Perspective

NMR-Based Metabolic Profiling and Metabonomic Approaches to Problems in Molecular Toxicology

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We have reviewed the main contributions to the development of NMR-based metabonomic and metabolic profiling approaches for toxicological assessment, biomarker discovery, and studies on toxic mechanisms. The metabonomic approach, (defined as the quantitative measurement of the multiparametric metabolic response of living systems to pathophysiological stimuli or genetic modification) was originally developed to assist interpretation in NMR-based toxicological studies. However, in recent years there has been extensive fusion with metabolomic and other metabolic profiling approaches developed in plant biology, and there is much wider coverage of the biomedical and environmental fields. Specifically, metabonomics involves the use of spectroscopic techniques with statistical and mathematical tools to elucidate dominant patterns and trends directly correlated with time-related metabolic fluctuations within spectral data sets usually derived from biofluids or tissue samples. Temporal multivariate metabolic signatures can be used to discover biomarkers of toxic effect, as general toxicity screening aids, or to provide novel mechanistic information. This approach is complementary to proteomics and genomics and is applicable to a wide range of problems, including disease diagnosis, evaluation of xenobiotic toxicity, functional genomics, and nutritional studies. The use of biological fluids as a source of whole organism metabolic information enhances the use of this approach in minimally invasive longitudinal studies.

Contents

1. Introduction to Metabolic Profiling

Broad spectrum metabolic profiling is now recognized as a powerful top-down systems biology tool that can provide a real world link to other omics sciences (*1–4*). The terms metabolomics (*5–7*) and metabonomics (*8*) are widely applied to these types of studies, and the terminology is often used interchangeably. Metabonomics provides a whole-organism biological description of time-related multivariate metabolic response to a treatment. It facilitates the study of the metabolic products and interactions of hundreds of cellular metabolomes (metabolic complements) and fluid compartments, which are unique to each cell type in the body but are coordinated in space and time, and this concept of the interacting metabolomes has been termed the metabonome (*9*). A variety of analytical technologies have been applied to metabolic profiling in toxicology, but most approaches utilize NMR spectroscopy or mass spectrometry as these instrumentalities can capture information on hundreds or even thousands of metabolites in a sample in a single analytical run. To date, there have been more publications reflecting the application of NMR spectroscopy in metabolic toxicology (excluding drug metabolism applications), but modern LC-MS methods are now being successfully utilized in this area (*10–12*), and the balance will change although we consider that there will always be a role for NMR in rapid multivariate metabolic profiling and in metabolic structural elucidation. As NMR spectroscopy (in its own right) comprises a wide range of analytical techniques (and can be applied to biofluids as well as intact tissues),

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we have concentrated our review on the role of NMR spectroscopy in the development of toxicological metabonomics, but we have also considered cognate biomedical applications as they will be of increasing importance in the future.

2. Background: The Early Days of Metabolic NMR spectroscopy

The development of Fourier transform NMR spectroscopy in the late 1960s, the introduction of superconducting magnets in the 1970s, and the consequent sensitivity increases resulted in the first applications of NMR spectroscopy for the metabolic profiling of biofluids and cells. Since then, numerous studies have concentrated on the use of ^{1}H NMR spectroscopy to characterize toxic response to drugs, as reflected in biofluid spectral signatures, and many novel metabolic markers of organ-specific toxicity have been discovered (*13*). The role of metabonomics in particular and magnetic resonance in general in the toxicological evaluation of drugs has developed extensively (*14*). ^{1}H NMR spectroscopy is well suited to the study of toxic events, as a biofluid fingerprint that reflects toxic response can be rapidly achieved without bias imposed by expectations of the type of toxin-induced metabolic changes. Moreover, many of the NMR-detectable metabolites are present at moderate to high concentrations and represent the products and intermediates of many important or hub pathways that are affected by many toxic or disease processes. Early applications of NMR spectroscopy found that quantitative changes in metabolite patterns gave information on the location and severity of toxic lesions, together with insights into the underlying molecular mechanisms of toxicity (*13, 15–17*).

Examples of early NMR studies of toxins include the effects of exposure to cadmium and mercury salts, which are both potent nephrotoxins (*18*), with acute cadmium exposure also causing profound testicular toxicity. NMR methods were applied successfully to analyze urine for novel metabolites caused by exposure of rats to acute cadmium and mercury dosing in dose−response studies. In the case of mercury, classical patterns of acquired Fanconi syndrome were observed, with markers of proximal tubular injury, including marked amino and organic aciduria, coupled with differential responses of citric acid cycle intermediate excretion (e.g., low citrate with high succinate) consistent with selective inhibition of mitochondrial enzymes such as succinate dehydrogenase and malate dehydrogenase (*15*). These studies showed for the first time that it was possible to capture site-specific severity and mechanistic information simultaneously. Studies by Gartland et al. (*19*) further demonstrated the value of such screening in determining the region-specific toxicity using a variety of experimental nephrotoxins. Many other proximal tubular toxins were studied around this time showing spectroscopic commonalities of time response and recovery from injury (*20–23*). The proximal tubular toxicity of para-amino phenol was extensively studied (*24, 25*) together with the protective effects of buthionine sulfoximine treatment and biliary cannulation (*26*), which showed that this compound worked effectively via a toxic thiol mechanism involving initial glutathione conjugation followed by further metabolism and the generation of a toxic thiol adduct that was transported to the kidney. In addition, the utility of the NMR approach in clinical toxicology was highlighted in a case study involving cutaneous exposure to phenol causing renal failure, the progression and recovery from which was followed by NMR spectroscopy (*27*). This study predates the metabolic trajectory analysis approaches (see below) for studying the development of lesions in experimental toxicology studies that were to become important later in the 1990s. In the case of cadmium toxicity, a similar approach was employed to show that urinary creatine was a highly sensitive early reporter of acute testicular injury with urinary creatine being elevated many hours before lesions became detectable by histopathology (*28, 29*). In later studies by Timbrell and co-workers (*30, 31*), it was found that creatinuria could also indicate liver damage, but this was only really indicative in the presence of concomitant taurinuria. Taurinuria was first demonstrated by NMR as a useful urinary marker for liver injury (*32, 33*), and a wide variety of hepatotoxins are now known to cause taurinuria, although this can be very variable depending on dietary sources (*34*). Other interesting subtoxic, but toxicologically significant metabolic effects of drugs could also be observed using this approach such as sugar acidurias caused by aldose reductase inhibitors (*35*). Ghauri et al. showed that chronic acetaminophen ingestion caused 5-oxoprolinuria in rats and that this could be completely eliminated using dietary methionine supplements indicating that oral drug dosing could deplete sulfur-containing amino acids and disrupt the glutathione cycle (*36*). It was later shown that even at therapeutic doses in humans, fractional 5-oxoprolinuria could be detected after acetaminophen treatment (*37*).

3. Pattern Recognition for Sample Classification and Biomarker Discovery

The use of chemometric methods to analyze complex spectral data sets was perhaps the single most important development in the practical application of metabonomics and has defined the development and progression of the field ever since. The first studies that used PR to classify biofluid samples used a simple scoring system to describe the fluctuating levels of 18 major endogenous metabolites in urine from rats that either were in a control group or had received a specific organ toxin that affected the liver, the testes, the renal cortex, or the renal medulla (*38, 39*). These studies showed that samples corresponding to different organ toxins mapped into distinctly different regions of the pattern recognition diagrams indicating that site-specific and severity information could be captured directly from the metabolic profile. Various refinements in data analysis were investigated, including taking scored data at three time points after toxin exposure for the nephrotoxins only as well as using a simple dual scoring system (the time and magnitude of the greatest change from control). The maps derived from the full time course information provided the best discrimination between toxin classes, emphasizing the importance of capturing dynamic information in the characterization of toxic lesions. This study was further extended (*40*) to incorporate actual metabolite NMR resonance intensities rather than simple scores. This was carried out for the nephrotoxins in the earlier group plus additional nephrotoxic compounds. A good separation of renal medullary and renal cortical toxins was achieved. In addition, it was possible to differentiate cortical toxins according to the region of the proximal tubule (S1, S2, and S3), which was affected, and also by the biochemical mechanism of the toxic effect. However, it was noted that absolute quantification of metabolites did not necessarily improve PR classification of toxicity over simple scoring systems.

The time course of metabolic urinary changes induced by two renal toxins was first investigated in detail by metabonomics using Fisher 344 rats administered a single acute dose of the renal cortical toxin, mercury II chloride, and the renal papillary toxin, 2-bromoethanamine (*41, 42*). Rat urine was collected for up to 9 days after dosing, and samples were analyzed using high resolution ^{1}H NMR spectroscopy. The onset, progression,

and recovery of the lesions were also followed using histopathology to provide a definitive classification of the toxic state relating to each urine sample, and the geometry of the trajectory generated information relating to the mechanism and sequential targets of the toxin. The concentrations of 20 endogenous urinary metabolites were measured at 8 time points after dosing and mapping methods were used to reduce the data dimensionality. These showed that the points on the plot could be related to the development of, and recovery from, the lesions.

Early pattern recognition studies on NMR data employed a reductionist approach of preselecting metabolite signals of interest. However, the NMR spectral results generated in a metabonomic study yield a unique metabolic fingerprint for each biofluid sample consisting of thousands of overlapping resonances, and measurement of a small set of signals will not reflect the full potential of the spectral profile. If the status of a given organism changes, such as in a diseased state or following exposure to a drug, the unique metabolic fingerprint or signature reflects this change (*2, 13*). Multivariate statistical methods provide an expert means of analyzing and maximizing information recovery from complex NMR spectral data sets. Detailed inspection of NMR spectra and integration of individual peaks can give valuable information on dominant biochemical changes; however, subtle variation in spectra may be overlooked, and it is difficult to envisage general effects as a function of both dose and time in a large cohort of samples with biological variability. Pattern recognition methods can be used to map the NMR spectra into a representative low dimensional space such that any clustering of the samples based on similarities of biochemical profiles can be determined and the biochemical basis of the pattern elucidated.

The initial objective in metabonomics is to classify a spectrum based on identification of its inherent patterns of peaks and, second, to identify those spectral features responsible for the classification (according to physiological or pathological status), which can be achieved via both supervised and unsupervised pattern recognition techniques. The NMR spectral data is preprocessed, which typically involves Fourier transformation, calibration of the chemical shift scale using an internal reference standard, and phase and polynomial baseline correction. To prepare the NMR data for multivariate modeling, the spectra are often divided into regions (along the chemical shift axis) whose areas are summed to provide an integral so that the intensities of peaks in such defined spectral regions are extracted, a process known as binning. This results in a data matrix (Figure 1a) consisting of rows that reflect observations/samples and columns that represent variables, for example, the spectral integrals of defined bins across the whole spectral width (*43*). Recent advances in chemometric approaches involve the utilization of full resolution NMR data, where each data point in an acquired spectrum is extracted as a variable for modeling. This approach has many advantages, for example, the spectral structure is retained, which enables the NMR user to identify metabolites with ease, and it also avoids searching within bins post data modeling to determine metabolites of discriminatory importance. The use of full resolution NMR data in chemometric modeling will be discussed in greater detail in a later section that encompasses orthogonal-projection on latent structures-discriminant analysis (O-PLS-DA).

Following the above preprocessing steps and the output of a data matrix consisting of samples and their associated variables, normalization is often applied to the rows (spectra). This adjusts spectral intensities so that concentration differences between samples are accounted for such that the samples are more

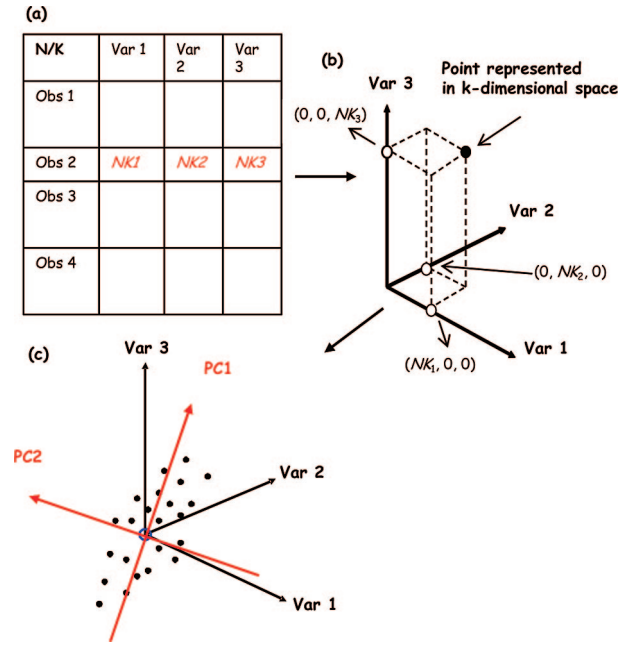

Figure 1. Principal components analysis. (a) Stylized data matrix consisting of N observations (spectra, $N = 4$) and K variables (spectral regions, $K = 3$). (b) Representation of the three variables placed in a 3D Cartesian coordinate system. (c) All observations in the data matrix are placed in 3D space, and the computed principal components are shown as vector arrows. Key: Obs, Observation; Var, Variable. (Reprinted with permission from Coen, M., and Kuchel, P.W. (2004) Metabonomics based on NMR spectroscopy. *Chem. Aust. 6*, 13–17. Copyright 2004 The Royal Australian Chemical Institute Inc.)

directly and reliably comparable. A commonly applied normalization method known as normalization to total area or constant sum sets the total spectral area of each spectrum to unity; therefore, the intensities of all data points are expressed relative to this. However, many other approaches are routinely used, and metabonomic studies that have investigated the effects of normalization routines on data modeling have been reported in the literature (*44, 45*). Scaling is the final preprocessing step typically applied to NMR spectral data prior to chemometric modeling and is a column operation that aims to reduce the noise in the data and hence improve model interpretability, for example, each column in a matrix can be set to have unit variance or a mean of zero (*44, 46*).

Principal components analysis (*47*) has been widely used in metabonomic studies and is an unsupervised approach in that it allows inherent clustering behavior of samples to be ascertained with no *a priori* knowledge of sample class membership. PCA reduces the dimensionality of a data set as it allows multidimensional data vectors to be projected onto a hyperplane of lower dimensions (typically 2 or 3), with this projection explaining as much of the variation as possible within the data. As previously introduced, the NMR data consists of a matrix of N observations (spectra) and K variables (spectral regions) (Figure 1a) so that a variable space of K dimensions is created. Each variable represents a numerical value on one coordinate axis, and each observation is placed in K-dimensional space. This situation is depicted in Figure 1b for the simple case of three variables in which all observations are added to the one coordinate system (Figure 1c), and then the first principal component (PC) is calculated by a standard method. The first PC (PC1) is a linear combination of the original input variables, and it describes the largest variation in the data set (Figure 1c). The second PC (PC2) is then calculated, and this is orthogonal to PC1 and describes the next highest degree of variation in

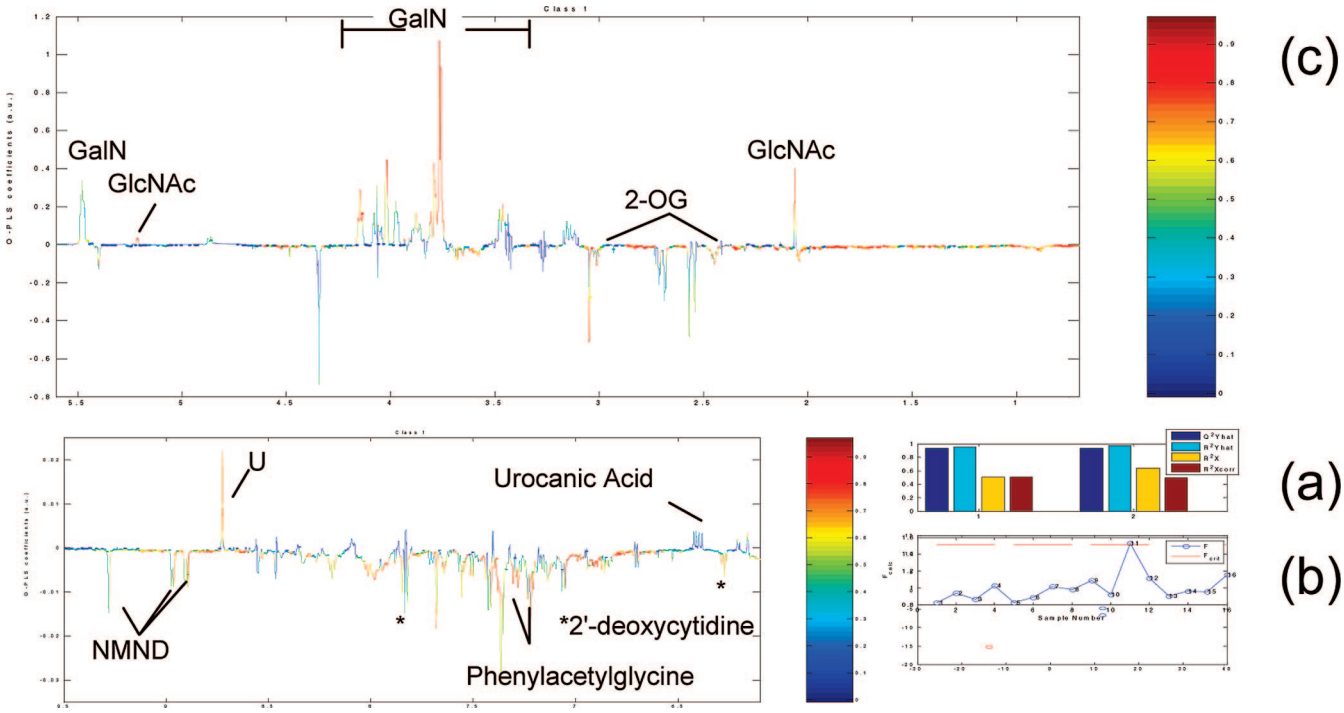

Figure 2. O-PLS-DA pair-wise modeling of urinary 600 MHz ^{1}H NMR spectra (acquired using the standard one-dimensional solvent suppression pulse sequence) from controls and galactosamine-treated classes to aid in identification of latent biomarkers within complex NMR data sets. (a) Model statistics, (b) cross-validated scores, and (c) O-PLS-DA coefficient loadings of full-resolution urinary NMR data revealing metabolites responsible for discrimination between controls and galN-treated samples. The color scale represents correlation (r^2) to the discriminant variable. The upper section of the loadings plots represents metabolites increased in the treated class, whereas the lower part represents metabolites decreased in intensity. (Reprinted with permission from ref *56*. Copyright 2007 American Chemical Society.)

the data set. When two PCs have been defined, they constitute a plane; hence, projection of the observation vectors (in 3-dimensions in Figure 1) in the multidimensional space onto this plane enables the data to be visualized in a two-dimensional (2D) map known as a scores plot. This plot reveals any inherent clustering of groups of data, based purely on the closeness or similarity of their input coordinates. Thus, the analysis provides a convenient and objective means of reducing the complexity of the original data and of visualizing groups and classifying them. A loadings plot is used to interpret the scores plot as it illustrates the spectral variables that contribute to the positioning of the samples on the scores plot and hence the variables which influence any observed separation in the data set. In addition, there are many other visualization (or unsupervised) methods, such as nonlinear mapping and hierarchical cluster analysis (*38*).

Alternatively, in what are known as supervised methods, data sets can be modeled so that the class of separate samples (a validation set) can be predicted on the basis of a series of mathematical models derived from the original data or training set. One widely used supervised method is partial least squares or projection to latent structures (PLS (*48*)), which relates a data matrix containing independent variables from samples, such as spectral intensity values (an X matrix), to a matrix containing dependent variables (e.g., measurements of response, such as toxicity scores) for those samples (a Y matrix). PLS-discriminant analysis (PLS-DA) uses a Y matrix that contains sample class membership information and hence is widely used for sample classification. PLS-DA has also been combined with a preprocessing filter called orthogonal signal correction (OSC), which removes structured class independent variation in data that may arise from analytical variation over the spectral acquisition time or from innate physiological variation (*49, 50*). The use of these and related technologies in metabonomics has recently been reviewed by Trygg et al. (*51*).

O-PLS-DA (*51–53*) is a relatively new approach that extends the traditional supervised algorithm of PLS by prefiltering classification-irrelevant orthogonal variation from data. This prefiltered, structured noise in the data set is modeled separately from the class variation and can also be further interpreted via the loading matrices. The application of O-PLS-DA improves the interpretability of spectral variation between classes and has been shown to successfully discriminate metabolic profiles in a wide range of studies, for example, the metabolic profiles of diets encompassing high-meat, low-meat, and vegetarian content (*54*).

A recent significant advance in chemometric modeling was the use of full-resolution NMR data sets that represented each data point in the spectrum rather than binned NMR data that represented summed segments of a spectrum. The use of full-resolution (all computer points in spectrum) NMR data allows the spectral structure to be retained, and this together with O-PLS-DA models that incorporate the correlation weight of the variables enabled loadings plots that are color coded and easily interpretable by the spectroscopist to be obtained (*55*). O-PLS-DA modeling together with this back-scaling step has been successfully applied to determine the metabolic consequences in multiple biofluid compartments of administration of the hepatotoxins, galactosamine and allyl formate (*56, 57*), and to characterize a model of irritable bowel syndrome together with the effects of the coadministration of probiotic bacteria (*58*). An example of such a back-scaled O-PLS loadings plot that differentiates between urine from control and galactosamine-treated animals is given in Figure 2. The increased and decreased levels of metabolites that discriminate between classes can clearly be identified as the spectral structure is retained, and the metabolites are color coded with respect to discriminatory significance. The application of O-PLS-DA to model full resolution ^{1}H NMR data sets also provides a means of dealing

with peak position variation and spectral misalignment issues as reliable models that are easily interpretable are produced (*55*). Peak positional variation is mainly caused by pH variation in a group of biological samples and a number of approaches to resolve the problem in urine NMR spectra have been published. These include the application of PCA to determine the misalignment for a given peak in a serial set of data (*59*), generalized fuzzy Hough transform (*60*), genetic algorithms and segment-wise peak alignment (*61*), and fuzzy warping (*62*). Reliable statistical modeling and peak alignment of LC-MS data is potentially a far greater problem, and efforts have also begun in this field to deal with these preprocessing problems (*63–65*).

Apart from the methods described above that use linear combinations of parameters for dimension reduction or classification, other nonlinear methods are widely used for multivariate data modeling such as neural networks (*66, 67*). Other novel data mining approaches include Bayesian modeling (*68*) and kernel-based partial least squares (*69*). A comprehensive review of the chemometric tools commonly applied to metabonomic data is provided in Trygg et al. and Eriksson et al. (*51, 53*).

As chromatographic and linked mass spectrometric tools increasingly find application in metabonomic and metabolomic studies, data mining methods that can deal with the high information content of these data are under investigation. A typical UPLC-MS spectrum will contain tens of thousands of peaks, which presents a new challenge in terms of extracting the relevant biomarker information. O-PLS-DA modeling has been applied to binned UPLC-MS data from a study of hydrazine toxicity to reduce the data to a number of candidate biomarker peaks, that is, those that are most significant in terms of class differentiation, these peaks then being further analyzed and structurally characterized (*70*). Further studies have utilized automatic peak detection, alignment, and data compression algorithms combined with PLS-DA for LC-MS data (*63*) and deconvolution approaches for GC-MS data sets (*71*).

4. Metabonomic and Integrated Metabonomic Applications in Toxicology

A wide range of toxins have now been investigated via metabonomic methods, including the kidney cortical toxins mercury II chloride (*15, 21, 41*), *p*-aminophenol (*24, 25, 72*), uranyl nitrate (*22, 23*), the anticancer drug ifosfamide (*73–75*), cephaloridine (*20, 76, 77*), the kidney medullary and papillary toxin, propylene imine (*19, 78*), and the renal papillary toxin 2-bromoethanamine hydrochloride (*19, 21, 46, 79–82*). A host of liver toxins have been studied using NMR-based metabonomics, including hydrazine (*33, 83–85*), allyl alcohol (*32, 46*), thioacetamide (*86*), 1-naphthylisothiocyanate (*87–89*), allyl formate (*57*), galactosamine (*56, 90–92*), bromobenzene (*93, 94*), acetaminophen (*95–100*), and carbon tetrachloride (*32*).

A recent publication (*101*) presents the results of an NMR-based metabonomic study, which investigated urinary profiles from rats dosed with two bisphosphonates, ibandronate and zoledronate, that are known to cause renal impairment in the clinical setting. This study was performed under fully blinded conditions and interestingly found diverse toxic responses for these compounds, which correlated with clinical chemistry and histopathology findings. Zoledronate was found to induce both nephrotoxicity and hepatotoxicity, and a previously unknown biomarker, namely, *N*-acetylfelinine was also discovered, this molecule previously being thought to only occur in feline species. *N*-acetylfelinine is directly reflective of the mode of action of bisphosphonates, which involves inhibition of farnesyl diphosphate synthase and hence can be used to monitor the *in vivo* inhibition activity of the drug. This study highlights the power of a nontargeted metabonomic approach in elucidating novel biomarkers of toxic response and in furthering the understanding of the mode of action of drugs.

Metabonomic evaluation of CI-1018, a selective type 4 phosphodiesterase inhibitor associated with vasculitis in rats, has also been undertaken and NMR spectral urinary profiles discriminated between rats with vascular lesions and those without (*102*). Vasculitis is encountered with several classes of drugs and the metabonomic approach has great potential for developing a rapid, noninvasive means of diagnosis.

The increasing application of NMR-based metabonomics for the diagnosis of environmental toxicology and in ecotoxicology assessment is evident in the published literature. An early study investigated the effects of multiple, low-level exposure to cadmium chloride (*103*), which is a chronic nephrotoxin as well as being an acute testicular toxin and revealed increased excretion of citrate and creatine. High-resolution ^{1}H magic angle spinning (MAS) NMR spectroscopy was used to profile kidney tissue and revealed altered lipid content in animals exposed to Cd^{2+}. This study also showed that unlike acute exposure, no testicular damage was evident following chronic subacute dosing. Exposure to metal contamination in earthworms has also been investigated by NMR (*104*), and a host of metabolic changes were determined, which included increased levels of maltose, a novel and potential biomarker for ecotoxicology assessment. This study has been further extended to encompass acquiring NMR spectra of tissue extracts of earthworms exposed to seven sites with diverse levels of metal contamination and soil type (*105*). Pattern recognition analysis of the data showed that the NMR spectral profiles of earthworm extracts from individual sites could be differentiated and that zinc was the major contaminant implicated in the differences in metabolic profiles. This study highlights the successful application of NMR-based metabonomics in ecotoxicological research in polluted field soils. ^{1}H NMR metabolomics has also been applied to determine the metabolic consequences of exposure of rainbow trout to the synthetic contraceptive estrogen ethinylestradiol (EE2) reflected in blood plasma and plasma lipid extracts (*106*). This study found that metabolites such as vitellogenin, alanine, phosphatidylcholine, phosphtidylethanolamine, polyunsaturated fatty acids, and cholesterol were altered in response to EE2 exposure, and these responses could be correlated with previous findings on the effects of estrogen in fish. Environmental metabonomics has been further extended to profile human urine in a control population and in those exposed to a high-selenium environment in China (*107*). The occurrence of renal and liver lesions related to overexposure to selenium correlated with increased urinary excretion of formate, lactate, acetate, hippurate, and alanine, and decreased excretion of citrate, creatine, and TMAO excretion when compared with that of the healthy human population. It is clear from these studies that the application of metabonomics in the field of environmental toxicology will continue to diversify and expand.

The development of integrative metabonomics where multiple biofluids and/or tissues are profiled simultaneously to try and capture multilevel or compartmental information is a powerful tool to determine a global systems response to a given toxin. This approach provides information on biological changes within different biological matrices, information that when considered as a whole at the biological pathway level often provides enhanced insight on a systems response, as changes in one compartment may be reflected in another or may highlight

mechanistic linkages. Spectroscopic data from multiple biofluids may also be statistically integrated at a data level via statistical correlation spectroscopy (section 8), which results in the computation of correlation maps that highlight correlation or anticorrelation of metabolite levels in biochemical pathways. This statistical spectroscopic approach provides a means of identifying biochemical linkages between compartments in a system and hence is key for isolating candidate biomarkers. The manual or statistical integration of multiple spectroscopic data that is reflective of a systems response to a pathophysiological stimulus allows isolation of the mechanistically relevant metabolic information within complex metabonomic data sets and will lead to new insights into mechanisms of toxicity. Recent examples of integrative metabonomics include the investigation of liver, plasma, and urinary biochemical changes following administration of 1-naphthylisothiocyanate (ANIT), a potent hepatotoxin (*89*). The ANIT-induced biochemical manifestations included a hepatic lipidosis associated with hyperlipidaemia, hyperglycaemia and glycosuria, increased urinary excretion of taurine and creatine, a shift in energy metabolism characterized by increased plasma ketone bodies with reduced urinary excretion of tricarboxylic acid cycle intermediates, and systematically raised hepatic bile acids leading to bileaciduria. Thus, the integration of metabolic data from several biological matrices provided a holistic approach to determine the toxic response of an intact system and enabled the characterization of key metabolic effects during the development and recovery from a toxic lesion.

Integrated metabonomics was also applied to elucidate the metabolic consequences of treatment with bromobenzene, a centrilobular hepatotoxin (*93*). Conventional solution-state NMR together with magic angle spinning NMR were used to obtain metabolic profiles of urine, plasma, and intact liver, which presented a broad systemic view of the effects of bromobenzene-induced hepatic toxicity. In addition, a number of putative protein targets of bromobenzene and its metabolites were identified including those enzymes of the glutathione cycle, exemplified by the presence of a novel biomarker, 5-oxoproline, in liver, plasma, and urine. This work highlighted the importance of metabonomics in novel biomarker discovery and in providing new insights into the mechanistic complexity of drug toxicity.

This integrated approach was also applied to determine the metabolic effects in urine, plasma, kidney, and liver of the well-known hepato and nephrotoxin, thioacetamide (*86*). The metabolic effects elucidated included renal and hepatic lipidosis and increased urinary levels of taurine and creatine together with elevated creatine in liver, kidney, and plasma. There was also evidence for a shift in energy metabolism as depleted levels of hepatic glucose and glycogen were apparent together with reduced urinary excretion of tricarboxylic acid cycle intermediates and ketosis. Furthermore, elevated levels of amino acids were seen in liver samples and blood plasma, which suggested protein degradation as a result of renal dysfunction, whereas elevated hepatic and urinary bile acids indicated secondary damage to the biliary system. This work showed the potential of metabolic profiling in differentiating the time-related systemic responses in a multiorgan toxicity model and, in addition, provided novel biochemical information on primary and secondary toxic responses. Multiple organ failure is a common life threatening complication of sepsis in man, and this study suggests a wider role of metabonomics in understanding systemic metabolic failure in critically ill patients.

A similar approach was also applied to a compound in development (MrkA) that had been shown to induce hepatotoxicity in several animal species (*108*). ^{1}H NMR spectra were acquired on urine and liver tissue samples, and pattern recognition analysis of the data enabled the metabolic effects of administration of MrkA to be determined; these included a urinary depletion in tricarboxylic acid cycle intermediates and the appearance of medium chain dicarboxylic acids. MAS NMR data revealed elevated triglyceride levels that were correlated with dicarboxylic acid uria and suggested the defective metabolism of fatty acids. This metabonomic result was confirmed by subsequent *in vitro* experiments that showed that MrkA impaired fatty acid metabolism, which highlighted the potential of an integrated metabonomics approach in defining an unknown mechanism of drug-induced toxicity. This study is an example of the successful testing and validation of a metabonomic generated hypothesis to determine a mechanism of toxicity and highlights the potential of metabonomics in the field of pharmaceutical research and development.

Integrative metabonomics was also utilized for the determination of time-related metabolic events in rat liver, plasma, and urine following hepatotoxic insult with allyl formate (*57*). The metabonomic results were compared with conventional clinical chemistry and histopathology analyses. The metabolic changes that were induced included hepatic lipidosis, decreased liver glycogen and glucose, decreased plasma lipids, increased plasma creatine and tyrosine, increased urinary taurine and creatine, and decreased urinary tricarboxylic acid cycle intermediates. These findings suggested mitochondrial impairment and consequential depletion of hepatic ATP with increases in plasma tyrosine, suggesting impaired protein synthesis, a known consequence of ATP depletion. This integrative approach was further extended by using partial least-squares modeling to correlate the metabolic profiles of liver and plasma, and the increased hepatic lipids were found to correlate with the reduced plasma lipids, which suggested disruption in lipid transport from the liver to plasma, which may have been caused by impaired apolipoprotein synthesis. This study highlighted the benefits of correlation of metabolic profiles from different biological samples such as liver and plasma, which added another dimension to biochemical information recovery at a systems level.

5. Metabolic Information from Intact Tissues: Magic Angle Spinning (MAS) NMR

MAS NMR is increasingly being utilized for the determination of metabolic profiles of intact tissue samples and for investigation of the dynamics and physicochemical properties of tissue. It provides a nondestructive analytical tool that requires small amounts of tissue (ca. 10 mg) that can be further analyzed by complementary technologies post spectral acquisition. The technique is based on spinning a tissue sample at the magic angle (54.7° relative to the magnetic field), which reduces anisotropic line-broadening effects and hence produces highly resolved spectra.

The technique opens up many diagnostic possibilities since information on a variety of metabolites in different cellular environments can be rapidly obtained, and specialized NMR experiments, such as those developed to measure molecular diffusion coefficients or longitudinal/transverse relaxation times, can be used to probe compartmentation. Confirmation of biochemical composition can be obtained using standard high-resolution NMR of both aqueous (protein-free) and methanolic extracts. This produces a comprehensive set of metabolic information that can be used in integrated metabonomics studies. Animal tissues can be examined after exposure to model organ-

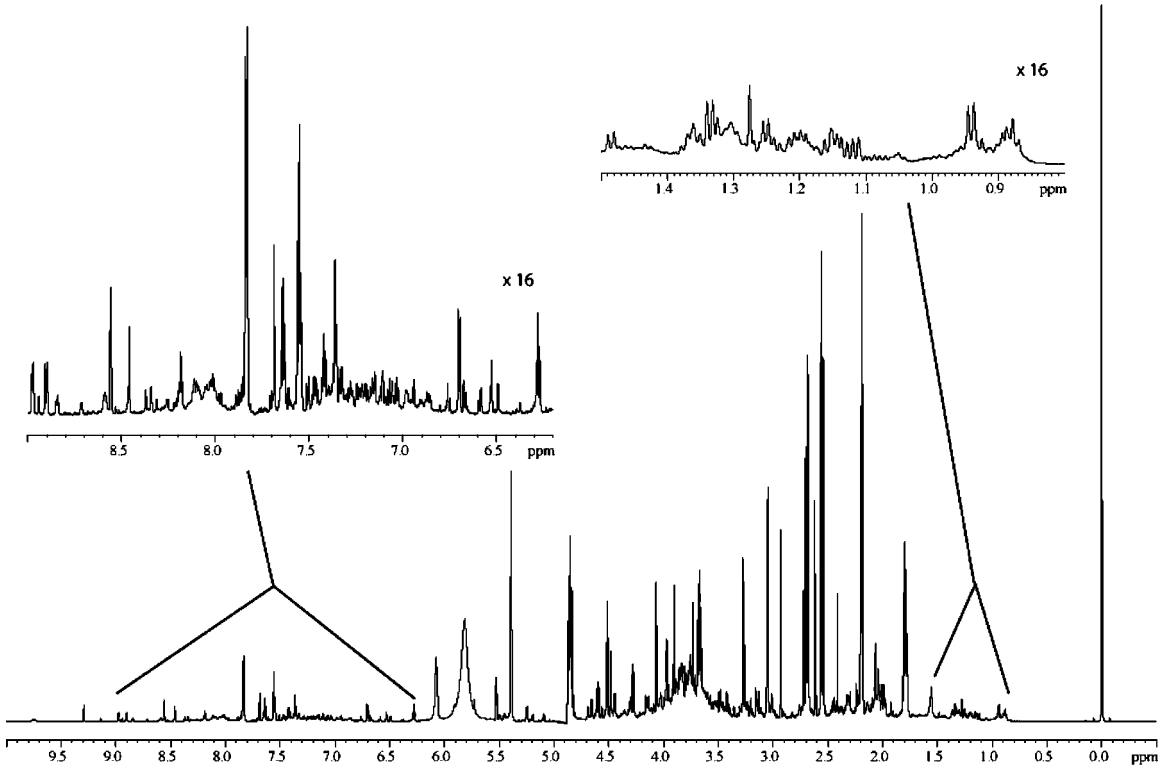

Figure 3. Typical ^{1}H 800 MHz cryo NMR spectrum of rat urine showing the high level of spectral detail including expansion of an aliphatic and aromatic region of the spectrum (Coen, M., unpublished data).

specific toxins and drugs using MAS NMR and standard NMR methods, allowing investigation of the time-related changes in biochemical profiles. MAS NMR data, like biofluid NMR spectra, can also be subjected to computer pattern recognition methods in order to classify toxicity type (target organ and biochemical mechanism) and to map time-related biochemical trajectories associated with drug-induced biochemical changes (*2, 8*). The ability to compare biofluid and tissue NMR spectra provides further insight into mechanisms of toxicity and target organ identification (section 4.0).

MAS NMR was first applied to profile cells and tissues (*109–116*). More recently, this approach has been successfully applied to metabolically profile the brain (*117, 118*), intestinal tissue (*58*), prostate (*119, 120*), kidney (*79, 84, 86, 121*), liver spheroids (*92*), liver (*56, 88, 89, 93, 95, 122*), whole-cell bacteria (*123*), hepatocellular carcinoma (*124*), and parasitic protozoa (*125*). In addition, the application of ^{31}P MAS NMR to study tissue has been reviewed (*126*). Recent advances in MAS NMR technologies include the use of pulse sequences, such as total sideband suppression (TOSS *119, 127*), phase-altered spinning sideband (PASS *119, 127*), and phase-corrected magic angle turning (PHORMAT (*128*), that enable high-resolution spectra to be generated at low spin rates so as to minimize sample degradation during spectral acquisition.

6. Ultrahigh Field NMR Spectroscopy of Biological Samples

As the role of NMR in metabonomics has evolved, an ever-increasing range of applications have opened, enabled by the developing technology. The advent of high-field NMR impacted significantly on the biological application areas as the much greater sensitivity that this afforded has significantly extended the NMR boundaries of detection and enabled a wider metabolic pool to be profiled. The availability of a 1 GHz magnet is imminent from manufacturers such as Bruker and Varian with 800, 900, and 950 MHz magnets now being widely available with cryoprobe platform capabilities. The improvement in NMR-based technologies has resulted in enhanced biological information recovery from metabonomic studies, which further extends the scope and ability of the platform.

A recent innovation that has further improved the analytical power of NMR is the availability of cryogenically cooled probes that cool the RF coils and preamplifier to approximately 20K with liquid helium boil off gas, and this has the effect of greatly reducing electronic/thermal noise, which results in an improvement factor in the spectral signal/noise ratio of up to 4-fold (*129*). Cryoprobes are thus ideally suited to weak and dilute samples, where the acquisition times can be much reduced when compared to data acquisition on a conventional room temperature probe. This technology has been used to rapidly generate information-rich ^{13}C NMR profiles of urine as the low natural abundance of ^{13}C nuclei are counteracted by the sensitivity gain (*129*). Cryogenic ^{1}H and ^{13}C NMR utilizing both 1D and 2D techniques have also been used to detect micromolar quantities of dimethylsulfone in cerebrospinal fluid in patients with methionine adenosyltransferase deficiency (*130*). A typical rat urine cryo ^{1}H NMR spectrum obtained on an 800 MHz instrument is shown in Figure 3. The high information content of this spectrum is evident with hundreds of resonances representing metabolites from a broad spectrum of chemical classes. Furthermore, as automated NMR technologies are continually developed and updated, large sample cohorts, such as those from epidemiological or population screening studies have become feasible as high-throughput, operator-independent spectral acquisition is achievable. Such automated systems involve the use of 96-well plates, robotic sample handling, and transfer to NMR flow probes; typically, up to 250 ^{1}H NMR

metabolic profiles can be acquired per day, but there is potential for running at much higher rates.

7. Improved Analytical Technologies for Metabolite Identification: Solid-Phase Extraction Chromatography, Liquid Chromatography, and Mass Spectrometry

A wide range of analytical platforms is increasingly being utilized either alone or in conjunction with NMR spectroscopy to detect and characterize a wider range of metabolites. Such platforms include capillary electrophoresis and high-performance and ultraperformance liquid chromatography (*11, 12*), which are coupled with mass spectrometry (MS). Chemical derivatization methods followed by gas chromatography and MS have been widely used to successfully generate metabolic profiles for a host of plant and microbial extracts (*5, 131–134*).

The development of directly coupled LC and NMR provided the ability to simplify complex biofluid spectra by use of a chromatographic separation step. This has proved particularly advantageous for structural elucidation of drug metabolites (*135–138*). An alternative sample extraction step that is often used that facilitates identification of potential biomarkers is solid-phase extraction chromatography (SPEC), which enables rapid extraction/fractionation/purification of a given analyte of interest from a complex matrix such as a biofluid sample (*139, 140*). The SPEC fraction can be profiled via NMR methods and the resonances of interest are less confounded by interfering, overlapped resonances, hence facilitating structural identification. Chromatographic methods can be also used to further extract the SPE fraction, and MS can also be used for complementary identification of the analyte(s) of interest. SPE followed by NMR has been successfully applied to assign *m*-(hydroxyphenyl)-propionic acid in rat urine (*141*), and LC-MS together with NMR spectroscopy has been utilized to assign phenotypic differences differentiated by urinary levels of dihydro-quinolinone glucuronide (*142*). Furthermore, the coupling of LC and NMR has been extended by using HPLC chromatography to fractionate a sample that is then simultaneously sent to a MS and NMR spectrometer for profiling. This routine has enabled a fully automated and more comprehensive analysis of biofluids than either platform used in isolation (*137, 143*).

MS is generally much more sensitive than NMR spectroscopy in terms of the inherent detection limit; however, this must be balanced with problems that are commonly encountered with reproducibility, matrix effects, and ion suppression. Furthermore, it is often necessary to target chromatographic methods to a given class of chemical. However, recent developments in the applications of MS coupled with liquid chromatographic methods to metabonomic data sets have resulted in the technologies providing complementary metabolic information to NMR-based analyses, and MS is rapidly gaining acceptance as a valuable biofluid metabolic profiling tool (*144–148*).

HPLC is typically performed on reversed-phase gradient chromatographic columns with HPLC using solvent gradients, and chromatography is often combined with electrospray ionization (ESI). MS is typically performed in both positive and negative modes for each sample to capture the most comprehensive metabolic information. Ultra performance liquid chromatography (UPLC) is a recently developed method (*12, 149–151*), which provides greatly improved chromatographic ability and hence a reduction in ion suppression problems. UPLC utilizes small particle sizes (1.7 μm) and high column pressures (>800 bar) for greater separation abilities and speed of data acquisition; typically, a 10-fold increase in speed and a 3- to 5-fold increase in sensitivity are seen compared to a conventional stationary phase. The main advantage of UPLC is in the reduction of ion-suppression in MS as the chromatographic separation is much improved. This technology coupled to mass spectrometry enables thousands of metabolites to be detected in a given sample in very short periods of time, hence realizing high-throughput data acquisition and has been applied to profile urine in a number of rodent studies (*12*). UPLC-MS has recently been applied to separate and identify amphetamine-type substances (*152*), rapidly quantify organic acid metabolites during microbial fermentation (*153*), detect lipophilic marine toxins (*154*), and determine troglitazone in mouse plasma (*155*) and oral contraceptives in human plasma (*156*). In metabonomic studies, UPLC-MS has successfully enabled the separation of Zucker rats on the basis of phenotypic differences (*149, 151*) and the sera from patients with intestinal fistulae from controls (*157*). UPLC-MS has also been successfully applied to differentiate metabolic profiles of urine from different strains of Zucker rats, namely, Zucker (fa/fa) obese, Zucker lean, and the lean/(fa) obese cross, and it was also possible to separate age-related effects in these animals (*158*). Metabolic profiles of plasma from the above strains of Zucker rat have previously been separated by UPLC-MS methods (*146, 151*), and taurocholate was found to significantly differentiate between the obese and lean strains, which provided further support for an involvement of taurine metabolism in diabetes. The creation of comprehensive mass spectral libraries (*133*) will be key to the continued expansion of these technologies in the field of metabonomics. A recent study (*159*) has used a multiplatform approach: both UPLC-MS and NMR to profile the hepatotoxic effects of pravastatin via metabolic profiling of urine. The two analytical platforms provided complementary information, and candidate biomarkers for pravastatin toxicity were identified such as elevated levels of taurine, creatine, and bile acids. The power of this approach was further exemplified in a study of the biochemical effects of mercury II chloride in the rat using ^{1}H NMR and HPLC-TOF/MS, which identified a host of biomarkers of toxicity by both NMR and MS (*160*). ^{1}H NMR revealed increased levels of lactate, alanine, acetate, succinate, trimethylamine, and glucose together with reduced levels of citrate and alpha-ketoglutarate postdosing. These findings were consistent with those previously reported in the literature and indicate proximal tubule nephrotoxicity (*19, 41, 42, 161*). The complementary use of HPLC-TOF/MS revealed decreases in kynurenic acid, xanthurenic acid, pantothenic acid, and 7-methylguanine postdosing, which provided further metabolic information on the toxic response to mercury II chloride.

The value of a combined NMR and MS metabolic profiling approach to monitor idiosyncratic hepatic response to ranitidine (RAN), a histamine-2 receptor antagonist, has been demonstrated (*162*). It is believed that environmental factors such as inflammation initiated by bacterial lipopolysaccharide (LPS) may play a role in idiosyncratic response, and hence, this metabonomic study involved the cotreatment of rats with RAN and LPS, in addition to treatment with RAN and LPS alone. Pattern recognition analysis of both NMR and MS data revealed the ability to differentiate metabolic profiles of the cotreated group from all others, which suggests the future potential of metabonomics in identification of drug candidates with the potential to cause idiosyncratic toxicity.

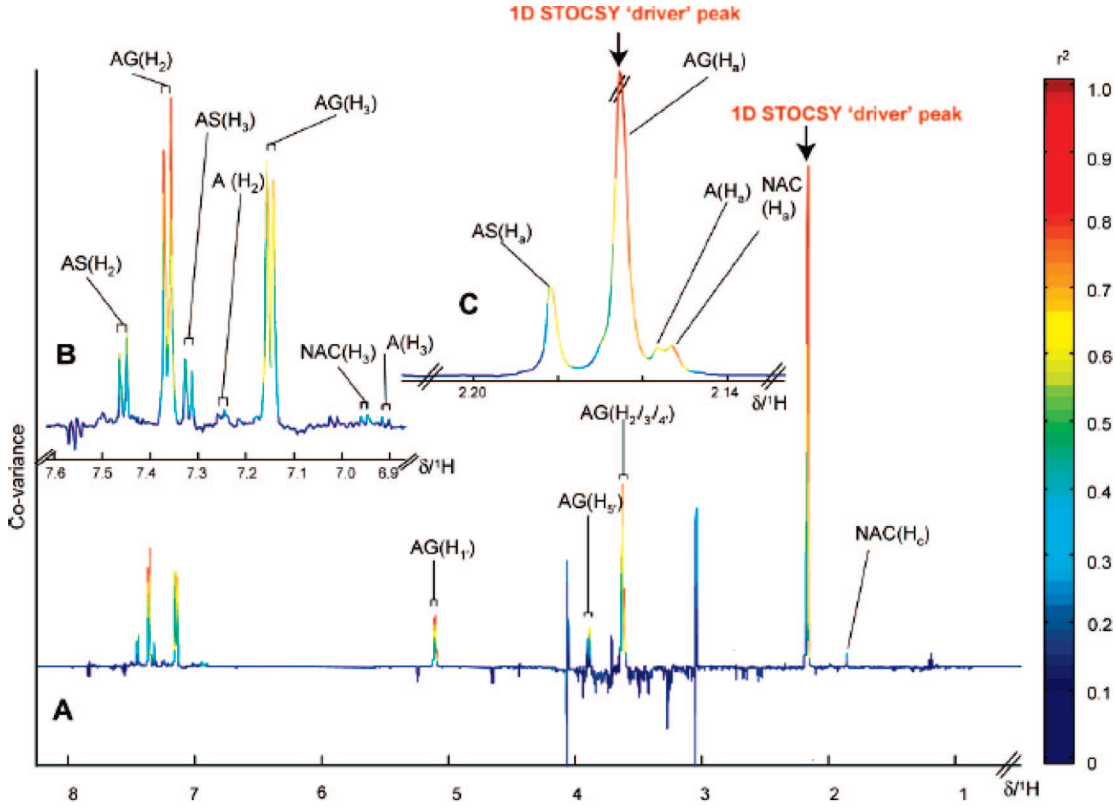

Figure 4. STOCSY involves computation of a correlation matrix between a chosen spectral data point (the maximum of a given peak) and all the other data points of the spectra. STOCSY is performed across a series of 1D NMR spectra and identifies correlated spectral resonances from the same molecule and also from molecules linked via metabolic pathways. The STOCSY approach facilitates molecular structural assignment and biomarker recovery. Color-coding is used to present the strength of the correlation (r2). The upper section of the STOCSY plot represents metabolites positively correlated with the driver peak, and the lower part represents metabolites that are negatively correlated. STOCSY plot derived from the correlation matrix calculated between the data point at the peak maxima of the N-acetyl proton signal of AG resonance (δ 2.17) and all other data points, as indicated by the arrow, showing strong correlation (red/orange data points) with resonances at 3.62, 3.89, 5.1, 7.13, and 7.36 ppm. Slightly weaker correlations are shown with A and AS. (B) Expansion for the aromatic region showing signals for acetaminophen and its related metabolites. (C) Expansions for the δ 2.17 N-acetyl resonance of acetaminophen metabolites. (Reprinted with permission from ref *164*. Copyright 2006 American Chemical Society.)

8. Statistical Spectroscopy and Biomarker Discovery

Recently developed statistical methods such as statistical correlation spectroscopy (STOCSY, (*163*)) have significantly enhanced information recovery from complex metabonomic data sets. STOCSY encompasses the computation of correlation statistics between the intensities of all computer points in a set of complex mixture spectra, thus generating connectivities between signals from molecules that vary in concentration between samples. This statistical method allows latent spectroscopic information of interest to be extracted from complex, highly overlapped spectra. STOCSY can be combined with supervised chemometric methods to provide linked information on those spectral features that best separate sample classes (*55, 163*).

STOCSY has been used to derive the assignment of biomarker metabolite NMR resonances in nephrotoxic states as a result of exposure to mercury chloride and provided an unbiased, sensitive approach to biomarker extraction and identification, and showed the potential for generating potential novel pathway connectivities (*164*). STOCSY has also been utilized for population-based identification of drug metabolites in human urine samples (*165*). ^{1}H NMR spectra were acquired for two groups of urine samples, and the application of STOCSY to the data enabled rapid identification of the major and minor drug metabolites in common use in the population, in particular, those from acetaminophen and ibuprofen. The work showed that statistical connectivities between drug metabolites could be established in routine high-throughput NMR screening of human samples from participants who had randomly self-administered drugs. Hence, the STOCSY approach provides a powerful tool in considering interpopulation patterns of drug metabolism in epidemiological and pharmacogenetic studies. An example of a STOCSY correlation plot driven from the N-acetyl resonance of acetaminophen glucuronide (AG) is given in Figure 4, where correlations to the other AG resonances are clearly seen. This plot also illustrates the information obtained on pathway connectivity relationships as weaker correlations are seen to the parent drug, acetaminophen, and to acetaminophen sulfate. Furthermore, STOCSY has recently been applied to enhance information recovery from LC-NMR data sets (*135*) and diffusion-edited NMR data sets (*166*) arising from complex biological mixtures. The STOCSY approach is generic and can be applied in both 1D and 2D forms and to homo or heteronuclear data to aid structural elucidation and determine pathway relationships in a given spectral sample set. Heterospectroscopic-STOCSY (HET-STOCSY) encompasses the statistical correlation of two different types of experimental data, for example, from heteronuclear experiments or any given parallel combination of experimental data. The successful application of HET-STOCSY to aid metabolic biomarker assignment in a metabonomic study of galactosamine-induced hepatotoxicity has recently been demonstrated for ^{1}H- ^{31}P MAS NMR spectra of intact liver (*167*).

Furthermore, the recent development of statistical heterospectroscopy (SHY) shows the power of computation of covariance matrices for successful interrogation of multispectroscopic data sets collected in series or parallel such as those from NMR and ultraperformance liquid chromatography–mass spectrometry (UPLC-MS) platforms. The potential of SHY has been demonstrated for a metabonomic data set representing hydrazine toxicity (*168*), where direct cross-correlation of chemical shifts (NMR) and *m*/*z* data (MS) has provided structural and metabolic pathway activity information. The application of SHY to MS and NMR data sets allows improved molecular biomarker identification capabilities as not only structural information but also higher level biological information on metabolic pathway activity and connectivities is obtained. This information is found within different levels of the NMR to MS correlation and anticorrelation matrices, and it should be noted that the SHY approach is equally applicable to any two independent spectroscopic data sets.

Related statistical cross-projection methods have also been applied to link proteomic with metabonomic data (*169*) on data arising from a human tumor xenograft mouse model of prostate tumor. Blood plasma from mice implanted with prostate tumor was profiled using both NMR spectroscopy and 2D-DIGE technologies. The data was integrated using OPLS modeling algorithms, and multiple correlations between metabolites and proteins were found, including associations between serotransferrin precursor and both tyrosine and D-3-hydroxybutyrate, and reduced levels of tyrosine were correlated with increased levels of gelsolin.

Furthermore, a recent publication highlights the successful integration of genome−phenotype data with metabonomic data (*170*) from a rat type II diabetes model. Plasma metabolic fingerprints derived from NMR spectroscopic profiling were used to map quantitative trait loci (QTL) in a cross between diabetic and control rats. Candidate metabolites were proposed for the most significant QTLs, one of which includes a gut microbial metabolite (benzoate) that can be explained by deletion of a uridine diphosphate glucuronosyltransferase. These trans-omic data projection methods allow systems biology integration of information for deeper mechanistic understanding of *in vivo* model systems and enhanced combination candidate metabolic biomarker recovery (*9, 171*).

9. Recent Consortium Projects Using NMR/MS Driven Metabonomics and Top-Down Systems Biology in Toxicology

The development of major new paradigms for drug screening requires the engagement and resources of the pharmaceutical industry coupled to academic research groups. Such initiatives involve experimental studies, database construction, and mathematical modeling. The utility of metabonomics in the evaluation of xenobiotic toxicity was comprehensively assessed by the Consortium for Metabonomic Toxicology (*172–174*), which was formed between five major pharmaceutical companies and Imperial College London, U.K. and ran between 2000 and 2004. The main objectives of COMET were to assess and develop methodologies to generate a metabonomic database using ^{1}H nuclear magnetic resonance (NMR) spectroscopy of rodent urine and blood serum for preclinical toxicological screening of candidate drugs and to build a predictive expert system for target organ toxicity. The analytical and biological variation that might arise through the use of metabonomics was evaluated at an early stage and a high degree of robustness demonstrated. The chosen COMET target compounds represented an extensive range of metabolic space; hence, structures and activities were diverse, but there was an emphasis on analysis of hepato and nephrotoxins. With the completion of 147, 7-day, low- and high-dose toxicological and physiological studies, the chief deliverables of a curated database of rodent biofluid NMR spectra and computer-based expert systems for the prediction of kidney or liver toxicity in rat and mouse on the basis of the spectral data have been generated and delivered to the sponsoring companies. The project, with its relatively modest resources by consortium standards, has exceeded all of its targets and was judged a success by the sponsoring companies who are, in some cases, already enhancing and making use of the spectral data in their in-house studies.

One of the modeling approaches using probabilistic temporal modeling (developed as part of the COMET project) was recently published (*174*). The COMET database was used to develop an expert system for prediction and classification of drug toxicity, which enabled collections of metabolic trajectories representing diverse responses to nephro and hepatotoxins to be compared (*174*) and the likely type of toxicity from new treatments to be predicted, thereby assisting in the elucidation of toxic mechanisms. A subset of the COMET database representing 80 hepato and nephrotoxins was used to build a modeling system that was capable of differentiating NMR urinary metabolic profiles from controls and treated animals. The CLOUDS approach (*94*) was then applied to determine similarities between treatments, which enabled the separation of kidney and liver toxins on the basis of their urinary metabolic profiles. This similarity matrix could then be used to predict the toxic outcomes of unknown treatments. The sensitivity and specificity of the expert system for liver toxins was 67 and 77% and for kidney toxins, 41% and 100%, respectively, whereas the predictive ability of the system had an error rate of 8%. This expert system uses the systems wide window provided by metabonomic data to successfully characterize toxic failure. The overall toxic response is driven by specific mechanisms and sites of action, but the metabolic fingerprint may be derived from multiple biological sources. This approach has provided the largest validation to date of the value of metabonomics in preclinical toxicology assessment and confirmed that the methodology offers practical utility for rapid *in vivo* drug toxicity screening.

Many novel modeling strategies, such as a method to test for homothetic geometry called scaled-to-maximum, aligned, and reduced trajectories (SMART) analysis, were developed as part of the COMET research effort (*94*). This method facilitates scaling of the differences between metabolic starting positions and hence enables multivariate response similarities to be visualized from two or more sets of metabonomic measurements. Metabolic principal component trajectories and SMART analysis were successfully applied to determine the interspecies variation between rats and mice exposed to hydrazine (*85*). This approach facilitated the comparison of the response geometries between the rat and mouse. A greater magnitude of metabolic effects were observed in the rat post hydrazine dosing, which was supported by the more pronounced effect on liver pathology in the rat than in the mouse. In addition, the COMET consortium successfully applied a host of spectral editing methods to profile intact liver tissue and combined this with PCA to elucidate the effects of hydrazine toxicity on rat liver biochemistry (*175*).

A further novel modeling method developed to classify large COMET data sets utilizes a density superposition approach, CLOUDS, which is a non-neural implementation of a classification technique developed from probabilistic neural net-

works (*176*). This approach was initially applied to NMR spectra of urine from rats from 19 different treatment groups, and the data were modeled according to organ of effect with >90% of the test samples classified as belonging to the correct group. These metabonomic data representing 19 toxins were also modeled using PCA, hierarchical cluster analysis (HCA), and k-nearest-neighbor (kNN) classification to reveal dose- and time-related effects (*177*). PCA and HCA provided valuable overviews of the data, highlighting characteristic metabolic perturbations representative of the organ of effect, and kNN analysis of the multivariate data successfully predicted all the different toxin classes with >85% success rate (training/test).

COMET also successfully assessed the analytical reproducibility of metabonomic protocols, which involved performing sample preparation and NMR data acquisition at two sites (one using a 500 MHz and the other using a 600 MHz system) using two identical (split) sets of urine samples from an 8-day acute study of hydrazine toxicity in the rat (*129*). Despite the difference in spectrometer operating frequency, both data sets were found to be extremely similar when analyzed using PCA and gave near-identical descriptions of the metabolic responses to hydrazine treatment. COMET also utilized an integrated metabonomic approach to establish the systemic metabolic response to liver regeneration, using the partial hepatectomy (PH) model in the rat (*179*). Male Sprague–Dawley rats were subjected to surgical removal of ~two-thirds of the liver, sham operated surgery, or no treatment, and urine, liver, and serum collected over a 7-day period were analyzed by NMR-based metabonomics. Several urinary perturbations, such as changes in 1-carbon and lipid metabolism, were observed, which were indicative of hepatocellular regeneration.

The follow-up second Consortium on Metabonomic Toxicology (COMET 2) is now underway, and it extends this linkage among pharmaceutical companies, a major instrument manufacturer, and academia and aims to utilize metabonomics as a top-down systems biology driver to direct research and experiments in the determination of mechanisms of toxicity and the assessment of risk factors (*1, 4, 171*). Hence, COMET 2 aims to significantly enhance the knowledge gained from COMET by detailed testing of metabonomic hypotheses using NMR, LC-MS, and appropriate *in vitro* and *in vivo* labeling studies and validation of results via follow-on biochemical studies with the ultimate aim of determination of mechanisms of action of a wide range of drugs. The initial efforts have concentrated on understanding model systems, for example, galactosamine hepatotoxicity, which despite being put forward as a model for viral hepatitis for many years still has many enigmatic features of its metabolism and elicits hyper-variable responses. A recently published study investigated the protective ability of glycine in a galactosamine-induced hepatotoxic model (*56*). This work demonstrated the utility of the new technologies in uncovering new metabolic facets of the mechanisms of supposedly well understood toxins.

Another ongoing COMET 2 problem relates to bromoethanamine (BEA) and understanding the development of renal papillary necrosis (RPN) using chemical models. Nonsteroidal anti-inflammatory drugs (NSAIDs) are the most widely recognized class of compounds known to cause this toxicity in rodents and in humans. However, it is difficult to study renal medullary function with noninvasive methods, and hence, there are no sensitive and selective biomarkers for evaluating RPN. Furthermore, it is planned that clinical samples of RPN will be analyzed so that biomarkers relevant to the clinical setting can be differentiated from those of relevance to general physiological function.

10. Clinical Metabonomics

Many of the earliest clinical studies that involved the application of NMR spectroscopy to profile urine and plasma have been reviewed previously, and concentration on the field of inborn errors of metabolism was evident (*180–182*). A recent and renewed surge has taken place in the application of NMR-based metabolic profiling together with multivariate statistical analysis to aid human disease diagnosis, which coincides with the more sensitive analytical platforms available and the enhanced statistical tools for biomarker discovery. An early and significant example of the application of NMR-based metabonomics in disease diagnosis was the study of human serum samples to ultimately develop a diagnostic method for coronary artery disease (*49*).

Recent clinical metabonomic studies include the investigation of type 1 diabetic nephropathy using plasma (*183*) and HPLC-MS methods to profile plasma phospholipid signatures in type II diabetes (*184*).

The application of NMR-based metabonomics in neurological disorders has been extensively reviewed (*118*), and recent studies have investigated neurological conditions, such as Alzheimer's disease, Huntington's disease, muscular dystrophy, cerebellar ataxia, meningitis (*185*), and schizophrenia (a detailed list of references is to be found in ref *118*).

One area of disease where progress is being made using NMR-based metabonomics studies of biofluids is cancer. Magic angle spinning NMR and multivariate data analysis methods have been used to successfully differentiate human hepatocellular carcinoma tumors from controls (*124*). A study that involved the diagnosis of epithelial ovarian cancer based on analysis of serum has also been published (*186*) together with the use of HPLC fingerprints to profile biofluids in patients with liver cancer (*187*).

Furthermore, a recent study has applied NMR-based metabonomic technologies to characterize inflammatory bowel diseases such as Crohn's disease and ulcerative colitis, and the published results show rapid and clear differentiation of fecal extracts between patients and controls (*188*). UPLC-MS methods have been used to profile sera from patients with intestinal fistula (*157*). An important study has been published that evaluates the effects of both sample preparation and storage on metabonomic profiles of human urine samples, and this leads the way toward standardized metabonomic practices for disease diagnosis (*189*).

The renal toxicity of aminoglycoside and glycopeptide antibiotics in a cohort of patients in an intensive care unit has also been studied using NMR-based metabonomic techniques (*190*). Urine from patients receiving an aminoglycoside and/or a glycopeptide and presenting with renal dysfunction were metabolically profiled, and a range of metabolite levels relative to creatinine were extracted. Dimethylamine was found to be highly correlated with clinical markers of renal dysfunction, and this suggested that nephrotoxicity from these antibiotics may involve both proximal tubular and medullary (loop of Henle and collecting duct) toxicity.

11. Molecular Epidemiology

The application of modern analytical and omics technologies to study large-scale population-based biological samples has

offered new opportunities to investigate many aspects of human biology and population phenotyping.

The International Study of Macro/Micronutrients and Blood Pressure (INTERMAP) was launched in 1996 to investigate the relationship of multiple dietary variables to blood pressure (*191, 192*). Urine samples ($n = 4680$) representing adult men and women (aged 40–59) from populations in Japan, the People's Republic of China, the United Kingdom, and the United States were analyzed using NMR-based metabonomic technologies. The robustness of the analytical platform together with sample handling/collection protocols was studied, and it was found that the reproducibility of the NMR screening platform was >98% (*194*). Furthermore, differences in urinary metabolites between populations were determined, which relate to genetic, dietary, and gut microbial factors. The U.S. human population ^{1}H NMR urinary data was also analyzed using a range of chemometric tools, such as PCA, STOCSY, and O-PLS-DA to characterize structural pathway connectivities of the metabolites of commonly used drugs (*165*). The concept of the xenometabolome was defined in this study as the "multivariate description of the xenobiotic (foreign compound) metabolic profile of an individual or sample from an individual that has been exposed through any route (either deliberately or accidentally) to drugs, environmental pollutants or dietary components, that cannot be completely catabolized by endogenous metabolic enzyme systems". Hence, the xenometabolome of an individual can be envisaged as the exogenous part of the metabolic phenotype that defines external, nongenomic, and environmental interaction, and this approach will clearly prove of importance in considering interpopulation patterns of drug metabolism in epidemiological and pharmacogenetic studies. Furthermore, the metabonomic urinary NMR data can be used to assess self-reported meta-data, which many epidemiological studies are based on, although these are often limited in reliability (publication in preparation). This work shows that it is possible to perform population pharmacology in molecular epidemiology studies, and capturing the xenometabolome as an environmental health factor represents a new metric to help understand population disease risk factors.

12. Integrated -Omic Applications

Metabonomics offers a complementary approach to alternative omics platforms such as genomics, transcriptomics, and proteomics, which involve the study of genetic complement, gene expression, and protein synthesis, respectively. Although transcriptomic/genomic and proteomic measurements can elucidate the response to the administration of toxic agents, it is difficult to relate findings to classical toxicological end points, whereas metabonomics can capture information on whole-organism functional integrity over time following pathophysiological stimuli. Multivariate temporal modeling of target biofluids and tissues provide information on characteristic changes in the pattern of concentrations of xenobiotic and endogenous metabolites that relate to the site and mechanism of toxicity. Metabonomics provides a useful connection between the omics platforms and actual tissue histology as real-world end points are observed and studied. There remains a strong imperative to be able to integrate information at the transcriptomic, proteomic, and metabonomic levels despite these different levels of biological control showing very different timescales of change. This is because some events can be rapid such as on/off gene switching, and other transcriptional and translational processes work on longer timescales, for example, protein synthesis and turnover, or, in the case of metabolic changes, can encompass enormous ranges of timescales (*171*). Counterintuitively, biochemical changes do not always occur in the order transcriptomic, proteomic, and metabolic because, for example, pharmacological or toxicological effects at the metabolic level can induce subsequent adaptation effects at the proteomic or transcriptomic levels (*2*). One important potential role for high-throughput and highly automated metabonomic methods, therefore, could be to direct the timing of more expensive or labor-intensive proteomic and transcriptomic analyses to maximize the probability of observing meaningful and relevant biochemical changes using those techniques.

Metabonomics will become increasingly important in connecting molecular events at the gene and protein level to those occurring at the macrosystem level including pathological end points, and recent multiomic studies include the analysis of methapyrilene- (*193*) and acetaminophen-induced (*96*) hepatotoxicity in the rat. In addition, integrated metabonomic and transcriptomic methods have been applied to elucidate the metabolic and genetic basis of insulin resistance and nonalcoholic fatty liver disease (*170, 195*) and to statistically integrate proteomic and metabonomic data representing a human tumor xenograft mouse model of prostate cancer (*169*).

An integrated metabonomic approach was also utilized to study the effects of peroxisome proliferator-activated receptor (PPAR) ligands on urine and plasma NMR and HPLC-based fingerprints, and two potential biomarkers of peroxisome proliferation in the rat were described, *N*-methylnicotinamide and *N*-methyl-4-pyridone-3-carboxamide, both endproducts of the tryptophan−nicotinamide adenine dinucleotide (NAD^+) pathway (*196*), suggesting the alteration of this pathway as a result of peroxisome proliferation. This study was extended to integrate metabonomic findings with genomic data, and the metabonomic derived mechanistic hypothesis was confirmed as the relevant genes encoding two key enzymes in the NAD^+ pathway, aminocarboxymuconate−semialdehyde decarboxylase (EC 4.1.1.45) and quinolinate phosphoribosyltransferase (EC 2.4.2.19), which were found to be significantly down-regulated (*197, 198*).

13. Pharmacometabonomics and Implications of the Extended Genome

For personalized healthcare, an individual's drug treatments must be tailored so as to achieve maximal efficacy and avoid adverse drug reactions. One of the approaches has been to understand the genetic makeup of different individuals (pharmacogenomics) and to relate these to their varying abilities to handle pharmaceuticals both for their beneficial effects and for identifying adverse effects. Recently, an alternative approach to understanding such intersubject variability has been developed using metabonomics to predict the metabolism and toxicity of a dosed substance, based solely on the analysis and modeling of a predose metabolic profile (*97*). Unlike pharmacogenomics, this approach, which has been termed pharmacometabonomics, is sensitive to both the genetic and modifying environmental influences that determine the metabolic fingerprint of an individual. This new approach has been illustrated with studies of the toxicity and metabolism of compounds with very different modes of action (allyl alcohol, galactosamine, and acetaminophen) administered to rats. The pharmacometabonomic principle has been illustrated in (*97*), where predose urinary profiles are used to predict the extent of liver damage sustained after acetaminophen administration as shown in Figure 5, where variation in the PCA scores of predose urinary data are

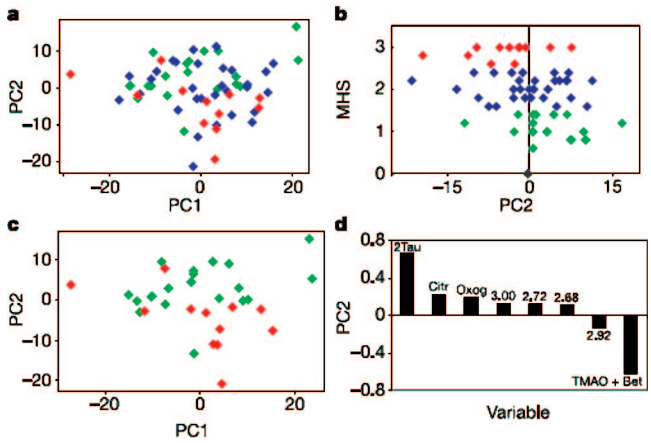

Figure 5. Predose discrimination of the degree of liver damage obtained in acetaminophen-dosed rats. (a) A scores plot from PCA of the predose NMR data. Each point represents a single rat and is color-coded by its histology class (with increasing severity of damage, class 1 is green, class 2 is blue, and class 3 is red). (b) Plot of mean histology score (MHS) vs the PC2 score obtained from the PCA. (c) A scores plot from PCA of the predose NMR data for rats in histology classes 1 and 3. Each point represents a single rat, with color-coding as described before. (d) A loadings plot corresponding to c, showing the variables making the largest contributions to PC2, and the direction of each contribution. Key: Tau, taurine; Citr, citrate; Oxog, 2-oxoglutarate; TMAO, trimethylamine-*N*-oxide; Bet, betaine. Reprinted by permission from Macmillan Publishers Ltd: *Nature* (http://www.nature.com), (ref *97*), Copyright (2006).

correlated with the postdose histopathology scores to reveal the discriminatory metabolites.

A further pharmacometabonomic study demonstrates a GC-MS-based approach to study two classical experimental models, the Streptozotocin-induced diabetic model in Wistar rats and the high-energy, diet-induced obesity model of Sprague–Dawley rats (*199*). Pre and postdose urine was collected together with serum clinical chemistry parameters and body weights so that animals could be classified via outcome as obesity-prone or obesity-resistant and diabetes-prone or diabetes-resistant. The variation in the predose/baseline urinary profiles of obesity-prone and obesity-resistant rats was found to correlate with outcome, and the discriminatory metabolites for both classes were related to gut microbial metabolism and energy metabolism. This work suggests that the pre-existing variation in the metabolic phenotype may provide a systems wide window to probe the metabolic basis for interanimal variation in response to xenobiotics, dietary intervention, or external stressors and may provide insight into varied metabolic phenotypes and the associated pathological outcomes. These pharmacometabonomic studies confirm that metabolic profiling methods are highly effective in understanding disease processes and drug responses in humans and model organisms and that they can be used to predict the effects of drug interventions.

As the metabolic phenotype both predicts and influences drug metabolism and toxic outcome (*97, 171*), we need to consider the response to drug intervention in the context of fundamental phenotype. The phenotype is modified by a wide range of factors that include subject genetics, diet, previous drug exposure, related enzyme induction, and the activities of the gut microflora (*200*). Indeed, the gut microbiotal activities may generate biochemically active substrates that can be absorbed by the host, and this may lead to induction of host enzymes that compete for metabolic substrates. Hence, the outcome of a drug intervention may be dependent on conditional probabilistic interactions between gut microbial metabolism and host metabolism, and such interactions may be in-part responsible for some idiosyncratic toxicological reactions (*9, 171, 200*). Given the enormous diversity of poorly understood microbial species in man, this may become an important area for study in future personalized healthcare scenarios. The variation in rodent metabolic phenotypes seen in some laboratories can be attributed to variation in animal microbiomes (*142, 201, 202*) and as such may be an important and previously unsuspected source of inter-laboratory and inter-study biological variation in toxic responses and metabolic fate.

14. Concluding Remarks

It is clear that the combined use of NMR spectroscopy and chemometric tools to generate and model information-rich metabolic profiles has resulted in the rapid development of a multifaceted field of metabonomics and new routes to biomarker discovery. Instrumental advances will continue to improve the sensitivity of the NMR platform, which will extend the detection limits and enable other metabolic pathways to be explored. The increased application of MS-based analytical platforms in the field of metabonomics will also widen the range of detectable metabolites and will prove particularly useful for targeted analyses aimed at specific groups of metabolites. Metabolic biomarkers provide real biological end points that have a high degree of commonality across many species and enable a global systems interpretation of biological effects. From the rapid rise in publication rates, it is clear that metabonomics can now be used for the validation of animal models of disease, preclinical evaluation of candidate drugs in safety studies, assessment of safety in humans in clinical trials, improved mechanistic understanding of idiosyncratic toxicity as well as pharmacometabonomics.

NMR-based metabonomics provides a robust and stable analytical platform with excellent analytical and biological reproducibility, whereas LC-MS based methods provide significant advantages in terms of increased sensitivity and greater dynamic range. The data sets generated are complex and challenging to reliably model and interpret, yet advances in statistical approaches to data mining continue to be made. The nontargeted and minimally invasive nature of whole-system metabonomic studies are ideal for biochemical and mechanistic hypothesis generation, although increased testing and validation of these hypotheses will be needed for even greater long-term impact of the field.

The information obtained from a typical NMR-based metabonomic study can be envisaged in terms of an analogy with an airline network or route map; the major hubs or cities are the major metabolic players and are vital for all biological activity. The absence or regulation of these major metabolites and pathways is critical to the health of the organism. It is these major hubs that are most easily followed with NMR spectroscopy, and it is the interactions, time-courses, and overall pattern of changes in these metabolites that has proven to be specific to a given toxin or disease state. However, MS methods can be envisaged as a potential means of studying all hubs (both minor and major) as the sensitivity of the analytical platform is higher, but major and minor hubs are not so easily differentiated as they are in NMR spectroscopy, and this potentially complicates the biological interpretation. This analogy clearly highlights the challenge in the interpretation of MS data, which is to identify and distinguish the variation of metabolic significance and specificity from the confounding multitude of metabolic information in the data. However, we envisage that the challenge

will be met in the near future and will further enhance the complementarities of NMR and MS platforms for biomarker discovery.

In summary, it is clear that metabonomics and its associated technologies will continue to develop in both sensitivity and in applications in as of yet under-explored areas and that metabonomics will form a fundamental part of all multiomics approaches where integrative systems biomarkers are sought.

Note Added after ASAP Publication. There were errors in refs *13*, *52*, *54*, *176*, and *177* in the version published ASAP January 3, 2008; the corrected version was published January 21, 2008.

References

(1) Nicholson, J. K. (2006) Global systems biology, personalized medicine and molecular epidemiology. *Mol. Syst. Biol. 2*, 52.

(2) Nicholson, J. K., Connelly, J., Lindon, J. C., and Holmes, E. (2002) Metabonomics: a platform for studying drug toxicity and gene function. *Nat. Rev. Drug Discovery 1* (2), 153–161.

(3) Lindon, J. C., Holmes, E., Bollard, M. E., Stanley, E. G., and Nicholson, J. K. (2004) Metabonomics technologies and their applications in physiological monitoring, drug safety assessment and disease diagnosis. *Biomarkers 9* (1), 1–31.

(4) Lindon, J. C., Holmes, E., and Nicholson, J. K. (2004) Metabonomics: systems biology in pharmaceutical research and development. *Curr. Opin. Mol. Ther. 6* (3), 265–272.

(5) Fiehn, O. (2002) Metabolomics--the link between genotypes and phenotypes. *Plant Mol. Biol. 48* (1–2), 155–171.

(6) Taylor, J., King, R. D., Altmann, T., and Fiehn, O. (2002) Application of metabolomics to plant genotype discrimination using statistics and machine learning. *Bioinformatics. 18* (2), S241–S248.

(7) Jenkins, H., Hardy, N., Beckmann, M., Draper, J., Smith, A. R., Taylor, J., Fiehn, O., Goodacre, R., Bino, R. J., Hall, R., Kopka, J., Lane, G. A., Lange, B. M., Liu, J. R., Mendes, P., Nikolau, B. J., Oliver, S. G., Paton, N. W., Rhee, S., Roessner-Tunali, U., Saito, K., Smedsgaard, J., Sumner, L. W., Wang, T., Walsh, S., Wurtele, E. S., and Kell, D. B. (2004) A proposed framework for the description of plant metabolomics experiments and their results. *Nat. Biotechnol. 22* (12), 1601–1606.

(8) Nicholson, J. K., Lindon, J. C., and Holmes, E. (1999) 'Metabonomics': understanding the metabolic responses of living systems to pathophysiological stimuli via multivariate statistical analysis of biological NMR spectroscopic data. *Xenobiotica 29* (11), 1181–1189.

(9) Nicholson, J. K., and Wilson, I. D. (2003) Opinion: understanding 'global' systems biology: metabonomics and the continuum of metabolism. *Nat. Rev. Drug Discov. 2* (8), 668–676.

(10) Lenz, E. M., and Wilson, I. D. (2007) Analytical strategies in metabonomics. *J. Proteome Res. 6* (2), 443–458.

(11) Wilson, I. D., Plumb, R., Granger, J., Major, H., Williams, R., and Lenz, E. M. (2005) HPLC-MS-based methods for the study of metabonomics. *J. Chromatogr., B 817* (1), 67–76.

(12) Wilson, I. D., Nicholson, J. K., Castro-Perez, J., Granger, J. H., Johnson, K. A., Smith, B. W., and Plumb, R. S. (2005) High resolution "ultra performance" liquid chromatography coupled to oa-TOF mass spectrometry as a tool for differential metabolic pathway profiling in functional genomic studies. *J. Proteome Res. 4* (2), 591–598.

(13) Nicholson, J. K., and Wilson, I. D. (1989) High resolution proton magnetic resonance spectroscopy of biological fluids. *Prog. Nucl. Magn. Reson. Spectrosc. 21*, 449–501.

(14) Lindon, J. C., Holmes, E., and Nicholson, J. K. (2007) Metabonomics in pharmaceutical R&D. *FEBS J. 274* (5), 1140–1151.

(15) Nicholson, J. K., Timbrell, J. A., and Sadler, P. J. (1985) Proton NMR spectra of urine as indicators of renal damage. Mercury-induced nephrotoxicity in rats. *Mol. Pharmacol. 27* (6), 644–651.

(16) Nicholson, J. K., Timbrell, J. A., Bales, J. R., and Sadler, P. J. (1985) A high resolution proton nuclear magnetic resonance approach to the study of hepatocyte and drug metabolism. Application to acetaminophen. *Mol. Pharmacol. 27* (6), 634–643.

(17) Nicholson, J. K., and Wilson, I. D. (1987) High resolution nuclear magnetic resonance spectroscopy of biological samples as an aid to drug development. *Prog. Drug Res. 31*, 427–479.

(18) Nicholson, J. K., Kendall, M. D., and Osborn, D. (1983) Cadmium and mercury nephrotoxicity. *Nature 304* (5927), 633–635.

(19) Gartland, K. P., Bonner, F. W., and Nicholson, J. K. (1989) Investigations into the biochemical effects of region-specific nephrotoxins. *Mol. Pharmacol. 35* (2), 242–250.

(20) Anthony, M. L., Gartland, K. P., Beddell, C. R., Lindon, J. C., and Nicholson, J. K. (1992) Cephaloridine-induced nephrotoxicity in the Fischer 344 rat: proton NMR spectroscopic studies of urine and plasma in relation to conventional clinical chemical and histopathological assessments of nephronal damage. *Arch. Toxicol. 66* (8), 525–537.

(21) Holmes, E., Bonner, F. W., Gartland, K. P., and Nicholson, J. K. (1990) Proton NMR monitoring of the onset and recovery of experimental renal damage. *J. Pharm. Biomed. Anal. 8* (8–12), 959–962.

(22) Gartland, K. P., Anthony, M. L., Beddell, C. R., Lindon, J. C., and Nicholson, J. K. (1990) Proton NMR studies on the effects of uranyl nitrate on the biochemical composition of rat urine and plasma. *J. Pharm. Biomed. Anal. 8* (8–12), 951–954.

(23) Anthony, M. L., Gartland, K. P., Beddell, C. R., Lindon, J. C., and Nicholson, J. K. (1994) Studies of the biochemical toxicology of uranyl nitrate in the rat. *Arch. Toxicol. 68* (1), 43–53.

(24) Gartland, K. P., Bonner, F. W., Timbrell, J. A., and Nicholson, J. K. (1989) Biochemical characterisation of para-aminophenol-induced nephrotoxic lesions in the F344 rat. *Arch. Toxicol. 63* (2), 97–106.

(25) Gartland, K. P., Eason, C. T., Wade, K. E., Bonner, F. W., and Nicholson, J. K. (1989) Proton NMR spectroscopy of bile for monitoring the excretion of endogenous and xenobiotic metabolites: application to para-aminophenol. *J. Pharm. Biomed. Anal. 7* (6), 699–707.

(26) Gartland, K. P., Eason, C. T., Bonner, F. W., and Nicholson, J. K. (1990) Effects of biliary cannulation and buthionine sulphoximine pretreatment on the nephrotoxicity of para-aminophenol in the Fischer 344 rat. *Arch. Toxicol. 64* (1), 14–25.

(27) Foxall, P. J., Bending, M. R., Gartland, K. P., and Nicholson, J. K. (1989) Acute renal failure following accidental cutaneous absorption of phenol: application of NMR urinalysis to monitor the disease process. *Hum. Toxicol. 8* (6), 491–496.

(28) Nicholson, J. K., Higham, D. P., Timbrell, J. A., and Sadler, P. J. (1989) Quantitative high resolution ^{1}H NMR urinalysis studies on the biochemical effects of cadmium in the rat. *Mol. Pharmacol. 36* (3), 398–404.

(29) Gray, J., Nicholson, J. K., Creasy, D. M., and Timbrell, J. A. (1990) Studies on the relationship between acute testicular damage and urinary and plasma creatine concentration. *Arch. Toxicol. 64* (6), 443–450.

(30) Timbrell, J. A. (1998) Biomarkers in toxicology. *Toxicology 129* (1), 1–12.

(31) Waterfield, C. J., Turton, J. A., Scales, M. D., and Timbrell, J. A. (1993) Investigations into the effects of various hepatotoxic compounds on urinary and liver taurine levels in rats. *Arch. Toxicol. 67* (4), 244–254.

(32) Sanins, S. M., Nicholson, J. K., Elcombe, C., and Timbrell, J. A. (1990) Hepatotoxin-induced hypertaurinuria: a proton NMR study. *Arch. Toxicol. 64* (5), 407–411.

(33) Sanins, S. M., Timbrell, J. A., Elcombe, C., and Nicholson, J. K. (1992) Proton NMR spectroscopic studies on the metabolism and biochemical effects of hydrazine in vivo. *Arch. Toxicol. 66* (7), 489–495.

(34) Timbrell, J. A., and Waterfield, C. J. (1996) Changes in taurine as an indicator of hepatic dysfunction and biochemical perturbations. Studies in vivo and in vitro. *Adv. Exp. Med. Biol. 403*, 125–134.

(35) Hoyle, V. R., Gilbert, P. J., Troke, J. A., Vose, C. W., and Nicholson, J. K. (1992) Studies on the biochemical effects of the aldose reductase inhibitor 2,7-difluorospirofluorene-9,5′-imidazolidine-2′,4′-dione (Al 1576, HOE 843). Detection of D-glucaric and D-glucuronic acid excretion by high resolution ^{1}H and ^{13}C NMR spectroscopy. *Biochem. Pharmacol. 44* (2), 231–241.

(36) Ghauri, F. Y., McLean, A. E., Beales, D., Wilson, I. D., and Nicholson, J. K. (1993) Induction of 5-oxoprolinuria in the rat following chronic feeding with N-acetyl 4-aminophenol (paracetamol). *Biochem. Pharmacol. 46* (5), 953–957.

(37) Pitt, J. J., and Hauser, S. (1998) Transient 5-oxoprolinuria and high anion gap metabolic acidosis: clinical and biochemical findings in eleven subjects. *Clin. Chem. 44* (7), 1497–1503.

(38) Gartland, K. P., Sanins, S. M., Nicholson, J. K., Sweatman, B. C., Beddell, C. R., and Lindon, J. C. (1990) Pattern recognition analysis of high resolution ^{1}H NMR spectra of urine. A nonlinear mapping approach to the classification of toxicological data. *NMR Biomed. 3* (4), 166–172.

(39) Gartland, K. P., Beddell, C. R., Lindon, J. C., and Nicholson, J. K. (1991) Application of pattern recognition methods to the analysis and classification of toxicological data derived from proton nuclear magnetic resonance spectroscopy of urine. *Mol. Pharmacol. 39* (5), 629–642.

(40) Anthony, M. L., Sweatman, B. C., Beddell, C. R., Lindon, J. C., and Nicholson, J. K. (1994) Pattern recognition classification of the site

of nephrotoxicity based on metabolic data derived from proton nuclear magnetic resonance spectra of urine. *Mol. Pharmacol. 46* (1), 199–211.

(41) Holmes, E., Bonner, F. W., Sweatman, B. C., Lindon, J. C., Beddell, C. R., Rahr, E., and Nicholson, J. K. (1992) Nuclear magnetic resonance spectroscopy and pattern recognition analysis of the biochemical processes associated with the progression of and recovery from nephrotoxic lesions in the rat induced by mercury(II) chloride and 2-bromoethanamine. *Mol. Pharmacol. 42* (5), 922–930.

(42) Holmes, E., Nicholson, J. K., Bonner, F. W., Sweatman, B. C., Beddell, C. R., Lindon, J. C., and Rahr, E. (1992) Mapping the biochemical trajectory of nephrotoxicity by pattern recognition of NMR urinanalysis. *NMR Biomed. 5* (6), 368–372.

(43) Holmes, E., Foxall, P. J., Nicholson, J. K., Neild, G. H., Brown, S. M., Beddell, C. R., Sweatman, B. C., Rahr, E., Lindon, J. C., and Spraul, M. (1994) Automatic data reduction and pattern recognition methods for analysis of ^{1}H nuclear magnetic resonance spectra of human urine from normal and pathological states. *Anal. Biochem. 220* (2), 284–296.

(44) Craig, A., Cloarec, O., Holmes, E., Nicholson, J. K., and Lindon, J. C. (2006) Scaling and normalization effects in NMR spectroscopic metabonomic data sets. *Anal. Chem. 78* (7), 2262–2267.

(45) Dieterle, F., Ross, A., Schlotterbeck, G., and Senn, H. (2006) Probabilistic quotient normalization as robust method to account for dilution of complex biological mixtures. Application in ^{1}H NMR metabonomics. *Anal. Chem. 78* (13), 4281–4290.

(46) Holmes, E., Nicholls, A. W., Lindon, J. C., Ramos, S., Spraul, M., Neidig, P., Connor, S. C., Connelly, J., Damment, S. J., Haselden, J., and Nicholson, J. K. (1998) Development of a model for classification of toxin-induced lesions using ^{1}H NMR spectroscopy of urine combined with pattern recognition. *NMR Biomed. 11* (4–5), 235–244.

(47) Jackson, J. E. A. (1991) *A Users Guide to Principal Components*, Wiley, New York.

(48) Wold, H. (1985) Partial Least Squares. In *Encyclopedia of Statistical Sciences* (Kotz S., and Johnson N. L., Eds.) pp 581–591, Wiley, New York.

(49) Brindle, J. T., Antti, H., Holmes, E., Tranter, G., Nicholson, J. K., Bethell, H. W., Clarke, S., Schofield, P. M., McKilligin, E., Mosedale, D. E., and Grainger, D. J. (2002) Rapid and noninvasive diagnosis of the presence and severity of coronary heart disease using ^{1}H-NMR-based metabonomics. *Nat. Med. 8* (12), 1439–1444.

(50) Gavaghan, C. L., Wilson, I. D., and Nicholson, J. K. (2002) Physiological variation in metabolic phenotyping and functional genomic studies: use of orthogonal signal correction and PLS-DA. *FEBS Lett. 530* (1–3), 191–196.

(51) Trygg, J., Holmes, E., and Lundstedt, T. (2007) Chemometrics in metabonomics. *J. Proteome Res. 6* (2), 469–479.

(52) Trygg, J., and Wold, S. (2003) O2-PLS, a two-block (X-Y) latent variable regression (LVR) method with an integral OSC filter. *J. Chemom. 17* (1), 53–64.

(53) Eriksson, L., Antti, H., Gottfries, J., Holmes, E., Johansson, E., Lindgren, F., Long, I., Lundstedt, T., Trygg, J., and Wold, S. (2004) Using chemometrics for navigating in the large data sets of genomics, proteomics, and metabonomics (gpm). *Anal. Bioanal. Chem. 380* (3), 419–429.

(54) Stella, C., Beckwith-Hall, B., Cloarec, O., Holmes, E., Lindon, J. C., Powell, J., van der Ouderaa, F., Bingham, S., Cross, A. J., and Nicholson, J. K. (2006) Susceptibility of human metabolic phenotypes to dietary modulation. *J. Proteome Res. 5* (10), 2780–2788.

(55) Cloarec, O., Dumas, M. E., Trygg, J., Craig, A., Barton, R. H., Lindon, J. C., Nicholson, J. K., and Holmes, E. (2005) Evaluation of the orthogonal projection on latent structure model limitations caused by chemical shift variability and improved visualization of biomarker changes in ^{1}H NMR spectroscopic metabonomic studies. *Anal. Chem. 77* (2), 517–526.

(56) Coen, M., Hong, Y. S., Clayton, T. A., Rohde, C. M., Pearce, J. T., Reily, M. D., Robertson, D. G., Holmes, E., Lindon, J. C., and Nicholson, J. K. (2007) The mechanism of galactosamine toxicity revisited; a metabonomic study. *J. Proteome Res. 6* (7), 2711–2719.

(57) Yap, I. K., Clayton, T. A., Tang, H., Everett, J. R., Hanton, G., Provost, J. P., Le Net, J. L., Charuel, C., Lindon, J. C., and Nicholson, J. K. (2006) An integrated metabonomic approach to describe temporal metabolic disregulation induced in the rat by the model hepatotoxin allyl formate. *J. Proteome Res. 5* (10), 2675–2684.

(58) Martin, F. P., Wang, Y., Sprenger, N., Holmes, E., Lindon, J. C., Kochhar, S., and Nicholson, J. K. (2007) Effects of probiotic *Lactobacillus paracasei* treatment on the host gut tissue metabolic profiles probed via magic-angle-spinning NMR spectroscopy. *J. Proteome Res. 6* (4), 1471–1481.

(59) Stoyanova, R., Nicholls, A. W., Nicholson, J. K., Lindon, J. C., and Brown, T. R. (2004) Automatic alignment of individual peaks in large high-resolution spectral data sets. *J. Magn. Reson. 170* (2), 329–335.

(60) Csenki, L., Alm, E., Torgrip, R. J., Aberg, K. M., Nord, L. I., Schuppe-Koistinen, I., and Lindberg, J. (2007) Proof of principle of a generalized fuzzy Hough transform approach to peak alignment of one-dimensional (1)H NMR data. *Anal. Bioanal. Chem. 389* (3), 875–885.

(61) Forshed, J., Torgrip, R. J., Aberg, K. M., Karlberg, B., Lindberg, J., and Jacobsson, S. P. (2005) A comparison of methods for alignment of NMR peaks in the context of cluster analysis. *J. Pharm. Biomed. Anal. 38* (5), 824–832.

(62) Wu, W., Daszykowski, M., Walczak, B., Sweatman, B. C., Connor, S. C., Haselden, J. N., Crowther, D. J., Gill, R. W., and Lutz, M. W. (2006) Peak alignment of urine NMR spectra using fuzzy warping. *J. Chem. Inf. Model. 46* (2), 863–875.

(63) Jonsson, P., Bruce, S. J., Moritz, T., Trygg, J., Sjostrom, M., Plumb, R., Granger, J., Maibaum, E., Nicholson, J. K., Holmes, E., and Antti, H. (2005) Extraction, interpretation and validation of information for comparing samples in metabolic LC/MS data sets. *Analyst 130* (5), 701–707.

(64) Katajamaa, M., and Oresic, M. (2007) Data processing for mass spectrometry-based metabolomics. *J. Chromatogr., A 1158* (1–2), 318–328.

(65) Smith, C. A., Want, E. J., O'Maille, G., Abagyan, R., and Siuzdak, G. (2006) XCMS: processing mass spectrometry data for metabolite profiling using nonlinear peak alignment, matching, and identification. *Anal. Chem. 78* (3), 779–787.

(66) Holmes, E., Nicholson, J. K., and Tranter, G. (2001) Metabonomic characterization of genetic variations in toxicological and metabolic responses using probabilistic neural networks. *Chem. Res. Toxicol. 14* (2), 182–191.

(67) Ott, K. H., Aranibar, N., Singh, B., and Stockton, G. W. (2003) Metabonomics classifies pathways affected by bioactive compounds. Artificial neural network classification of NMR spectra of plant extracts. *Phytochemistry 62* (6), 971–985.

(68) Vehtari, A., Makinen, V. P., Soininen, P., Ingman, P., Makela, S. M., Savolainen, M. J., Hannuksela, M. L., Kaski, K., and Ala-Korpela, M. (2007) A novel Bayesian approach to quantify clinical variables and to determine their spectroscopic counterparts in ^{1}H NMR metabonomic data. *BMC Bioinf. 8* (2), S8.

(69) Embrechts, M. J., and Ekins, S. (2007) Classification of metabolites with kernel-partial least squares (K-PLS). *Drug Metab. Dispos. 35* (3), 325–327.

(70) Crockford, D. J., Lindon, J. C., Cloarec, O., Plumb, R. S., Bruce, S. J., Zirah, S., Rainville, P., Stumpf, C. L., Johnson, K., Holmes, E., and Nicholson, J. K. (2006) Statistical search space reduction and two-dimensional data display approaches for UPLC-MS in biomarker discovery and pathway analysis. *Anal. Chem. 78* (13), 4398–4408.

(71) Jonsson, P., Johansson, A. I., Gullberg, J., Trygg, J., A. J., Grung, B., Marklund, S., Sjostrom, M., Antti, H., and Moritz, T. (2005) High-throughput data analysis for detecting and identifying differences between samples in GC/MS-based metabolomic analyses. *Anal. Chem. 77* (17), 5635–5642.

(72) Robertson, D. G., Reily, M. D., Sigler, R. E., Wells, D. F., Paterson, D. A., and Braden, T. K. (2000) Metabonomics: evaluation of nuclear magnetic resonance (NMR) and pattern recognition technology for rapid in vivo screening of liver and kidney toxicants. *Toxicol. Sci. 57* (2), 326–337.

(73) Foxall, P. J., Singer, J. M., Hartley, J. M., Neild, G. H., Lapsley, M., Nicholson, J. K., and Souhami, R. L. (1997) Urinary proton magnetic resonance studies of early ifosfamide-induced nephrotoxicity and encephalopathy. *Clin. Cancer Res. 3* (9), 1507–1518.

(74) Foxall, P. J., Lenz, E. M., Lindon, J. C., Neild, G. H., Wilson, I. D., and Nicholson, J. K. (1996) Nuclear magnetic resonance and high-performance liquid chromatography-nuclear magnetic resonance studies on the toxicity and metabolism of ifosfamide. *Ther. Drug Monit. 18* (4), 498–505.

(75) Vilasi, A., Cutillas, P. R., Maher, A. D., Zirah, S. F., Capasso, G., Norden, A. W., Holmes, E., Nicholson, J. K., and Unwin, R. J. (2007) Combined proteomic and metabonomic studies in three genetic forms of the renal Fanconi syndrome. *Am. J. Physiol. Renal Physiol. 293* (2), F456–F467.

(76) Murgatroyd, L. B., Pickford, R. J., Smith, I. K., Wilson, I. D., and Middleton, B. J. (1992) ^{1}H-NMR spectroscopy as a means of monitoring nephrotoxicity as exemplified by studies with cephaloridine. *Hum. Exp. Toxicol. 11* (1), 35–41.

(77) Halligan, S., Byard, S. J., Spencer, A. J., Gray, T. J., Harpur, E. S., and Bonner, F. W. (1995) A study of the nephrotoxicity of three cephalosporins in rabbits using ^{1}H NMR spectroscopy. *Toxicol. Lett. 81* (1), 15–21.

(78) Holmes, E., Bonner, F. W., and Nicholson, J. K. (1997) ^{1}H NMR spectroscopic and histopathological studies on propyleneimine-induced renal papillary necrosis in the rat and the multimammate desert mouse (*Mastomys natalensis*). *Comp. Biochem. Physiol C 116* (2), 125–134.

(79) Garrod, S., Humpher, E., Connor, S. C., Connelly, J. C., Spraul, M., Nicholson, J. K., and Holmes, E. (2001) High-resolution (1)H NMR and magic angle spinning NMR spectroscopic investigation of the biochemical effects of 2-bromoethanamine in intact renal and hepatic tissue. *Magn. Reson. Med. 45* (5), 781–790.

(80) Holmes, E., Caddick, S., Lindon, J. C., Wilson, I. D., Kryvawych, S., and Nicholson, J. K. (1995) ^{1}H and ^{2}H NMR spectroscopic studies on the metabolism and biochemical effects of 2-bromoethanamine in the rat. *Biochem. Pharmacol. 49* (10), 1349–1359.

(81) Holmes, E., Bonner, F. W., and Nicholson, J. K. (1995) Comparative studies on the nephrotoxicity of 2-bromoethanamine hydrobromide in the Fischer 344 rat and the multimammate desert mouse (*Mastomys natalensis*). *Arch. Toxicol. 70* (2), 89–95.

(82) Anthony, M. L., Holmes, E., McDowell, P. C., Gray, T. J., Blackmore, M., and Nicholson, J. K. (1995) ^{1}H NMR spectroscopic studies on the reactions of haloalkylamines with bicarbonate ions: formation of N-carbamates and 2-oxazolidones in cell culture media and blood plasma. *Chem. Res. Toxicol. 8* (8), 1046–1053.

(83) Nicholls, A. W., Holmes, E., Lindon, J. C., Shockcor, J. P., Farrant, R. D., Haselden, J. N., Damment, S. J., Waterfield, C. J., and Nicholson, J. K. (2001) Metabonomic investigations into hydrazine toxicity in the rat. *Chem. Res. Toxicol. 14* (8), 975–987.

(84) Garrod, S., Bollard, M. E., Nicholls, A. W., Connor, S. C., Connelly, J., Nicholson, J. K., and Holmes, E. (2005) Integrated metabonomic analysis of the multiorgan effects of hydrazine toxicity in the rat. *Chem. Res. Toxicol. 18* (2), 115–122.

(85) Bollard, M. E., Keun, H. C., Beckonert, O., Ebbels, T. M., Antti, H., Nicholls, A. W., Shockcor, J. P., Cantor, G. H., Stevens, G., Lindon, J. C., Holmes, E., and Nicholson, J. K. (2005) Comparative metabonomics of differential hydrazine toxicity in the rat and mouse. *Toxicol. Appl. Pharmacol. 204* (2), 135–151.

(86) Waters, N. J., Waterfield, C. J., Farrant, R. D., Holmes, E., and Nicholson, J. K. (2005) Metabonomic deconvolution of embedded toxicity: application to thioacetamide hepato- and nephrotoxicity. *Chem. Res. Toxicol. 18* (4), 639–654.

(87) Azmi, J., Griffin, J. L., Shore, R. F., Holmes, E., and Nicholson, J. K. (2005) Chemometric analysis of biofluids following toxicant induced hepatotoxicity: a metabonomic approach to distinguish the effects of 1-naphthylisothiocyanate from its products. *Xenobiotica 35* (8), 839–852.

(88) Waters, N. J., Holmes, E., Waterfield, C. J., Farrant, R. D., and Nicholson, J. K. (2002) NMR and pattern recognition studies on liver extracts and intact livers from rats treated with alpha-naphthylisothiocyanate. *Biochem. Pharmacol. 64* (1), 67–77.

(89) Waters, N. J., Holmes, E., Williams, A., Waterfield, C. J., Farrant, R. D., and Nicholson, J. K. (2001) NMR and pattern recognition studies on the time-related metabolic effects of alpha-naphthylisothiocyanate on liver, urine, and plasma in the rat: an integrative metabonomic approach. *Chem. Res. Toxicol. 14* (10), 1401–1412.

(90) Clayton, T. A., Lindon, J. C., Everett, J. R., Charuel, C., Hanton, G., Le Net, J. L., Provost, J. P., and Nicholson, J. K. (2007) Hepatotoxin-induced hypertyrosinemia and its toxicological significance. *Arch. Toxicol. 81* (3), 201–210.

(91) Beckwith-Hall, B. M., Nicholson, J. K., Nicholls, A. W., Foxall, P. J., Lindon, J. C., Connor, S. C., Abdi, M., Connelly, J., and Holmes, E. (1998) Nuclear magnetic resonance spectroscopic and principal components analysis investigations into biochemical effects of three model hepatotoxins. *Chem. Res. Toxicol. 11* (4), 260–272.

(92) Bollard, M. E., Xu, J., Purcell, W., Griffin, J. L., Quirk, C., Holmes, E., and Nicholson, J. K. (2002) Metabolic profiling of the effects of D-galactosamine in liver spheroids using (1)H NMR and MAS-NMR spectroscopy. *Chem. Res. Toxicol. 15* (11), 1351–1359.

(93) Waters, N. J., Waterfield, C. J., Farrant, R. D., Holmes, E., and Nicholson, J. K. (2006) Integrated metabonomic analysis of bromobenzene-induced hepatotoxicity: novel induction of 5-oxoprolinosis. *J. Proteome Res. 5* (6), 1448–1459.

(94) Keun, H. C., Ebbels, T. M., Bollard, M. E., Beckonert, O., Antti, H., Holmes, E., Lindon, J. C., and Nicholson, J. K. (2004) Geometric trajectory analysis of metabolic responses to toxicity can define treatment specific profiles. *Chem. Res. Toxicol. 17* (5), 579–587.

(95) Coen, M., Lenz, E. M., Nicholson, J. K., Wilson, I. D., Pognan, F., and Lindon, J. C. (2003) An integrated metabonomic investigation of acetaminophen toxicity in the mouse using NMR spectroscopy. *Chem. Res. Toxicol. 16* (3), 295–303.

(96) Coen, M., Ruepp, S. U., Lindon, J. C., Nicholson, J. K., Pognan, F., Lenz, E. M., and Wilson, I. D. (2004) Integrated application of transcriptomics and metabonomics yields new insight into the toxicity due to paracetamol in the mouse. *J. Pharm. Biomed. Anal. 35* (1), 93–105.

(97) Clayton, T. A., Lindon, J. C., Cloarec, O., Antti, H., Charuel, C., Hanton, G., Provost, J. P., Le Net, J. L., Baker, D., Walley, R. J., Everett, J. R., and Nicholson, J. K. (2006) Pharmaco-metabonomic phenotyping and personalized drug treatment. *Nature 440* (7087), 1073–1077.

(98) Bales, J. R., Nicholson, J. K., and Sadler, P. J. (1985) Two-dimensional proton nuclear magnetic resonance "maps" of acetaminophen metabolites in human urine. *Clin. Chem. 31* (5), 757–762.

(99) Bales, J. R., Sadler, P. J., Nicholson, J. K., and Timbrell, J. A. (1984) Urinary excretion of acetaminophen and its metabolites as studied by proton NMR spectroscopy. *Clin. Chem. 30* (10), 1631–1636.

(100) Nicholls, A. W., Caddick, S., Wilson, I. D., Farrant, R. D., Lindon, J. C., and Nicholson, J. K. (1995) High resolution NMR spectroscopic studies on the metabolism and futile deacetylation of 4-hydroxyacetanilide (paracetamol) in the rat. *Biochem. Pharmacol. 49* (8), 1155–1164.

(101) Dieterle, F., Schlotterbeck, G., Binder, M., Ross, A., Suter, L., and Senn, H. (2007) Application of metabonomics in a comparative profiling study reveals N-acetylfelinine excretion as a biomarker for inhibition of the farnesyl pathway by bisphosphonates. *Chem. Res. Toxicol. 20* (9), 1291–1299.

(102) Robertson, D. G., Reily, M. D., Albassam, M., and Dethloff, L. A. (2001) Metabonomic assessment of vasculitis in rats. *Cardiovasc. Toxicol. 1* (1), 7–19.

(103) Griffin, J. L., Walker, L. A., Shore, R. F., and Nicholson, J. K. (2001) Metabolic profiling of chronic cadmium exposure in the rat. *Chem. Res. Toxicol. 14* (10), 1428–1434.

(104) Bundy, J. G., Spurgeon, D. J., Svendsen, C., Hankard, P. K., Weeks, J. M., Osborn, D., Lindon, J. C., and Nicholson, J. K. (2004) Environmental metabonomics: applying combination biomarker analysis in earthworms at a metal contaminated site. *Ecotoxicology 13* (8), 797–806.

(105) Bundy, J. G., Keun, H. C., Sidhu, J. K., Spurgeon, D. J., Svendsen, C., Kille, P., and Morgan, A. J. (2007) Metabolic profile biomarkers of metal contamination in a sentinel terrestrial species are applicable across multiple sites. *Environ. Sci. Technol. 41* (12), 4458–4464.

(106) Samuelsson, L. M., Forlin, L., Karlsson, G., dolfsson-Erici, M., and Larsson, D. G. (2006) Using NMR metabolomics to identify responses of an environmental estrogen in blood plasma of fish. *Aquat. Toxicol. 78* (4), 341–349.

(107) Zhu, Y. S., Xu, H. B., Huang, K. X., Hu, W. H., and Liu, M. L. (2002) A study on human urine in a high-selenium area of China by ^{1}H-NMR spectroscopy. *Biol. Trace Elem. Res. 89* (2), 155–163.

(108) Mortishire-Smith, R. J., Skiles, G. L., Lawrence, J. W., Spence, S., Nicholls, A. W., Johnson, B. A., and Nicholson, J. K. (2004) Use of metabonomics to identify impaired fatty acid metabolism as the mechanism of a drug-induced toxicity. *Chem. Res. Toxicol. 17* (2), 165–173.

(109) Cheng, L. L., Lean, C. L., Bogdanova, A., Wright, S. C., Jr., Ackerman, J. L., Brady, T. J., and Garrido, L. (1996) Enhanced resolution of proton NMR spectra of malignant lymph nodes using magic-angle spinning. *Magn Reson. Med. 36* (5), 653–658.

(110) Cheng, L. L., Ma, M. J., Becerra, L., Ptak, T., Tracey, I., Lackner, A., and Gonzalez, R. G. (1997) Quantitative neuropathology by high resolution magic angle spinning proton magnetic resonance spectroscopy. *Proc. Natl. Acad. Sci. U.S.A. 94* (12), 6408–6413.

(111) Cheng, L. L., Chang, I. W., Smith, B. L., and Gonzalez, R. G. (1998) Evaluating human breast ductal carcinomas with high-resolution magic-angle spinning proton magnetic resonance spectroscopy. *J. Magn. Reson. 135* (1), 194–202.

(112) Cheng, L. L., Chang, I. W., Louis, D. N., and Gonzalez, R. G. (1998) Correlation of high-resolution magic angle spinning proton magnetic resonance spectroscopy with histopathology of intact human brain tumor specimens. *Cancer Res. 58* (9), 1825–1832.

(113) Millis, K. K., Maas, W. E., Cory, D. G., and Singer, S. (1997) Gradient, high-resolution, magic-angle spinning nuclear magnetic resonance spectroscopy of human adipocyte tissue. *Magn. Reson. Med. 38* (3), 399–403.

(114) Moka, D., Vorreuther, R., Schicha, H., Spraul, M., Humpfer, E., Lipinski, M., Foxall, P. J., Nicholson, J. K., and Lindon, J. C. (1998) Biochemical classification of kidney carcinoma biopsy samples using magic-angle-spinning ^{1}H nuclear magnetic resonance spectroscopy. *J. Pharm. Biomed. Anal. 17* (1), 125–132.

(115) Tomlins, A. M., Foxall, P. J., Lynch, M. J., Parkinson, J., Everett, J. R., and Nicholson, J. K. (1998) High resolution ^{1}H NMR spectroscopic studies on dynamic biochemical processes in incubated human seminal fluid samples. *Biochim. Biophys. Acta 1379* (3), 367–380.

(116) Weybright, P., Millis, K., Campbell, N., Cory, D. G., and Singer, S. (1998) Gradient, high-resolution, magic angle spinning ^{1}H nuclear

magnetic resonance spectroscopy of intact cells. *Magn. Reson. Med. 39* (3), 337–345.
(117) Mao, H., Toufexis, D., Wang, X., Lacreuse, A., and Wu, S. (2007) Changes of metabolite profile in kainic acid induced hippocampal injury in rats measured by HRMAS NMR. *Exp. Brain Res. 183* (4), 477–485.
(118) Holmes, E., Tsang, T. M., and Tabrizi, S. J. (2006) The application of NMR-based metabonomics in neurological disorders. *NeuroRx. 3* (3), 358–372.
(119) Taylor, J. L., Wu, C. L., Cory, D., Gonzalez, R. G., Bielecki, A., and Cheng, L. L. (2003) High-resolution magic angle spinning proton NMR analysis of human prostate tissue with slow spinning rates. *Magn. Reson. Med. 50* (3), 627–632.
(120) Swanson, M. G., Zektzer, A. S., Tabatabai, Z. L., Simko, J., Jarso, S., Keshari, K. R., Schmitt, L., Carroll, P. R., Shinohara, K., Vigneron, D. B., and Kurhanewicz, J. (2006) Quantitative analysis of prostate metabolites using ^{1}H HR-MAS spectroscopy. *Magn. Reson. Med. 55* (6), 1257–1264.
(121) Garrod, S., Humpfer, E., Spraul, M., Connor, S. C., Polley, S., Connelly, J., Lindon, J. C., Nicholson, J. K., and Holmes, E. (1999) High-resolution magic angle spinning ^{1}H NMR spectroscopic studies on intact rat renal cortex and medulla. *Magn. Reson. Med. 41* (6), 1108–1118.
(122) Bollard, M. E., Garrod, S., Holmes, E., Lindon, J. C., Humpfer, E., Spraul, M., and Nicholson, J. K. (2000) High-resolution (1)H and (1)H-(13)C magic angle spinning NMR spectroscopy of rat liver. *Magn. Reson. Med. 44* (2), 201–207.
(123) Li, W., Lee, R. E., Lee, R. E., and Li, J. (2005) Methods for acquisition and assignment of multidimensional high-resolution magic angle spinning NMR of whole cell bacteria. *Anal. Chem. 77* (18), 5785–5792.
(124) Yang, Y., Li, C., Nie, X., Feng, X., Chen, W., Yue, Y., Tang, H., and Deng, F. (2007) Metabonomic studies of human hepatocellular carcinoma using high-resolution magic-angle spinning (1)H NMR spectroscopy in conjunction with multivariate data analysis. *J. Proteome Res. 6* (7), 2605–2614.
(125) Moreno, B., Rodrigues, C. O., Bailey, B. N., Urbina, J. A., Moreno, S. N., Docampo, R., and Oldfield, E. (2002) Magic-angle spinning (31)P NMR spectroscopy of condensed phosphates in parasitic protozoa: visualizing the invisible. *FEBS Lett. 523* (1–3), 207–212.
(126) Payne, G. S., Troy, H., Vaidya, S. J., Griffiths, J. R., Leach, M. O., and Chung, Y. L. (2006) Evaluation of ^{31}P high-resolution magic angle spinning of intact tissue samples. *NMR Biomed. 19* (5), 593–598.
(127) Hu, J. Z., Wind, R. A., and Rommereim, D. N. (2006) (1)H relaxation times of metabolites in biological samples obtained with nondestructive ex-vivo slow-MAS NMR. *Magn. Reson. Chem. 44* (3), 269–275.
(128) Wind, R. A., Hu, J. Z., and Majors, P. D. (2006) Localized in vivo isotropic-anisotropic correlation ^{1}H NMR spectroscopy using ultraslow magic angle spinning. *Magn. Reson. Med. 55* (1), 41–49.
(129) Keun, H. C., Beckonert, O., Griffin, J. L., Richter, C., Moskau, D., Lindon, J. C., and Nicholson, J. K. (2002) Cryogenic probe ^{13}C NMR spectroscopy of urine for metabonomic studies. *Anal. Chem. 74* (17), 4588–4593.
(130) Engelke, U. F., Tangerman, A., Willemsen, M. A., Moskau, D., Loss, S., Mudd, S. H., and Wevers, R. A. (2005) Dimethyl sulfone in human cerebrospinal fluid and blood plasma confirmed by one-dimensional (1)H and two-dimensional (1)H-(13)C NMR. *NMR Biomed. 18* (5), 331–336.
(131) Fiehn, O., and Kind, T. (2007) Metabolite profiling in blood plasma. *Methods Mol. Biol. 358*, 3–17.
(132) Fiehn, O., Kopka, J., Dormann, P., Altmann, T., Trethewey, R. N., and Willmitzer, L. (2000) Metabolite profiling for plant functional genomics. *Nat. Biotechnol. 18* (11), 1157–1161.
(133) Halket, J. M., Waterman, D., Przyborowska, A. M., Patel, R. K., Fraser, P. D., and Bramley, P. M. (2005) Chemical derivatization and mass spectral libraries in metabolic profiling by GC/MS and LC/MS/MS. *J. Exp. Bot. 56* (410), 219–243.
(134) Mashego, M. R., Rumbold, K., De, M. M., Vandamme, E., Soetaert, W., and Heijnen, J. J. (2007) Microbial metabolomics: past, present and future methodologies. *Biotechnol. Lett. 29* (1), 1–16.
(135) Cloarec, O., Campbell, A., Tseng, L. H., Braumann, U., Spraul, M., Scarfe, G., Weaver, R., and Nicholson, J. K. (2007) Virtual chromatographic resolution enhancement in cryoflow LC-NMR experiments via statistical total correlation spectroscopy. *Anal. Chem. 79* (9), 3304–3311.
(136) Nicholls, A. W., Lindon, J. C., Farrant, R. D., Shockcor, J. P., Wilson, I. D., and Nicholson, J. K. (1999) Directly-coupled HPLC-NMR spectroscopic studies of metabolism and futile deacetylation of phenacetin in the rat. *J. Pharm. Biomed. Anal. 20* (6), 865–873.
(137) Sidelmann, U. G., Bjornsdottir, I., Shockcor, J. P., Hansen, S. H., Lindon, J. C., and Nicholson, J. K. (2001) Directly coupled HPLC-NMR and HPLC-MS approaches for the rapid characterisation of drug metabolites in urine: application to the human metabolism of naproxen. *J. Pharm. Biomed. Anal. 24* (4), 569–579.
(138) Spraul, M., Hofmann, M., Lindon, J. C., Farrant, R. D., Seddon, M. J., Nicholson, J. K., and Wilson, I. D. (1994) Evaluation of liquid chromatography coupled with high-field ^{1}H NMR spectroscopy for drug metabolite detection and characterization: the identification of paracetamol metabolites in urine and bile. *NMR Biomed. 7* (7), 295–303.
(139) Wilson, I. D., and Nicholson, J. K. (1987) Solid-phase extraction chromatography and nuclear magnetic resonance spectrometry for the identification and isolation of drug metabolites in urine. *Anal. Chem. 59* (23), 2830–2832.
(140) Wilson, I. D., and Nicholson, J. K. (1988) Solid phase extraction chromatography and NMR spectroscopy (SPEC-NMR) for the rapid identification of drug metabolites in urine. *J. Pharm. Biomed. Anal. 6* (2), 151–165.
(141) Phipps, A. N., Stewart, J., Wright, B., and Wilson, I. D. (1998) Effect of diet on the urinary excretion of hippuric acid and other dietary-derived aromatics in rat. A complex interaction between diet, gut microflora and substrate specificity. *Xenobiotica 28* (5), 527–537.
(142) Robosky, L. C., Wells, D. F., Egnash, L. A., Manning, M. L., Reily, M. D., and Robertson, D. G. (2005) Metabonomic identification of two distinct phenotypes in Sprague-Dawley (Crl:CD(SD)) rats. *Toxicol. Sci. 87* (1), 277–284.
(143) Shockcor, J. P., Unger, S. E., Wilson, I. D., Foxall, P. J., Nicholson, J. K., and Lindon, J. C. (1996) Combined HPLC, NMR spectroscopy, and ion-trap mass spectrometry with application to the detection and characterization of xenobiotic and endogenous metabolites in human urine. *Anal. Chem. 68* (24), 4431–4435.
(144) Lenz, E. M., Bright, J., Knight, R., Westwood, F. R., Davies, D., Major, H., and Wilson, I. D. (2005) Metabonomics with ^{1}H-NMR spectroscopy and liquid chromatography-mass spectrometry applied to the investigation of metabolic changes caused by gentamicin-induced nephrotoxicity in the rat. *Biomarkers 10* (2–3), 173–187.
(145) Want, E. J., Nordstrom, A., Morita, H., and Siuzdak, G. (2007) From exogenous to endogenous: the inevitable imprint of mass spectrometry in metabolomics. *J. Proteome Res. 6* (2), 459–468.
(146) Williams, R., Lenz, E. M., Wilson, A. J., Granger, J., Wilson, I. D., Major, H., Stumpf, C., and Plumb, R. (2006) A multi-analytical platform approach to the metabonomic analysis of plasma from normal and Zucker (fa/fa) obese rats. *Mol. Biosyst. 2* (3–4), 174–183.
(147) Williams, R. E., Major, H., Lock, E. A., Lenz, E. M., and Wilson, I. D. (2005) D-Serine-induced nephrotoxicity: a HPLC-TOF/MS-based metabonomics approach. *Toxicology 207* (2), 179–190.
(148) Williams, R. E., Lenz, E. M., Lowden, J. S., Rantalainen, M., and Wilson, I. D. (2005) The metabonomics of aging and development in the rat: an investigation into the effect of age on the profile of endogenous metabolites in the urine of male rats using ^{1}H NMR and HPLC-TOF MS. *Mol. Biosyst. 1* (2), 166–175.
(149) Plumb, R. S., Granger, J. H., Stumpf, C. L., Johnson, K. A., Smith, B. W., Gaulitz, S., Wilson, I. D., and Castro-Perez, J. (2005) A rapid screening approach to metabonomics using UPLC and oa-TOF mass spectrometry: application to age, gender and diurnal variation in normal/Zucker obese rats and black, white and nude mice. *Analyst 130* (6), 844–849.
(150) Plumb, R. S., Johnson, K. A., Rainville, P., Smith, B. W., Wilson, I. D., Castro-Perez, J. M., and Nicholson, J. K. (2006) UPLC/MS(E); a new approach for generating molecular fragment information for biomarker structure elucidation. *Rapid Commun. Mass Spectrom. 20* (13), 1989–1994.
(151) Plumb, R. S., Johnson, K. A., Rainville, P., Shockcor, J. P., Williams, R., Granger, J. H., and Wilson, I. D. (2006) The detection of phenotypic differences in the metabolic plasma profile of three strains of Zucker rats at 20 weeks of age using ultra-performance liquid chromatography/orthogonal acceleration time-of-flight mass spectrometry. *Rapid Commun. Mass Spectrom. 20* (19), 2800–2806.
(152) Apollonio, L. G., Pianca, D. J., Whittall, I. R., Maher, W. A., and Kyd, J. M. (2006) A demonstration of the use of ultra-performance liquid chromatography-mass spectrometry UPLC/MS in the determination of amphetamine-type substances and ketamine for forensic and toxicological analysis. *J. Chromatogr., B 836* (1–2), 111–115.
(153) Ross, K. L., Tu, T. T., Smith, S., and Dalluge, J. J. (2007) Profiling of organic acids during fermentation by ultraperformance liquid chromatography-tandem mass spectrometry. *Anal. Chem. 79* (13), 4840–4844.
(154) Fux, E., McMillan, D., Bire, R., and Hess, P. (2007) Development of an ultra-performance liquid chromatography-mass spectrometry method for the detection of lipophilic marine toxins. *J. Chromatogr., A. 1157* (1), 273–280.
(155) New, L. S., Saha, S., Ong, M. M., Boelsterli, U. A., and Chan, E. C. (2007) Pharmacokinetic study of intraperitoneally administered

troglitazone in mice using ultra-performance liquid chromatography/tandem mass spectrometry. *Rapid Commun. Mass Spectrom. 21* (6), 982–988.

(156) Licea-Perez, H., Wang, S., Bowen, C. L., and Yang, E. (2007) A semi-automated 96-well plate method for the simultaneous determination of oral contraceptives concentrations in human plasma using ultra performance liquid chromatography coupled with tandem mass spectrometry. *J. Chromatogr., B 852* (1–2), 69–76.

(157) Yin, P., Zhao, X., Li, Q., Wang, J., Li, J., and Xu, G. (2006) Metabonomics study of intestinal fistulas based on ultraperformance liquid chromatography coupled with Q-TOF mass spectrometry (UPLC/Q-TOF MS). *J. Proteome Res. 5* (9), 2135–2143.

(158) Granger, J. H., Williams, R., Lenz, E. M., Plumb, R. S., Stumpf, C. L., and Wilson, I. D. (2007) A metabonomic study of strain- and age-related differences in the Zucker rat. *Rapid Commun. Mass Spectrom. 21* (13), 2039–2045.

(159) Lenz, E. M., Williams, R. E., Sidaway, J., Smith, B. W., Plumb, R. S., Johnson, K. A., Rainville, P., Shockcor, J., Stumpf, C. L., Granger, J. H., and Wilson, I. D. (2007) The application of microbore UPLC/oa-TOF-MS and (1)H NMR spectroscopy to the metabonomic analysis of rat urine following the intravenous administration of pravastatin. *J. Pharm. Biomed. Anal. 44* (4), 845–852.

(160) Lenz, E. M., Bright, J., Knight, R., Wilson, I. D., and Major, H. (2004) A metabonomic investigation of the biochemical effects of mercuric chloride in the rat using ^{1}H NMR and HPLC-TOF/MS: time dependent changes in the urinary profile of endogenous metabolites as a result of nephrotoxicity. *Analyst 129* (6), 535–541.

(161) Holmes, E., Nicholls, A. W., Lindon, J. C., Connor, S. C., Connelly, J. C., Haselden, J. N., Damment, S. J., Spraul, M., Neidig, P., and Nicholson, J. K. (2000) Chemometric models for toxicity classification based on NMR spectra of biofluids. *Chem. Res. Toxicol. 13* (6), 471–478.

(162) Maddox, J. F., Luyendyk, J. P., Cosma, G. N., Breau, A. P., Bible, R. H., Jr., Harrigan, G. G., Goodacre, R., Ganey, P. E., Cantor, G. H., Cockerell, G. L., and Roth, R. A. (2006) Metabonomic evaluation of idiosyncrasy-like liver injury in rats cotreated with ranitidine and lipopolysaccharide. *Toxicol. Appl. Pharmacol. 212* (1), 35–44.

(163) Cloarec, O., Dumas, M. E., Craig, A., Barton, R. H., Trygg, J., Hudson, J., Blancher, C., Gauguier, D., Lindon, J. C., Holmes, E., and Nicholson, J. (2005) Statistical total correlation spectroscopy: an exploratory approach for latent biomarker identification from metabolic ^{1}H NMR data sets. *Anal. Chem. 77* (5), 1282–1289.

(164) Holmes, E., Cloarec, O., and Nicholson, J. K. (2006) Probing latent biomarker signatures and in vivo pathway activity in experimental disease states via statistical total correlation spectroscopy (STOCSY) of biofluids: application to $HgCl_2$ toxicity. *J. Proteome Res. 5* (6), 1313–1320.

(165) Holmes, E., Loo, R. L., Cloarec, O., Coen, M., Tang, H., Maibaum, E., Bruce, S., Chan, Q., Elliott, P., Stamler, J., Wilson, I. D., Lindon, J. C., and Nicholson, J. K. (2007) Detection of urinary drug metabolite (xenometabolome) signatures in molecular epidemiology studies via statistical total correlation (NMR) spectroscopy. *Anal. Chem. 79* (7), 2629–2640.

(166) Smith, L. M., Maher, A. D., Cloarec, O., Rantalainen, M., Tang, H., Elliott, P., Stamler, J., Lindon, J. C., Holmes, E., and Nicholson, J. K. (2007) Statistical correlation and projection methods for improved information recovery from diffusion-edited NMR spectra of biological samples. *Anal. Chem. 79* (15), 5682–5689.

(167) Coen, M., Hong, Y. S., Cloarec, O., Rohde, C. M., Reily, M. D., Robertson, D. G., Holmes, E., Lindon, J. C., and Nicholson, J. K. (2007) Heteronuclear ^{1}H - ^{31}P Statistical Ttal Correlation NMR SpectroscopY (HET-STOCSY) of intact liver for metabolic biomarker assignment: Application to galactosamine-induced hepatotoxicity. *Anal. Chem. 79* (23), 8956–8966.

(168) Crockford, D. J., Holmes, E., Lindon, J. C., Plumb, R. S., Zirah, S., Bruce, S. J., Rainville, P., Stumpf, C. L., and Nicholson, J. K. (2006) Statistical heterospectroscopy, an approach to the integrated analysis of NMR and UPLC-MS data sets: application in metabonomic toxicology studies. *Anal. Chem. 78* (2), 363–371.

(169) Rantalainen, M., Cloarec, O., Beckonert, O., Wilson, I. D., Jackson, D., Tonge, R., Rowlinson, R., Rayner, S., Nickson, J., Wilkinson, R. W., Mills, J. D., Trygg, J., Nicholson, J. K., and Holmes, E. (2006) Statistically integrated metabonomic-proteomic studies on a human prostate cancer xenograft model in mice. *J. Proteome Res. 5* (10), 2642–2655.

(170) Dumas, M. E., Wilder, S. P., Bihoreau, M. T., Barton, R. H., Fearnside, J. F., Argoud, K., D'Amato, L., Wallis, R. H., Blancher, C., Keun, H. C., Baunsgaard, D., Scott, J., Sidelmann, U. G., Nicholson, J. K., and Gauguier, D. (2007) Direct quantitative trait locus mapping of mammalian metabolic phenotypes in diabetic and normoglycemic rat models. *Nat. Genet. 39* (5), 666–672.

(171) Nicholson, J. K., Holmes, E., Lindon, J. C., and Wilson, I. D. (2004) The challenges of modeling mammalian biocomplexity. *Nat. Biotechnol. 22* (10), 1268–1274.

(172) Lindon, J. C., Keun, H. C., Ebbels, T. M., Pearce, J. M., Holmes, E., and Nicholson, J. K. (2005) The Consortium for Metabonomic Toxicology (COMET): aims, activities and achievements. *Pharmacogenomics. 6* (7), 691–699.

(173) Lindon, J. C., Nicholson, J. K., Holmes, E., Antti, H., Bollard, M. E., Keun, H., Beckonert, O., Ebbels, T. M., Reily, M. D., Robertson, D., Stevens, G. J., Luke, P., Breau, A. P., Cantor, G. H., Bible, R. H., Niederhauser, U., Senn, H., Schlotterbeck, G., Sidelmann, U. G., Laursen, S. M., Tymiak, A., Car, B. D., Lehman-McKeeman, L., Colet, J. M., Loukaci, A., and Thomas, C. (2003) Contemporary issues in toxicology the role of metabonomics in toxicology and its evaluation by the COMET project. *Toxicol. Appl. Pharmacol. 187* (3), 137–146.

(174) Ebbels, T. M., Keun, H. C., Beckonert, O. P., Bollard, M. E., Lindon, J. C., Holmes, E., and Nicholson, J. K. (2007) Prediction and classification of drug toxicity using probabilistic modeling of temporal metabolic data: the consortium on metabonomic toxicology screening approach. *J. Proteome Res. 6* (11), 4407–4422.

(175) Wang, Y., Bollard, M. E., Keun, H., Antti, H., Beckonert, O., Ebbels, T. M., Lindon, J. C., Holmes, E., Tang, H., and Nicholson, J. K. (2003) Spectral editing and pattern recognition methods applied to high-resolution magic-angle spinning ^{1}H nuclear magnetic resonance spectroscopy of liver tissues. *Anal. Biochem. 323* (1), 26–32.

(176) Ebbels, T. M. D., Keun, H. C., Beckonert, O. P., Antti, H., Bollard, M. E., Lindon, J. C., Holmes, E. C., and Nicholson, J. K. (2003) Toxicity classification from metabonomic data usinga density superposition approach: 'CLOUDS'. *Anal. Chim. Acta 490*, 109–122.

(177) Beckonert, O. P., Bollard, M. E., Ebbels, T. M. D., Keun, H. C., Antti, H., Lindon, J. C., Holmes, E. C., and Nicholson, J. K. (2003) NMR-based metabonomic toxicity classification: hierarchical cluster analysis and k-nearest-neighbour approaches. *Anal. Chim. Acta 490*, 3–15.

(178) Keun, H. C., Ebbels, T. M., Antti, H., Bollard, M. E., Beckonert, O., Schlotterbeck, G., Senn, H., Niederhauser, U., Holmes, E., Lindon, J. C., and Nicholson, J. K. (2002) Analytical reproducibility in (1)H NMR-based metabonomic urinalysis. *Chem. Res. Toxicol. 15* (11), 1380–1386.

(179) Bollard, M. E., Contel, N. R., Ebbels T. M. D., Smith L., Beckonert O., Cantor G. H., Lehman-McKeeman L. D., Holmes E. H., Lindon J. C., Nicholson J. K., and Keun, H. C. (2007) NMR-based metabolic profiling reveals biomarkers of liver regeneration following partial hepatectomy in the rat. *J. Proteome Res.*, submitted for publication.

(180) Lindon, J. C., Nicholson, J. K., and Everett, J. R. (1999) NMR spectroscopy of biofluids. In *Annual Reports on NMR Spectroscopy* (Webb, G. A., Ed.) p 1Y88, Academic Press, Oxford, U.K.

(181) Moolenaar, S. H., Engelke, U. F., and Wevers, R. A. (2003) Proton nuclear magnetic resonance spectroscopy of body fluids in the field of inborn errors of metabolism. *Ann. Clin. Biochem. 40* (Pt 1), 16–24.

(182) Vangala, S., and Tonelli, A. (2007) Biomarkers, metabonomics, and drug development: can inborn errors of metabolism help in understanding drug toxicity? *AAPS. J. 9* (3), E284–E297.

(183) Makinen, V. P., Soininen, P., Forsblom, C., Parkkonen, M., Ingman, P., Kaski, K., Groop, P. H., and la-Korpela, M. (2006) Diagnosing diabetic nephropathy by ^{1}H NMR metabonomics of serum. *MAGMA. 19* (6), 281–296.

(184) Wang, C., Kong, H., Guan, Y., Yang, J., Gu, J., Yang, S., and Xu, G. (2005) Plasma phospholipid metabolic profiling and biomarkers of type 2 diabetes mellitus based on high-performance liquid chromatography/electrospray mass spectrometry and multivariate statistical analysis. *Anal. Chem. 77* (13), 4108–4116.

(185) Coen, M., O'Sullivan, M., Bubb, W. A., Kuchel, P. W., and Sorrell, T. (2005) Proton nuclear magnetic resonance-based metabonomics for rapid diagnosis of meningitis and ventriculitis. *Clin. Infect. Dis. 41* (11), 1582–1590.

(186) Odunsi, K., Wollman, R. M., Ambrosone, C. B., Hutson, A., McCann, S. E., Tammela, J., Geisler, J. P., Miller, G., Sellers, T., Cliby, W., Qian, F., Keitz, B., Intengan, M., Lele, S., and Alderfer, J. L. (2005) Detection of epithelial ovarian cancer using ^{1}H-NMR-based metabonomics. *Int. J. Cancer 113* (5), 782–788.

(187) Yang, J., Xu, G., Zheng, Y., Kong, H., Pang, T., Lv, S., and Yang, Q. (2004) Diagnosis of liver cancer using HPLC-based metabonomics avoiding false-positive result from hepatitis and hepatocirrhosis diseases. *J. Chromatogr., B 813* (1–2), 59–65.

(188) Marchesi, J. R., Holmes, E., Khan, F., Kochhar, S., Scanlan, P., Shanahan, F., Wilson, I. D., and Wang, Y. (2007) Rapid and noninvasive metabonomic characterization of inflammatory bowel disease. *J. Proteome Res. 6* (2), 546–551.

(189) Lauridsen, M., Hansen, S. H., Jaroszewski, J. W., and Cornett, C. (2007) Human urine as test material in ^{1}H NMR-based metabonom-

ics: recommendations for sample preparation and storage. *Anal. Chem. 79* (3), 1181–1186.

(190) Le, M. L., Racine, S., Le, T. P., Adnet, F., Larue, V., Cohen, Y., Leroux, Y., Cupa, M., and Hantz, E. (2002) Aminoglycoside and glycopeptide renal toxicity in intensive care patients studied by proton magnetic resonance spectroscopy of urine. *Crit. Care Med. 30* (6), 1242–1245.

(191) Stamler, J., Elliott, P., Appel, L., Chan, Q., Buzzard, M., Dennis, B., Dyer, A. R., Elmer, P., Greenland, P., Jones, D., Kesteloot, H., Kuller, L., Labarthe, D., Liu, K., Moag-Stahlberg, A., Nichaman, M., Okayama, A., Okuda, N., Robertson, C., Rodriguez, B., Stevens, M., Ueshima, H., Horn, L. V., and Zhou, B. (2003) Higher blood pressure in middle-aged American adults with less education-role of multiple dietary factors: the INTERMAP study. *J. Hum. Hypertens. 17* (9), 655–775.

(192) Dennis, B., Stamler, J., Buzzard, M., Conway, R., Elliott, P., Moag-Stahlberg, A., Okayama, A., Okuda, N., Robertson, C., Robinson, F., Schakel, S., Stevens, M., Van Heel, N., Zhao, L., and Zhou, B. F. (2003) INTERMAP: the dietary data: process and quality control. *J. Hum. Hypertens. 17* (9), 609–622.

(193) Craig, A., Sidaway, J., Holmes, E., Orton, T., Jackson, D., Rowlinson, R., Nickson, J., Tonge, R., Wilson, I., and Nicholson, J. (2006) Systems toxicology: integrated genomic, proteomic and metabonomic analysis of methapyrilene induced hepatotoxicity in the rat. *J. Proteome Res. 5* (7), 1586–1601.

(194) Dumas, M. E., Maibaum, E. C., Teague, C., Ueshima, H., Zhou, B., Lindon, J. C., Nicholson, J. K., Stamler, J., Elliott, P., Chan, Q., and Holmes, E. (2006) Assessment of analytical reproducibility of ^{1}H NMR spectroscopy based metabonomics for large-scale epidemiological research: the INTERMAP Study. *Anal. Chem. 78* (7), 2199–2208.

(195) Toye, A. A., Dumas, M. E., Blancher, C., Rothwell, A. R., Fearnside, J. F., Wilder, S. P., Bihoreau, M. T., Cloarec, O., Azzouzi, I., Young, S., Barton, R. H., Holmes, E., McCarthy, M. I., Tatoud, R., Nicholson, J. K., Scott, J., and Gauguier, D. (2007) Subtle metabolic and liver gene transcriptional changes underlie diet-induced fatty liver susceptibility in insulin-resistant mice. *Diabetologia. 50* (9), 1867–1879.

(196) Ringeissen, S., Connor, S. C., Brown, H. R., Sweatman, B. C., Hodson, M. P., Kenny, S. P., Haworth, R. I., McGill, P., Price, M. A., Aylott, M. C., Nunez, D. J., Haselden, J. N., and Waterfield, C. J. (2003) Potential urinary and plasma biomarkers of peroxisome proliferation in the rat: identification of N-methylnicotinamide and N-methyl-4-pyridone-3-carboxamide by ^{1}H nuclear magnetic resonance and high performance liquid chromatography. *Biomarkers 8* (3–4), 240–271.

(197) Connor, S. C., Hodson, M. P., Ringeissen, S., Sweatman, B. C., McGill, P. J., Waterfield, C. J., and Haselden, J. N. (2004) Development of a multivariate statistical model to predict peroxisome proliferation in the rat, based on urinary ^{1}H-NMR spectral patterns. *Biomarkers 9* (4–5), 364–385.

(198) Delaney, J., Hodson, M. P., Thakkar, H., Connor, S. C., Sweatman, B. C., Kenny, S. P., McGill, P. J., Holder, J. C., Hutton, K. A., Haselden, J. N., and Waterfield, C. J. (2005) Tryptophan-NAD+ pathway metabolites as putative biomarkers and predictors of peroxisome proliferation. *Arch. Toxicol. 79* (4), 208–223.

(199) Li, H., Ni, Y., Su, M., Qiu, Y., Zhou, M., Qiu, M., Zhao, A., Zhao, L., and Jia, W. (2007) Pharmacometabonomic phenotyping reveals different responses to xenobiotic intervention in rats. *J. Proteome Res. 6* (4), 1364–1370.

(200) Nicholson, J. K., Holmes, E., and Wilson, I. D. (2005) Gut microorganisms, mammalian metabolism and personalized health care. *Nat. Rev. Microbiol. 3*, 431–438.

(201) Holmes, E., and Nicholson, J. K. (2005) Variation in gut microbiota strongly influences individual rodent phenotypes. *J. Toxicol. Sci. 87*, 1–2.

(202) Rohde, C. M., Wells, D. F., Robosky, L. C., Manning, M. L., Clifford, C. B., Reily, M. D., and Robertson, D. G. (2007) Metabonomic evaluation of Schaedler altered microflora rats. *Chem. Res. Toxicol. 20*, 1388–1392. .

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