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REVIEW

Current metabolomics: Practical applications

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The field of metabolomics continues to grow rapidly over the last decade and has been proven to be a powerful technology in predicting and explaining complex phenotypes in diverse biological systems. Metabolomics complements other omics, such as transcriptomics and proteomics and since it is a 'downstream' result of gene expression, changes in the metabolome is considered to best reflect the activities of the cell at a functional level. Thus far, metabolomics might be the sole technology capable of detecting complex, biologically essential changes. As one of the omics technology, metabolomics has exciting applications in varied fields, including medical science, synthetic biology, medicine, and predictive modeling of plant, animal and microbial systems. In addition, integrated applications with genomics, transcriptomics, and proteomics provide greater understanding of global system biology. In this review, we discuss recent applications of metabolomics in microbiology, plant, animal, food, and medical science.

[**Key words:** Metabolomics; Metabolome; Mass spectrometry; Chemometrics; Multivariate analysis]

The rapidly growing field of metabolomics complements data derived from genomics, transcriptomics and proteomics in providing a systematic approach to the study of biological systems and networks (1). Metabolomics has been an application-driven science with broad range of applications in various fields, including medical science, synthetic biology, medicine, and predictive modeling of plant, animal and microbial systems. The high applicability of metabolomics to various fields partly stems from the close association of the metabolome to the physiology of the cell. While the genome, transcriptome and proteome can be seen as mediums in the flow of gene expression, the metabolome represents the final omic level in a biological system, and reflects changes in phenotype and function (2). The metabolomics workflow includes sample preparation, analysis using various instruments, data processing and data analysis. The power of metabolomics lies on the acquisition of analytical data in which metabolites in a cellular system are quantified, and the extraction of the most meaningful elements of the data by using various data analysis tool. In this article, we discuss the application of metabolomics in various fields, namely in microbiology, and plant, animal, medical and food sciences.

MICROBIAL METABOLOMICS

Microbe is an important sample for the field of metabolomics since it has been used for development of experimental procedures and construction of research tactics. Because metabolomics is a high-resolution analysis, it requires controllable experimental conditions, such as growth conditions and surrounding environment to validate methods. Therefore, checking method validation is one important application of microbial metabolomics, especially sample preparation and metabolite measurement.

Several studies have been conducted to optimize sample preparation using microorganisms for metabolomics (3–7). There are two important processes in sample preparation: quenching and extraction. Quenching is the process of stopping biological reactions in a cell, and extraction is the process of obtaining metabolites from the cell. In the early stages of its development, metabolomics was used for the discrimination of strains possessing different characteristics. Owing to its multicomponent data, high-resolution information for each cell type could be successfully obtained (8). In such study, high reproducibility is an important consideration for sample preparation. However, recent applications have been gradually focusing on metabolism *per se*. Thus, sample preparation, especially sample quenching, has been focused on stopping metabolism at a specific period to measure the true quantity of metabolites at a given time. For quenching, the following are required to accurately measure the quantity of metabolite in a cell: short time frame during which the biological reaction is stopped, limited leakage of metabolite and reproducibility. A summary of quenching procedures can be seen in Table 1 (9–15).

The appropriate extraction method must be chosen considering the following: cell properties, such as robustness of the cell membrane, chemical properties of the target analyte and reactivity of enzymes. The most common method is organic solvent

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TABLE 1. Summary of common quenching methods in microbial metabolomics.

Method	Features	Advantage	Disadvantage	Application	Reference
Filtration quenching	Sample is collected with a filter membrane and quenched by rapid cooling or chemicals	Good reproducibility	Time-consuming, requires manual operation	Recommended for prokaryotes, i.e., *E. coli*	9–11
Injection quenching	Sample is directly injected to a pre-cooled quenching solution and collected by centrifugation	Rapid	Metabolite leakage (particularly in gram-negative bacteria) and leftover sample (can be overcome by additional washing step but may result in significant metabolite leakage)	Commonly used in *S. cerevisiae*	3,4,12–14

extraction, using methanol and chloroform but physical stresses, such as high temperature or freeze-thaw, are sometimes applied. In an early study of metabolite extraction from *Escherichia coli* for global analysis, cold (−40°C) methanol extraction produced good results compared with hot ethanol, hot methanol, perchloric acid, alkaline or methanol/chloroform extraction in terms of metabolite coverage, reproducibility and simplicity (15). A version of this protocol, using 100% cold methanol at −48°C with multiple freeze–thaw cycles, has also been developed (4). Although the authors concluded that this method has global applicability, they noted that some other extraction methods were also suitable for some target analyses. For *Saccharomyces cerevisiae*, the boiling ethanol and chloroform-methanol extraction methods exhibited the best efficacy and highest metabolite recovery, compared with hot water, freezing-thawing in methanol and acidic acetonitrile-methanol extraction (16).

One of the groundbreaking works in early microbial metabolomics was the phenotyping of silent gene mutants (8). In this work, silent gene knockout mutants were distinguished by metabolic snapshots and multivariate analysis. This suggested a possible benefit of metabolomics phenotyping with high resolution for gene functioning. The recent advancement in metabolic phenotyping has succeeded in the semi-rational screening of aging-related genes (17). For phenotyping, relative quantification is usually sufficient, while absolute quantification can be used to study metabolic dynamics, e.g., using the K_m of enzymes, within a particular pathway.

Although absolute quantification is difficult to achieve by MS-based metabolomics, it has been made possible by the combination of isotopically full labeled cells and commercially available compounds (18). Stable isotopes are also used in the study of microbial metabolomics. Recently, an interesting study, using ^{13}C, discovered riboneogenesis in *S. cerevisiae* by target profiling of isotopomers in knockout mutants (19). Furthermore, metabolomics-based molecular network analysis has been performed using systematic *in vivo* metabolite–protein interactions in the ergosterol biosynthesis pathway (20). Footprinting, which is analysis of culture medium, is also used to distinguish genotype (21). And recent study showed the potential of footprinting for study of co-culture system (22).

The most studied bacterial species in metabolomics is *E. coli*. In early studies, thin-layer chromatography and ^{14}C-glucose were used to reveal the relationship between cellular metabolism and global regulation (23). This report demonstrated that multiple metabolites, such as glutamate, aspartate, trehalose, adenosine, UDP-sugar and putrescine level varied with different specific growth rate under chemostat condition. More recently, metabolic dynamics was studied by using absolute quantification and K_m of related enzymes to clarify that the pair of most detected metabolite intercellular concentration exceeded the K_m value of its related enzyme (18). In addition, the relationship between ammonia assimilation and nutrient perturbation was studied by combination of absolute quantification and flux simulation (24). This study reported two important intermediates, 2-oxoglutarate and glutamine, for ammonia assimilation and more interestingly the authors showed that despite having weak enzymatic inhibition, some active-site inhibitors play important role in metabolic dynamics (24). Rapid analysis using MALDI has been used to obtain detailed time course data to study the dynamics of intracellular metabolism (25). An approach using ^{13}C-labeled intermediates in central metabolism for metabolic flux analysis has also been developed for non-stationary metabolic flux analysis (26).

Some innovative methodological studies in eukaryotes have already been described above (17,19–21). In *S. cerevisiae*, the relationship between concentration of metabolites and enzyme abundance was studied to reveal the link between metabolic flux and global metabolism (27). In metabolic engineering, several studies have been conducted to improve yields of important metabolites, such as high production of SO_2 in the bottom-fermenting yeast *Saccharomyces pastorianus* (28), through a combination of metabolomics and transcriptomics. In addition, the development of metabolic engineering strategies to confer resistance to stresses has been studied using xylose-fermenting *S. cerevisiae* to find target genes for optimization of fermentation (29). The discovery of a new synthetic pathway for glyoxylate from glycine was achieved using metabolomics and isotope labeling analysis (30).

Co-culture system also has been studied (22,31). Zhou et al. studied the cooperation between *Bacillus megaterium* and *Ketogulonicigenium vulgare*, which is used for production of precursor of vitamin C, by time course sampling and discovered symbiotic relation at early growth stage and antagonism at later stage (31). Nakanishi et al. (22) studied more dynamic state of metabolism under co-culture system using ^{13}C-substrate and nuclear magnetic resonance, and cooperation between *Bifidobacterium longum* and *E. coli* O157. The author demonstrated that aspartate and serine from *B. longum* is utilized by *E. coli*.

Metabolomics for the study of biological systems still has a long period of development ahead before reaching maturity. In the future, metabolome analysis of microorganisms will remain invaluable for the development of methods for the study of higher organisms.

PLANT METABOLOMICS

The application of metabolomics techniques to plant science was pioneered by groups at the Max Planck Institute. Weckwerth and coworkers obtained a series of metabolome data from potato tubers using GC/MS (32). A pair-wise comparison of metabolite levels showed that many pairs of metabolites exist, and that their levels show high correlation coefficients despite fluctuations in biological replicates. The investigators also demonstrated that the structure of the correlation network could be changed in a mutant with a silent phenotype, in which metabolic changes were observed despite a lack of visible changes. Theoretical studies revealed that factors such as equilibrium between neighboring metabolites shared biosynthetic enzymes and coordinated regulation of gene expression are likely responsible for the correlation between metabolite levels (33). These pioneering works

demonstrated that a novel relationship among metabolites can be determined using statistical analysis of a metabolome dataset, from which information on the plant metabolic system can be obtained. These investigations also indicate that the metabolome represents the ultimate phenotype of cells. Due to the technical advantages, metabolomics has played a key role in plant science, helping researchers to understand cellular systems and decode the function of genes (34).

It is known that the metabolic state of plants is dynamically controlled by transcriptional regulation in response to environmental and developmental conditions. The regulatory mechanisms between gene expression and the resulting metabolic phenotype is, however, still a black box. Thus, detailed investigation of the dynamic behavior of metabolic systems has been a big challenge for plant systems biologists. One promising strategy is a global survey of gene expression and metabolite accumulation to estimate mechanisms. In order to address this issue, an integrated analysis of transcriptome and metabolome data combined with metabolic pathway information was performed. The early applications of this strategy included an investigation of the reprogramming of gene expression and metabolism triggered by nutritional stress, such as sulfur starvation. A major breakthrough provided by this strategy was the identification of a gene-to-metabolite network regulating plant metabolism against environmental stress. Integrated analysis of time course data revealed that groups of metabolites and genes related to primary and secondary metabolites are coordinately modulated by stress induced by sulfur deficiency (35,36). A similar analysis was performed for various plants, including pathogen-infected *Medicago* and metabolically engineered rice (37,38). Another major achievement was the prediction of novel gene function by using the rules governing the link between gene expression and metabolite accumulation. The integrated analysis allowed the prediction of genes involved in glucosinolate biosynthesis [e.g., genes coding for two MYB transcription factors (39) and side chain elongation (40)] in a comprehensive manner. This strategy has also been applied to other plant species (41,42).

The integrated analysis of gene expression and secondary metabolites in the model plant *Arabidopsis thaliana* was intensively performed for construction of the AtMetExpress development dataset, which is part of the AtMetExpress metabolite accumulation atlas (43). Genome sequence analysis of *A. thaliana* revealed that it has many metabolic genes, possibly for the production of many phytochemicals. However, phytochemical diversity in plant species, and the underlying metabolic systems are unclear. Thus, the phytochemicals produced during development in *A. thaliana* were investigated in samples covering many growth stages and organs. Based on the dataset, 1589 metabolite signals were detected, from which the structures of 167 metabolites were elucidated. Analysis of the dataset demonstrated that *Arabidopsis* produces various phytochemicals in a highly tissue-specific manner. Integrated analyses with transcriptome data revealed that tissue-specific accumulation often accompanies the expression of key biosynthesis-related genes (44). These results indicate that the functional differentiation of secondary metabolite biosynthesis among the various tissues was achieved by controlling the expression of a small number of key regulatory genes. It is also postulated that a simple mode of regulation, transcript-to-metabolite, is the origin of the dynamics and diversity of plant secondary metabolism.

Metabolomics techniques have also been applied to the crop and breeding sciences. Genetic factors regulating metabolite levels have been intensively investigated because nutritional quality is an important quantitative trait of crops. For example, quantitative trait locus (QTL) analysis of m-traits revealed the loci responsible for the control of vitamin E levels (45). Recently, metabolome QTL analyses have been conducted for a comprehensive understanding of the genetic background controlling the plant metabolome (46–51). QTL analysis of tomato fruit traits indicated that levels of primary metabolites, including amino acids and sugars, were under the control of many QTLs (52), and also showed that the modes of inheritance differ for the various metabolites (53). The analysis of *Arabidopsis* revealed that QTLs are unevenly distributed in the genome, suggesting that the overall composition of the plant metabolome can be controlled by small genomic regions (48). In addition, the relationship between metabolite composition and other important traits, such as yield and biomass, has been investigated since these traits are likely to interact closely with metabolism in plants. The analysis of tomato fruit traits with metabolome data indicated that there are certain correlations among these traits, although in most cases, only weak correlations were observed (46). Regression analysis of metabolome data for *Arabidopsis* biomass traits demonstrated that the growth rate of *Arabidopsis* seedlings is predictable from the metabolome signature to a degree (48). These studies suggest that the interactions between metabolites and other traits should be predictable using metabolome data.

Molecular breeding by the introduction of various transgenes into plants is an efficient way to produce genetically modified (GM) crops with improved performance. Metabolomics plays a key role in the public acceptance of GM crops by providing data necessary for risk management (52). Similarities between the metabolic profiles of tissues from GM plants, non-transformants and other cultivars have been examined using multivariate analysis (53,54). Substantial equivalence is verified when the perturbation of the metabolic profile caused by transgene expression is within the variance that exists among the various cultivars.

Technical improvements in metabolomics have been spurred by the need of plant scientists to detect and identify diverse phytochemicals. The ability to produce various phytochemicals has evolved in plants for the purpose of self-defense, environmental adaptation and interaction with other organisms. Because humans utilize phytochemicals for numerous purposes, including the production of pharmaceuticals, further understanding of the genetic background behind the diversity of secondary metabolites produced by plants will facilitate a more intensive application of these compounds. However, the current bottleneck in metabolome analysis of plant metabolites is the annotation of metabolite signals (55–57). It has recently been recognized that three types of data and infrastructure are required for database-assisted elucidation of metabolite structures (58,59). First, tandem mass (MS/MS) spectra data for structural elucidation of metabolites (60). Second, a comprehensive mass spectral database of phytochemicals. Although great effort has been put into construction of the MS/MS spectral databases (61), further enrichment is required for structural elucidation of a wider range of metabolites. Third, a method to determine the false discovery rate in large-scale search results (62). Further development of these databases and methodology is required to explore the diversity of plant secondary metabolites. This is the next challenge in the development of metabolomics technology.

ANIMAL METABOLOMICS

Metabolomics technology has been applied to study biological phenomena in several model organisms, including zebra fish and fruit fly. Comprehensive analysis of metabolites in these organisms has provided a wealth of information on physiological, developmental and pathological processes. Metabolomics can therefore provide novel insight that can be developed and applied to research in other species. A summary of application of metabolomics in animal science can be seen in Table 2.

Zebra fish (*Danio rerio*) The zebra fish (*D. rerio*) is a very popular model organism for biological, behavioral and biomedical

TABLE 2. Summary of animal metabolomics applications cited in this review.

Species	Material	Method	Approach	Application	Reference
Zebra fish	Egg	GC/MS	Non-targeted	Prediction of vertebrate development	66
Zebra fish	Egg	LC/MS	Non-targeted	Prediction of vertebrate development	67
Zebra fish	Liver	GC/MS, LC/MS/MS, NMR	Targeted	Metabolic profiling of male and female zebra fish liver	68
Nematode	Whole body	LC/MS, NMR	Targeted/non-targeted	Metabolic responses to cadmium	75
Nematode	Whole body	LC/fluorescence detection	Targeted	Metabolic profiling of mitochondrial respiratory chain mutants	77
Fruit fly	Whole body	NMR	Non-targeted	Metabolomic signatures of inbreeding	85
Fruit fly	Whole body	NMR	Non-targeted	Metabolic profiling of heat stress	86
Fruit fly	Whole body	NMR	Non-targeted	Metabolomic profiling of rapid cold hardening and cold shock	87
Fruit fly	Thorax	NMR	Non-targeted	Metabolomic of age-related decline of hypoxia tolerance	88
Fruit fly	Whole body	LC/FT-MS	Targeted	Metabonomic profiling of different strains	89

research, particularly for studies on embryogenesis, organogenesis and general vertebrate development. The zebra fish is now gaining popularity as a model for research into disease and drug discovery. This is because, compared with rodents, they are easily bred in large numbers with a relatively low maintenance cost. In addition, the small size of this fish makes it highly suitable for whole organism assessment of changes in global gene expression caused by chemicals and drugs (63–65).

Hayashi et al. (66) successfully demonstrated the dynamic changes involved in early vertebrate development by the metabolomics approach. They used a metabolomics as a novel strategy in order to understand embryogenesis in zebra fish. The metabolome during the different stages of embryogenesis exhibited dynamic changes, indicating that the types and quantities of metabolites are correlated with biological activities during development because they could successfully predict embryonic stages using metabolomic information. On the basis of the robust correlation between the metabolome and embryogenesis, they concluded that the metabolome can be used as a fingerprint for a particular developmental process.

Metabolomics has been used to study single zebra fish embryos (67). Highly accurate PLS models of developmental stages were constructed using single-embryo metabolic nontarget fingerprinting. This model was implemented on different developmental stages under standard rearing conditions, but it was necessary to apply different rearing conditions to demonstrate the applicability and versatility of this single-embryo metabolomics approach. This metabolomics approach should enable the identification of the developmental stage of embryos exhibiting no visible defects and help clarify the metabolic differences between wild-type and mutant animals or drug treatments and so on.

Ong et al. (68) performed a multiple platform approach, incorporating GC/MS, LC/MS and ^{1}H NMR to study the biochemical profiles of livers from female and male zebra fish. Their findings showed that ^{1}H NMR provided comprehensive information on glucose, amino acids, pyruvate and other biochemical constituents of the zebra fish liver. GC/MS spectrometry was able to analyze cholesterol, as well as saturated and unsaturated fatty acids. LC/MS spectrometry was ideal for the analysis of lipids and phospholipids. This multiple techniques approach showed significant differences in the liver of female and male zebra fish. The biomolecules observed to be different included amino acid, unsaturated fatty acids, cholesterol, phospholipids and other small molecules. The overall findings suggest that the multiplatform approach offers a much better coverage of the metabolome.

Nematode (*Caenorhabditis elegans*) The nematode (*C. elegans*) has been characterized in great detail in terms of its development, morphology and physiology at the cellular level, and its genome has been completely sequenced (69). A powerful genetic tool for *C. elegans* is available, as well as a large collection of mutants. In addition, *C. elegans* is one of the few animals for which high-throughput *in vivo* RNA interference (RNAi) screening has been established (70). *C. elegans* is a good platform, for example, to understand the physiology of lifespan and aging, for the study of genetic diseases, drug toxicity screening and pharmacological studies (71–74).

Hughes et al. (75) generated different mutants and performed NMR- and UPLC-MS-based metabolomic analyses. Their results imply that the main physiological responses to cadmium are independent of metallothionein status (at least in phytochelatin synthase-normal nematodes) and result in an increased production of phytochelatins by altered flux through the methionine transsulfonation pathway. They confirmed earlier observations that phytochelatin synthase is required in *C. elegans* for protection against cadmium by demonstrating the production of phytochelatins (phytochelatin-2 and phytochelatin-3) directly in tissue extracts of cadmium-exposed *C. elegans*.

C. elegans, which has greater than 83% genetic homology with humans (76), affords a model of primary mitochondrial dysfunction that provides insight into cellular adaptations that accompany mutations in nuclear genes encoding mitochondrial proteins. Falk et al. (77) applied metabolomics to mutants of the mitochondrial respiratory chain. Their work showed that primary mitochondrial disease is associated with gene expression alterations in multiple metabolic pathways. Specific pathways that were significantly upregulated in primary mitochondrial respiratory chain disease included those involved in oxidative phosphorylation and the tricarboxylic acid cycle, and those participating in carbohydrate, amino acid and fatty acid metabolism.

A powerful genetic toolset for *C. elegans* exists and a large collection of mutants is available. Therefore, *C. elegans* is an ideal model organism to explore the correlation between metabolites and gene function.

Fruit fly (*Drosophila melanogaster*) The fruit fly (*D. melanogaster*) is another principal model organism used for studying the genetics of aging and physiology, for a number of reasons. Flies develop to adulthood quickly, have a short lifespan, are easy to breed and undergo functional senescence with similarities to human aging (78). Transcriptional expression studies of *D. melanogaster* that investigate the extent to which gene transcription varies between phenotypes are increasingly common, including studies of pesticide resistance (79), developmental biology (80), oxidative stress (81,82), starvation, sugar feeding and the effects of phenobarbital (83,84). Metabolomics approach of *D. melanogaster* was performed in order to investigate the relationship between metabolite and phenotype.

Pedersen et al. (85) applied metabolomics using a multiple platform approach to study the effects of breeding of Drosophila, including mutations and stressful temperatures. They found that the metabolite fingerprints of inbred and outbred flies were clearly distinguishable. The approach used in this study to examine the association between metabolite and gene expression levels is likely to be generally applicable for the integration of multivariate data

from different omic technologies. Integration of information across multi-omic technologies has the potential to broaden the limited window provided by individual technologies, thus enhancing the understanding of biological systems.

Malmendal et al. (86) confirmed that NMR metabolomic profiling could be used to understand the perturbations (and the homeostatic response) caused by heat stress in *D. melanogaster*. The metabolite profile was analyzed during recovery after exposure to different thermal stress treatments and compared with untreated controls with time course. They demonstrated that the analytical method used to understand the metabolic trajectory caused by a biological stress is a powerful tool for monitoring effects on homeostasis, which could be widely applied to cellular/molecular physiology and biochemistry. The data revealed changes in several specific metabolites that are likely to be related to stress, and both the overall metabolomic response and changes in the specific metabolites varied with the intensity of the heat stress applied to the flies. In addition, *D. melanogaster* has features that make it an ideal organism for the development of metabolomics models for the study of physiology. Indeed, metabolomics has been successfully applied for the study of cold shock (87), hypoxia (88) and mutations (89) in this species.

Although the application of metabolomics for the study of animal physiology and biochemistry is still relatively limited in comparison with plant science and microbiology (less than 30 articles for each study organism, i.e., zebra fish, nematode and fruit fly as shown by the number of hit articles in PubMed, August 1, 2012), the number of reports is steadily increasing in recent years.

Metabolomics approach will enable identification of mutants exhibiting no visible defects and clarification of novel aspects of known mutants. Integration of information across multi omics technologies has the potential to broaden the limited window provided by individual technologies, thus enhancing our understanding of biological systems. Accumulation of metabolome data for various model organisms should further animal science.

MEDICAL METABOLOMICS

With the recent breakthrough in metabolomics technologies, application of metabolomics has been increasing in the medical field. There are two major purposes for its use in medicine; the first is to acquire knowledge on the mechanisms of drug action or the disease itself, and another is to explore biomarkers. Aside from diagnostic purposes, biomarkers have been used as indicators of toxicity and therapeutic effects. This chapter discusses the different approaches used for various diseases (Table 3).

Metabolic syndromes and related diseases Life-threatening diseases caused by disorders of lipid and sugar metabolism, such as cerebrovascular and cardiovascular disease, are a major cause of death in developed countries. Therefore, it is important to detect changes in metabolites for the acquisition of pathophysiological knowledge and discovery of biomarkers for early diagnosis of these fatal diseases. Sabatine et al. examined changes in the metabolome caused by exercise stress in inducible ischemia patients and a control group to identify novel biomarkers of myocardial ischemia (90). LC/MS-based metabolomic analysis revealed that 6 out of the 23 metabolites that varied after exercise in patients were involved in the citric acid metabolic pathway. Furthermore, a significant difference in changes of six metabolites, including citric acid, between patients and controls was observed. Teul and coworkers used GC/MS in combination with ^{1}H NMR and identified 24 metabolites involved in insulin-resistance that were important for distinguishing patients with stable carotid atherosclerosis from healthy subjects (91).

Recently, research has been conducted to clarify the detailed pathogenic mechanisms of hyperlipidemia and diabetes, which occur prior to these diseases. Connor et al. applied an integrated metabolome/transcriptome analysis to model type 2 diabetes in db/db mice (92). As a result, differences in 24 pathways related to lipid metabolism, including gluconeogenesis, mitochondrial dysfunction, oxidative stress and branched chain amino acid biosynthesis, were observed between db/db and db/+ (control) mice. Metabolomic analysis was also applied to human subjects. Suhre et al. applied multiplatform metabolomics using GC, LC, MS/MS and NMR to subjects with self-reported diabetes and controls and identified 420 metabolites from the sera of these subjects (93). They found perturbations in pathways linked to kidney dysfunction, lipid metabolism and interactions with gut microflora. Two independent studies of obesity using metabolomics were also

TABLE 3. Summary of medical metabolomics applications cited in this review.

Disease	Species	Material	Method	Approach	Specific biomarker species	Reference
Myocardial ischemia	Human	Plasma	LC/MS/MS	Targeted	Gamma-amino-butyric acid, uric acid, citrate	90,91
Type 2 diabetes	Mouse	Urine	NMR	Targeted	Mannose, 1,5-anhydroglucitol, phenylacetylglutamine	92
Type 2 diabetes	Human	Plasma	GC/MS, LC/MS/MS, NMR	Targeted	3-Indoxyl sulfate, glycerophospholipids, bile acids	93
Obesity	Human	Serum	LC/MS/MS	Non-targeted	Lysophosphatidylcholine	94
Obesity	Human	Serum	MS/MS	Targeted	Phosphatidylcholine	95
Cardiovascular disease	Human	Plasma	LC/MS/MS	Non-targeted	Trimethylamine *N*-oxide, choline, betaine	96
Ovarian carcinoma	Human	Tumor tissue	GC/MS	Non-targeted	Alpha-glycerolphosphate, uracil, glycine	97
Lung cancer	Human	Tissue, plasma	GC/MS, NMR	Stable isotope resolved analysis	(^{13}C-enrichment in lactate, alanine, succinate)	98
Pancreatic cancer	Human	Serum	GC/MS	Targeted	Thiodiglycolic acid, lactic acid, 7-hydroxyoctanoic acid	99
Hepatocellular carcinoma	Human	Urine	GC/MS	Non-targeted	Xylitol, urea, hydroxy proline dipeptide	100
Colorectal cancer	Human/rat	Urine/tissue	GC/MS	Targeted	Succinate, *N*-acetyl-aspartate, 2-hydroxyhippurate	101
Oral cancer	Human	Saliva	CE/MS	Non-targeted	Pyrroline, leucine + isoleucine, taurine	102
Breast cancer	Human	Saliva	CE/MS	Non-targeted	Taurine, putrescine, leucine + isoleucine	102
Pancreatic cancer	Human	Saliva	CE/MS	Non-targeted	Leucine + isoleucine, phenylalanine, alpha-amino butyric acid	102
Schizophrenia	Human	Cerebrospinal fluid	NMR	Non-targeted	Lactate, citrate, glucose	103
Parkinson's disease	Human	Plasma	NMR	Targeted	Threonate, myoinositol, suberate	104
Huntington's disease	Human/mouse	Serum	GC/MS	Non-targeted	Glycerol, urea, valine	105
Schizophrenia	Human	Plasma	LC/MS/MS, GC/MS	Targeted	Free fatty acids, triglycerides, phosphatidylethanolamine	106
Depression	Rat	Plasma	GC/MS	Targeted	Glucose, glutamine, butanedioic acid	107

conducted. Differences in the fatty acids lysophosphatidylcholine (94), phosphatidylcholine (95) and acylcarnitine were observed between obese and lean subjects. Their results partially revealed the mechanism underlying hyperlipidemia.

More recently, a large-scale human study of cardiovascular disease (CVD) was conducted using LC/MS/MS-based metabolomics (96). The authors chose trimethylamine oxide (TMAO) as a candidate biomarker. The effectiveness of the marker was confirmed by analyzing TMAO in serum of nearly 2000 patients. TMAO levels exhibited a dose-dependent association with the risk of CVD and multiple individual CVD phenotypes, including peripheral artery disease, coronary artery disease and history of myocardial infarction. This study has shown the potential for clinical application of biomarkers discovered by metabolomics and is a milestone in the history of metabolomics.

Cancer Cancer is a leading cause of death worldwide and accounted for 7.6 million deaths in 2008. Deaths from cancer are projected to rise to over 11 million by 2030. Because early diagnosis is required to improve prognosis, discovery of sensitive cancer biomarkers has become one of the priorities of cancer research.

Denkert and coworkers analyzed invasive carcinomas and borderline tumors (97) by GC/TOF-MS, and detected 291 metabolites, of which more than a hundred were annotated. Furthermore, 51 metabolites were significantly different between carcinomas and borderline tumors, and 88% of borderline tumor cells could be identified using a supervised prediction model. The authors used the Kyoto Encyclopedia of Genes and Genomes for correlating the metabolic changes to different pathways and key enzymes. They found perturbations in the metabolic pathways for glycerolipids, fatty acids and amino acids in the carcinomas. Metabolomics helps provide an accurate diagnosis of cancer and provides insight into the pathogenesis of the disease. Fan et al. used ^{13}C stable isotope metabolomics using uniformly labeled ^{13}C-glucose as tracers to study metabolite flux in lung cancer cells (98). Their analysis of lung cancer tissue from ^{13}C-infused patients revealed that lung cancer cells contained high amounts of ^{13}C-labeled lactate, alanine, indicating enhancement of energy production *via* glycolysis. Generally, glucose for glycolysis and glutamine for glutaminolysis *via* the Krebs cycle are thought to be the major energy resources in cancer cell. However, the elevated levels of the aspartate isotopomer with three ^{13}C-labeled carbons and the ^{13}C-2,3-glutamate isotopomer in lung tumor tissues provides definitive evidence of the increased production of aspartate and glutamate from ^{13}C-glucose *via* glycolysis, anaplerotic pyruvate carboxylation and the Krebs cycle. Furthermore, the authors confirmed pyruvate carboxylation activation in tumor tissues by an increased level of pyruvate carboxylase mRNA and protein. These findings suggest that isotopomer-based metabolomics is useful for understanding the biochemical changes in cancer cells.

Numerous studies have been performed to identify cancer-specific biomarkers in less-invasive samples. Nishiumi et al. sought to identify serum biomarkers of pancreatic cancer, which is difficult to detect and diagnose early (99). The levels of 18 out of 60 metabolites identified in patients' sera by GC/MS was significantly different from that of healthy subjects. Furthermore, this difference was observed even in stage I patients. These results suggest that screening of early stage pancreatic cancer can be realized by using serum metabolomics. Researchers have been keen on seeking biomarkers in non-invasive samples, such as urine (100,101) and saliva (102). In these studies, in the same fashion of most metabolomic studies of diagnostic method, multivariable analysis and/or multiple biomarkers were used to enhance sensitivity and specificity of the method. Taken together, metabolomics is a promising non-invasive, highly sensitive diagnostic method for cancer.

Central nervous system diseases and psychiatric disorders In diseases of the central nervous system, it is difficult to establish quantitative diagnostic criteria. Therefore, the most urgent task for these diseases is to build a biomarker panel for diagnosis and confirmation of treatment effects.

Holmes et al. applied ^{1}H NMR-based metabolomics to the study of cerebrospinal fluid (CSF) (103). CSF circulates throughout the entire central nervous system, thus it is an appropriate target of neural metabolomics. Their results suggest that there is a dysregulation of glucose homeostasis in the CSF of drug-naive schizophrenics. Application of PLS-DA clearly distinguished the schizophrenic patients from healthy subjects. Furthermore, the ^{1}H NMR spectra of over 50% of the patients returned to normal after antipsychotic drug treatment.

Numerous studies have been conducted using blood, which can be collected in a less-invasive manner. However, the biochemical properties of the cerebrospinal fluid are quite different from that of blood due to the blood-cerebrospinal barrier. The matter of concern is how perturbations of the brain affect the blood metabolic profile. Ahmed et al. applied ^{1}H NMR-based metabolomics to Parkinson's disease patients (104), revealing that 22 metabolites showed significantly different concentrations compared with healthy subjects. According to the PLS-DA loading plot, pyruvate is the most important variable for cluster separation, and the results of gene expression studies suggest that there is altered expression of enzymes related to pyruvate metabolism. Furthermore, the application of ^{1}H NMR spectra to neural network algorithms showed $>$90% accuracy for prediction of disease progression. Metabolomic profiling using GC/TOF-MS has been applied to Huntington disease patients and transgenic mice (105). The results indicate that there are alterations in fatty acid and aliphatic amino acid metabolism in patients and in a mouse model of the disease. These results clearly reveal that degeneration in the brain affects the metabolite profile of blood, despite the blood-cerebrospinal barrier.

A number of researchers have focused on psychiatric diseases, such as schizophrenia and depression. Schizophrenia is partly caused by alterations in neuronal membrane phospholipids and neurotransmitter systems. Weight increase and hypertriglyceridemia are major side effects of anti-schizophrenic medication. Therefore, lipid metabolism seems to be important in schizophrenia pathogenesis. Kaddurah-Daouk and coworkers applied the lipidomic method to schizophrenia, targeting $>$300 lipids, including a wide range of polar and nonpolar molecules (106). The profile of many lipid species was affected by anti-schizophrenic drugs. Elevated levels of free fatty acids and triglycerides in plasma were observed, and the level of phosphatidylethanolamine was lower, in drug-naive patients compared with healthy controls. Interestingly, plasma phosphatidylethanolamine was increased by anti-schizophrenic medication. These results indicate that lipidomic analysis has the potential to identify biomarkers that define drug-response phenotypes. Further study with larger sample set will enable us to elucidate the relationship between therapeutic benefit and the plasma lipid changes. Metabolomics has also been applied to depression, for which the etiology is unclear. Li et al. used chronic unpredictable mild stress (CUMS) rats as a model for depression, and performed GC/MS-based metabolomic analysis of the plasma (107). The control and CUMS groups were well discriminated by PCA, and the loading indicated that the CUMS rats were perturbed in amino acid metabolism, energy metabolism and glycometabolism. These results suggest that metabolomic analysis can detect the biochemical alterations in mood disorders. Thus, metabolomics is a powerful tool for understanding the pathogenesis of these diseases, and for identifying biomarkers for early diagnosis, therapeutics and drug toxicity.

Metabolomic analysis of tissues from patients and/or animal models is a promising approach to reveal pathogenesis of diseases. Integration of metabolomics with other omics science, more specifically, genomics, transcriptomics and proteomics will lead to

a better understanding of the link between changes that occur in body and pathological condition. Clarification of pathogenic mechanism by metabolomics is expected to be directly connected with discovery of drug targets. Discovery of highly sensitive, specific biomarkers is also one of the defined goals of medical metabolomics. A number of researches have shown that some of diagnostic indexes based on multiple metabolite concentration constricted by metabolomics are superior to conventional biochemical markers in terms of specificity and/or sensitivity (108). Metabolomic approach is expected to discover biomarkers for diseases which are difficult to set definite diagnostic criteria such as psychiatric disease and to diagnose cancer in its early stages contributing to improve prognosis. Validation of these markers on a larger cohort of patients will confirm the utility of metabolomics in clinical science.

METABOLOMICS OF FOOD AND HERBAL MEDICINES

In the fields of food science and herbal medicines, metabolomics has emerged as an important tool for evaluating quality and safety (109). The quality of final food products are greatly affected by pre-harvest (genetic origin, cultivation area and growing environment) and post-harvest (milling, modified/controlled and atmosphere storage) processes (110). Recently, metabolomics has been employed for quality control, because it is useful for evaluating multiple factors simultaneously. Although metabolomics approaches have recently gained popularity in the area of foods and herbal medicines, sensory evaluation is still the main method used to assess the quality and taste of foods and herbal medicines. Sensory evaluation is defined as a scientific method used to evoke, measure, analyze and interpret responses to products, as perceived through the senses of sight, smell, touch, taste and hearing (111). Sensory evaluation holds great importance in food industry as it greatly affects the prices and quality of various food products. However, the reproducibility of sensory evaluation highly depends on the skill of sensory panels. As training and maintenance of well-trained sensory experts are time-consuming and expensive, an alternative instrument-based method that is less subjective and more efficient is needed to complement sensory evaluation. In this section, we describe the recent applications of metabolomics techniques for the quality evaluation of food and herbal medicine, particularly in relation to human senses.

Metabolomics have been applied for the analysis of major crops [potato (112), tomato (113), cereals (114) and fruits (115)] and popular beverages (wine (116) and beer (117)). A summary of references of this part can be seen in Table 4 (117–127). There are two concepts on applications of food metabolomics, mimicking human senses and cataloging taste-active compounds. In order to mimic five canonical senses: sight, hearing, touch, taste and smell, electronic sensors have achieved a remarkable breakthrough (128). Main target of food assessment by electronic sensors are alcohols, such as beer (129) and wine (130). Recently, electric sensors conjunction with MS-based instruments (118) has been employed to evaluate food quality. Combinations of electronic sensors and MS-based instruments will be important tools toward improvement of food metabolomics. Another growing application of food metabolomics is *sensomics*, in which the main objective is to discover the key players imparting the attractive taste of foods (119,131). In order to identify and catalog taste-active metabolites, omics technology has been frequently employed. Metabolomics technology helps to understand the complexity of human sense to evaluate food quality.

Multivariate analyses were used to identify the relationship between metabolite profiles of food and quality as assessed by professional sensory panels. For example, PLS-based models have been used to examine the relationship between green tea metabolites and quality, with FT/NIR (120), NMR (121), GC/MS (122), and UPLC/MS (123) techniques being employed. In addition, the electronic tongue was used in combination with principal component analysis (PCA) for the classification of Chinese tea samples of different geographical origins and quality grades by professional tasters (132). Various analytical instruments and multivariate analyses have been used to characterize the sensory properties of green tea. These reports show that combinations of instrument data and multivariate analyses are promising tools for the future of sensory chemistry.

In the area of herbal medicines, which is closely related with food, metabolomics is expected to resolve the special issues of traditional Chinese medicines (TCM) and focus on biomarker discovery (133). In the market, it is difficult to assess quality of herbal medicines by the quantities of the various constituents because of interaction like synergistic effect. Therefore, sensory tests remain practically important for deciding quality and price. However, only a few studies (134–137) have been conducted to assess sensory quality of herbal medicines. Therefore, further research on sensory analysis is still required for the herbal medicine industry.

A combination of sensory and instrumental data has the potential to improve quality control procedures for herbal medicines. Thus, to study the relationship between metabolic profiling and quality ranking, metabolomics analyses using NMR (124), GC/MS (125), pyrolysis GC/MS (126) and UPLC/MS (127) have been applied to *Angelica acutiloba*. Recently, the relationship between content of pharmacological components and the grade of *Angelicae Radix* was documented, although only some ligustilides and furocoumarin were assessed (138). Comprehensive analyses of herbal

TABLE 4. Summary of food and herbal medicine metabolomics applications cited in this review.

Material	Method	Approach	Application	Reference
Potato	LC/MS	Random forest regression	Construction of networks integrating gene expression, metabolites and phenotypic traits	112
Tomato	GC/MS, LC/MS	PCA, PLS	Determination of key metabolites as /efficient predictors	113
Cereals (rice)	GC/MS	PCA	Distinction of biological variability	114
Fruits (melon)	GC/MS	PCA	Distinction and characterization of varieties	115
Wine	HPLC/MS	PCA, PLS-DA	Distinction of varieties and geographical origins	116
Beer	NMR	PCA, PLS-DA	Determination of the chemical changes	117
Wine	EN/MS	PCA, PLS-DA, SLDA	Prediction of the geographical origin	118
Beer	LC-MS/MS	PCA	Determination of the change of bitter compounds during storage	119
Green tea	FT/NIR	PLS	Classification of quality	120
Green tea	NMR	PCA, PLS	Classification and search of biomarkers of quality	121
Green tea	Pyrolysis GC/MS	PLS	Classification and search of biomarkers of quality	122
Green tea	UPLC/MS	PCA, PLS	Classification and search of biomarkers of quality	123
Angelica acutiloba	NMR	PLS, PLS-DA	Classification of geographical origins, variety differences and quality	124
A. acutiloba	GC/MS	PCA	Classification of geographical origins, variety differences and quality	125
A. acutiloba	Pyrolysis GC/MS	PCA, PLS-DA	Classification of geographical origins, variety differences and quality	126
A. acutiloba	UPLC/MS	PCA, PLS-DA	Classification of geographical origins, variety differences and quality	127

medicines, e.g., using metabolomics techniques, are required for both pharmaceutical and industrial purposes. Further research is required, not only to determine genetic origin and cultivation conditions, but also to identify compounds that affect quality.

Metabolomics techniques are effective tools to assess food quality. The studies of food metabolomics include sensory evaluation, investigation of taste-active compounds and the development of instruments mimicking the human senses. In particular, metabolomics can identify important components assessed by sensory evaluation by combining the metabolome information derived from instruments and professional sensory evaluation scores. Products, such as wine, coffee and green tea, that are usually ranked by sensory evaluation, are of particular interest for such studies. Metabolomics techniques will continue to assist researchers in finding a scientific method to determine the real quality of food. In assessment of herbal medicines, one of the most important goals is to analyze pharmacological and adverse (toxic) effects. Currently, it is a challenging task to technically to assess and define all active compounds in all raw materials. Therefore, prediction of sensory tests by metabolomics technology is essential to evaluate quality of herbal medicine because sensory test is a practical method in the market. Ultimately metabolomics should lead to the establishment of a robust and reliable method for quality control. In the future, technological advances in instrument development and data processing methods in metabolomics should lead to the establishment of a more practical scientific assessment of sensory chemistry.

CONCLUSION

This review describes in detail the recent progress in metabolomics application in various fields. Snapshot metabolomics is a high-resolution phenotype analysis tool that is very useful for revealing silent phenotypes in microorganisms. Stable isotope dilution is also used in quantitative analysis and for dynamic observation. Studies focusing on development of methods in microbes will remain invaluable for method development in the study of higher organisms.

Plant metabolomics is a key technology for understanding cellular systems and for decoding the function of genes. In many cases, plant metabolomics is integrated with genomics, transcriptomics and proteomics. Metabolomics technology has also been applied to the breeding of commercially important crops. Metabolomics should be useful for QTL analysis of m-traits, such as the level of variable constituents. Annotation of metabolomics signals is one of the remaining problems to be resolved for further progression of metabolomics research. Particularly, in plant metabolomics, secondary metabolite annotation is a very important procedure and technical improvements are expected in this area.

In animal metabolomics, discoveries in early development are thoroughly discussed. Zebra fish, fruit fly and nematode are given as examples of model organisms. Accumulation of metabolome data for various model organisms should further animal science. Metabolomics is obviously useful for medical research. The relationship between metabolic perturbations and disease is discussed, with a focus on cancer, the most significant research target of medical metabolomics. Metabolomic approach is expected to discover biomarkers for diseases which are difficult to set definite diagnostic criteria such as psychiatric disease and to diagnose cancer in its early stages contributing to improve prognosis.

Finally, application of metabolomics for foods and herbal medicines are reviewed. Chemometrics techniques have been used for food characterization, in which gas chromatography and near infrared spectroscopy are employed. Recently, the newest metabolomics technologies have been applied to food characterization. In particular, sensory assessment of food by skilled human evaluators has been examined using metabolic fingerprinting methodology.

In recent years, the usefulness of metabolomics as applied to various fields of life science has become apparent. In addition to providing experimental data for identifying unknown and underappreciated reactions in the metabolic network, metabolomics has also emerged as a powerful tool for quantitatively predicting physiological traits. By identifying the metabolites important to the trait, crucial information can be gleaned regarding the underlying molecular mechanisms contributing to the phenotype in question. Elucidation of such molecular mechanisms or pathways will be of tremendous use in metabolic engineering aiming at modifying these phenotypes in various target organisms.

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