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Metabolomic and its approach in food science

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Abstract
Metabolomics detects and quantifies the low molecular weight molecules, known as metabolites (constituents of the metabolome), produced by active, living cells under different conditions and times in their life cycles. NMR is playing an important role in metabolomics because of its ability to observe mixtures of small molecules in living cells or in cell extracts. Metabolomics, a new technology, is a promising tool for food processors, food quality and safety laboratories, food chain providers, and also plant breeders. Metabolomics involves the rapid, high throughput characterization of the small molecule metabolites found in an organism. Metabolome is closely tied to the genotype of an organism, its physiology and its environment and offers a unique opportunity to look at genotype-phenotype relationships. Metabolomics is increasingly being used in a variety of health applications including pharmacology, pre-clinical drug trials, toxicology, transplant monitoring, newborn screening and clinical chemistry. However, a key limitation to metabolomics is the fact that the human metabolome is not at all well characterized.

Keywords: Metabolomic, food science, human metabolome

Introduction
Metabolomics is a newly emerging science which can be seen as an advanced, specialized form of analytical biochemistry. Metabolomics is a relatively new field of ‘‘omics’’research concerned with the high-throughput identification and quantification of small molecule (<1500 Da) called as metabolites in the metabolome (German, Hammock, & Watkins, 2005) [19]. The metabolome is formally defined as the collection of all small molecule or metabolites or chemicals that can be found in a cell, organ or organism This technology is centred around the detection of small molecules and, by definition, excludes the organic biopolymers such as proteins and fatty acids. Important small metabolites include e.g. amino and other organic acids, sugars, volatile metabolites and most of the diverse secondary metabolites found in plants such as alkaloids, phenolic components and coloured metabolites such as carotenoids and anthocyanins.

Metabolomics approach is the aim to gain the broadest overview possible of the biochemical composition of complex biological samples in just one or a small number of analyses. Liquid or gas chromatography (LC or GC) are usually used to separate the individual components in complex organic extracts after which either Mass Spectrometry (MS) is employed to detect the metabolites present or Nuclear Megnetic Resonance (NMR) may be used. Metabolomics, which is also known as metabonomics (Holmes, 1999) [37] or metabolic profiling is usually used either for ‘fingerprinting’ samples to perform comparative analyses to detect differences of for ‘profiling’ where individual differential metabolites are identified for further analysis.

Metabolomic analyses have been generally classified as targeted or untargeted (Fig. 1). Targeted analyses focus on a specific group of intended metabolites with most cases requiring identification and quantification of as many metabolites as possible within the group (Ramautar, Demirci, & Jong, 2006) [44]. Targeted analyses are important for assessing the behavior of a specific group of compounds in the sample under determined conditions. Targeted metabolomics typically requires higher level of purification and a selective extraction of metabolites. In contrast, untargeted metabolomics focuses on the detection of as many groups of metabolites as possible to obtain patterns or fingerprints without necessarily identifying nor quantifying a specific compound(s) (Monton & Soga, 2007) [36]. Untargeted analyses have been used in the identification of possible fingerprints of biological phenomena such as plant diseases.

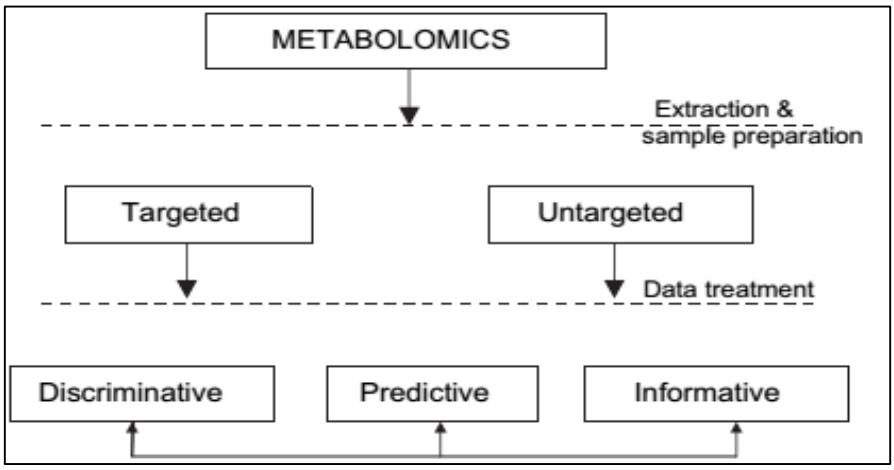

Fig 1: General classification of metabolomics

Based on the specific objective of the analysis and data manipulation, most metabolomic studies can also be classified as discriminative, informative, and/or predictive (Fig. 1). Discriminative analyses have been aimed to find differences between sample populations without necessarily creating statistical models or evaluating possible pathways that may elucidate such differences. Wine has been classified by grape variety and production area by metabolomic techniques (Son *et al.*, 2008). Discrimination is usually achieved by the use of multivariate data analysis (MVDA) techniques intended to maximize classification, principal components analysis (PCA) being the most used tool.

Informative metabolomic analyses have focused on the identification and quantification of targeted or untargeted metabolites to obtain sample intrinsic information. Informative metabolomics has been used in the development and continuous update of metabolite databases such as the human metabolome database (Wishart *et al.*, 2007) [58]. Possible pathways, discovery of novel bioactive compounds, discovery of biomarkers, creation of specialized metabolite databases, and metabolites functionality studies can also be carried out by informative metabolomics. Finally, some metabolomics reports have been predictive. In this case, statistical models based on metabolite profile and abundance are created to predict a variable that is difficult to quantify by other means. Metabolite-based models for prediction of green tea sensory quality have been developed (Ikeda, Kanaya, Yonetani, Kobayashi, & Fukusaki, 2007). These models are usually produced by partial least square (PLS) regression.

The process of metabolomic analysis

Metabolomic analyses consist of a sequence of steps including sample preparation, metabolite extraction, derivatization, metabolite separation, detection, and data treatment (Fig. 2). However, not every steps is always needed. Only detection and data analysis have been essential steps in all reported metabolomics studies. The selection of the steps depends on the type of study (untargeted vs. targeted), kind of sample (e.g. solids vs. liquids), instrumentation to be used for separation (e.g. GC vs. LC) and detection method (e.g. MSvs. NMR).Table 1 summarizes recent metabolomics studies used for food analysis.

Sample Preparation

Solid samples such as apple peel (Rudell, Mattheis, & Curry, 2008) [45] and potatoes are typically ground under liquid nitrogen or after freeze-drying. Proper grinding enhances the release of metabolites during extraction.

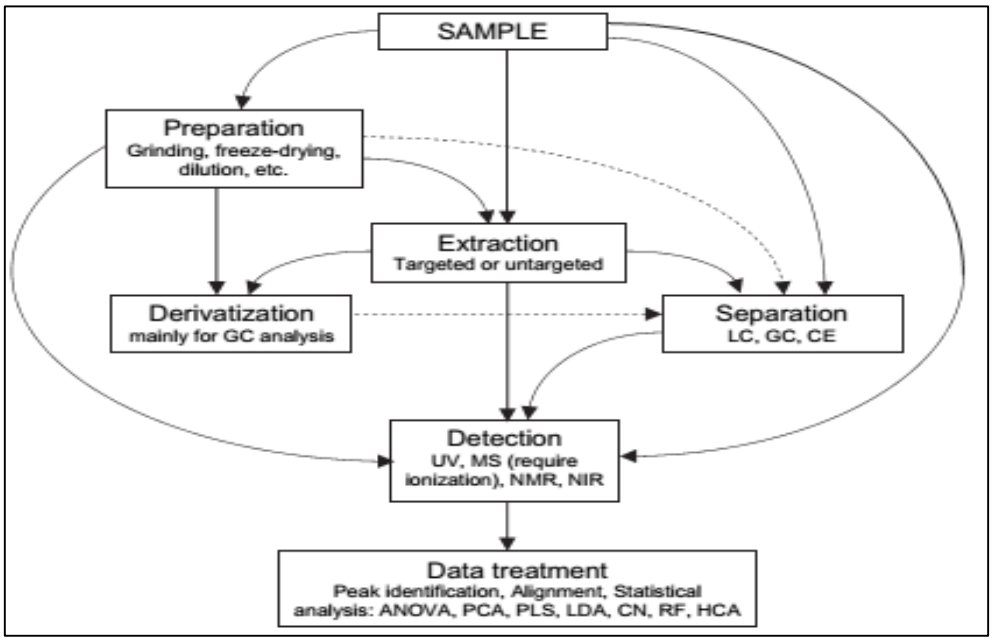

Fig 2: Schematic representation of the process of metabolomics analysis

Freeze-drying acts as a concentration step and minimizes possible differences in metabolites due to dissimilarities in moisture content between groups of sample. Other concentrated liquid samples such as honey can be diluted as a preliminary step. However, to maximize the amount of information to be collected, concentration steps are more suitable. For example, metabolites in wine (Son *et al.*, 2008) and volatiles in olive oil (Cavaliere *et al.*, 2007) [10] have been concentrated by lyophilization and solid phase microextraction (SPME) respectively.

Extraction

The initial extraction procedure is aimed at maximizing the amount and concentration of the compounds of interest. For this reason, extraction is probably the most critical step in metabolomics. In untargeted metabolomics, the nature of compounds of interest is mostly unknown. Hence, several solvents and extraction methods should be tested and compared between the groups of samples. Most reports on untargeted food analysis do not describe preliminary comparisons among extraction solvents tested. However, the extraction methods used in foods have been similar to those found optimal in comparable research fields such as non-food plant metabolomics. For instance, the combination methanol-water-chloroform (MeOH-H2O-CHCl3) in different proportions was shown to be superior to other solvents for untargeted studies in plants such as Arabidopsis thaliana (Gullberg, Jonsson, Nordstrom, Sjostrom, & Moritz, 2004) [22] because of its capacity of extracting both hydrophilic and hydrophobic compounds. Therefore, the effectiveness of MeOH-H2O-CHCl3 in green tea (Pongsuwan *et al.*, 2008), potatoes and other foods was anticipated.

Table 1: Most common metabolomics processes in food analysis

Sample: Purpose of analysis	Type	Extraction and preparation	Separation–detection	Data treatment	Reference
Apples: light induced changes in peel	Untargeted/discriminative	MeOH Derivatization for GC–MS	GC–MS LC–MS	PCA	Rudell *et al.*, 2008
Berries: polyphenol composition	Targeted/informative	Acetic acid + water C18 and Sephadex LH 20 columns	LC–MS DIMS	Compound identification	McDougall *et al.*, 2008
Broccoli, mustard, and brassica: glucosinolates composition	Targeted/informative	Hot water (90 °C) + sonication	$LC–MS^n$	Compound identification	Rochfort *et al.*, 2008
Broccoli: variety differentiation	Untargeted/discriminative	Freeze dried MeOH + H_2O	LC–UV–MS DIMS	PCA, ANOVA	Luthria *et al.*, 2008
Cheese: Production control	Untargeted/informative	–	IMS	Compound identification	Vautz *et al.*, 2006
E. coli: glycolisis metabolites	Targeted/informative	Indirect thermal treatment	LC–MS	Compound identification	Schaub & Reuss, 2008
Ginseng: variety differentiation	Untargeted/discriminative	Deuterated MeOH + buffered water	NMR	PCA	Kang *et al.*, 2008
Green: tea quality	Untargeted/predictive	Freeze dried MeOH + H_2O + $CHCl_3$	UPLC–TOF–MS	PCA, PLS	Pongsuwan *et al.*, 2008
Honey: origin verification	Untargeted/discriminative/ predictive	Buffered water	NMR	PLS–GP	Donarski *et al.*, 2008
Maize: GMO identification	Untargeted/discriminative	MeOH + water + ultrasonication	CE–TOF–MS	Student's t, PCA	Levandi *et al.*, 2008
Meat: quality/safety	Untargeted/discriminative	Neutral desorption	EESI–MS	PCA	Chen *et al.*, 2007
Olive oil: origin differentiation	Targeted/discriminative	SPME	GC–CI–MS	LDA Kruskal–Wallis and Wald–Wolfowitz tests	Cavaliere *et al.*, 2007
Pine mushrooms: quality differentiation	Untargeted/discriminative	MeOH + H_2O + $CHCl_3$	NMR	PCA	Cho *et al.*, 2007
Potato: GM differentiation	Untargeted/discriminative	MeOH + H_2O + $CHCl_3$ Derivatization for GC–MS	GC–MS DIMS	PCA	Catchpole *et al.*, 2005
Potato: identification of cultivars	Untargeted/discriminative/ informative	Freeze dried + MeOH + water + chloroform + derivatization	GC–TOF–MS	ANOVA, PCA	Dobson *et al.*, 2008
Potato: variety differentiation	Untargeted/discriminative/ informative	MeOH + H_2O + $CHCl_3$ Derivatization for GC–MS	GC–MS DIMS	RF	Beckmann *et al.*, 2007
Soybean: GMO differentiation	Untargeted/informative	MeOH–EtOH–H_2O	CE–TOF–MS	Compound identification	Garcia-Villalba *et al.*, 2008
Spinach: *E. coli* contamination	Untargeted/discriminative	Neutral desorption	EESI–MS	PCA	Chen *et al.*, 2007
Tomato paste: changes during production	Targeted to antioxidants/ informative Untargeted/informative	Targeted: H_2O–MeOH and MeOH–$CHCl_3$ Untargeted: Formic acid–MeOH–H_2O	LC–antioxidant detector LC–TOF–MS	ANOVA, PCA	Capanoglu *et al.*, 2008
Tomato: metabolite correlations	Untargeted/predictive	Volatiles: EDTA–NaOH–H_2O + SPME Sugars and organic acids: MeOH + derivatization	GC–MS	PCA, LDA, CN	Ursem *et al.*, 2008
Tomato: variety differentiation	Untargeted/discriminative	Lyophilization + MeOH + sonication	LC–TOF–MS NMR	PCA	Moco *et al.*, 2008
Tomato: volatiles analysis	Targeted/discriminative	EDTA–NaOH–H_2O + SPME	GC–MS	PCA, HCA	Tikunov *et al.*, 2005
Watermelon: quality evaluation	Untargeted/predictive	Buffered D_2O	NMR	PLS–LDA	Tarachiwin *et al.*, 2008
Wine: metabolite characterization	Untargeted/discriminative	Lyophilized + buffered D_2O	NMR	PCA, PLS	Son *et al.*, 2008
Yeast: aroma compounds production	Targeted/discriminative	Diethyl ether	GC–FID	PCA, PLS	Rossouw *et al.*, 2008
Yeast: strain differentiation	Untargeted/discriminative	Lyophilization + derivatization	GC–TOF–MS	PCA, HCA	MacKenzie *et al.*, 2008
Yeast: strain differentiation	Untargeted/discriminative	–	NIR	PCA LDA	Cozzolino *et al.*, 2006

For untargeted analysis, the use of sequential and selective extractions followed by metabolite analysis of each extract was previously recommended (Dixon *et al.*, 2006) [15]. Usually, an initial hydrophilic extraction (typically with MeOH-H2O) followed by centrifugation and hydrophobic extraction (typically with CHCl3) of the pellet achieves this purpose. Sequential extraction maximized the amount of metabolites from tomato paste (Capanoglu, Beekwilder, Boyacioglu, Hall, & De Vos, 2008) [9] finding discriminating compounds in both hydrophilic and hydrophobic fractions.

Derivatization

In food metabolomics, derivatization is commonly used prior to GC analysis in order to increase volatility of analytes.

Derivatization is usually a two-step process starting with oximation (conversion of aldehydes and ketones to oximes) of the sample to reduce tautomerism (especially from monosaccharide), followed by silylation to increase volatility by reducing hydrophilicity of functional groups OH, SH or NH (Gullberg *et al.*, 2004) [22]. Several oximation and silylation reagents have been tested in the past. Gullberg *et al.* (2004) [22] reviewed previous comparisons among derivatization reagents and reported that methoxiamine hydrochloride in pyridine and N-methyl-N-trimethyl silyltrifluoro acetamide were the most appropriate reagents for oximation and silylation respectively. In food analyses, these reagents have shown to improve GC separation of metabolites from potato and other products. Both derivatization time and temperature affect the metabolite independently with major changes at the beginning of the reaction (Ma, Wang, Lu, Xu, & Liu, 2008). Therefore, preliminary experiments should be done to determine optimum derivatization times and temperatures that maximize the detection of compounds of interest. In food metabolomic analysis, several silylation reactions have been carried out at 37°C for 90 min with good results.

Separation and detection

Separation and detection of the metabolites have been considered the key steps in metabolic profiling. Particular attention has been given to separation techniques such as liquid chromatography (LC) in its high performance (HPLC) or ultra performance (UPLC) forms, gas chromatography (GC), capillary electrophoresis (CE), as well as the coupling of these instruments to detection techniques such as mass spectrometry (MS), nuclear magnetic resonance (NMR), and near infrared spectrometry (NIR). Working principle as well as individual and hyphenated suitability of these techniques in metabolomics have been broadly discussed (Bedair & Sumner, 2008; Toyo'oka, 2008; Wishart, 2008b) [3, 55. 60]. In food metabolomics most separation analyses have been achieved by GC, CE, and LC).

Detection methods are mostly based on NIR, MS, or NMR techniques. In food metabolomics MS and NMR have been used the most. A greater amount of data is generally obtained by using MS accompanied by high throughput separation techniques such as HPLC or UPLC. For instance, the quality of green tea has been evaluated by NMR (Tarachiwin, Ute, Kobayashi, & Fukusakii, 2007) [52] and UPLC-MS and statistical models from UPLC-MS yielded a higher prediction coefficient than models from NMR, probably due to the higher number of peaks detected by UPLC-MS. However, other factors such as sample variability should also be considered. Although not as sensitive as the other detection techniques, NIR has provided a fast non-destructive fingerprint in several metabolomic analyses such as strain differentiation of wine yeast (Cozzolino, Flood, Bellon, Gishen, & Lopes, 2006) [11].

Data Treatment

Metabolomic data have usually been submitted to compound identification and MVDA. Compound identification has been mainly achieved by database matching and comparison with pure standards ran under same conditions. Data analysis in food metabolomics is largely carried out by several chemometrics tools. Typically, metabolomics data have been aligned before comparison to correct for instrumental deviations on retention/migration times. Alignment has been shown to drastically improve the performance of MVDA techniques (Son *et al.*, 2008) [50].

Discriminative metabolomics usually relies on multivariate methods such as PCA for sample grouping. PCA creates new variables (principal components) by linear combinations of the metabolites detected while maximizing sample variation. Grouping occurs when comparing the values of two or more principal components of each sample as used for discrimination of broccoli varieties. On the contrary, PLS (Partial Least Square) is a MVDA technique that allows sample discrimination by reduction of dimensionality while maximizing correlation between variables. PLS has been the main technique used for predictive metabolomics studies such as the creation of a metabolite-based model for sensory evaluation of watermelon (Tarachiwin, Masako, & Fukusaki, 2008) [43]. Similarly, linear discriminant analysis (LDA) with classification hypothesis was used for discrimination of olive oil according to origin (Cavaliere *et al.*, 2007) [10].

Metabolomics in Food Component Analysis

Traditionally food component analysis involves identifying and classifying food constituents into very broad categories such as proteins, fats, carbohydrates, fiber, vitamins, trace elements, solids and/or ash. However, with the advent of metabolomics, foods and beverages are now being analyzed with considerably more chemical detail. From the perspective of a metabolomics researcher, most foods can essentially be viewed as complex chemical mixtures consisting of various metabolites and chemical additives in a solid, semi-solid or liquid matrix. Some ''manufactured'' foods consist of just 10-20 different chemicals (artificial juices, soft drinks, purified vegetable oils) while other foods consist of hundreds of compounds (milk, cheese) and still others may have thousands of compounds (fruits, meats and most prepared foods). The number and diversity of chemical compounds in foods can be better appreciated if we consider the following numbers: (1) the human metabolome (which is expected to be similar to what could be found in most red meats) contains more than 3000 detectable (>1 pM) endogenous metabolites (Wishart *et al.*, 2007) [58]; (2) the milk metabolome contains more than 200 different oligosaccharides (3) the edible plant metabolome likely consists of more than 10,000 different detectable compounds with more than 2000 nutrient metabolites and more than 8000 non-nutrient phytochemicals having already been identified (Ehrman *et al.*, 2007) [16]; and (4) the U.S. FDA maintains a list of more than 3000 approved food additives (of which 2000 are well-defined chemicals) that can be found in processed foods. This compilation is often referred to as ''Everything'' Added to Food in the United States (EAFUS). Taken together, this collection of 15,000+ food components (which consists of both artificial and natural nutrients as well as artificial and natural non-nutrients) is often referred to as the nutrition metabolome or simply the food metabolome. The food metabolome is characterized not only by considerable chemical diversity (>100 major chemical classes), but also by considerable variations in chemical abundance. Detecting, identifying and quantifying so many different compounds over such enormous (1 trillion-fold) concentration ranges represents a significant technical and computational challenge. Nevertheless, with the advent of new technologies and expanded spectral libraries, metabolomics could add significant new knowledge to the study of health and disease processes.

In particular, an impressive number of natural foods, spices and beverages have already been the subject of detailed metabolomic-based component analysis, including milk grapes (Mattivi, Guzzon, Vrhovsek, Stefanini, & Velasco, 2006; Pereira *et al.*, 2006) [34, 41], tomatoes and tomato juice (Tiziani, Schwartz, & Vodovotz, 2006) [54], rhubarb (Ye, Han, Chen, Zheng, & Guo, 2007) [62], beer (Almeida *et al.*, 2006; Nord, Vaag, & Duus, 2004) [1, 38], celery seeds (Kitajima, Ishikawa, & Satoh, 2003) [25], coriander (Ishikawa, Kondo, & Kitajima, 2003) [25] as well as many other herbs and spices (Ishikawa, Takayanagi, & Kitajima, 2002; Matsumura, Ishikawa, & Kitajima, 2002) [26]. These analyses used a combination of NMR, GC-MS, LC-MS and CE techniques to identify up to 100 different phytochemicals (Ye *et al.*, 2007) [62] or 200 different carbohydrates in selected fruit, vegetable or beverage samples. The use of multiple separation and detection technologies generally allows for a far broader range of metabolites to be detected, with NMR being particularly useful for detecting high abundance polar metabolites and GC-MS or LCMS being best for low abundance non-polar or semi-polar metabolites. It is also worth noting that the majority of food-based metabolomics studies published to date have focused on liquids or liquid extracts (i.e. beverages) rather than an analysis of food solids. This is primarily because most nutrients can be easily extracted into liquids and that most chemical detection technologies are optimized for working with liquids.

Studies of tomatoes and tomato juices using LC-MS methods and NMR (Tiziani *et al.*, 2006) [54] have shown how these two methods can reveal complementary information about different constituents in tomatoes. LC-MS methods, in combination with appropriate reference databases, allow the ready identification of many (>60) non-polar, low abundance secondary metabolites such as polyphenols, flavo-glucosides and alkaloids. On the other hand, NMR was found to be particularly useful in the identification and quantification of the lycopene and carotenoid isomers that could not be distinguished by LC or LC-MS methods (Tiziani *et al.*, 2006) [54]. Other NMR studies have identified and quantified many of the more abundant (mostly polar) compounds in tomatoes including sugars, flavanoid glycosides, amino acids and organic acids (Le Gall, Colquhoun, Davis, Collins, & Verhoeyen, 2003) [30]. Variations in some of these tomato-specific phytochemicals are largely responsible for the subtle variations in flavors, aromas, ripening stages and colors in tomatoes. They are also responsible for their potential prostate cancer-fighting properties (Tiziani *et al.*, 2006) [54]. Variations in the more abundant, polar metabolites (sugars, acids, etc.) are responsible for the more obvious tomato taste variations.

Over the coming years it is likely that food composition studies will become much more common with far more comprehensive metabolomic analyses being done of many of the economically or pharmaceutically more important fruits (apples, bananas, oranges, strawberries, pineapples, mangos, dates, pomegranates, blueberries), vegetables (potatoes, cabbage, carrots, avacados, spinach, broccoli, cauliflower, onions, corn), grains (wheat, barley, oats, flaxseed, rye), nuts (almonds, walnuts, peanuts, cashews), processed foods (cheeses, yogurt, wines, vegetable oils, fish oils), meats (beef, chicken, fishes and crustaceans) and, of course, many nutraceutical foods or beverages (coffee, chocolate, gingko, green tea, ginger, ginseng, garlic). It is also likely metabolomic-based food component studies will allow food scientists to more precisely follow the consequences of different preparation (frying vs. baking; steaming vs. boiling) and preservation (freezing, drying, smoking, refrigerating) processes on key food components. These studies will also helps in guiding food scientists in the breeding, selection or modification of better crops, the breeding and feeding of livestock or fishstock as well as in the preparation of many processed foods.

Metabolomics in Food Quality/Authenticity Assessment

The capacity to identify food constituents using metabolic profiling can also be used to assess both food adulteration and food quality. In particular, the detection of adulterent on contaminated food products exploits the fact that certain chemicals or certain concentrations of chemicals are quite characteristic of certain types of juices, extracts and oils (Ogrinc, Kosir, Spangenberg, & Kidric, 2003) [39]. Similarly chemical composition characteristics can also be exploited to distinguish between food products with desirable characteristics that otherwise cannot be detected by flavor, aroma or color (such as unsaturated fat content, a favorable amino acid profile, increased vitamin content, enriched phytochemical content). Food quality assessment also impacts food quality control. In fact, metabolomic techniques may find their greatest use in the food industry in monitoring quality control or batch-to batch product reproducibility.

To date most reported metabolomic studies on food adulteration and food quality have focused on characterizing oils or beverages, such as vegetable oils (Hutton, Garbow, & Hayes, 1999; Ogrinc *et al.*, 2003; Prestes *et al.*, 2007) [23, 39], fish oils (Aursand, Standal, & Axelson, 2007) [2], fruit juices (Cuny *et al.*, 2008; Cuny, Le Gall, Colquhoun, Lees, & Rutledge, 2007; Le Gall, Puaud, & Colquhoun, 2001) [13, 12], wines (Aursand *et al.*, 2007) [2] and beers (Almeida *et al.*, 2006) [1].

Fruit juice adulteration is relatively common as it is very difficult to detect via taste or color. For instance, orange juice can be blended with lower-cost grapefruit juice without any obvious changes to flavor or color. However, the presence of grapefruit juice in a presumptively pure orange juice product can have serious consequences to individuals on certain medications. Grapefruit juice has a number of coumarin-like flavanoids and other powerful CYP450 inhibitors (Griennavar, Poulose, Jayaprakasha, Bhat, & Patil, 2006) [21]. Therefore, the detection and prevention of this kind of adulteration is particularly important. Traditional approach to detect this kind of juice blending use fairly lengthy and labor intensive HPLC methods. Recently an NMR-based chemometric approach has been developed that uses Independent Component Analysis, a variant of PCA (Cuny *et al.*, 2007) [12]. This approach is fundamentally based on analyzing selected regions of the ^{1}H NMR spectrum that are known to contain distinguishing flavanoid glycoside signals The method was tested on 92 different juices, including 59 pure orange juices, 23 grapefruit juices and 10 blends. It was found to be quite accurate, with 98% of the samples being correctly classified. Furthermore, this NMR method took less than half the time of the standard chromatographic approach.

NMR has also been successfully used in monitoring batch-to-batch quality and production site differences in beer (Almeida *et al.*, 2006) [1]. Large multi-national breweries must prepare their beers at many different geographic locations. Given the desire to produce similar tasting and similar quality beers at these different sites, it is important to develop ways that can perform quality control not just at the level of color, alcohol

content or specific gravity, but at a more detailed molecular level. The study by Almeida *et al.* (2006) [1] used in ^{1}H NMR combination with principal component analysis to reveal that different production sites, presumably producing the same beer, could be easily distinguished based on their content of lactic acid, pyruvic acid, dextrans, adenosine, inosine, uridine, tyrosine and 2-phenylethanol. Quantifying these compounds also allowed the authors to identify one production site that exhibited much greater variability in these compounds (and therefore had less quality control) than the other production sites. These methods could also be adapted by other multinational corporations to improve quality control in soft drink production, juice production and vegetable oil manufacturing. Similar methods might also be used to monitor the quality control of functional foods and nutraceuticals (i.e. food extracts that are claimed to have positive medicinal effects) that are harvested from different geographic locations.

To date, most published food/beverage adulteration studies have employed NMR in combination with PCA, artificial neural networks (ANNs) or other multivariate statistical techniques to identify and classify samples. However, it is also possible to use mass spectrometry to detect food adulteration. In particular, the use of isotope ratio mass spectrometry (IRMS) has long been used to detect the adulteration of wines and olive oils, as well as the detection of added beet sugar and exogenous citric acid to fruit juices (Ogrinc *et al.*, 2003) [39].

Given the multinational nature of food production and processing along with the growing concerns over food safety and food quality, it is likely that more stringent and more precise food monitoring systems will have to be put in place over the coming years. The demonstrated potential of metabolomic techniques to rapidly and inexpensively identify adulterated beverages and oils, as well as their potential to accurately monitor previously undetectable quality control issues certainly suggests that metabolomics could soon play a major role in many aspects of food quality assessment and quality control.

Metabolomics in food consumption monitoring

Monitoring or measuring food consumption is one of the most important and challenging tasks for nutritional scientists. In an ideal world, nutrition scientists would like to control precisely what a person eats or monitor everything that a person has consumed, much the same way that we can control or monitor the diets of caged lab animals. This approach would certainly allow for important diet-response findings to be made. Most of the published findings on the positive or negative consequences of certain foods or diets are derived from epidemiological studies of large population cohorts who fill out food frequency questionnaires (FFQs). Unfortunately FFQ compliance for prospective diet studies and FFQ recall for retrospective diet studies has not been particularly good (Brantsaeter *et al.*, 2007) [7].

Given the shortcomings of FFQs, an emerging application of metabolomics is in food consumption monitoring. This particular approach takes advantage of the knowledge being acquired in food component analysis and food quality measurement and applies it to the analysis of blood, urine or saliva from volunteers in diet intervention studies. This is very similar, both in design and concept, to using metabolomics in drug safety or drug efficacy assessment (Lindon *et al.*, 2004; Gibney *et al.*, 2005) [20]. However, in the case of nutritional monitoring, the ‘‘drugs’’ are relatively ill-defined chemical mixtures of nutrients and non-nutrients (i.e. food).

The utility of metabolomics-based food consumption monitoring comes not only through an improved read-out of food consumption patterns, but also through better interpretation of the true physiological consequences of a given diet (or drug). Metabolomic studies aimed at identifying physiological effects of food intervention trials must be able to distinguish spectral changes in the urine or blood arising from diet-specific non-nutrient ‘‘spill over’’ (such as peaks arising from flavanoids, alkaloids or food additives) from those spectral changes arising from diet-induced physiological changes, such as peaks arising from changed endogenous metabolites or endogenous signaling molecules. Failure to distinguish these differences will lead to serious misinterpretation of any diet intervention study. Indeed, if the diet-derived signals are not properly accounted for, all that one may learn from an extensive metabolomic study is the fact that two groups ate differently — an obvious fact that needs no secondary confirmation.

As a general rule, the best biofluid to study nutrient intake or to identify food-specific biomarkers is urine (Gibney *et al.*, 2005; Walsh, Brennan, Malthouse, Roche, & Gibney, 2006) [20]. Urine is essentially the body’s liquid waste repository and any nutrient or non-nutrient that is not needed or present in excess will find its way into the urine. Indeed, a major difference between urine and blood is the ratio of metabolites to non-metabolites (i.e. phytochemicals, cooking by-products, xenobiotics and other compounds that cannot be productively metabolized by the body). For instance, a recent study on biomarkers of onion consumption found that 11 quercitin metabolites were detectable in urine at relatively high abundance, whereas only 5 lower abundance quercitin metabolites could be found in blood (Mullen, Boitier, Stewart, & Crozier, 2004).

Table 2 provides a list of a number of the food-specific biomarkers that can be found in blood or urine. In some cases the mere presence of the compound indicates consumption of a certain food, in other cases, the concentration of the compound is significantly elevated for a period of time. Most food-specific biomarkers are present in the blood or urine for 5-10 hours, with some persisting as long as 48 hours. Many of these compounds are phytochemicals or non-nutrients that are not metabolized by the body. Others are actually secondary metabolites having been tagged for elimination as glycosylated, glucuronidated or sulfated conjugates. In many respects, the body treats these compounds much the same way as it treats drugs or other xenobiotics. Hence the same concepts behind drug testing and drug monitoring can be used in food consumption monitoring. The difference is that drugs are usually relatively pure compounds with relatively few metabolites. Foods are not pure compounds and consequently have many more constituents and many more metabolites. Some, however, are quite unique to certain foods or food processing events and these are the ones that are listed in Table 2.

In the not-too-distant future, this list will no doubt expand quite significantly as more compounds are identified and more foods are studied through different metabolomic approach. It is also likely that food consumption biomarkers will evolve from being just one or two relatively non-specific compounds to a far more specific ‘‘profile’’ consisting of multiple compounds. Likewise the use of concentration data

in conjunction with information on the time since, or the amount of, consumption should allow much more precise measures of metabolite flow and flux as well as the identification of individual differences in metabolite processing or absorption. In time, metabolomic techniques might be able to replace some of the short-term food consumption questionnaires used in prospective diet challenge or food intervention studies

Table 2: Summary of food consumption and diet biomarkers

Food/diet	Biomarker	Biofluid (Level)	Reference(s)
Black tea	Gallic acid	Urine (increased)	Mennen et al., 2006
	4-O-methylgallic acid	Urine (increased)	Mennen et al., 2006
	Epicatechin	Urine (increased)	Ito et al., 2005
	Kaempferol	Urine (increased)	Brantsaeter et al., 2007
Wine	Gallic acid	Urine (increased)	Mennen et al., 2006
	4-O-methylgallic acid	Urine (increased)	Mennen et al., 2006
Coffee	Caffeic acid	Urine (increased)	Mennen et al., 2006
	Chlorogenic acid	Urine (increased)	Mennen et al., 2006
Cocoa	Naringenin	Urine (increased)	Ito et al., 2005
Alcohol	Ethyl glucuronide	Urine (increased)	Bicker et al., 2006
	Ethyl phosphate	Urine (increased)	Bicker et al., 2006
	Ethyl sulfate	Urine (increased)	Bicker et al., 2006
Milk	Hippuric acid	Urine (decrease)	Bertram et al., 2007
Apple	Phloretin	Urine (increased)	Mennen et al., 2006
Grapefruit	Naringenin	Urine (increased)	Mennen et al., 2006
Orange	Hesperetin	Urine (increased)	Mennen et al., 2006
Pomegranate	Ellagic acid	Blood (increase)	Seeram et al., 2006
	Dimethylellagic acid glucuronide	Blood (increase)	Seeram et al., 2006
Citrus fruit	Naringenin	Urine (increased)	Mennen et al., 2006
	Hesperetin		Brantsaeter et al., 2007
Garlic	Allylmercapturic acid	Urine (increased)	De Rooij, Boogaard, Rijksen, Commandeur, & Vermeulen, 1996
Cooked protein	Lysinoalanine	Urine (increased)	Somoza et al., 2006
	N(e)-fructolysine		Somoza et al., 2006
	N(e)-carboxymethllysine		Somoza et al., 2006
Cooked vegetables	Alpha-carotene	Blood (increase)	Brantsaeter et al., 2007
Cooked onions	Quercetin glucuronides	Urine (increased)	Hong & Mitchell 2004
	Quercetin	Urine (increased)	Hong & Mitchell 2004
	Methyl quercitin	Urine (increased)	Hong & Mitchell 2004
Fish	Trimethylamine	Urine (increased)	Lee et al., 2006
	Trimethylamine-oxide	Urine (increased)	Lee et al., 2006
Meat-rich diet	1-methylhistidine	Urine (increased)	Myint et al., 2000
	Creatine	Urine (increased)	Stella et al., 2006
	Carnitine	Urine (increased)	Stella et al., 2006
	Trimethylamine-oxide	Urine (increased)	Stella et al., 2006
Vegetarian diet	Salicyluric acid	Urine (increased)	Lawrence et al., 2003
	Salicylic acid	Urine (increased)	Lawrence et al., 2003
Carbohydrate rich diet	Lactate	Urine (increased)	Zuppi et al., 1998
	Alanine	Urine (increased)	Zuppi et al., 1998
	Citrate	Urine (increased)	Zuppi et al., 1998

Metabolomics in physiological monitoring of diet and nutrition studies

Over the past century great strides have been made by food scientists and biochemists in identifying the essential nutrients needed for human growth and viability. Improved dietary guidelines and mandated food supplementation with essential minerals and vitamins has been remarkably successful in treating most nutritional ‘‘deficiencies’’. Today nutritional scientists are challenged with finding new ways of treating or preventing diseases brought on by nutritional ‘‘oversufficiencies’’ such as obesity, diabetes, chronic inflammation and cardiovascular disease (German *et al.*, 2005) [19]. They are also being asked to identify bioactive food components that potentially increase life expectancy, reduce weight, enhance physical or mental performance and prevent diseases such as atherosclerosis, heart disease, cancer and arthritis.

In addressing these challenges, nutrition scientists may choose one of two routes to explore the effect of diet or bioactive food components on human health. One is through retrospective epidemiological studies that attempt to correlate long-term food consumption with health status or disease susceptibility. The other is through shorter-term prospective food intervention or diet challenge experiments. While many

encouraging correlations between diet and improved health status have been identified through epidemiological studies (German *et al.*, 2005; Gibney *et al.*, 2005) [19, 20], these correlations do not necessarily imply causation. Consequently diet-challenge experiments, with a focus on quantitatively measuring molecular biomarkers and physiological consequences, have become the preferred route to exploring the causes behind the beneficial or detrimental effects of key nutrients.

Genomic, proteomic and, most recently, metabolomic techniques are now commonly used in many diet-challenge or food intervention experiments (Gibney *et al.*, 2005) [20]. Depending on the physiological response being measured, metabolomic assays can be used to measure small molecule biomarkers of oxidative stress, redox potential, anti-oxidant activity, inflammation and cardiovascular disease risk (Table 3). While proteomic and transcriptomic markers also exist for tracking these responses, the advantage of using small molecule biomarkers is that the same small molecule monitoring techniques can also be used to look at the short-term concentration changes of diet related chemicals or metabolites. In other words, only one technology platform needs to be employed to measure food inputs and physiological outputs.

In contrast to the urinary polar compounds that can be used as biomarkers of food consumption (Table 2) the compounds that are used as biomarkers of physiological response to foods (Table 3) are typically nonpolar and most are found in blood. This is because blood is generally a more stable and reliable indicator of physiological processes. Furthermore, blood carries many more non-polar molecules than urine. Not all of the compounds listed in Table 3 have been traditionally detected by NMR or MS methods. In fact, some are frequently detected using immunoassays (thromboxanes, leukotrienes) or measured via centrifugation protocols (LDL, HDL). However, advances in MS-based lipidomic technologies (Wiest & Watkins, 2007) and lipoprotein characterization (Otvos, 2002) [40] are now allowing these molecules to be detected and/or quantified using relatively standard, high-throughput MS or NMR techniques. One of the first studies to use metabolomic techniques in a diet-intervention trial involved studying the effects of soy-derived proteins and soy isoflavones (Solansky *et al.*, 2003) [48]. ^{1}H NMR and chemometric techniques were used to analyze the blood plasma changes in pre-menopausal women on a multi-week soy diet. The results showed that significant differences could be detected in lipoproteins, amino acid and carbohydrate profiles that related to metabolic pathways responsible for osmolyte fluctuation and energy metabolism. A similar study by Fardet *et al.* (2007) [17] explored the influence of whole-grain and wheat flour diets on rats using both NMR-based chemometric methods. These studies showed that the whole-grain fed animals had higher urinary levels of Kreb's cycle basal metabolic rate as well as an alteration to the gut microflora metabolism.

Table 3: Physiological response biomarkers in blood and urine

Compound	Biofluid (level)	Indication	Reference(s)
8-isoprostane F2a	Urine (increased)	Lipid peroxidation Oxidative stress	Thompson et al., 2006
8-hydroxy-2-deoxyguanosine	Lymphocytes/Urine (increased)	DNA oxidative damage	Thompson et al., 2006
Malondialdehyde (MDA)	Urine (increased)	Oxidative stress	Fardet et al., 2007
Glutathione (reduced)	Plasma (decreased)	Oxidative stress	Fardet et al., 2007
Hydrogen peroxide	Urine (increased)	Oxidative stress	Bogani et al., 2007
Low density lipoprotein (LDL)	Plasma (increased)	Increased risk for CVD	Castro, Barroso, & Sinnecker, 2005
High density lipoprotein (HDL)	Plasma (decreased)	Increased risk for CVD	Castro et al., 2005
Triacylglycerol (TAG)	Plasma (increased)	Increased risk for CVD	Castro et al, 2005
Homocysteine	Plasma (increased)	Increased risk for CVD	Chiuve et al., 2007
Total cholesterol	Plasma (increased)	Increased risk for CVD	Castro et al., 2005
alpha-tocopherol (Vitamin E)	Plasma (decreased)	Decreased anti-oxidant potential	Castilla et al., 2006
Ascorbic acid (Vitamin C)	Plasma (decreased)	Decreased anti-oxidant potential	Castilla et al., 2006
Thromboxane B2	Plasma (increased)	Inflammation	Bogani et al., 2007
Leukotriene B4	Plasma (increased)	Inflammation	Garg et al., 2007
Prostaglandin E2	Plasma (increased)	Inflammation	Sánchez-Moreno et al., 2004
Uric Acid	Plasma (increased)	Inflammation; Oxidative stress	Sánchez-Moreno et al., 2004

NMR studies on plasma and liver showed increases in glutathione (reduced) and betaine, which are general indicators of good redox status and lower oxidative stress.

Because many of the effects of diet intervention are likely to be more subtle and the biomarkers of interest will be at lower concentrations, many of the more recent studies have focused on using more sensitive MS methods or immunoassays. These include studies on the effects of fruit and vegetable diversity on the levels of oxidative biomarkers (Thompson *et al.*, 2006) [53], the influence of concentrated red grape juice on oxidative stress, lipidemic and inflammatory markers in blood, the effect of extra virgin olive oil on plasma inflammatory and oxidative stress markers (Bogani, Galli, Villa, & Visioli, 2007) [6], the consequences of macadamia nut consumption on markers for cardiovascular disease (Garg, Blake, Wills, & Clayton, 2007) [18] and the influence of gazpacho soup consumption on plasma biomarkers of oxidative and inflammatory stress (Sa´nchez-Moreno *et al.*, 2004) [46]. All of these studies showed some statistically significant, positive effect largely confirming the results of previous retrospective epidemiological studies. Many of these studies used both small molecule and large molecule protein biomarkers to

measure the physiological effects of interest.

While human health and disease are ultimately influenced by long-term exposure to diet, the use of short-term diet challenges to measure their projected effects on long-term health outcomes is perhaps the most effective (and cost-efficient) route to using metabolomics in understanding diet health relationships. The use of glucose, fructose (especially high fructose corn syrup), fat (cholesterol and/or trans-fatty acid) and high-calorie diet-challenge experiments along with their monitoring via sensitive metabolomic techniques will likely reveal some key insights into the temporal development of chronic diseases such as obesity, diabetes, cardiovascular disease and metabolic syndrome. Likewise, the use of fish/olive oil, flaxseed, cruciferous vegetable, polyphenol rich fruit and low-calorie diet-challenge experiments may reveal equally important insights into their efficacy for treating metabolic syndrome, combating cardiovascular disease and extending life expectancy.

Diet-challenge experiments, if properly and comprehensively monitored by the more sensitive (i.e. GC-MS and LC-MS) metabolomic methods, will also help to reveal the breadth of individual responses to these different dietary perturbations. No doubt some ‘‘high-risk’’ individuals will show much greater or longer lasting perturbations for certain disease-risk biomarkers or metabolites whereas other ‘‘low-risk’’ individuals will show almost none. While metabolomics promises to provide a far more quantitative and chemically detailed understanding of phenotypic changes, the integrated use of transcriptomic, SNP-profiling, copy-number variation (CNV), gut metagenomic and proteomic/enyzmatic studies in these diet-challenge experiments will also allow for a much more complete understanding of the inter-individual differences or proclivities due to genetic and epigenetic effects.

As noted earlier, there can be considerable diversity in individual responses to diet along with the considerable diversity of diets within the human population. This diversity certainly makes metabolic profiling and metabolite ‘‘standardization’’ difficult. Standardization and inter-subject comparison is also made more complicated by the fact that age, gender and diurnal effects also influence the concentrations and even the presence of certain metabolites. The creation of dedicated metabolite or metabolomic databases that include these gender-specific ranges, age specific variations or temporal effects could greatly help in this regard. Likewise the creation and sharing of comprehensive, longitudinal metabolomic (and other omic) data sets collected from well-designed and well-documented diet-challenge experiments would definitely make this sort of database development (and maintenance) much easier. It could also make group comparisons and metabolite calibrations much more robust and their conclusions much more reproducible.

Conclusions

Metabolomics has shown to be an important tool for the progress of the many areas of food science such as compliance of regulations, processing, quality, safety, and microbiology. Recent studies suggest that the potential of metabolomics in food science can be expanded to the area of food product development by determining the compounds responsible for consumers’ taste preferences. The development of rapid technologies such as DIMS (Direct Infusion Mass Spectrometry), IMS (Ion Mobility Spectrometry), and EESI (Extractive Electrospray Ionization) has helped the growth of metabolomics in food science. However, further improvement on these techniques is necessary to overcome sensitivity and compound identification issues. Most of the data analyses in food have relied in linear MVDA tools, not considering possible non-linear aspects of the samples. Future trends should involve the use of non-linear tools for dimensionality reduction in food metabolomics. Even though metabolomic analyses in food have been much diversified, most studies can be considered as discriminative with very few compounds identified. Therefore, the development of a food metabolome database, as suggested by Wishart (2008b) [60] is needed in order to facilitate compound identification and the development of informative metabolomics. In addition, most reports have focused on fruit and vegetables leaving the meat, seafood, and related areas still underexplored. Because of some metabolic similarities, identification of many compounds in red meat can be carried out by using available human metabolome databases (Wishart, 2008b) [60]. Metabolomics’ successful association to other analytical areas such as genomics has been demonstrated, showing the potential of metabolite profiling to be linked to other areas as well.

Metabolomics as a first step for sensor development has the potential to introduce a series of new rapid methods for food analysis. In this area, bacteria biomarkers can be discovered by metabolomic techniques and sensors can be developed for rapid detection of the selected biomarkers. To achieve this purpose, studies on microbial biomarkers identification involving different levels of bacterial contamination, accompanying flora, and biomarker response in food are needed. The rapid growth of metabolomics in the food science area suggests the potential of discriminative, predictive, and informative analyses to solve the most important problems and provide important information to the food industry.While promising advances have been made, current techniques are only capable of detecting perhaps 1/10th of the relevant metabolome. This expanded breadth and depth of coverage is particularly important in food and nutrition studies. While these kinds of endeavors may take years to complete and cost millions of dollars, hopefully the food science community (and its funding agencies) will find a way of coordinating its activities to complete these efforts. Indeed, having public resource like a food metabolome database or a nutritional phenotype database could be as valuable to food scientists as Gen Bank has been to molecular biologists.

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