separation method. Underwood et al. applied metabolic profiling by GC/TOFMS to serum samples obtained from Huntington's disease patients and a transgenic mouse model [71]. The findings in the study indicated clear differences in metabolic profiles between the transgenic mice and wild-type littermates. Similar differences in metabolic profiles of human patients and control subjects were also observed [71]. Similar metabonomic platform was utilized to identify several novel biomarkers of heart failure in serum samples [89]. Apart from metabolic profiling in urine and blood, GC/MS has also been extensively utilized for metabolic profiling in plants [2,40,90], in cell culture media samples [91] and tissue extracts [92–94].

4. GC/MS methodology in metabonomic research

In most of the metabonomic applications, 0.5–2 μL of derivatized sample extract is introduced into a heated injector (200–250 °C), where rapid vaporization and mixing with the carrier gas occurs (usually helium), followed by chromatographic separation of metabolites on the GC column and subsequent MS detection [13,93]. Metabolites are predominantly analyzed as TMS derivatives. Therefore, it is preferable to deactivate the inlet by injecting MSTFA or BSTFA before the analysis of metabonomic samples. Glass injector ports should always be used when working with silylating reagents. Erratic and irreproducible results frequently begin to occur when stainless steel injection ports are used for the analysis of TMS derivatives. Sample can be injected in either split or splitless mode. In a splitless system, the advantage is that a larger amount of sample can be introduced into the column. However, a split system is preferred when the detector is sensitive to trace amounts of analyte and there is concern about sample overloading of the column. Therefore, in metabonomic studies, split mode is generally preferred because metabolites are present in wide range of concentrations. Chromatograms in metabonomic studies are complex due to large number of metabolite peaks as wells as multiple derivatization products. Therefore, long analysis time (up to 60 min) may be needed for satisfactory chromatographic separation. The most important factors which influence chromatographic separation include column properties (length of the column, stationary phase, internal diameter (i.d.)), carrier gas type, carrier gas velocity and oven temperature program. In this paper, both column properties and MS parameters that are adopted in metabonomic research are discussed.

4.1. Column properties

Capillary GC columns made of fused silica are commonly employed in GC/MS based metabonomic studies. These columns have a thin film of liquid phase bonded to the walls of a narrow i.d. (0.25 mm [13] or 1.8 mm [9]) column. Capillary GC columns can operate at very higher temperatures and provide significantly higher chromatographic resolution. Because of the small i.d. of these columns, the sample capacity of the 0.25 mm columns is limited to about 50–100 ng per component of a mixture. Columns with varying polarity (DB-1 to DB-50) [13,40,46,95], varying chemical composition of stationary phases, and varying lengths (10 to 60 meters) have been utilized in metabonomic analysis [96]. However, DB-5MS columns or columns with equivalent stationary phase (HP-5MS and RTX-5MS) are typically used in metabonomic studies [14,41,54]. A DB-5MS column is a fused silica capillary column, chemically bonded with a 5% diphenyl cross-linked 95% dimethylpolysiloxane stationary phase (0.25 μm film thickness). The capillary column is held in an oven that can be ramped continuously or in steps to achieve desired separation. As the temperature increases, those compounds that have low boiling points elute from the column sooner than those that have higher boiling points. Therefore, there are actually two distinct separation forces, temperature and stationary phase interactions. Typical column oven temperatures range from 40 to 325 °C [12,13]. Maximum temperature that can be used on a particular column should always be verified with the manufacturer's instructions. The rate at which a sample passes through the column is directly proportional to the temperature of the column. The higher the column temperature, the faster the sample moves through the column. However, the faster a sample moves through the column, the less it interacts with the stationary phase, and the chromatographic separation is poor. Similarly, the carrier gas flow rate also affects the analysis. The higher the flow rates the faster the analysis, but the lower the separation between analytes. Selecting the flow rate is therefore the same compromise between the level of separation and length of analysis as selecting the column temperature. Column flow rate between 0.8 and 2 mL/min is commonly used for metabolic profiling [40,54]. By optimizing various GC parameters, it is possible to separate most if not all of the endogenous metabolites before they enter the mass spectrometer for detection.

4.2. MS parameters

EI is the most commonly used method of ionization in metabonomic studies. EI is performed in a high-vacuum ion source (10^{-7} to 10^{-5} mbar, 200–250 °C), where analytes in vapor state are bombarded with electrons at 70 eV [40]. This gives the sample molecules a great deal of excess energy and many fragment ions are formed. Fragmentation pattern is characteristic to a particular molecule and therefore can be useful in determining the structure of the analyte. Unfortunately, some compounds fragment completely and do not give molecular ions. Therefore, chemical ionization (CI) has also been utilized in some of the metabonomic studies [70]. CI is a relatively softer ionization technique. Thus, CI produces less

fragmentation compared to EI. CI can produce molecular ions for some volatile compounds that do not give molecular ions in EI. For metabonomic applications, MS is typically utilized in full scan mode. Broad range mass fragments of m/z from 50 to 700 are generally monitored [40]. MS should not be set to scan for small mass fragments (less than m/z 50) or else one may encounter high amount of noise due to the detection of interferences like nitrogen (m/z of 28) in air. When metabolites are analyzed as TMS derivatives, the MS detector should be turned off until the TMS by-products (mono (trimethylsilyl)trifluoro-acetamide and trifluoroacetamide) are completely eluted. These by-products are present in high concentrations and therefore can saturate MS detector. TMS by-products are highly volatile and usually elute within 5 min of sample injection. However, this MS "switch-off" period should be carefully optimized because some of the low boiling point TMS-derivatized amino acids elute immediately after the TMS by-products. Therefore, switching off the detector for longer time may result in reduction in number of compounds detected.

5. Data analysis

5.1. Metabolite identification

Following GC/MS analysis, metabolite identification or confirmation is performed by retention time or index comparison with pure standard compounds or comparison using the retention index of mass spectral library databases. Retention index (RI) of a particular compound is calculated by relating the retention time of the compound to retention times of standard n-alkanes analyzed under same analytical condition. Some of the instrument manufacturers' datasystems can automatically calculate retention index. For example, Shimadzu GCMSsolution (Version 2.5) can automatically calculate RI of compounds, based on RT of n-alkanes. Another alternative approach to calculate RI automatically is by using Automated Mass Spectral Deconvolution and Identification System (AMDIS) [97] from the National Institute of Standards and Technology (NIST). In addition, the software can also perform spectral deconvolution and library searching against the NIST database (www.hdscience.com, http://www.nist.gov/srd/nist1.htm). The ADMIS software has been successfully applied in metabolic profiling [98]. A useful and important information on library searching for compound identification is presented by Halket et al. in his recent review [99] and in other publications [100]. NIST 2005 is the latest edition of the most popular NIST spectrum library used for compound identification by GC/MS, with an expanded collection of 190,825 spectra and 121,112 retention indices. It is either available alone, or combined with the Wiley Registry (Wiley Registry 7/NIST 2005) for the complete mass spectral library solution (www.hdscience.com). Although these libraries are extensive, one has to bear in mind that they do not contain a large number of endogenous metabolites that are found in the biological metabolic pathway.

5.2. Peak alignment

While retention time (RT) shifts are less apparent in GC compared to LC, even small changes in RT can complicate data processing and lead to misinterpretation of data. In GC/MS analysis, RT may also change as a result of capillary columns being trimmed during maintenance or when the column is exchanged with a new one from a different batch. The RT shifts can pose greater challenge when large number of samples are analyzed and when the chromatographic resolution of metabolite peaks is poor. For this reason, the use of signal-alignment software has become a routine procedure for comparing chromatograms or spectra. Programs that are optimized for GC/MS peak picking, such as AMDIS, usually perform better during deconvolution procedures, but these programs have no inbuilt peak alignment algorithms. XCMS [54,101], MZmine [54], MetAlign [102] and MET-IDEA [103], are freely available software that can be explored for noise reduction, baseline correction and peak alignment. An overlay of our in-house GC/MS chromatograms of human urine samples before and after noise reduction, baseline correction and peak alignment using MetAlign has been presented in Fig. 3. All the peak alignment programs use the open source MS exchange format netCDF, which is readily available as export format for most mass spectrometers. However, input parameters have to be carefully optimized and peak alignment results should always be double-checked. If input parameters are not carefully optimized, the software used for peak alignment and peak matching can lead to false positive and false negative results [54]. Subsequently, different data preprocessing steps are applied in order to generate 'clean' data in the form of normalized peak areas that reflect the metabolite concentrations.

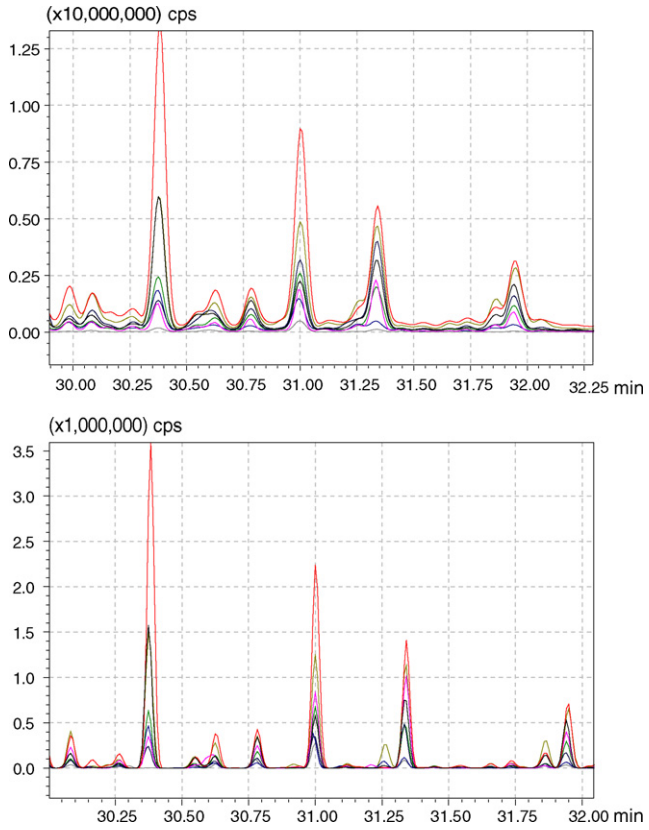

Fig. 3. An overlay of GC/MS chromatograms of healthy male urine sample before (upper) and after (lower) noise reduction, baseline correction and peak alignment using MetAlign.

5.3. Chemometric data analysis

Metabonomic analysis generates large and complex datasets. Therefore, chemometric analysis has become an integral part of metabolic profiling techniques due to its ability to provide interpretable models for complex inter-correlated data [104]. Multivariate projection methods such as Principal component analysis (PCA) allow the identification of groups of variables that are interrelated via phenomena that cannot be directly observed. Selecting a proper data pretreatment method prior to PCA analysis is an important step in the analysis of metabonomics data and can greatly

affect the metabolites that are identified to be the most important. Effect of different data pretreatment methods (centering, autoscaling, pareto scaling, range scaling, vast scaling, log transformation, and power transformation) on biological interpretation of GC/MS metabonomic datasets was investigated by van den Berg et al. [73]. PCA gives a simplified representation of the information contained in the spectra and cannot generally use additional information about the data, such as class information. Therefore, PCA is often followed by a supervised analysis technique such as Partial Least Squares Discriminant Analysis (PLS-DA) or O-PLS-DA that can aid in obtaining a list of potential biomarkers which are statistically significant and which separate one class from another [96,105]. The main benefit in the interpretation of data using OPLS-DA compared to PLS-DA lies in the ability of OPLS-DA to separate predictive from non-predictive (orthogonal) variation [84]. Concentrations of potential biomarkers in control and treatment groups should be further subjected to Welch *t*-test to check whether identified biomarkers are statistically significant. Few other multivariate data analysis techniques have also been explored for aiding biomarker detection in metabonomic studies [106,107].

6. Recent advances

Comprehensive two-dimensional GC (GC × GC) coupled to TOFMS is a recent addition to wide range of chromatographic techniques utilized for metabonomic analysis. GC × GC/TOFMS uses two capillary columns with complementary stationary phases. All components eluting from first column are subjected to separation in a second column through a modulator device. This device is typically a cryogenic modulator [108]. While the first column is usually nonselective like HP-5MS (or equivalent e.g. DB-5MS, RTX-5MS), the second column is shorter and separates compounds based on polarity. Koek et al. had evaluated the separation efficiency of GC × GC/TOFMS using HP-5MS as first column which was coupled to three different columns (different length, diameter and film thickness) with polar phases in the second dimension [109]. One of the key advantages of GC × GC/TOFMS is that it provides high separation capacity along with enhanced detection limit. The peak capacity of GC × GC separation is approximately the multiple of peak capacities obtained by two individual columns. Moreover, the analysis time is shorter when compared to 'normal' GC and yet it generates much higher amount of information per sample per unit time. GC × GC/TOFMS has already shown promise in metabolic profiling [109–113]. Welthagen et al. compared metabolic profiles of tissue extracts obtained from obese and lean mice using GC × GC/TOFMS to identify biomarkers [110]. In the same study, GC/TOFMS was also compared to GC × GC/TOFMS to analyze mouse spleen extracts [110]. Approximately 1227 compounds were detected using GC × GC/TOFMS as compared to 538 compounds detected using GC/TOFMS. Apart from the increased number of detectable peaks, spectral purity was much improved in GC × GC/TOFMS, which in turn improves mass spectral deconvolution and compound identification [110]. A GC × GC/TOFMS total ion chromatogram of silylated pig colon sample prepared in our laboratory is illustrated in Fig. 4. This representation of the data is known as the contour plot. In this display, the peaks found by the software (LECO Inc., USA) are indicated as black dots. In this chromatogram, it can be seen that many of the components are overloaded, with consequent streaking of the chromatographic peaks. This has been done deliberately to identify more of the metabolites at lower level. As shown in Fig. 4, more than 900 peaks were located. Despite the many advantages of using GC × GC/TOFMS, one of its biggest challenges in metabonomic analysis is the complexity and large volume of the three dimensional data. Although the separation of

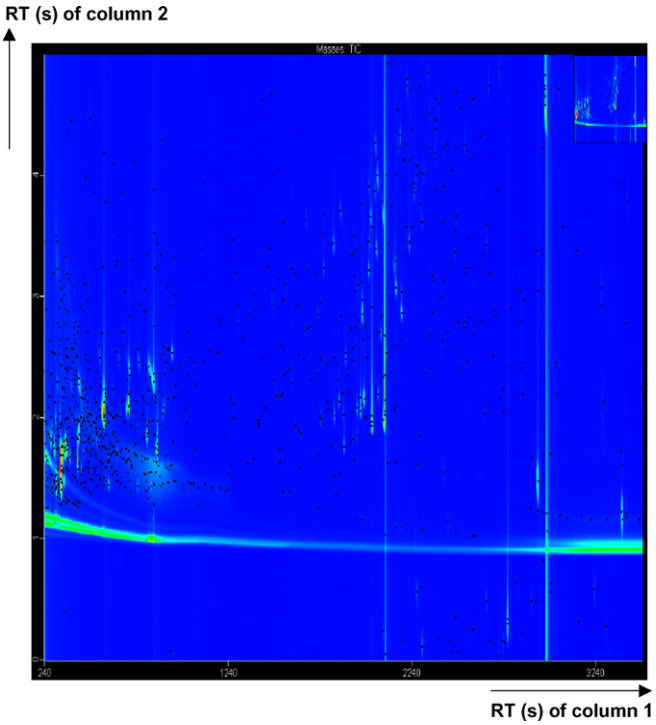

Fig. 4. GC × GC/TOFMS total ion chromatogram showing the metabolic profile of silylated pig colon sample. The peaks are indicated by black dots.

compounds is improved as compared to one dimension GC, one has to note that the GC × GC separation space still contains a large number of overlapping peaks. While current commercially available software such as ChromaTOF (LECO Inc., USA) can perform complex peak detection and spectral deconvolution, several other approaches are also being investigated for processing of data generated by GC × GC/TOFMS in metabonomic studies [87,113]. Certainly, the GC × GC/TOFMS technology shows great promise in the global metabolic profiling of biofluids in the near future.

7. Conclusions

GC/MS is gaining popularity in metabonomic studies, as it offers high chromatographic resolution, high sensitivity, and reproducibility. Availability of EI spectral libraries further facilitates the identification of diagnostic biomarkers and aids the subsequent mechanistic elucidation of the biological or pathological variations. In metabonomic studies, it is important to detect subtle differences in the complex mixtures found in biofluids, such as urine and blood, or in tissue extracts. Metabonomic studies performed to date demonstrate clearly that GC/MS is a promising technique in metabolic profiling of biofluids. Further, the use of GC × GC/TOFMS instruments combined with advances in chemometric data analysis will greatly enhance the coverage of the metabolome. Although GC/MS based metabolic profiling generates data with very rich information, subsequent interpretation of data and correlation of the results to biological function requires great experience and expertise. Nevertheless, a range of complementary analytical methods (LC/MS, NMR, CE/MS, and FTIR) should be explored to achieve the best coverage of metabolites that are being profiled. In conclusion, the future trend in metabolic profiling will involve the use of several analytical techniques in combination to explore the metabolome in biofluids and certainly, GC/MS is poised to play an important role.

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