Published in final edited form as:

Chem Res Toxicol. 2010 May 17; 23(5): 851–860. doi:10.1021/tx100020p.

XENOBIOTIC METABOLISM – A VIEW THROUGH THE METABOLOMETER

Andrew D. Patterson^a, Frank J. Gonzalez^a, and Jeffrey R. Idle^{a,b}

^aLaboratory of Metabolism, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892

^bInstitute of Pharmacology, First Faculty of Medicine, Charles University, Praha, Czech Republic

Abstract

The combination of advanced ultra-performance liquid chromatography coupled with mass spectrometry, chemometrics, and genetically modified mice provide an attractive raft of technologies with which to examine the metabolism of xenobiotics. Here a reexamination of the metabolism of the food mutagen PhIP (2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine), the suspect carcinogen areca alkaloids (arecoline, arecaidine, and arecoline 1-oxide), the hormone supplement melatonin, and the metabolism of the experimental cancer therapeutic agent aminoflavone is presented. In all cases, the metabolic maps of the xenobiotics were considerably enlarged, providing new insights into their toxicology. The inclusion of transgenic mice permitted unequivocal attribution of individual and often novel metabolic pathways to particular enzymes. Lastly, a future perspective for xenobiotic metabolomics is discussed and its impact on the metabolome is described. The studies reviewed here are not specific to the mouse, and can be adapted to study xenobiotic metabolism in any animal species, including Man. The view through the metabolometer is unique and visualizes a metabolic space that contains both established and unknown metabolites of a xenobiotic thereby enhancing knowledge of their modes of toxic action.

Keywords

Drug metabolism; metabolomics; mass spectrometry; chemometrics

INTRODUCTION

Xenobiotic is a term used to describe chemical substances that are foreign to animal life and thus includes such examples as plant constituents, drugs, pesticides, cosmetics, flavorings, fragrances, food additives, industrial chemicals and environmental pollutants. It has been estimated that humans are exposed to 1-3 million xenobiotics in their lifetimes (1). Most of these chemicals that gain access to the body via the diet, air, drinking water, drug administration, and lifestyle choices, undergo a broad range of processes of detoxication that in general render them less toxic, more polar, and readily excretable. Detoxication reactions in lower animals may also impact on human health. Incomplete detoxication in organisms in the food chain may determine the extent of human exposure to environmental toxins, for example hepatotoxic microcystins from algal blooms (2) and mycotoxins ingested by agricultural species (3). While the general principles of xenobiotic detoxication were delineated long ago (4), it was also recognized that xenobiotic metabolism could modify the

pharmacological properties of drugs (4,5) or even activate inert chemicals into biologically reactive species (6). Moreover, the cancer chemotherapeutic drugs stilboestrol diphosphate (7) and cyclophosphamide (8) were the first to be specifically designed to undergo xenobiotic metabolism and activation in the human body to generate pharmacologically active species. Clearly, it is important to understand both the qualitative and quantitative aspects of xenobiotic metabolism in both Man and lower species to gain insights into mechanistic aspects of toxicity profiles.

The study of xenobiotic metabolism over the decades has hinged on technical developments in the field of analytical chemistry. Xenobiotic metabolism has its origins in the mid-1800s and for almost a century its practice involved isolation, purification, and simple chemical investigation of urinary constituents. The advent of UV/visible and fluorescence spectroscopy, radiolabelled compounds, and partition chromatography catalyzed a major expansion of activities in the field. However, the biggest single advance of benefit to the study of xenobiotic metabolism was development of biomedical mass spectrometry, at first GC-MS and subsequently the range of LC-MS and NMR technologies that are ubiquitous today. Drug metabolism may now be conducted by what may be called drug metabolomics. During its short history, metabolomics has generally been thought of as a tool for either discovering cellular responses to external stimuli such as toxicant administration (9,10), or cataloging metabolic pathways in health and disease (11,12). In addition to examining endogenous molecular changes in response to stimuli, metabolomics can equally be applied to the examination of xenobiotic metabolites (1,13,14), the footprints of cellular metabolism that are left on xenobiotic molecules.

In this review we will examine the advances in xenobiotic metabolism that have been made using metabolomics, a science that combines advanced analytical chemistry with chemometric analyses such as multivariate data analysis. Because of the doses employed, when xenobiotics are administered to experimental animals, their metabolites occur generally at concentrations in excess of most endogenous metabolites, i.e. the majority of the metabolome. This alone makes them relatively easy to find using metabolomic protocols (summarized in Fig. 1). Additionally, the chemometric methods [reviewed in 15, 16] that compare control animals with dosed animals, or fluids collected prior to xenobiotic dosing versus post, greatly assist in the identification of metabolites deriving from biotransformation of the administered xenobiotic, as opposed to those that comprise the unperturbed metabolome (Table 1). Finally, use of mass spectrometry with accurate mass determination yields a restricted number of empirical formulae for each xenobiotic-related ion because it is known that each of these ions must have derived from the metabolism of the administered xenobiotic. Furthermore, there are also software packages that can assist in generating a table of metabolites directly from the mass spectral data matrix (17,18).

Metabolism of xenobiotics using metabolomics

More than 52 million organic and inorganic substances have been synthesized of which over 39 million are commercially available (19) and thus represent potential human exposures. The proportion of these xenobiotics whose metabolism has been established in Man and laboratory animals is trivial. It is well established that xenobiotics can elicit toxic reactions through mechanisms involving their biotransformation to reactive chemical species (20). To understand their toxicologic potential, it is vital to determine the detailed metabolic pathways of xenobiotics that have known human exposures.

The studies revisited below follow a similar experimental design. Simply stated, urine from vehicle- and xenobiotic-treated mice was analyzed using ultra-performance liquid chromatography coupled with mass spectrometry and compared using advanced chemometrics. This approach was first introduced by Plumb and colleagues (21) and, recently,

has been expertly reviewed (13). The metabolomic approach is not to be confused with metabolite profiling in which, for example, drug and drug metabolites are identified using methods such as mass defect filtering (17,22) or by scanning (LC/MS/MS) for known drug metabolites such as glutathione conjugates or glucuronides (23). In other words, in a true metabolomics study no emphasis is placed on measuring a particular metabolite or group of metabolites, but rather on measuring as many metabolites as possible and then using chemometrics to identify xenobiotic metabolites. While the studies presented below represent, to the best of our knowledge, some of the first successful applications of the xenobiotic metabolomic approach, other recent examples include the identification of novel tolcapone (a catechol-*O*-methyl transferase inhibitor) metabolites in rats (24) and new metabolites of fenofibrate in *Cynomolgus* monkeys (25). In both cases, the metabolic maps of tolcapone (6 novel metabolites identified) and fenofibrate (5 novel metabolites identified) were expanded.

The heterocyclic amine food mutagen 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP)—PhIP is the most abundant heterocyclic amine formed during the cooking of meat and fish (26). Only food that is boiled or cooked below 200°C is relatively free of PhIP and related heterocyclic amines. Metabolic activation of PhIP to *N*²-acetoxy-PhIP and *N*²-sulfonyloxy-PhIP largely involves *N*-hydroxylation by CYP1A2 followed by conjugation of the hydroxylamine by *N*-acetyltransferase and sulfotransferase, respectively. Interspecies differences in the patterns of PhIP metabolism have been reported, making it difficult to extrapolate laboratory data to human risk assessment for PhIP exposure. In essence, rodent P450 enzymes predominantly carry out a detoxication reaction (4'-hydroxylation) while the human counterparts produce the *N*-hydroxy metabolite that leads to esterification and production of the active electrophilic metabolite. There were a total of nine known metabolites, six arising from the detoxication reactions of *N*-glucuronidation, 4'-hydroxylation and subsequent sulfate conjugation. In contrast, three activated metabolites were reported in the literature, *N*²-hydroxy-PhIP and its *O*-acetyl and sulfate conjugates (Fig. 2). Because of the rodent-human metabolic differences, transgenic mice humanized for the *CYP1A2* gene was employed to understand better the metabolism and toxicology of PhIP in mice with a human pattern of PhIP metabolism (27). It should be noted that this class of heterocyclic amines is among the most mutagenic group of substances yet tested (26) and there is a real concern for cancer risk to humans from their dietary exposure (28).

The metabolomics of PhIP metabolism in the mouse has been undertaken from a number of different standpoints. Firstly, it was clear that other metabolites of PhIP might exist. Secondly, by applying metabolomic methods in different transgenic mouse lines, the role of various enzymes in the detoxication and activation of PhIP, and also in the formation of DNA adducts, should become clearer. PhIP (10 mg/kg p.o.) was administered to wild-type (*mCyp1a2*^{+/+}), *Cyp1a2*-null (*mCyp1a2*^{-/-}), and *CYP1A2*-humanized (*hCYP1A2*^{+/+}, *mCyp1a2*^{-/-}) male 129/SvJ strain mice and 0-24 h urine collected and subjected to metabolomic analysis using ultraperformance liquid chromatography-coupled electrospray ionization quadrupole time-of-flight mass spectrometry (UPLC-ESI-QTOFMS) and principal components analysis (PCA) (29). This dose, while many times greater than the dose a typical human would receive from cooked meat (30), was used to maximize drug metabolite discovery. The mass spectrometry data best fitted a three-component model in the PCA analysis, with the control (untreated), wild-type, null, and humanized animals all clustering in the scores space (Fig. 3A). Interestingly, control, wild-type, and humanized mice clustered on a plane defined by components 1 and 2 in the three-dimensional scores space, with the null animals lying far above in component 3. Both known and unknown metabolites of PhIP were visible in the three-dimensional loadings space (Fig. 3B), including PhIP itself (1) and PhIP *N*²-glucuronide (12), a known metabolite (Fig. 2) that is not formed by CYP1A2, and two unknowns that were identified by tandem mass spectrometry experiments as *N*²-methyl-PhIP (2) and 4'-hydroxy-*N*²-methyl-PhIP (6). The other metabolites labeled in Fig. 3B appear to be distributed close to

or on the surface of the plane defined by the control, wild-type, and humanized mice. These metabolites of PhIP are therefore likely to be formed by mouse and/or human CYP1A2 and include the known metabolites (Fig. 2) 4'-hydroxy-PhIP (**3**), 5-hydroxy-PhIP (**5**), 4'-hydroxy-PhIP 4'-*O*-sulfate (**7**), PhIP *N*³-glucuronide (**11**), *N*²-hydroxy-PhIP *N*²-glucuronide (**16**), and *N*²-hydroxy-PhIP *N*³-glucuronide (**17**). Other novel metabolites confirmed by mass fragmentography were 5-hydroxy-PhIP 5-*O*-sulfate (**8**), *N*²,4'-dihydroxy-PhIP 4'-*O*-sulfate (**9**), 4',5-dihydroxy-PhIP 4'-*O*-sulfate (**10**), 5-hydroxy-PhIP 5-*O*-β-D-glucuronide (**14**), *N*²,4'-dihydroxy-PhIP 4'-*O*-β-D-glucuronide (**18**) and 4'-hydroxy-PhIP *N*²-β-D-glucuronide (**19**). Thus, of the 19 excretory products of PhIP in the mouse, eight were novel metabolites discovered by metabolomics (29). Finally, consistent with the distribution of scores and loadings in the three-dimensional space (Fig. 3), the relative urinary excretion of metabolites in the three mouse lines was: wild-type, **1**≈**3**≈**17**>>**12** (**6** not detected); *Cyp1a2*-null, **1**>**12**>**6**>**17**>**3**; *CYP1A2*-humanized, **17**>>**1**>**3**≈**12**>**6** (29). These insights assist in understanding the genotoxicity of PhIP to target organs such as colon and breast (29). They also shed insight into the value of genetically modified mice in human risk assessment for carcinogen exposure and suggest that in the case of PhIP, *CYP1A2*-humanized mice might be more predictive.

The areca alkaloids arecoline, arecaidine, and arecoline 1-oxide—It has been estimated that some 600 million people are exposed to areca alkaloids arecoline, arecaidine, guvacine and guvacoline through the habit of areca nut chewing (31), the fourth most popular habituated chemical group after tobacco, alcohol, and caffeine (32). Areca nut chewing has been associated with oral cancer in India, Pakistan, Taiwan, and China (33). The prevailing theory attributes this to the formation of nitrosamines from areca alkaloids in the mouth, in particular guvacine and guvacoline. However, it is not known what role the predominant alkaloids arecoline and arecaidine might play in this process. The metabolism of the principal alkaloids was largely unknown despite their toxicological importance to about one-sixth of the world's adult population. Fig. 4A shows the metabolic map of arecoline and arecaidine that was available to the International Agency for Research on Cancer (IARC) at the time of its evaluation of the carcinogenic risk of areca alkaloids to humans (33). As tertiary amines, these metabolites are unlikely to form nitrosamines in the mouth. No other obvious cancer causing mechanism emerges from perusal of the chemistry of these compounds.

In order to understand better the biotransformation of the areca alkaloids, two metabolomic studies have been undertaken in the mouse, one with arecoline and arecaidine (34) and the other with the principal metabolite of arecoline, arecoline 1-oxide (Fig. 4) (35). Groups of male strain FVB mice were administered arecoline hydrobromide (20 mg/kg, both p.o. and i.p.) and others arecaidine (20 mg/kg, both p.o. and i.p.). Urines were collected for 0-12 h, both prior to dosing and immediately post-dose and subjected to UPLC-ESI-QTOFMS analysis in positive ion mode and by PLS-DA (projection to latent structures-discriminant analysis). The two-component PLS-DA scores plots showed clustering of the treated and control animals with a clear separation, principally in component 1 (34). Fig. 5 displays the PLS-DA loadings plot for arecoline-treated (p.o.) *versus* control mice. The mouse urinary metabolome in positive ion mode corresponds to approximately 5,000 ions, which derive from considerably less chemical constituents, the difference being due to in-source fragment ions, dimers, isotopes, and adducts with Na⁺, NH₄⁺, etc. These phenomena have been discussed in greater detail elsewhere (36). The constellation of ions comprising the mouse metabolome can be clearly seen in the PLS-DA loadings plot (Fig. 5). Administration of arecoline perturbed this distribution of ions in the loadings space by adding a significant number of new ions that were deviated in both component 1 (abscissa) and component 2 (ordinate) of the variance. Two far removed ions are shown that represent ions deriving from the two most abundant arecoline metabolites in urine, 1-methyl-nipecotic acid (X) and arecoline 1-oxide (Y). It is worth noting that the former had not been previously reported. Other novel metabolites (Fig. 4B), together with all the previously

reported metabolites (Fig. 4A), emerged from analysis of the ions shown in the shaded ellipse in Fig. 5. The studies with arecaidine also yielded the carboxylic acid metabolites shown in Fig. 4B (34), while those with ($\pm$)-arecoline 1-oxide administration yielded the novel metabolites shown in Fig. 4C (35). Overall, the four known metabolites of these areca alkaloids were confirmed and nine novel metabolites described (34,35). The phenomenon of apparent double-bond reduction *per se* to yield nipecotic acid derivatives is without precedent. It was considered that the nine saturated metabolites (i.e. without the double bond) all arose due to glutathione conjugation and further degradation of the resultant mercapturic acids (34,35). Were any of these reactions to occur in the tissues of the oral cavity, they might place a burden on the antioxidant defenses that depend upon reduced glutathione and other cellular thiols that are not abundantly produced in this tissue. This might therefore open new avenues of research into potential toxicologic processes associated with the common exposure to areca nut alkaloids.

Melatonin—Although melatonin is an endogenous hormone produced in μ g quantities per day in humans by the pineal gland, retina, and the gut (37), it is now widely sold as a dietary supplement to be self administered at pharmacological doses, about two orders of magnitude above physiological amounts (38). Under these conditions melatonin should be treated as a xenobiotic. One of the principal rationales for the widespread use of melatonin is the evidence that it can act as an antioxidant (39). Therefore, melatonin has been subjected to research in animal models of human disease, such as hepatic, renal, and brain ischemia (39). Most interest, however, has centered around the potential of melatonin as an antioxidant to prevent cancer, particularly breast and endometrial cancer. Available data suggest that melatonin is not only an antioxidant but also has antimitotic and antiangiogenic activity (40). It may also enhance the immune system in the elderly by stimulating production of progenitor cells for granulocytes and macrophages, by invigorating the production of natural killer cells and CD4+ cells, while inhibiting CD8+ cells. Moreover, melatonin stimulates natural killer cells and T helper lymphocytes to release various cytokines (41). There are therefore many reasons to believe that physiological depletion of melatonin may lead to breast, endometrial, and colorectal cancer and that administration of melatonin may have cancer preventative effects (42). The situation is more complex due to the potential contributions of melatonin metabolites. The major metabolite 6-hydroxymelatonin has been reported to be genotoxic under *in vitro* conditions (43). Contrary reports state that 6-hydroxymelatonin shares antioxidant properties with melatonin (44,45). Certain metabolites of melatonin, such as AMK and AFMK, that are detected *in vivo*, are thought to arise from the reaction of melatonin with reactive oxygen species (ROS) and, in a strict sense, are not metabolites, but rather antioxidant products (39). Given the uncertainties regarding the role of melatonin metabolites in cancer prevention, a detailed metabolomic investigation of melatonin metabolism was deemed necessary.

A complex metabolomic study of melatonin was undertaken that asked a range of questions and employed C57BL/6, CBA, and 129/SvJ strain mice, together with the transgenic *Cyp1a2*-null and *CYP1A2*-humanized mouse lines (38). For the production of the metabolic map of melatonin, mice were administered melatonin (4 mg/kg i.p.) and 0-24 h urine collected. This dose is 60-times the average human (70 kg) would expect to receive from a 2 mg melatonin supplement. Control urines from the same mice were collected 2 days previously. These urines were subjected to UPLC-ESI-QTOFMS analysis in positive ion mode and the data obtained analyzed by PCA and by OPLS (orthogonal projection to latent structures). PCA showed separate clustering of the treated and untreated animals in a two-dimensional scores space with the separation occurring exclusively in component 1 (38). Further analysis with OPLS yielded a loadings S-plot (Fig. 6), which is a form of data presentation that can give insights into the relative abundance ($w[2]$) of individual metabolites (strictly, ions derived from them) and how well they correlated to the OPLS model ($p(\text{corr})[1]$). Clearly, administration of xenobiotics should produce metabolite signals that are highly correlated to the model that involves a

comparison with urines from control untreated animals. The shaded ellipse in Fig. 6 represents ions that are upregulated after melatonin administration and that have a correlation to the OPLS model of 0.85 to 1.0. Additional experiments involving tandem mass spectrometry confirmed the presence of known melatonin metabolites in mouse urine, but also revealed a number of novel metabolites. Fig. 7 shows the new metabolic map of melatonin in the mouse with six of the seven novel metabolites shown in the shaded boxes. The seventh novel metabolite was the glucuronide of dihydroxymelatonin (**6**), whose exact structure could not be determined and was reasoned to be 4,6-, 6,7-, or 2,6-dihydroxymelatonin (38). The novel metabolites shown are melatonin *N*-glucuronide (**4**), cyclic melatonin (**14**), cyclic *N*-acetylserotonin 5-*O*-glucuronide (**13**), cyclic 6-hydroxymelatonin (**12**), 5-hydroxyindole acetaldehyde (**15**), and the unidentified dihydroxymelatonin (**6**). As stated earlier, compounds **16** (AFMK) and **17** (AMK) are not metabolites, but were products of the reaction of melatonin with ROS. It is noteworthy that neither AFMK or AMK were observed in this study, despite earlier reports that they each contributed 0.01% of the administered dose in mice (39) and that 50% of the administered dose of radioactive melatonin was recovered in rat urine as AMF and AFMK combined (46). Perhaps there exists an unreported major species difference in melatonin disposition between the rat and the mouse. One further controversy that was settled by this metabolomics study concerned the conjugation of the primary major metabolite of melatonin in the mouse, 6-hydroxymelatonin. This metabolite is conjugated with sulfate in humans and rats, but two groups had reported starkly differing findings, one that the mouse conjugated 6-hydroxymelatonin also with sulfate and the other with glucuronic acid. This metabolomic study demonstrated unequivocally that 6-hydroxymelatonin is conjugated almost exclusively with glucuronic acid in the mouse (38). Thus, the metabolomic investigation of melatonin metabolism produced new data that may help understand better the physiology, biochemistry, endocrinology, toxicology and cancer preventative effects of melatonin.

Aminoflavone (NSC 686288)—During the search for novel breast cancer chemotherapeutic agents, 5,4'-diaminoflavone and some of its congeners demonstrated high activity against the breast cancer cell line MCF-7 that is known to possess the estrogen receptor (ER) and be estrogen-responsive (47). Interest focused on 5-amino-2-(4'-amino-3'-fluorophenyl)-6,8-difluoro-7-methyl-4*H*-1-benzopyran-4-one (DAF; NSC 686288; aminoflavone) and it was reported that rats dosed with aminoflavone had a single metabolite in their blood, which was shown to be the 4'-acetyl amino compound (48). Aminoflavone was extensively metabolized to a number of metabolites by CYP1A1 and CYP1A2 in rat and human liver microsomes, one of which was reported to be a potentially reactive hydroxylamine (49). In addition, induction of CYP1A1 and CYP1A2 in cell lines increased the DNA binding and cytotoxicity of aminoflavone. Furthermore, it was shown that aminoflavone was itself a CYP1A inducer and could thus induce its own metabolism and enhance its cytotoxicity (49). Given the role of metabolism in pharmacology and toxicology of this promising experimental drug and the uncertainties surrounding its metabolism *in vivo*, it was decided to undertake a metabolomic investigation of aminoflavone metabolism in the mouse.

Because of the reported role of CYP1A isozymes in the *in vitro* metabolism and activation of aminoflavone, a complex protocol was evolved that employed wild-type 129/SvJ mice, *Cyp1a2*-null mice, and *CYP1A2*-humanized mice (50). Aminoflavone was administered (50 mg/kg p.o. in corn oil) and urine collected for 0-24 h. Prior control urines were collected for each mouse. Urines were subjected to UPLC-ESI-QTOFMS analysis in positive ion mode and the resultant data matrix of ions analyzed by PCA. As shown in Fig. 8A, control and aminoflavone treated mouse urines clustered and separated in the PCA scores space, largely along component 1. Accordingly, a metabolic space was created in the PCA loadings plot that defined the aminoflavone metabolites (Fig. 8B). As with the case of arecoline (Fig. 5), there were ions derived from xenobiotic metabolites that were clearly displaced from the mouse urinary metabolome. These ions were subjected to mass fragmentography by tandem mass

spectrometry to identify their chemical structures (13,50). The resultant metabolic map for aminoflavone is shown in Fig. 9. The only aminoflavone metabolite that was known to be produced *in vivo*, *N*⁴-acetyl-aminoflavone (48), was not present in mouse urine (50). All the other metabolites discovered were novel, although the exact nature of some of the glucuronic acid and sulfate conjugates was not established. Nevertheless, the amount and quality of new metabolic information emerging from this metabolomic survey was considerable and established that aminoflavone is principally *N*-hydroxylated at *N*⁵ and this hydroxylamine, presumably the same metabolite that was detected with human and rat liver microsomes but never identified (49), is further conjugated with glucuronic acid (Fig. 9). Clear evidence of both activation and detoxication of aminoflavone was found and the role of CYP1A2 in these pathways established through the use of genetically modified mice combined with metabolomics (50). Subsequent to this work, it was reported that activation of aminoflavone by sulfotransferase was necessary for its genotoxicity and antiproliferative effects (51). Aminoflavone is now in clinical trials and much more has been established regarding its molecular mechanisms of action (52). Similar LC-MS based methods to those used in the metabolomics study are now employed to determine its concentration in human plasma (53).

CONCLUSIONS AND FUTURE PERSPECTIVES

As has been expertly addressed (54), it is without doubt that xenobiotic metabolism directly contributes, in both number (e.g., PhIP exposure results in 19 detectable urinary metabolites) and relative abundance (e.g., xenobiotics are some of the most highly concentrated metabolites) to the metabolome (Fig. 10). It is for these reasons that the metabolomic approaches discussed in this review have been successful. Xenobiotic metabolism can also influence endogenous metabolism, and while the focus of this review has been primarily on xenobiotic metabolism, additional insight into changes in host metabolism can be gleaned by purging drug and drug-related metabolites from the dataset, and then performing a new chemometric analysis. This approach has been recently demonstrated to understand fenofibrate effects in humans (55). The influence of xenobiotic metabolism on the host metabolome also raises important questions about attempts to catalog or even define the metabolome. For example, should the metabolome be quarantined from other “omes,” existing simply as a collection of endogenous chemicals involved in processes such as glycolysis, the citric acid cycle, or fatty acid β -oxidation pathway? Or, would it be more appropriate to consider the metabolome as an amalgam of compounds comprised of the products of all endogenous and xenobiotic metabolism given that complete isolation from the exposure to compounds found in food, pesticides, cosmetics, environmental pollutants, or drugs is seemingly unavoidable in any human study? While some may dismiss this as semantics, this discussion has important implications for any *in vivo* metabolomics (unbiased, not targeted) study, where, most certainly, xenobiotics and their metabolites will be interspersed and inseparable from the endogenous biochemicals. In our opinion, it would be premature, particularly if one envisions a clinical setting where limited patient history might be available, to state that the endogenous metabolome would provide more information than say the xenobiome, and vice versa. Technically speaking, high-throughput measurement of a wide-range of compounds would be relatively simple and inexpensive given the sophistication of current technologies such as mass spectrometry. Furthermore, having a global, unbiased perspective of the complex interplay between endogenous and xenobiotic metabolism would add substantially to our understanding of biochemical, pharmacological and toxicological processes and might eventually identify useful biomarkers for assessing risk and exposure.

As a step in the right direction, particularly for xenobiotic metabolomics and toxicology, the Human Metabolome Project (<http://www.metabolomics.ca/>) has extended their cataloging efforts far beyond endogenous compounds, and now include detailed information on xenobiotics commonly found in food (FooDB), those taken as drugs (DrugBank), or those

received through exposure (t3db) such as pollutants and pesticides (56–60). However, metabolite information, especially toxic metabolites such as those from acetaminophen including *N*-acetyl-*p*-benzoquinone imine, remains limited. A centralized repository for drug metabolite data, much like how FooDB, DrugBank, and t3db are organized, would be a valuable and convenient resource for drug (and other xenobiotics) metabolism investigations of existing or candidate drugs.

It has been reported here that application of advanced mass spectrometry coupled with multivariate data analyses is able to provide a new perspective on xenobiotic metabolism and therefore toxicology. This view through the metabolometer provides an unprecedented level of metabolic detail for drugs and chemicals that would not be seen through other methodologies. This technology is by no means restricted to the study of the mouse and has been used, for example, to re-examine the metabolism of fenofibrate in monkeys (25). However, in the case of the mouse, there exists a very powerful combination of technologies, genetically modified mice combined with metabolomics, uniquely suited to delineate the role of specific genes and their proteins in discrete metabolic pathways. These observations can ultimately be extended to heterogeneous human populations where other factors such as age, gender, and race are likely to impact and thus complicate metabolomic studies (61). Further discussion of this subject falls beyond the scope of this review. Through the metabolometer, visualization of a metabolic space (see Figs. 3, 5, 6, and 8) in a loadings plot of ions derived from the analysis of biofluids from xenobiotic-treated animals gives immediate insights into both qualitative and quantitative components of xenobiotic transformation. Providing that investigations are carefully designed, controlled, and executed, the view presented here is possible for any organic molecule in any animal species.

Acknowledgments

We thank Drs. Chi Chen, Sarbani Giri, and Xiaochao Ma for their extensive contributions to the xenobiotic metabolomics paradigm. This work was funded by the National Cancer Institute, Intramural Research Program, Center for Cancer Research. A.D.P. was supported by a Pharmacology Research Associate in Training Fellowship from the National Institute of General Medical Sciences. J.R.I. is grateful to U.S. Smokeless Tobacco Company for a Grant for collaborative research.

List of abbreviations used

CYP	cytochrome P450
LC-MS	liquid chromatography coupled mass spectrometry
GC-MS	gas chromatography coupled mass spectrometry
UPLC	ultra-performance liquid chromatography
ESI	electrospray ionization
QTOFMS	quadrupole time-of-flight mass spectrometry
OPLS	orthogonal projection to latent structures
PCA	principal components analysis
PhIP	2-amino-1-methyl-6-phenylimidazo[4,5- <i>b</i>]pyridine
PLS-DA	projection to latent structures-discriminant analysis

REFERENCES

1. Idle JR, Gonzalez FJ. Metabolomics. *Cell Metab* 2007;6:348–351. [PubMed: 17983580]

2. Martins JC, Vasconcelos VM. Microcystin dynamics in aquatic organisms. *J Toxicol Environ Health B Crit Rev* 2009;12:65–82. [PubMed: 19117210]
3. Zinedine A, Soriano JM, Molto JC, Manes J. Review on the toxicity, occurrence, metabolism, detoxification, regulations and intake of zearalenone: an oestrogenic mycotoxin. *Food Chem Toxicol* 2007;45:1–18. [PubMed: 17045381]
4. Williams, RT. Detoxication Mechanisms. *The Metabolism and Detoxication of Drugs, Toxic Substances and Other Organic Compounds*. Second Edition ed.. John Wiley & Sons Inc.; New York: 1959.
5. Williams RT. Detoxication mechanisms in man. *Clin Pharmacol Ther* 1963;4:234–254. [PubMed: 14000932]
6. Miller JA, Miller EC. Metabolic activation and reactivity of chemical carcinogens. *Mutat Res* 1975;33:25–26. [PubMed: 1202335]
7. Persky L, Krohmer JS, Storaasli JP. Clinical studies of excretion and localization of diethyl stilbestrol diphosphate labelled with radioactive phosphorus (P32). *Surg Forum* 1957;8:610–613. [PubMed: 13529696]
8. Arnold H, Bourseaux F, Brock N. Chemotherapeutic action of a cyclic nitrogen mustard phosphamide ester (B 518-ASTA) in experimental tumours of the rat. *Nature* 1958;181:931. [PubMed: 13526741]
9. Holmes E, Nicholson JK, Tranter G. Metabonomic characterization of genetic variations in toxicological and metabolic responses using probabilistic neural networks. *Chem Res Toxicol* 2001;14:182–191. [PubMed: 11258967]
10. Nicholson JK, Lindon JC, Holmes E. 'Metabonomics': understanding the metabolic responses of living systems to pathophysiological stimuli via multivariate statistical analysis of biological NMR spectroscopic data. *Xenobiotica* 1999;29:1181–1189. [PubMed: 10598751]
11. Kaddurah-Daouk R, Kristal BS, Weinshilboum RM. Metabolomics: a global biochemical approach to drug response and disease. *Annu Rev Pharmacol Toxicol* 2008;48:653–683. [PubMed: 18184107]
12. Griffin JL. Understanding mouse models of disease through metabolomics. *Curr Opin Chem Biol* 2006;10:309–315. [PubMed: 16815732]
13. Chen C, Gonzalez FJ, Idle JR. LC-MS-based metabolomics in drug metabolism. *Drug Metab Rev* 2007;39:581–597. [PubMed: 17786640]
14. Griffin JL, Bollard ME. Metabonomics: its potential as a tool in toxicology for safety assessment and data integration. *Curr Drug Metab* 2004;5:389–398. [PubMed: 15544433]
15. Wishart DS. Computational approaches to metabolomics. *Methods Mol Biol* 2010;593:283–313. [PubMed: 19957155]
16. Trygg J, Holmes E, Lundstedt T. Chemometrics in metabonomics. *J Proteome Res* 2007;6:469–479. [PubMed: 17269704]
17. Mortishire-Smith RJ, Castro-Perez JM, Yu K, Shockcor JP, Goshawk J, Hartshorn MJ, Hill A. Generic dealkylation: a tool for increasing the hit-rate of metabolite rationalization, and automatic customization of mass defect filters. *Rapid Commun Mass Spectrom* 2009;23:939–948. [PubMed: 19241416]
18. Mortishire-Smith RJ, O'Connor D, Castro-Perez JM, Kirby J. Accelerated throughput metabolic route screening in early drug discovery using high-resolution liquid chromatography/quadrupole time-of-flight mass spectrometry and automated data analysis. *Rapid Commun Mass Spectrom* 2005;19:2659–2670. [PubMed: 16124034]
19. CAS. The Latest CAS Registry Number and Substance Count, Chemical Abstracts Service. 2009.
20. Wogan GN, Hecht SS, Felton JS, Conney AH, Loeb LA. Environmental and chemical carcinogenesis. *Semin Cancer Biol* 2004;14:473–486. [PubMed: 15489140]
21. Plumb RS, Stumpf CL, Granger JH, Castro-Perez J, Haselden JN, Dear GJ. Use of liquid chromatography/time-of-flight mass spectrometry and multivariate statistical analysis shows promise for the detection of drug metabolites in biological fluids. *Rapid Commun Mass Spectrom* 2003;17:2632–2638. [PubMed: 14648901]
22. Bateman KP, Castro-Perez J, Wrona M, Shockcor JP, Yu K, Oballa R, Nicoll-Griffith DA. MSE with mass defect filtering for in vitro and in vivo metabolite identification. *Rapid Commun Mass Spectrom* 2007;21:1485–1496. [PubMed: 17394128]

23. Plumb RS, Rainville PD, Potts WB 3rd, Castro-Perez JM, Johnson KA, Wilson ID. High-temperature ultra-performance liquid chromatography coupled to hybrid quadrupole time-of-flight mass spectrometry applied to ibuprofen metabolites in human urine. *Rapid Commun Mass Spectrom* 2007;21:4079–4085. [PubMed: 18022959]
24. Sun J, Von Tungeln LS, Hines W, Beger RD. Identification of metabolite profiles of the catechol-O-methyl transferase inhibitor tolcapone in rat urine using LC/MS-based metabolomics analysis. *J Chromatogr B Analyt Technol Biomed Life Sci* 2009;877:2557–2565.
25. Liu A, Patterson AD, Yang Z, Zhang X, Liu W, Qiu F, Sun H, Krausz KW, Idle JR, Gonzalez FJ, Dai R. Fenofibrate metabolism in the cynomolgus monkey using ultraperformance liquid chromatography-quadrupole time-of-flight mass spectrometry-based metabolomics. *Drug Metab Dispos* 2009;37:1157–1163. [PubMed: 19251819]
26. Felton JS, Knize MG, Wu RW, Colvin ME, Hatch FT, Malfatti MA. Mutagenic potency of food-derived heterocyclic amines. *Mutat Res* 2007;616:90–94. [PubMed: 17161439]
27. Cheung C, Ma X, Krausz KW, Kimura S, Feigenbaum L, Dalton TP, Nebert DW, Idle JR, Gonzalez FJ. Differential metabolism of 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) in mice humanized for CYP1A1 and CYP1A2. *Chem Res Toxicol* 2005;18:1471–1478. [PubMed: 16167840]
28. Sinha R. An epidemiologic approach to studying heterocyclic amines. *Mutat Res* 2002;506-507:197–204. [PubMed: 12351159]
29. Chen C, Ma X, Malfatti MA, Krausz KW, Kimura S, Felton JS, Idle JR, Gonzalez FJ. A comprehensive investigation of 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) metabolism in the mouse using a multivariate data analysis approach. *Chem Res Toxicol* 2007;20:531–542. [PubMed: 17279779]
30. Gu D, McNaughton L, Lemaster D, Lake BG, Gooderham NJ, Kadlubar FF, Turesky RJ. A Comprehensive Approach to the Profiling of the Cooked Meat Carcinogens 2-Amino-3,8-dimethylimidazo[4,5-f]quinoxaline, 2-Amino-1-methyl-6-phenylimidazo[4,5-b]pyridine, and Their Metabolites in Human Urine. *Chem Res Toxicol*. 2010
31. Gupta PC, Warnakulasuriya S. Global epidemiology of areca nut usage. *Addict Biol* 2002;7:77–83. [PubMed: 11900626]
32. Winstock A. Areca nut-abuse liability, dependence and public health. *Addict Biol* 2002;7:133–138. [PubMed: 11900633]
33. IARC. Betel-quid and areca-nut chewing and some areca-nut-derived nitrosamines. Lyon: 2004.
34. Giri S, Idle JR, Chen C, Zabriskie TM, Krausz KW, Gonzalez FJ. A metabolomic approach to the metabolism of the areca nut alkaloids arecoline and arecaidine in the mouse. *Chem Res Toxicol* 2006;19:818–827. [PubMed: 16780361]
35. Giri S, Krausz KW, Idle JR, Gonzalez FJ. The metabolomics of (+/-)-arecoline 1-oxide in the mouse and its formation by human flavin-containing monooxygenases. *Biochem Pharmacol* 2007;73:561–573. [PubMed: 17123469]
36. Patterson AD, Lanz C, Gonzalez FJ, Idle JR. The role of mass spectrometry-based metabolomics in medical countermeasures against radiation. *Mass Spectrom Rev*. 2009 published online, doi: 10.1002/mas.20272.
37. Bubenik GA. Thirty four years since the discovery of gastrointestinal melatonin. *J Physiol Pharmacol* 2008;59(Suppl 2):33–51. [PubMed: 18812627]
38. Ma X, Chen C, Krausz KW, Idle JR, Gonzalez FJ. A metabolomic perspective of melatonin metabolism in the mouse. *Endocrinology* 2008;149:1869–1879. [PubMed: 18187545]
39. Ma X, Idle JR, Krausz KW, Tan DX, Ceraulo L, Gonzalez FJ. Urinary metabolites and antioxidant products of exogenous melatonin in the mouse. *J Pineal Res* 2006;40:343–349. [PubMed: 16635022]
40. Viswanathan AN, Schernhammer ES. Circulating melatonin and the risk of breast and endometrial cancer in women. *Cancer Lett* 2009;281:1–7. [PubMed: 19070424]
41. Cardinali DP, Esquifino AI, Srinivasan V, Pandi-Perumal SR. Melatonin and the immune system in aging. *Neuroimmunomodulation* 2008;15:272–278. [PubMed: 19047804]
42. Srinivasan V, Spence DW, Pandi-Perumal SR, Trakht I, Cardinali DP. Therapeutic actions of melatonin in cancer: possible mechanisms. *Integr Cancer Ther* 2008;7:189–203. [PubMed: 18815150]

43. Sakano K, Oikawa S, Hiraku Y, Kawanishi S. Oxidative DNA damage induced by a melatonin metabolite, 6-hydroxymelatonin, via a unique non-o-quinone type of redox cycle. *Biochem Pharmacol* 2004;68:1869–1878. [PubMed: 15450952]
44. Maharaj DS, Maharaj H, Antunes EM, Maree DM, Nyokong T, Glass BD, Daya S. 6-Hydroxymelatonin protects against quinolinic-acid-induced oxidative neurotoxicity in the rat hippocampus. *J Pharm Pharmacol* 2005;57:877–881. [PubMed: 15969947]
45. Maharaj DS, Maharaj H, Daya S, Glass BD. Melatonin and 6-hydroxymelatonin protect against iron-induced neurotoxicity. *J Neurochem* 2006;96:78–81. [PubMed: 16300638]
46. Hirata F, Hayaishi O, Tokuyama T, Seno S. In vitro and in vivo formation of two new metabolites of melatonin. *J Biol Chem* 1974;249:1311–1313. [PubMed: 4814344]
47. Akama T, Shida Y, Sugaya T, Ishida H, Gomi K, Kasai M. Novel 5-aminoflavone derivatives as specific antitumor agents in breast cancer. *J Med Chem* 1996;39:3461–3469. [PubMed: 8784443]
48. Phillips LR, Bramhall C, Buckley J, Wolfe T, House TL, Stinson SF. Identification of the principal circulating metabolite of a synthetic 5,4'-diaminoflavone (NSC 686288), an antitumor agent, in the rat. *J Chromatogr B Biomed Sci Appl* 2000;741:205–211. [PubMed: 10872590]
49. Kuffel MJ, Schroeder JC, Pobst LJ, Naylor S, Reid JM, Kaufmann SH, Ames MM. Activation of the antitumor agent aminoflavone (NSC 686288) is mediated by induction of tumor cell cytochrome P450 1A1/1A2. *Mol Pharmacol* 2002;62:143–153. [PubMed: 12065765]
50. Chen C, Meng L, Ma X, Krausz KW, Pommier Y, Idle JR, Gonzalez FJ. Urinary metabolite profiling reveals CYP1A2-mediated metabolism of NSC686288 (aminoflavone). *J Pharmacol Exp Ther* 2006;318:1330–1342. [PubMed: 16775196]
51. Meng LH, Shankavaram U, Chen C, Agama K, Fu HQ, Gonzalez FJ, Weinstein J, Pommier Y. Activation of aminoflavone (NSC 686288) by a sulfotransferase is required for the antiproliferative effect of the drug and for induction of histone gamma-H2AX. *Cancer Res* 2006;66:9656–9664. [PubMed: 17018623]
52. Meng LH, Meng Z, Miao ZH, Veenstra TD, Pommier Y. Cytokeratin-RNA cross-linking mediated by the antitumor aminoflavone, 5-amino-2,3-fluorophenyl-6,8-difluoro-7-methyl-4H-1-benzopyran-4-one. *J Pharmacol Exp Ther* 2008;325:674–680. [PubMed: 18287208]
53. Wiegand R, Wu J, Sha X, LoRusso P, Heath E, Li J. Validation and implementation of a liquid chromatography/tandem mass spectrometry assay to quantitate aminoflavone (NSC 686288) in human plasma. *J Chromatogr B Analyt Technol Biomed Life Sci* 2009;877:1460–1464.
54. Nicholson JK, Wilson ID. Opinion: understanding 'global' systems biology: metabonomics and the continuum of metabolism. *Nat Rev Drug Discov* 2003;2:668–676. [PubMed: 12904817]
55. Patterson AD, Slanar O, Krausz KW, Li F, Hofer CC, Perlik F, Gonzalez FJ, Idle JR. Human urinary metabolomic profile of PPARalpha induced fatty acid beta-oxidation. *J Proteome Res* 2009;8:4293–4300. [PubMed: 19569716]
56. Frolkis A, Knox C, Lim E, Jewison T, Law V, Hau DD, Liu P, Gautam B, Ly S, Guo AC, Xia J, Liang Y, Shrivastava S, Wishart DS. SMPDB: The Small Molecule Pathway Database. *Nucleic Acids Res* 2010;38:D480–487. [PubMed: 19948758]
57. Lim E, Pon A, Djoumbou Y, Knox C, Shrivastava S, Guo AC, Neveu V, Wishart DS. T3DB: a comprehensively annotated database of common toxins and their targets. *Nucleic Acids Res* 2010;38:D781–786. [PubMed: 19897546]
58. Forsythe IJ, Wishart DS. Exploring human metabolites using the human metabolome database. *Curr Protoc Bioinformatics*. 2009 Chapter 14, Unit14 18.
59. Wishart DS, Knox C, Guo AC, Eisner R, Young N, Gautam B, Hau DD, Psychogios N, Dong E, Bouatra S, Mandal R, Sinelnikov I, Xia J, Jia L, Cruz JA, Lim E, Sobsey CA, Shrivastava S, Huang P, Liu P, Fang L, Peng J, Fradette R, Cheng D, Tzur D, Clements M, Lewis A, De Souza A, Zuniga A, Dawe M, Xiong Y, Clive D, Greiner R, Nazzyrova A, Shaykhtudinov R, Li L, Vogel HJ, Forsythe I. HMDB: a knowledgebase for the human metabolome. *Nucleic Acids Res* 2009;37:D603–610. [PubMed: 18953024]
60. Wishart DS, Tzur D, Knox C, Eisner R, Guo AC, Young N, Cheng D, Jewell K, Arndt D, Sawhney S, Fung C, Nikolai L, Lewis M, Coutouly MA, Forsythe I, Tang P, Shrivastava S, Jeroncio K, Stothard P, Amegbey G, Block D, Hau DD, Wagner J, Miniaci J, Clements M, Gebremedhin M, Guo N, Zhang Y, Duggan GE, Macinnis GD, Weljie AM, Dowlatabadi R, Bamforth F, Clive D, Greiner R, Li L,

- Marrie T, Sykes BD, Vogel HJ, Querengesser L. HMDB: the Human Metabolome Database. *Nucleic Acids Res* 2007;35:D521–526. [PubMed: 17202168]
61. Lawton KA, Berger A, Mitchell M, Milgram KE, Evans AM, Guo L, Hanson RW, Kalhan SC, Ryals JA, Milburn MV. Analysis of the adult human plasma metabolome. *Pharmacogenomics* 2008;9:383–397. [PubMed: 18384253]

Fig. 1.

The multivariate data analysis approach to identifying potential drug metabolites. Beginning with an S-Plot (center), suspected drug metabolites can be identified using: (1) the trend plot feature in SIMCA-P to demonstrate the absence of the suspected drug metabolite in vehicle-treated animals; (2) comparing extracted ion chromatograms for the presence (drug-treated) and absence (vehicle-treated) of the suspected drug metabolite; and (3) tandem MS fragmentography of the suspected drug metabolite and an authentic compound (synthesized or obtained from commercial sources) to confirm the drug metabolite identity. Other methods to identify drug metabolites including isotope analysis may be informative for xenobiotics containing chlorine (isotope ratio = 3:1 assuming a single chlorine), for example.

Fig. 2. The known metabolism of PhIP prior to metabolomic analysis. Nine metabolites were known, six detoxication products (green segment) and three active metabolites that lead to DNA adducts (red segment).

Fig. 3. Metabolomics of PhIP metabolism in the mouse. **Panel A.** Clustering of wild-type, *CYP1A2*-humanized (*hCYP1A2*), *Cyp1a2*-null, and control mouse urines in a three-dimensional scores space. **Panel B.** Distribution of ions in a three-dimensional metabolic space defined by a PCA loadings plot. Numbers represent metabolite IDs. Not all 18 metabolites discovered are visible. The chemical structures of all eight novel PhIP metabolites are shown underneath. Adapted from (29).

Fig. 4. Metabolomics of the metabolism of the areca alkaloids arecoline, arecaidine, and arecoline 1-oxide in the mouse. Panel A (red). The known metabolites prior to metabolomic investigation. Panel B (blue). Novel metabolites of arecoline and arecaidine in the mouse discovered through metabolomics. Panel C (green). Novel metabolites of arecoline 1-oxide in the mouse discovered through metabolomics.

Fig. 5. Metabolomics of arecoline metabolism in the mouse. The mouse urinary metabolome can be clearly seen as a large cloud of ions centered on the 0,0 intersection of the PLS-DA loadings space. The shaded ellipse represents the metabolic space for arecoline, from which both the known and novel metabolites were identified. Adapted from (34).

Fig. 6. Metabolomics of melatonin metabolism in the mouse. The shaded ellipse represents the metabolic space for melatonin defined as that segment of the OPLS loadings S-plot that contains ions that correlated highly (0.85 to 1.0) to the OPLS model. Arrows indicate model fit (Y-axis) and abundance (X-axis). Adapted from (38).

Fig. 7. The metabolic map for melatonin in the mouse showing six of the seven novel metabolites (blue segments). The seventh novel metabolite was a glucuronide of dihydroxymelatonin (6) whose exact structure was not determined. Adapted from (38).

Fig. 8. Metabolomics of aminoflavone metabolism in the mouse. **Panel A.** Clustering of the control and aminoflavone-treated mice in a PCA scores plot. **Panel B.** Visualization of the aminoflavone metabolic space (red circle) in a PCA loadings plot. Note that the mouse urinary metabolome is clearly shown as a large cloud of ions centered on the 0,0 intersection of the PCA loadings space. Adapted from (13).

Fig. 9.

Metabolomics of aminoflavone metabolism in the mouse. Only one *in vivo* metabolite of aminoflavone had been reported, *N*^{4'}-acetyl-aminoflavone in rat plasma (red segment). This metabolite was not found in this study. However, a large number of novel metabolites of aminoflavone were discovered in the mouse, including hydroxylation at the *N*⁵-, *N*^{4'}-, and *C*⁵ positions, together with various glucuronide and sulfate conjugates.

Fig. 10.

Xenobiotic (and gut microbiota) metabolism including that from food, pesticides, cosmetics, pollutants, and drugs can add, in some cases, substantially, both in number and abundance, to the metabolome.

Table 1

Popular Chemometric Approaches to Xenobiotic Metabolism

	Description
Principal Components Analysis (PCA)	PCA is a typical first-pass visual approach to extract information from high-dimensional metabolomic datasets. The first principal component represents the maximal variation in the dataset. Each subsequent principal component explains the remaining variation. PCA is unsupervised, that is, no class information is provided. Sample grouping and outliers are displayed in the scores while the variables influencing the observed groupings are displayed in the loadings.
Projection to Latent Structures Discriminant Analysis (PLS-DA)	PLS-DA models are created by rotating the PCA model to maximize class separation. Thus, PLS-DA models are supervised since information regarding class membership (e.g., vehicle and treated) is provided by the user. Like PCA, information is displayed in the scores and loadings. Additional steps may be required to validate the models such as cross-validation.
Orthogonal Projection to Latent Structures (OPLS)	OPLS is intended to simplify data interpretation by concentrating related variation into the first component while placing unrelated (orthogonal) variation into the second component. Like PLS-DA, OPLS models are supervised and may require additional validation steps to assist interpretation.