REVIEW

Metabolomics for measuring phytochemicals, and assessing human and animal responses to phytochemicals, in food science

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Metabolomics, comprehensive metabolite analysis, is finding increasing application as a tool to measure and enable the manipulation of the phytochemical content of foods, to identify the measures of dietary intake, and to understand human and animal responses to phytochemicals in the diet. Recent applications of metabolomics directed toward understanding the role of phytochemicals in food and nutrition are reviewed.

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1 Introduction

In the contemporary science paradigm, the functioning of an organism is increasingly being studied by exhaustive analysis of the interactions of its genome, transcriptome, proteome, and metabolome with the environment. Modern technology provides the ability to characterize all three ''omes,'' with the result that vast amounts of ''omics'' data are being produced together with an increased understanding of the composition and functioning of living organisms.

The ''metabolome'' may be defined as the total complement of metabolites produced by and usually contained within an organism, and ''metabolomics'' as the untargeted measurement of these metabolites with the overall objective of measuring and identifying all the metabolites present in a chemical sample prepared from some living organism [1]. Plants produce a very large number of chemically diverse metabolites. The total number of metabolites produced by plants has been estimated to exceed 200 000 [2] or to be up to 1 000 000 [3]. Approximately, 10 000 plus metabolites are currently detectable in the edible plant metabolome [4]; this is a more modest count, but still provides an immense challenge for metabolomic analysis in terms of metabolite extraction, identification, and measurement. While the metabolites produced by an organism could be viewed merely as end points of biosynthesis, it is increasingly obvious that the knowledge of metabolites (identity, concentrations, and biosynthesis) is vital to understanding the functioning of whole organisms within their ecological environment, and that such knowledge is an integral component of systems biology [3, 5]. By providing such knowledge, metabolomics can play an important role in the improvement of agricultural systems and of plant-based foods. Areas of application include the understanding of metabolic pathways, food and environmental safety, dietary nutrition and health [5].

The modern field of ''metabolomics'' is little older than a decade and the underpinning methodologies and processes are now well established [3, 5, 6]. Metabolomic studies aim to capture snapshots of the metabolome and relate the observed metabolic profiles to physiological states that may be of genetic or environmental origin. In nutrition research, the aim is to understand the effects of diet on human health and in this case the interaction between multiple complex metabolomes is investigated [7]. This requires the measurement and analysis of the metabolomes of both the

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Abbreviations: DIMS, direct infusion MS; **GM,** genetically modified; **PCA,** principal component analysis; **PLE,** partial least squares; **SPME,** solid-phase microextraction

food and the animal or human consuming the food, with the aim of generating a hypothesis that relates to a nutritional, or anti-nutritional, effect of the food on the consumer. In Fig. 1, we attempt to summarize the relationships between the different aspects of a food and the nutrition metabolomic workflow.

A number of recent publications have reviewed current progress in the metabolomics of food and [4, 5, 8–12] nutrition. In this review, we will focus on the application of metabolomics to plant-based foods, specifically how metabolomic analysis can be used to measure the phytochemical composition of foods, biotransformation of ingested phytochemicals by animals (including humans), and animal responses to the ingestion of phytochemicals. Our intention is not to provide an exhaustive review of metabolomics per se, but rather to give a broad overview of the technology and then present selected examples to show how metabolomics has been applied in food and nutrition research and to demonstrate the promise of this approach.

2 Experimental methods in metabolomic analysis

While the aim of metabolome analysis may be the measurement of all metabolites in a sample, realistically this ambitious goal is not possible, as every step in the metabolomic workflow necessarily places constraints on either the types of metabolites that are extracted from the sample or on those that can be measured. In addition, biological samples represent snapshots of metabolism taken at the moment of sample collection and therefore the design of the sampling regime is a crucial part of a metabolomic experiment. A typical workflow for metabolomic analysis has recently been described [9] and the steps include metabolite extraction from the biological matrix, extract cleanup (purification) in the preparation for analysis, instrumental analysis, data processing, and statistical analysis. The particular procedures adopted for each of these steps constrain the types of metabolites that are extracted, detected, and measured. For example, the widely used GC-MS-based metabolic profiling of primary metabolites as trimethylsilyl derivatives [13] excludes the measurement of highly volatile flavor compounds and of nonvolatile-charged compounds including many plant pigments such as anthocyanins. At a practical level, when designing a metabolomic experiment, analytical options are often (but not ideally) selected based on underlying assumptions about the types of metabolites that are most likely to be of interest to the experimenter.

2.1 Metabolite extraction and sample preparation

Most plant and food samples contain a considerable number of metabolites dispersed within a complex solid and a liquid matrix such as a plant leaf. As the most widely used analytical instruments all require liquid samples, the goal of sample extraction and preparation is to transfer the metabolites of interest from the complex plant matrix into a homogeneous liquid phase suitable for instrumental analysis. Kim and Verpoorte [14] recently summarized the solvents used for sample extraction. Aqueous alcohol (methanol or ethanol) are the mixtures being most frequently used; alternatively, sample extraction with chloroform/methanol mixtures followed by a phase partitioning step, implemented by adding water, to separate the polar and nonpolar metabolites is also frequently employed. Once again, the choices made for sample extraction and preparation determine the range of metabolites that will be

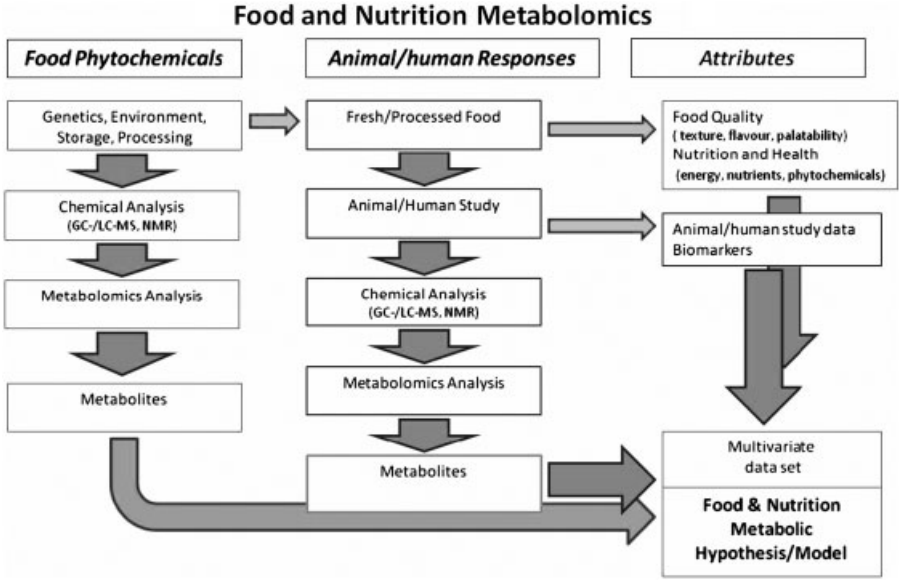

Figure 1. An overview of a food science–metabolomic work flow.

detected and measured in the later stages of metabolomic analysis.

2.2 Instrumental analysis of metabolites

Metabolite characterization of prepared sample extracts can be achieved by a variety of instrumental approaches, most commonly mass spectrometry (MS) or nuclear magnetic resonance (NMR) spectroscopy.

2.2.1 NMR spectroscopy

Typically, in NMR metabolomic analysis, sample extracts are dissolved in a deuterated solvent and the resonance frequencies of the hydrogen nuclei in each metabolite are measured. NMR involves minimal sample preparation and is ideal for aqueous samples such as urine and blood serum. However, with the diverse range of metabolites commonly encountered in plant extracts finding a suitable and inexpensive deuterated solvent can be difficult and often selective solvent extraction or partial purification of a plant extract is required to give a stable soluble sample. NMR analysis is quantitative under appropriate experimental conditions; however, each metabolite typically gives multiple signals arising from the chemically different hydrogen atoms contained within each metabolite. The multiple signals from different metabolites usually overlap, so that unique signals, or combinations of signals, must be found for each individual metabolite, something that is not always possible. A further consequence of overlapping signals is that, in the NMR analysis of complex plant extracts, actual compound identification of many metabolites may not be possible. Perhaps for these reasons, NMR analysis has not been as widely used in plant metabolomics as MS-based methods.

2.2.2 GC-MS

GC-MS involves the coupling of a gas chromatograph (GC) with a mass spectrometer and has been widely used in metabolomic analysis. Compound volatility is an absolute requirement for GC, making GC-MS the ideal technique for the measurement of volatile flavor metabolites. GC-MS has also been widely used to measure primary metabolites, such as sugars and amino and organic acids. However, as these compounds are usually polar and nonvolatile, conversion to volatile trimethylsilyl or *t*-butyldimethylsilyl derivatives is necessary before analysis [13]. These additional chemical manipulations of the sample add complexity to sample preparation and often make the identification of unknown metabolites more difficult, as the mass spectral fingerprint of each metabolite can be dominated by ions derived from the chemical derivatizing reagent. Despite these limitations, GC-MS analysis is a robust, sensitive, and relatively inexpensive method for the analysis of plant primary metabolites [13], as well as the analysis of volatiles, terpenoids, fatty acids and lipids, plus simple glycosides and phenolics.

2.2.3 LC-MS

Improvements in the efficiency of analyte separation by LC, combined with new accurate high-resolution mass spectrometers, enable improved compound identification capabilities, and increasingly sophisticated data systems have made LC-MS a valuable adjunct to GC-MS. LC-MS involves the coupling of LC with MS and like GC-MS, metabolite measurement involves a physical separation of metabolites using a chromatographic step followed by a mass separation using MS. The eluent from the chromatography column is transferred into the mass spectrometer through an ion source where the dissolved metabolites are converted into gas-phase charged species (ions). Ion formation by electrospray ionization (ESI) is most often used for metabolite analysis although not all compounds are ionizable by this method. Increasingly, sophisticated instrument control and data analysis systems have made LC-MS the method of choice for comprehensive metabolic profiling and biomarker discovery.

Typical workflows for metabolome analysis by LC-MS have been described in detail elsewhere [15, 16]. LC-MS employs a number of variants including chromatography columns with different stationary phases (reversed phase, normal phase, and HILIC) to provide alternative methods for metabolite separation, and the use of alternative ion sources such as atmospheric pressure chemical ionization (APCI) to generate ions from a different range of metabolites. In some cases, the LC stage is modified to effect little or no separation of metabolites: this is known as direct infusion MS (DIMS) [17]. The particular advantage of DIMS is the short analysis time for each sample. Some compounds, however, cannot be analysed in the presence of others because of ion-suppression effects that occur with ESI. Usually, some form of chromatographic separation is required, particularly if the aim is to identify individual metabolites.

Although LC-MS is widely utilized for metabolomic research, there are a number of limitations associated with the use of LC-MS for metabolomic approaches to plant and nutrition research [11]. These include, but are not limited to: inadequate coverage of the metabolome; detection of numerous unidentified metabolites; inability to extract data reliably from raw MS data files; variable quality of MS data due to ion-suppression effects; and the inability to interpret the changes in metabolic profiles adequately. It can be expected that the significance of these issues will decline as metabolomic research develops and they are addressed [11].

As the instrumentation for metabolomics continues to advance, the challenge for metabolomic analysis will increasingly move to the interpretation of metabolomic

results and the chemical identification of significant metabolites. A number of recent reviews provide overviews of methods for data processing and analysis [18] and of database resources available to assist with metabolite identification and pathway analysis [19, 20].

3 Metabolomics of food derived from plants

A selection of published plant and food metabolomic studies is summarized in Table 1.

Metabolomic studies have been conducted in plant and plant-based foods for a variety of reasons, including breeding (cultivar development), plant nutrition, postharvest storage, and quality (flavor) [21].

3.1 Phenotypic and metabolite diversity for cultivar development

Cultivar development is the manipulation and introduction of traits to improve the phenotypes of plants. Phenotypic diversity within a species is required for successful plant breeding [22] and consequently, plant breeders are concerned with both maintaining diversity within germplasm collections used for breeding, and ensuring that a desired trait is sufficiently heritable, before attempting to breed for it. Furthermore, the genetic and metabolite diversity found between domesticated and wild relatives provides resources for breeders to introduce new traits into commercial cultivars [23]. The phenotype of an individual genotype is related to its metabolome. Metabolome analysis and comparison of the metabolomes of individual genotypes in a germplasm collection is an excellent way to measure metabolite diversity and to locate individuals with metabolomes that differ from the ''typical'' individual for the species. For example, Schauer et al. using introgressed tomato lines showed that metabolite loci are associated with whole-plant phenotypes including yield-associated traits [24]. This type of study demonstrates that metabolome analysis can be used for the discovery and monitoring of phenotypic traits, including those associated with nutritional value.

Berryfruits, including raspberries (*Rubus idaeus*), are considered to confer health benefits to human consumers [25] and the identification of the bioactive constituents of the fruit and development of new raspberry cultivars with enhanced concentrations of these phytonutrients are therefore key goals for plant breeders. Stewart et al. [26] used a direct infusion (DI)LC-MS approach to measure metabolites in fruit from 300 individual plants of a raspberry population segregating for a variety of traits and grown in two different environments. Multivariate statistical analysis of the MS data identified significant environmental and genetic effects on metabolite concentrations within the population with major effects on the anthocyanin components. The identification of the principal contributors to metabolite diversity and heritability of metabolites within this raspberry population could be used to focus selection and recrossing on specific individuals with high concentrations and a diversity of anthocyanin metabolites, to enable the development of new fruit cultivars with tailored phytochemical composition and the potential for enhanced human health attributes. Further examples of crops where phytochemical diversity has been explored by a metabolomic profiling approach include tomato [24], potato [27–29], apple [30], and maize [31].

Metabolite analysis of individuals in a population to discover metabolite variation is a promising approach for breeders. As suggested by Giovannoni [32] and Harrigan et al. [23], the application of metabolomic approaches to measure metabolite diversity will lead to a better understanding of the biology underpinning important plant food-quality traits. However, combining genetic information (genetic maps) with metabolite data sets appears to hold even more promise, especially when the target traits are complex and vary in a quantitative manner as defined by quantitative trait loci (QTL) [33]. The combination of genomic breeding strategies, such as marker-assisted selection with metabolome analysis, could dramatically shorten the breeding cycle time and lead to the faster development of improved cultivars.

3.2 Environmental responses

The phenotype of a plant is a combination of its genetics and the environment in which the plant is grown, that is phenotype = genotype × environment. Most, but not all, major crops have consistent genetics and it is normally assumed that all individuals of a specific cultivar have the same or very similar genetics. The differences observed in phenotype, and therefore metabolite composition in a particular species or cultivar, are therefore largely due to environmental influences. These environmental differences can be on a micro scale in food plants, such as within a fruit tree (between the outer and the inner canopy), a medium scale such as within a field (exposed to different soil types), or on a macro scale such as found in variation in climatic conditions between growing regions. Furthermore, the degree to which a specific cultivar is changed by its environment appears to be genetically determined. For example, we have found that, among apples grown in three climatically distinct regions of New Zealand, some cultivars produced apples with similar polyphenolic compositions, whereas for other cultivars the polyphenolic content varied substantively with growing region [34]. Numerous metabolic pathways may be altered by environmental conditions, such as climate (temperature, light, and water), physical (soil), and attack by pathogens and pests. The metabolomic approach is therefore an attractive way of understanding the changes in the phytochemical composition of a food plant which occur in response to changing environmental conditions.

Table 1. A selection of metabolomic studies on common foods summarizing the purpose of these studies, the methodologies used and the major conclusions reached

Purpose	Plant/food	Sample preparation	Analysis	Data analysis	Outcome	Reference
Metabolite diversity/ breeding	Raspberries	Acetic acid/acetonitrile	DIMS-LC-MS	PCA	Variation attributed to anthocyanins	Stewart et al. [26]
Metabolite diversity/ breeding	Tomatoes	MeOH with H_2O/ chloroform partition	GC-MS	PCA	Identification of 889 metabolic loci, metabolites associated with yield trait	Schauer et al. [24]
Metabolite diversity/ breeding	Potatoes	MeOH with H_2O/ chloroform partition	GC-MS	PCA Correlation	Detected variation between cultivars and landraces	Dobson et al. [27, 28]
Geographic origin	Grapes/wine	95% EtOH	NMR	PCA/PLS-DA	Grapes from different regions can be distinguished	Son et al. [69]
Geographic origin	Coffee	MeOH/H_2O	GC-FID/LCMS	PCA	Samples from Asia, Africa, and S. America could be distinguished	Choi et al [35]
Geographic origin	Green tea	Hot H_2O	NMR	PCA/OPLS-DA	Growing region with high temperature, rainfall and sun increased the anine concentrations	Lee et al. [70]
Geographic origin	Olive oil	SPME	GC-MS	Linear discriminant analysis	Distinguished from different productions areas within a region	Cavaliere et al. [36]
Quality	Green tea	MeOH/H_2O/chloroform	GC-MS/LC-MS	PCA/OPLS-DA	Tea grown on greater shade had higher umami and less astringency and these attributes were related to sugars, amino acid and phenolics	Ku et al. [71]
Postharvest disorder	Apples		GC-MS			Rudell et al. [38]
Sensory	Tomatoes	Lyophilisation/SPME/ extraction H_2O:MeOH	GC-MS and LC-MS	PCA/PLS (with validation)		Thissen et al. [21]
Sensory	Wine/grapes	Wine directly added to NMR tubes	NMR	PCA/PLS-DA	Metabolites related to sun/shade treatments and mouth-feel parameters	Rochfort et al. [39]
GM assessment	Tomatoes	80% MeOH/H_2O	GC-MS, LC-MS, CE-MS	ANOVA/PCA	GM lines were within traditional cultivar variation	Kusano et al. [41]

3.3 Geographic origin of foods

Geographic origin can be an important attribute of commercial plant-derived foods as it gives an indication of quality, phytochemical composition, the amount of phytochemical variation expected and appeals to certain values of consumers. Additionally, where regions produce crops with specific ''terroir,'' being able to classify geographic origin is a way of protecting the reputation and value of specific products as is specifically recognized in the European Union under ''Protected Designation of Origin'' and ''Protected Geographical Indication'' classifications.

The quality of coffee is determined by the metabolites contained within the coffee bean, which in turn are determined by environmental and genetic factors. Choi et al. [35] used a combination of targeted (protein, carbohydrate, monosaccharide, and amine) and nontargeted (GC-FID and LC-MS) analytical methods to distinguish effectively between coffee beans originating from three different production regions (Asia, South America, and Africa). Coffee quality has been well studied and it is well established that flavor and taste, the most important attributes, are strongly influenced by the environmental factors of soil, climate, and genotype. Extensions of this type of study may be able to identify the combinations of genotype, soil, or climate that could yield a predetermined set of quality attributes.

Olive oil is an important component of the ''Mediterranean diet'' and has variable attributes across different production regions. Cavaliere et al. [36] used solid-phase microextraction (SPME) and chemical ionization GC-MS to investigate the lipoxygenase cascade in olive oils produced in different areas of Calabria, Italy. Linear discriminant analysis showed that oils from four production areas within Calabria could be distinguished in a robust, statistically significant way. In this case, the aldehyde components contributed most to the ability to discriminate the oils by production area. The ability to identify and classify oils from different areas might be used for commercial product differentiation and in support of Protected Designation of Origin classifications.

3.4 Postharvest and food production

From a global perspective, plant-derived foods are typically either processed or subjected to postharvest storage before consumption by humans. The changes in metabolite composition that occur during these processes are immensely important both for the acceptability and for the nutritional content of the food that reaches consumers. Plants contain a complex mixture of metabolites and predicting how the concentrations of these metabolites will change during postharvest storage and processing is extremely difficult. Furthermore, fresh plant foods are living organisms, and hence biosynthetic and catabolic metabolite pathways continue to operate during storage. Nonoptimal storage conditions can not only accelerate product breakdown but may also result in microbial infections and the occurrence of physiological disorders resulting in shrunken, discolored, or rotted products. Postharvest disorders have their genesis in complex metabolic processes occurring both before and after harvesting and make foods unacceptable to consumers.

Superficial scald is a postharvest disorder of apples and pears resulting in a blackened skin and is attributed to chilling stress following low-temperature storage [37]. Superficial scald is particularly prevalent in apple cultivars such as ''Granny Smith'' and although the mechanism is only partly understood, it is thought to involve the oxidation of lipid metabolites, such as α-farnesene, in the natural layers of wax on the skin. Using untargeted metabolic profiling of ''Granny Smith'' apples and various scald suppressing treatments, Rudell et al. [38] found that there were extensive metabolite changes that preceded the development of the symptoms of superficial scald. A number of known oxidation products of the sesquiterpene α-farnesene were associated with the initiation of superficial scald. In addition, some new and interesting associations with unidentified peel triterpenoids similar to ursolic acid were identified. By identifying new metabolites and pathways associated with this major disorder of apples, the metabolomic approach used here has expanded our understanding of the induction and of symptom development in superficial scald and may ultimately lead to improved storage management options for fruit.

3.5 Sensory and quality attributes

Sensory and quality attributes of plant foods are determined by the metabolites they contain [8, 21] and an understanding of the components of the metabolome is necessary to optimize these attributes. Sensory properties are particularly important to primary production sectors and processed food industries and are usually the major focus in the development of new and improved products. Most sensory attributes are associated with known metabolites and traditional research often focuses on the targeted analysis of a small number of metabolites or ''flavor impact'' compounds. The metabolomic approach offers the opportunity to discover new sensory-associated metabolites or the combinations of metabolites with new sensory outcomes for consumers. Like metabolomic analysis, sensory analysis can yield an extensive data set with many complex interactions between attributes. Statistical analysis is required to discover the relationships between these two complex data sets. Thissen et al. [21] recently used a data set of tomato metabolites comprising data from three analytical platforms (SPME GC-MS, GC-MS, and LC-MS) and compared this with a sensory data set of 22 attributes for 19 tomato samples. In this case, principal component analysis (PCA) proved inadequate for identifying the relationships between the metabolite and the sensory data sets and hence a partial least squares (PLS)

approach followed by substantial model validation was used. Using PLS regression, specific metabolites, which were either chemically identified or of known elemental composition, were found to be associated with sensory attributes such as aftertaste duration, sourness, sweetness, and taste intensity. An important observation was that the associated metabolites did not always exhibit sensory properties. This may be a consequence of this statistical method or that the metabolites may be biosynthetic precursors, may have masking or synergistic interactions, or may regulate biosynthesis. A metabolomic strategy allows researchers to focus on the topic of the study unimpeded by the limitations of incomplete or incorrect prior knowledge.

Rochfort et al. [39] analyzed wine samples directly by NMR (without sample preparation) and were able to distinguish between wine made from grapes with different degrees of exposure to the sun. These wines also varied in flavor and aroma, and in characters related to mouth feel. The statistical model developed from this study was able to classify wines in a manner similar to sensory analysis. As sensory analysis is an expensive technique involving trained human panellists, the possibility of using metabolomic analysis to provide a simpler and less expensive approach for sensory classification is attractive.

3.6 Genetic modification of food plants

A number of genetically modified (GM) crops are in commercial production, and more are likely to be introduced to the human food supply in the coming years. As there is substantial opposition to genetically engineered foods, the ability to measure (by quantitative assessment) the actual difference between a GM crop and a non-GM crop is important, but very challenging and is ideally suited to a metabolomic approach.

When GM plants are produced, a new gene is inserted into the existing plant genome with additional DNA sequences that include a gene promoter; a gene terminator; a selectable marker (to isolate transformed plant cells following the transformation procedure); and, when *Agrobacterium tumefaciens* infection is used for transformation, additional DNA related to the bacterial vector. Furthermore, genes may be introduced by infection: with a DNA-transferring bacterium; shot into the cell by force (biolistics); or by migration into plant cell protoplasts by electroporation. These processes can insert the new gene into random locations and in multiple copy numbers. Therefore, the transformation procedure, the additional DNA, plant tissue culture, and unrecognized biosynthetic pathways may all contribute to the development of unexpected, and possibly undesirable, phenotypes in the resulting GM plants. The untargeted analysis of metabolites is therefore an ideal approach for determining both the unexpected effects of the genetic transformation, and the holistic ''equivalence'' of the GM plant compared with the original.

Acceptance of GM plant-derived foods involves detailed risk assessment, an aspect of which is the estimation of substantial equivalence [40]. In essence, this is proving that the traits exhibited by GM plants are comparable with those of traditional cultivars of the specific crop. Kusano et al. recently used three metabolomic platforms (GC-MS, LC-MS, and CE-MS) to study the metabolome of two tomato lines expressing a gene for the protein miraculin (a sweetness enhancer) and to compare them with metabolomes from five standard tomato cultivars [41]. The combined data sets from the three platforms contained 166 identified metabolites, which represented 85% of the chemical diversity contained in the LycoCyc database (www.gramene.org/pathway/lycocyc.html). In total, 1376 peaks were detected, and of these 92% were within acceptable deviation, which was defined as within the symmetric boundary determined by the non-GM cultivar furthest from the control line. Interestingly, there was only small variance (4–6%) between the two GM lines and the control and this difference was similar to that measured between GM and non-GM controls for maize and soybean [42]. These GM effects on variable metabolites were attributed to tissue culturing, the transformation procedure, the position of the insert, and the marker gene used for selection. Overall, the metabolite variance for the GM tomato lines was within natural cultivar variance; as also found for potato [43].

4 Metabolomics: A tool for measuring animal and human responses to food phytochemicals

Consumption of plant metabolites present in food may result either in their excretion from the body unmodified [44, 45] or, more commonly, after biotransformation by the body and/or by gut microflora into further metabolites that appear in the serum, urine, or feces [46–49]. Common modes of biotransformation include the oxidative degradation of dietary phenolics by the intestinal microflora [50] and their subsequent conjugation as glucuronides or sulfate esters before excretion in the urine [48]. Metabolomic analysis of serum, urine, or feces can therefore provide a snapshot of the state of an organism's metabolic processes, of recent dietary intake or exposure to exogenous chemicals, and of the biological activity of the intestinal microflora [51, 52]. Reduced or elevated concentrations of particular metabolites may provide indicators (biomarkers) of the status of particular metabolic processes or tissue states and indirectly of the health status of the organism [53].

4.1 Human and animal studies of biomarkers of dietary intake

Phytochemicals ingested as part of the diet may appear in the serum, feces, or urine, either unchanged or chemically

modified by microbial or mammalian metabolism. Such metabolites can serve as markers of dietary intake complementing and validating information obtained by dietary surveys or providing checks of compliance by human subjects within experimental protocols [46, 52]. For example, consumption of apple, grapefruit, and orange results in elevated concentration of the phenolic compounds, phloretin, naringenin and hesperetin, respectively, in the urine [4, 54]. Consumption of dry grape juice and wine by men and women resulted in increased urinary excretion of 18 phenolic acids consistent with extensive gut microbial metabolism [55]. Many of the metabolites found in urine or serum, may, however, be produced by multiple biosynthetic routes [46, 47]. Thus, hippuric acid is produced by the intestinal microbial metabolism of phenolic dietary compounds such as chlorogenic acid [48] and also from metabolism of aromatic amino acids. Furthermore, the amount of hippuric acid excreted also depends on the basal diet itself [56], making interpretation of dietary markers from uncontrolled diets uncertain. Importantly, Primrose et al. very recently summarized the results from several current studies aimed at determining the potential of using metabolite biomarkers for dietary intake [52]. Results from the MEtabolomics to characterize Dietary Exposure (MEDE) study demonstrated that postprandial (3 h) urine contained metabolites indicative of recent dietary intake. Other key findings of MEDE were that the same metabolites are associated with particular meals (i.e. a standard breakfast), there is metabolite variation among study participants, and several physiological, and behavioral factors had little effect on dietary metabolite biomarkers. These results ''demonstrated the considerable potential of metabolomics as a route to identify and develop novel and robust biomarkers of dietary exposure.''

Markers of phytochemical dietary intake would also be valuable to have for grazing animals, where diet choice may be important in determining nutritional intake and value, geographic origin, and to the future sustainability and species diversity of the plant ecosystem. In a study with sheep, nine heath-land plant groups and three mixed forage diets containing heather (*Calluna vulgaris*) (10, 20, and 30%) were analyzed by GC-MS [57]. Additional GC-MS metabolic profiling of plasma and feces from sheep fed the heather-containing diets allowed discrimination between diets and between animals maintained on these diets, with identification of the plant metabolites responsible for these differences.

The ability of antioxidant polyphenols to counteract undesirable physiological effects (e.g. oxidative stress) associated with high-fat diets was tested using the common dietary flavanol, catechin, fed to male Wistar rats on high (15 and 25%) and normal (5%) fat diets over 6 wk [58]. Urinary metabolites were analyzed by LC-MS. While supplementation with catechin did not prevent the increase in metabolite biomarkers of oxidative stress induced by the high-fat diets, a number of other metabolites perturbed by the high-fat diet were restored to normal concentrations. These results show that the metabolomic approach may lead to a better understanding of the biological system, even where the expected results were not obtained.

The health status of experimental animal may affect the adsorption, biotransformation, and excretion of dietary metabolites. The interleukin-10-deficient ($IL10^{-/-}$) mouse shows increased intestinal permeability and develops intestinal inflammation in the presence of normal intestinal microflora. Thus, $IL10^{-/-}$ mice, fed diets containing kiwifruit (*Actinidia* spp.) extracts, excreted more dietary-associated metabolites in their urine than did control mice on the same diets [59].

4.2 Animal and human responses to phytochemical intake studies

While the NMR metabolic profile of human urine and serum has been extensively studied in terms of human gender, age, disease, and ethnicity, there has been less emphasis on the effects of diet on NMR metabolic profiles [60]. Studies with humans are complicated by the variable genetic background, unrestricted diet, and personal history of human subjects. Plant-derived metabolites measured in urine or serum may arise from a combination of microbial and/or mammalian metabolism [61]. Metabolic analysis, however, provides the opportunity to detect subtle metabolic effects associated with states of ''healthiness'' rather than disease, and has revealed evidence for the existence of different metabolic phenotypes associated with individual humans [62].

Humans may ingest phytochemicals either as part of their normal diet or, in a more concentrated form, as herbal supplements in an attempt to obtain health benefits. The ability of herbal supplements and plant extracts to act as nutraceuticals and restore normal metabolic function has been tested in a number of studies using animal models of disease [59, 63, 64]. A rosemary extract containing folic acid was fed to streptozocin-induced diabetic rats in an acute study, resulting in detectable differences in some urinary metabolites [63]. The synergy between *Panax ginseng* and *Salvia miltiorrhiza* (7:3), used in the traditional Chinese medicine *Shuanglong* Formula, was tested in Sprague–Dawley rats using a surgical model of acute myocardial infarction [64]. After oral gavage with the herbal extract, 24-h urine samples were collected over the 21 days of the experiment and analyzed by LC-MS. A combined analysis of changes in urinary metabolites, serum biochemistry, and histopathology led the authors to suggest that the combined herbal extract was more effective at reducing cardiac injury, possibly through the regulation of myocardial energy metabolism.

Application of these approaches to humans is considered unethical and human studies are restricted to working with preselected cohorts of overtly healthy subjects exhibiting

preliminary signs of an adverse health condition. A human trial with subjects described as being ''at high risk for cardiovascular disease'' tested the effect of consumption of cocoa (*Theobroma cacao*) polyphenols or a control diet over 2, 4-wk periods [65]. The study involved targeted LC-MS profiling of phenolics in 24-h urine and fasting plasma, and identified increased phase II metabolites (glucuronides and sulfate conjugates of phenolics) as biomarkers resulting from the consumption of cacao or flavanol-rich foods. Although the measurement of protein biomarkers supported an anti-inflammatory effect of cocoa [66], no evidence for metabolic effects relevant to health outcomes of the human subjects appears to have been reported.

The metabolic responses of free-living subjects to daily consumption of chocolate has been reported [67] in a study in which subjects were classified into two groups according to the self-reported anxiety scores. Urine and serum samples were taken at the beginning, during, and at the end of the 2-wk study and analyzed by NMR and GC- and LC-MS. Dark chocolate was identified as reducing the urinary excretion of cortisol and catecholamines, reducing stress markers and partially normalizing stress-related differences in energy and gut microflora metabolism in those individuals reporting high anxiety. In a second study, a dietary cocktail consisting of resveratrol, tomato and green tea extract, antioxidants and ω-3 polyunsaturated fatty acids was tested on 36 healthy overweight men with low-grade chronic inflammation as evidenced by mildly elevated concentrations of plasma C-reactive protein in a double-blind, placebo-controlled, crossover trial over a period of 5 wk [68]. In addition to quantification of recognized inflammatory and oxidative stress biomarkers in plasma and urine, this study included proteomic and transcriptomic analysis, and metabolomic analysis of 274 plasma metabolites by GC- and LC-MS. In contrast to the results obtained using the recognized biomarkers, integration of the data obtained from these various approaches allowed ''the detection of multiple subtle health effects of a mix of dietary components in relatively healthy overweight subjects.'' These studies demonstrate the potential of metabolomic analysis to provide evidence for the health benefits of foods in ''healthy'' free-living humans.

5 Concluding remarks

Metabolomics is proving to be a powerful approach for the analysis and comparison of the metabolomes of plants, plant-derived foods, and animals and has been demonstrated to have wide applicability to food and nutrition science. The nontargeted, holistic aim of metabolomics allows the visualization of the metabolome and enables the discovery of new associations between metabolites and phenotypes and new understandings of complex biological processes. This is particularly true for nutrition, where there are intricate interactions between the complex food metabolome and the consumer metabolome.

Although targeted analysis has been widely used in the past, the concept of nontargeted, holistic measurement of metabolites is relatively new and for many of the applications discussed above the results obtained so far represent only an indication of what may be achieved as analytical technology plus techniques for data analysis and interpretation develop further. As metabolomic analysis matures, new technology will increase the extent of the metabolome that can be measured. Increasingly, the studies are using multiple platforms to extend metabolite coverage. Currently, many of the metabolites that can be measured do not have an actual chemical identity assigned to them. The ability to place metabolites into metabolic pathways and networks and hence assign functional roles to them will be enhanced as more metabolites are chemically identified and this information becomes added to publicly accessible databases [20]. Metabolomic analysis is likely to become increasingly less expensive and better able to produce comprehensive metabolite coverage across multiple metabolomes. The systems biology approach, integrating genomics, proteomics, and metabolomics, is a powerful approach for developing an increased understanding of complex biological processes.

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