Applications of Metabonomics in Pesticide Toxicology

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Abstract: Metabonomic studies quantitatively measure the small molecule metabolites and their intermediates in the biological samples (serum, urine or tissue extracts) and have gained wide applications in many fields, especially in toxicology. Pesticides are extensively used around the world and pesticide toxicity has become a serious threat to human health. Metabonomic approach has been applied in many aspects of pesticide toxicology research such as eco-environmental toxicity studies, biomarker identification, and mechanism of toxicity studies. Both whole organism animal models and cell culture models are used for metabonomic studies on pesticide toxicology. In the literature, metabonomic analyses on the toxicity of over thirty common pesticides, including insecticides, herbicides and fungicides, have been carried out using magnetic resonance spectroscopy or mass spectrometry. The combined toxicity of pesticides or pesticide with heavy metals was also investigated with metabonomic approach. In this article, recent progresses made in applying metabonomic approach in pesticide toxicology are thoroughly reviewed and the challenges with application of this approach are also discussed.

Keywords: Biomarker, environmental toxicity, metabonomics, pesticide, risk assessment, toxicology.

1. BRIEF INTRODUCTION TO METABONOMICS AND ITS APPLICATION IN TOXICOLOGY

Metabonomics quantitatively measures the dynamic change in low-molecular-weight metabolites and their intermediates in the living organisms as response to external stimuli or genetic modification. It is also noticed in some literature that there is another similar term “Metabolomics”. In this review, the term "metabonomics" is employed. The major tools used in metabonomics include nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS) coupled with the chemometric approach for the multivariate statistical analysis on the metabolites data. Several review articles have discussed the recent advances in the analytical methods [1, 2] and the chemometric approaches [3-5] used in the metabonomic studies.

With metabonomics, a large number of metabolites can be simultaneously monitored, which provides a detailed phenotype of a particular chemical’s effect on a living organism [6]. Metabonomic approaches have been proved highly useful in pharmacology and toxicology as complementary to the traditional approaches [7-12], especially in toxicology [13-17]. The Consortium for Metabonomic Toxicology is the milestone international collaboration for toxicological studies [18, 19]. A major use of metabonomics in toxicology is to identify patterns of metabolite changes and biomarkers as signatures of toxicity, which can be used to predict hazard manifestation. This new approach has also been applied in the ecological risk assessment of pesticides and other hazardous chemicals used in household or industry [20].

2. APPLICATION OF METABONOMICS IN PESTICIDE TOXICOLOGY-OVERVIEW

Pesticides are a class of biocides used to incapacitate, kill or mitigate any pest, especially in the agriculture practice. Pesticides can be classified by target organisms such as herbicides, insecticides and fungicides, or grouped as chemical families such as organophosphorus compounds, carbamates, and organochlorines. Despite the profound benefit of pesticides use in agriculture, pesticides may cause acute and delayed health effects. It was estimated by WHO that 3 million people are hospitalized by pesticide poisoning each year [21]. In addition, pesticides may also persist in the environment and cause serious pollution in air, water, soil and food [22]. Thus, the toxicological study on pesticides has attracted much attention with about three hundred publications each year. In the recent two to three years, metabonomics have been used extensively in the pesticide toxicological study. This review aims to summarize the recent progress in the pesticide toxicology using metabonomics methodologies.

2.1. Areas of Pesticide Toxicological Studies Using Metabonomic Approach

Metabonomics is able to systematically detect the dynamic physiological changes upon exposure to toxicants. Since the subtle metabolic changes often precede the biochemical changes induced by xenobiotics, metabonomics can detect the exposure to hazardous chemicals at an earlier stage. Generally speaking, applications of metabonomics in toxicological study include safety screening, biomarker identification and study on mechanism of toxicity [16]. The major applications of metabonomics in pesticide toxicology fall into the following three categories.
2.1.1. Environmental and Eco-Toxicological Study

A large number of organic pesticides can persist in the environment for a long time and cause harm to aquatic and soil animals. Metabonomic approach can help us better understand the toxicity of chronic exposure to environmentally relevant doses of pesticides (reviewed in [23]). For example, a study determined metabonomics of male roach (Rutilus rutilus) exposed to fenitrothion at environmentally realistic concentration (2 μg/L in water) for 28 days [24]. Results showed significant alterations in the hepatic phosphagen system and phenylalanine metabolism in liver and testes, which were proposed to be the off-target toxicities [24]. In addition, metabonomics is a promising tool for eco-toxicological studies to detect pesticide residues in both water and soil using earthworms and other sentinel animals [25-34].

2.1.2. Biomarker Identification

It has been well accepted that metabonomics could be used to identify novel metabolite biomarkers for exposure to various toxic compounds [15]. A number of pesticide toxicological studies have proposed different set of biomarkers by using metabonomic analyses [24, 30, 35-40]. Generally speaking, biomarkers may serve as “biological indicator” for chemical’s exposure, effect, and disease susceptibility [41]. Thus, the proposed toxicity biomarkers can be applied in the molecular mode of action identification as well as the dose-response analysis and exposure quantification in risk assessment. For example, nicotine metabolite cotinine has been successfully applied to quantitatively estimate the smoking status in epidemiological studies [42]. Another example is that biomarkers in the heme biosynthetic pathway were successfully used in risk assessment practice to predict toxic outcomes from lead exposure and develop lowest observable adverse effect levels (LOAELs) for exposure to lead [43].

2.1.3. Mechanism of Toxicity

Metabonomics is powerful for dissecting the detailed mechanisms of toxicity [44]. Changes in various metabolic pathways identified by metabonomics could shed light on the mechanism of action study of xenobiotics [15, 45, 46]. Pesticides have a wide variety of toxicity, such as neurotoxicity, hepatotoxicity, nephrotoxicity, etc. Some metabolic pathways, such as aromatic metabolism [47-49] and fatty acid metabolism [40, 50], were found to be involved in the pesticide toxicity as revealed by metabonomic analyses.

2.2. Model Systems of Pesticide Toxicological Studies Using Metabonomic Approach

Both *in vivo* and *in vitro* approaches have been used in the pesticide safety assessment and toxicity testing.

2.2.1. Whole Animal Toxicology

Currently, experimental animal models are the major model systems in toxicological studies [9, 51]. In majority of pesticide toxicity studies, rats were used as animal model to represent mammals [35-40, 48, 52-56]. In some environmental eco-toxicological studies, different fish species such as rainbow trout and salmon were used to study the pesticide toxicity to aquatic animals [26, 28, 29, 32, 57, 58]. Earthworms were frequently used to study the pesticide toxicity to soil invertebrates [25, 27, 30, 31, 33, 34]. Since pesticides are often used on agricultural crops and vegetables, plants such as tomato were also used in pesticide toxicity study [59-62].

2.2.2. Cell Culture Toxicology

Cell models such as immortalized cell lines or primary culture cells are used more and more often in the safety assessment and toxicological test [63]. Metabonomics can be used for evaluating *in vitro* toxicity in cells [64, 65]. Cell lines have advantages over the whole organisms, as cells are more economic to maintain and there are less variables during the experiment and data interpretation. In addition, metabonomics data can be directly correlated with genomics and proteomics data [63]. The use of human cells can provide information about human toxicity. In addition, variations due to inter-species extrapolation can be minimized. The challenges to the *in vitro* metabonomics are differences in growth conditions such as variations in growth medium composition and passage numbers of the culture, as well as difficulties in obtaining sufficient cultured medium or cells for metabonomic analysis. Several studies have used *ex vivo* insect tissue culture [66] to detect the metabolic changes induced by pesticide exposure.

2.2.3. Epidemiological Studies

Metabonomics has been used to identify exposure biomarkers and modified metabolic pathways in human populations in order to relate the environmental exposure to the etiology of diseases (reviewed in [67]). NMR-based metabonomics were carried out with urine samples of eighty-three pregnant women working on farmland, which had the potential to be exposed to various pesticides. Glycine, lactate, threonine, and glycerophosphocholine were induced and citrate level was decreased, which suggested disturbance in energy metabolism and induction of oxidative stress [68].

Next we will introduce the progress of metabonomic applications in pesticide toxicological studies according to the classification of pesticides as their target organisms.

3. APPLICATIONS OF METABONOMICS IN THE INSECTICIDE TOXICOLOGY

Agricultural insects are a major class of pest insects. Thus insecticides use contributes greatly to the increase of agricultural productivity. In 2007, insecticides accounted for the second largest portion (about 30%) of total expenditures on pesticides around the world [69]. Recent progresses on application of metabonomics in the insecticide toxicological studies are summarized in Table 1.

3.1. Organophosphorus Pesticides and Carbamates

Organophosphorus compounds and carbamates are important groups of pesticides, which inhibit acetylcholinesterase and disrupt synaptic neurotransmission in insects, humans, and many other animals. These pesticides have high acute toxicity due to the parasympathetic over-stimulation.

Dichlorvos (DDVP) is a common organophosphorus insecticide used in agriculture. Changes in the rat urine metabolic profiles upon
### Table 1. Summary of metabonomic studies on insecticides.

<table>
<thead>
<tr>
<th>Class</th>
<th>Pesticide</th>
<th>Model organism</th>
<th>Analysis</th>
<th>Biomarkers identified</th>
<th>Altered biological pathways</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Organophosphorus compounds</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Dichlorvos</td>
<td>Rat (urine)</td>
<td>UPLC-MS</td>
<td>Dimethyl phosphate</td>
<td>Carbohydrate and fatty acid metabolism, the antioxidant system</td>
<td>[40]</td>
</tr>
<tr>
<td></td>
<td>Dichlorvos</td>
<td>Rat (plasma)</td>
<td>UPLC-MS</td>
<td>Lysophosphocholine</td>
<td>Fatty acid metabolism</td>
<td>[56]</td>
</tr>
<tr>
<td></td>
<td>Malathion</td>
<td>Japanese medaka juveniles</td>
<td>GC-MS</td>
<td></td>
<td>Gluconeogenesis protein synthesis and degradation</td>
<td>[32]</td>
</tr>
<tr>
<td></td>
<td>Diazinon</td>
<td>Salmon embryos and alevins</td>
<td>NMR</td>
<td>Phosphocreatine</td>
<td></td>
<td>[58]</td>
</tr>
<tr>
<td></td>
<td>Fenitrothion</td>
<td>Roaches</td>
<td>NMR, Direct Infusion MS</td>
<td>Acetocholine, 11-ketotestosteron, cortisone, creatine, phosphocreatine, N-acetylphenylalanine</td>
<td>Hepatic phosphagen system, phenylalanine metabolism</td>
<td>[24]</td>
</tr>
<tr>
<td></td>
<td>Dimethoate</td>
<td>Rat (urine and plasma)</td>
<td>UPLC-MS</td>
<td>Dimethylthiophosphate (DMTP), dimethyldithiophosphate (DMDTP), Lipid metabolism, glucose, fatty acids, amino acids, and collagen metabolism</td>
<td></td>
<td>[36]</td>
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<tr>
<td></td>
<td>Acephate</td>
<td>Rat (urine)</td>
<td>UPLC-MS</td>
<td>DMTP Glucose, nucleic acid, and protein metabolism</td>
<td></td>
<td>[37]</td>
</tr>
<tr>
<td></td>
<td>Chlorpyrifos</td>
<td>Earthworm</td>
<td>NMR</td>
<td></td>
<td></td>
<td>[30]</td>
</tr>
<tr>
<td><strong>Carbamates</strong></td>
<td>Carbaryl</td>
<td>Earthworm</td>
<td>NMR</td>
<td>Tyrosine</td>
<td>Amino acid metabolism</td>
<td>[30]</td>
</tr>
<tr>
<td></td>
<td>Carbofuran</td>
<td>Earthworm</td>
<td>GC-MS</td>
<td></td>
<td>Amino acid and carbohydrate metabolism</td>
<td>[31]</td>
</tr>
<tr>
<td></td>
<td>Carbofuran</td>
<td>Tomato (fruits)</td>
<td>LC-MS</td>
<td></td>
<td></td>
<td>[61, 62]</td>
</tr>
<tr>
<td></td>
<td>Propoxur</td>
<td>Rat (serum and urine)</td>
<td>NMR</td>
<td>Oxidative stress, impair liver function, enhance ketogenesis and fatty acid β-oxidation, and increase glycolysis</td>
<td></td>
<td>[54]</td>
</tr>
<tr>
<td><strong>Pyrethroids</strong></td>
<td>Fenvalerate</td>
<td>Daphnia magna</td>
<td>FT-ICR MS</td>
<td></td>
<td>Amino sugar metabolism</td>
<td>[50]</td>
</tr>
<tr>
<td></td>
<td>Lambda-cyhalothrin</td>
<td>Goldfish</td>
<td>NMR</td>
<td></td>
<td>Neurotransmitters and osmoregulators metabolism, oxidative stress, metabolisms of energy and amino acids</td>
<td>[29]</td>
</tr>
<tr>
<td><strong>Organochlorine</strong></td>
<td>Endosulfan</td>
<td>Earthworm (coelomic fluid and tissue)</td>
<td>NMR</td>
<td></td>
<td>Glutamate metabolism, apoptosis</td>
<td>[33, 34]</td>
</tr>
<tr>
<td></td>
<td>Endosulfan</td>
<td>Mice (plasma, liver)</td>
<td>NMR</td>
<td>Glucose, lactate</td>
<td>Energy metabolism, choline metabolism, oxidative stress</td>
<td>[70]</td>
</tr>
<tr>
<td></td>
<td>Methoxychlor</td>
<td>Rat (urine)</td>
<td>NMR</td>
<td>Acetate, alanine, benzo-ate, lactate, glycine</td>
<td>Disruption of endocrine system</td>
<td>[39]</td>
</tr>
<tr>
<td></td>
<td>Pentachlorophenol</td>
<td>Marine mussels</td>
<td>NMR</td>
<td>Allantoine, alanine, valine</td>
<td>Purine metabolism, amino acid metabolism</td>
<td>[71]</td>
</tr>
<tr>
<td><strong>Natural compounds</strong></td>
<td>Rotenone</td>
<td>Myotube cells</td>
<td>NMR</td>
<td></td>
<td>Tricarboxylic acid cycle</td>
<td>[72]</td>
</tr>
</tbody>
</table>
chronic exposure to low dose DDVP (2.4, 7.2, and 21.6 mg/kg body weight (bw) /d given in drinking water over 24 weeks) were studied by ultra performance liquid chromatography (UPLC)-MS [40]. Dimethyl phosphate (DMP) was identified as a sensitive biomarker of exposure. In addition, an increase in estrone sulfate, lactobionic acid, indoxyl sulfic and a decrease in citric acid, creatinine, gulonic acid, uric acid and urea were observed, which suggested the disturbance in carbohydrate and fatty acid metabolism, and imbalance of the antioxidant system induced by chronic exposure to low level DDVP [40]. UPLC-MS was also used to assess the metabolic profile changes of rat plasma upon chronic low dose exposure to DDVP with the same dose and treatment [56]. Some lipids level decreased in plasma such as LysoPC, which indicated liver damage [56].

Malathion toxicity to Japanese medaka juveniles was studied by using a gas chromatography (GC)-MS-based metabonomic approach. The levels of almost all the amino acids increased in treated group, suggesting that the proteolysis occurred in the treated group (20 and 2,000 μg/L in water for 96 hours). Thus, malathion exposure may have altered the balance between protein synthesis and degradation and induced gluconeogenesis in medaka [32]. Salmon embryos and alevins exposed to diazinon had significant metabolic changes revealed by NMR spectroscopy. Phosphocreatine was found to decrease significantly in eyed eggs treated with diazinon (100 ppb for 96 hours) [58]. NMR- and MS-based metabolomics were used to detect the off-target toxicity of fenitrothion to roaches (2, 20, and 200 μg/L for 28 days). The metabolomic results showed that the hepatic phosphagen system and phenylalanine metabolism were altered [24]. Metabonomic analysis by UPLC-MS on urine and plasma of rats chronically administered with low dose dimethoate (0.04, 0.12, and 0.36 mg/kg bw/d given through drinking water for 24 weeks) showed changes in citric acid, uric acid, suberic acid, isovalerylglutamic acid, L-tyrosine, glycylyproline, allantoin, dimethylthiophosphate (DMTP) and dimethylthiophosphate (DMDTP). DMTP and DMDTP were identified as biomarkers for dimethoate exposure [36]. The nephrotoxicity of chronic exposure to low dose acephate (0.5, 1.5, and 4.5 mg/kg bw/d for 24 weeks) in Wistar rats was studied using UPLC-MS. DMTP was identified as biomarker for acephate exposure [37]. NMR-based metabonomics was used to study the metabolic changes in earthworms (Eisenia fetida) after exposure to carbaryl (a carbamate pesticide, 10 μg/cm² on filter paper for 48 hours) and chlorpyrifos (an organophosphorus pesticide, 2.7 μg/cm² on filter paper for 48 hours). Different sets of biomarkers were identified for carbaryl and chlorpyrifos, which indicated that metabolomics could distinguish the responses resulted from exposure to pesticides with different toxic modes of action [30].

Carbofuran is one of the most commonly used and most toxic carbamate pesticides in agriculture. To assess its soil ecotoxicity, tissue specific metabonomic changes of earthworm after carbofuran exposure (0.15, 0.3, and 0.6 mg/kg in soil) were identified by GC-MS and seventeen metabolites were identified [31]. Metabonomic analysis in tomato fruits (cultivar Rambo) exposed to carbofuran (20% carbofuran was applied at 4 L/ha in irrigating water for 21 days) was carried out using LC-MS. Pesticide treatment caused changes in a number of endogenous tomato metabolites as a response to physiological stress [61, 62]. NMR spectroscopy in rats showed that exposure to low dose propoxur (0.85, 1.70, and 8.51 mg/kg bw/d for 28 consecutive days) led to induced oxidative stress, enhanced fatty acid β-oxidation and ketogenesis, and increased glycolysis in urine, which contributed to its hepatotoxicity in rats [54].

3.2. Pyrethroids

Pyrethroids were developed by synthetic modification of pyrethin, which is a naturally occurring pesticide found in chrysanthemums. They are widely used as agricultural and household insecticides with deltamethrin as the most popular pyrethroid.

Metabolic profiling of Daphnia magna after fenvalerate exposure (0.6 μg/L in culture medium for 24 hours) was assessed using direct infusion Fourier transform ion cyclotron resonance (FT-ICR)-MS-based metabolomics. The results showed that amino sugar metabolism was disrupted by fenvalerate treatment [50]. NMR-based metabolomic approach was applied to investigate the toxicity of lambda-cyhalothrin (LCT, 0.012 μg/L in water for 7 days) in goldfish (Carassius auratus). LCT exposure affected levels of many metabolites (e.g., leucine, isoleucine and valine in brain and kidney; lactate in brain, heart and kidney), and broke the balance of neurotransmitters and osmoregulators, evoked oxidative stress, disturbed energy and amino acids metabolism [29].

3.3. Organochlorine

Organochlorine pesticides are largely banned in North America and Europe, but are used extensively in many developing nations. These chemicals are difficult to degrade and could persist in the environment for a long time.

Toxic mode of action (MOA) of endosulfan (0.1, 1.0, and 10.0 mg/kg in soil for 7 days) to earthworm (Eisenia fetida) was studied by NMR-based metabolomics. Significant changes in glutamine/γ-aminobutyric acid (GABA)-glutamate metabolites and spermidine were detected in coelomic fluid (CF), which suggested that MOA was mainly mediated by glutamate neurotoxicity and apoptosis [34]. Upon endosulfan exposure (1.0 μg/cm² and 2.0 μg/cm² on filter paper for 48 hours), malate, alanine, glycine, myo-inositol, α-ketoglutarate, lactate, succinate, betaine, and spermidine in the earthworm CF and alanine, glutamate, glutamine, malose, fumarate, lactate, and melibiose in earthworm tissue extract were significantly altered and detected by NMR-based metabolomics [33]. An NMR-based metabonomic approach was used to study the metabolic changes induced by dietary exposure to low dose endosulfan (30 μg/kg of food for 14 weeks) in mice. Metabolites with significant changes suggested induction of oxidative stress in liver, which was consistent with the prooxidant activities of endosulfan. Some gender specific metabolic changes were also identified [70].

Methoxychlor is an endocrine disruptor with estrogenic and anti-androgenic activities. NMR spectroscopy study in female rats identified potential biomarkers for methoxychlor exposure (50, 100, or 200 mg/kg bw/d, orally or subcutaneously for 3 days): benzoate, acetate, lactate, alanine, and glycine [39]. Marine mussels (Mytilus edulis) were treated with pentachlorophenol (50 μg/L and 350 μg/L water for 7 days) and metabolic profiles were identified using
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3.4. Natural Compounds

Rotenone is a naturally occurring insecticide in jicama vine plant. NMR-based metabolomic analysis on in vitro myotube cells treated with rotenone (0.1 μM in cell culture medium for 8 hours) revealed changes in metabolites involved in tricarboxylic acid (TCA) cycle, providing a detailed mechanism of rotenone’s inhibition on mitochondrial function [72]. Isolates of fungi in genus Metarhizium and Beauveria are used as biological pesticides. Metabolomic analysis using HPLC-MS/MS on ex vivo cultured insect tissue exposed to these two fungi showed significantly different sets of secondary metabolites secreted by these two fungi during different stages of growth [66].

4. APPLICATIONS OF METABONOMICS IN THE HERBICIDE TOXICOLOGY

Herbicides are widely used in agriculture and landscape turf management. In 2007, herbicides accounted for the largest portion (about 40%) of total expenditures on pesticides around the world with glyphosate and atrazine being the most popular herbicides [69].

NMR fingerprinting was used to study metabolic changes in common duckweed (Lemma minor) after treatment by each of the four herbicides glyphosate, mesotrione, norflurazon and paraquat (296, 152, 111, and 0.19 μM, respectively, in culture medium for 72 hours). Macroscopical signs of phytotoxicity were not observed due to the low dose of herbicides used. However, NMR spectroscopy was able to detect prominent changes of metabolites [59]. Rapeseed seedlings were exposed to low doses of glyphosate (1.0, 5.0, 10, 20, 30, and 50 μM in nutrient solution for 9 days) and the metabolites in the plant crudes were studied with UPLC-Diode-Array-Detection. Plants treated with as low as 5 μM glycosate had distinct metabolic profiles compared to untreated plants [60].

Atrazine is among the most widely distributed contaminants in the U.S. Hyallela azteca was exposed to atrazine (30 μg/L in water for 42 days) and its metabolites were detected by 2D GC/LC-time of flight (TOF) MS. By-products of β-oxidation of fatty acids were the major metabolites detected, which suggested that energy metabolism was disrupted. Eicosanoids levels were induced in treated females, which suggested that neuropeptide system may be disturbed [48]. In Arabidopsis thaliana, metabolomics, combined with transcriptomics, identified tyrosine amino-transferase as the target for cinmethylin exposure [73].

A study was conducted using Japanese medaka (Oryzias latipes) to investigate the effect of dinoseb (50 ppb and 75 ppb for 110 hours), a substituted dinitrophenol herbicide, on the development of fish embryos [57]. Levels of alanine, ATP and tyrosine were decreased, and lactate level was induced after dinoseb treatment in medaka embryo as determined by NMR. In addition, dose-dependent metabolic changes were detected in the embryos and alevins of Chinook salmon (Oncorhynchus tshawytscha) upon exposure to pesticides dinoseb (250 ppb for 96 hours) by NMR spectroscopy [58]. Metabolic profiling of Daphnia magna exposed to dinitrophenol (1.5 mg/L in culture medium for 24 hours) was studied using FT-ICR MS metabolomics. Metabolic changes in whole organism homogenates were found to be more profound compared to those in hemolymph [50].

5. APPLICATIONS OF METABONOMICS IN THE FUNGI CIDE TOXICOLOGY

Fungicides are widely used to treat fungus infection both in agriculture and in animals. In 2007, fungicides accounted for the third largest portion (about 20%) of total expenditures on pesticides around the world [69].

NMR metabonomics studies showed that different triadimefon enantiomers (via oral gavage at 144 or 720 mg/kg bw) induced different metabolic changes in rainbow trout liver [28]. Raman spectroscopy was found to better differentiate metabolic changes in rat urine upon exposure to each of the three triazole fungicides, myclobutanil, propiconazole, and triadimefon (daily via oral gavage at doses of 300, 300, and 175 mg/kg bw/d, respectively, for 5 days by oral gavage), compared to NMR spectroscopy [52].

Vinclozolin, a dicarboximide fungicide, has anti-androgenic effects. A metabolic study with GC-MS and LC-MS/MS showed that the metabolite profile of rats after vinclozolin exposure (1000 ppm and 3000 ppm in diet for 28 days) was similar to androgen receptor antagonists such as flutamide [55]. This result suggested that future screening for endocrine disrupters could be improved by making use of metabolomics. Male Fischer F344 rats were exposed to each of the two benzimidazole fungicides (carbendazim and thiabendazole) and two bipyridylidium herbicides (chloromequat and mepiquat) at four different doses for each pesticides (0.5, 100, 375, and 750 mg/kg bw for carbendazim; 0.1, 40, 120, and 300 mg/kg bw for thiabendazole; 0.05, 50, 75, and 100 mg/kg bw for chlormequat; 0.6, 50, 75, and 150 mg/kg bw for mepiquat with one single dose by oral gavage). There were no obvious toxic signs such as changes in growth and organ weights. However, metabolic changes were detected by NMR spectroscopy in 24 h after exposure [53]. In lettuce (Lactuca sativa L.) leaves, exposure to mancozeb (sprayed with 2 mg/L mancozeb for 7 days) induced metabolic adaptions in TCA cycle and anti-oxidant defense mechanisms. Changes in phenylalanine, dehydroascorbate, tartrate and formate were observed by NMR-based metabonomics. Mature leaves appeared to be more extensively affected by exposure to mancozeb than younger leaves [74].

Urirines from seven workers in Italy vineyard with dermal exposure to tebuconazole (TEB) (with total dermal exposure 1950 μg) were analyzed by LC/triple quadrupole MS. TEB-OH was identified to be the major metabolite. TEB-OH and TEB-COOH were found to be potential exposure biomarkers for TEB exposure in human urine [75].

6. APPLICATIONS OF METABONOMICS IN STUDYING COMBINED TOXICITY OF MULTIPLE PESTICIDES

In the classical experimental studies for pesticide toxicity, only individual pesticide was administrated to animals each time. Actually, multiple pesticides are accumulated in the environment after long-term use due to their high stability. In addition, when using chemical pesticides for crop protection, farmers usually use a mix-
nature of two or more pesticides to apply on the plant. In order to better understand the actions of multiple pesticides, applications of metabonomics have been carried out with both earthworms and rodents as animal models.

Earthworms (Lumbricus rubellus) were exposed to the mixtures of imidacloprid/thiacloprid (0.365, 0.73, and 1.095 mg/kg in soil for imidacloprid and 0.32, 0.64, and 0.96 mg/kg in soil for thiacloprid) and chlorpyrifos/nicel (4.73 and 8.07 mg/kg in soil for chlorpyrifos). NMR and GC-MS analysis showed that metabolic changes induced by equitoxic combination of imidacloprid and thiacloprid were intermediate between those induced by each pesticide alone, which indicated that these two pesticides had an independent joint interaction. Metabolic changes induced by chlorpyrifos and nicel were distinct, which suggested that they had different modes of action [25]. Nematode Caenorhabditis elegans was exposed to the mixture of nickel and chlorpyrifos. NMR spectroscopy and GC-MS identified metabolic changes in TCA cycle intermediates and branch chain amino acids [27].

NMR spectroscopy-based metabolomic analysis showed that exposure to either deltamethrin (1.02 and 6.40 mg/kg bw/d for 60 consecutive days) or permethrin (12 and 75 mg/kg bw/d for 60 consecutive days) and their mixtures induced elevated levels of dimethylglycine, dimethylamine, acetate, trimethylamine in urine and free amino acids in serum, and decreased 2-oxoglutarate in urine, all of which indicated nephrotoxicity [47]. The energy metabolism was disturbed as TCA cycle intermediates were reduced. 3-D-hydroxybutyrate, acetate, and lactate were induced in treated rats, which suggested that anaerobic glycolysis, ketogenesis, and fatty acid β-oxidation were induced [47].

Metabolomic analysis using NMR spectroscopy revealed that an increase in acetate, formate and alanine in urine could serve as a potential biomarker for the combined chronic exposure of propoxur (0.68, 1.70, and 4.25 mg/kg bw/d for 90 days by oral gavage) and permethrin (12, 30, and 75 mg/kg bw/d for 90 days by oral gavage) [38]. Metabolomic analysis using NMR spectroscopy indicated that exposure to a mixture of dichlorvos (0.64, 1.60, and 4.00 mg/kg bw/d for 90 days by oral gavage) and deltamethrin (1.02, 2.56, and 6.40 mg/kg bw/d for 90 days by oral gavage) resulted in the increases of urinary dimethylamine, lactate, glycine and N-glycoprotein in rats, suggesting the disruption of liver energy metabolism and kidney function. More prominent changes in serum trimethylamine-N-oxide, choline, alanine and acetone were found in mixture treatment group compared to either dichlorvos or deltamethrin [49].

Metabolic changes in rats induced by chronic exposure to the mixture of four organophosphorus pesticides acephate (0.5, 1.5, and 4.5 mg/kg bw/d in drinking water for 24 weeks), dichlorvos (2.4, 7.2, and 21.6 mg/kg bw/d in drinking water for 24 weeks), dimethoate (0.04, 0.12, and 0.36 mg/kg bw/d in drinking water for 24 weeks), and phorate (0.05, 0.15, and 0.45 mg/kg/d in drinking water for 24 weeks) were studied by UPLC-MS. These pesticides interfered with the TCA cycle, disturbed the lipid metabolism and induced DNA damage and oxidative stress [35].

NMR-based metabolomic approach was performed to study the effect of the mixture of alachlor (0.01 μg), captan (2 μg), diazinon (0.004 μg), endosulfan (0.12 μg), mancozeb (1 μg), and maneb (1 μg) administered to mice by oral gavage three times per week for four weeks. Metabolites involved in regulation of glucose metabolism and oxidative stress in liver were significantly changed [76]. The same pesticides both alone or in combination were used to treat female mice throughout gestation and lactation. NMR-based metabolomic approach showed that glucose, choline, lactate, phosphocholine and glycerophosphocholine levels changed in plasma of adult offspring [77].

A low-dose mixture of 8 pesticides representing the main pesticides used in cereal crops in Brittany (France) in 2004 (acetochlor, bromoxynil, carbofuran, chlormequat, ethephon, fenpropimorph, glyphosate, and imidacloprid) was administered to pregnant rats from early pregnancy to delivery. 1H NMR-based metabolomics analyses identified changed metabolites in liver and brain of the dams and offspring (lipids, ketone bodies, ATP, ADP/AMP, alanine, glutamate, aspartate, N-acetylaspartate, and other amino-acids, glucose, glycogen, glycerol, taurine, and glycerophosphocholine), which suggest that the pesticide mixture induces an oxidative stress and impairment of the glucose metabolism in both the mother and the offspring [78].

NMR-based metabolomics have been successfully applied to epidemiological studies [67]. This is especially relevant in the metabolomics on mixture of pesticides due to the fact that people are often exposed to a wide variety of pesticides. One study analyzed urine samples of eighty-three pregnant women working on a farmland, which may be exposed to a variety of pesticides. Glycine, lactate, threonine, and glycerophosphocholine were elevated and citrate level was decreased, which suggested disturbance in energy metabolism and induction of oxidative stress [68].

7. DISCUSSIONS AND CONCLUDING REMARKS

All of these reviewed studies supported the idea that metabolomics was able to quantitatively measure the dynamic changes in metabolites after pesticide exposure, which may be applied in environmental and ecotoxicological studies to estimate the exposure and help elucidate the toxic mode of action for pesticides. However, it is to be noted that the changes in metabolites may not be directly linked to the specific toxic effect due to the fact that adaptive response may also cause changes in metabolites [68]. Use of different doses in the animal studies may help to determine whether the changes in metabolites reflect the early toxic effects.

Although there has been much progress in the field of metabolomics, its application in pesticide toxicological laboratories is still limited, partly due to the technical difficulty. Like other omics approach, metabolomic analysis detects levels of hundreds or even thousands of metabolites. To annotate and analyze this huge amount of data remains a challenge. Softwares have been developed, including those that integrate metabolomics with transcriptomics, but they are far from user friendly [79]. The whole metabolomics protocol, including sample preparation, detection and data processing methods, need to be standardized to generate repeatable and effective data, especially for regulatory purposes.

Challenges remain to be tackled for applications of metabolomics in pesticide toxicity studies. The first issue is biomarker validation [23]. As we can see from Table 1, some of the biomarkers such
as DMTP and lactate were identified in different studies exposed to different pesticides. Thus, often a biomarker panel with multiple metabolites is desirable in order to better discriminate the different pesticides. Comparing with different databases of biomarkers identified from NMR and MS-based metabonomics is also necessary [67]. In addition, a detailed dose response analysis on biomarkers needs to be carried out in order to apply the biomarkers for quantitative estimate of exposure and ultimately the toxicity risk assessment.

Another important issue is that studies directly target human populations, such as clinical studies and epidemiological studies on pesticides are scarce. The potential for the application of metabonomics in environmental epidemiology and toxicology needs to be further exploited [67]. In addition, human cell culture can be used as a promising alternative for animal experiment. Recently, the human-on-a-chip technology was developed using multiple cell culture chambers to study multi-organ interactions [80]. The approach has been applied in metabonomics using co-cultured liver and kidney cells to study organ specific toxicity [81]. As the technology advances, we should be able to further exploit the potential applications of metabonomics in the field of pesticide toxicology.

ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>CF</td>
<td>Coelomic fluid</td>
</tr>
<tr>
<td>DDVP</td>
<td>Dichlorvos</td>
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<tr>
<td>DMP</td>
<td>Dimethyl phosphate</td>
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<tr>
<td>DMTP</td>
<td>Dimethylthiophosphate</td>
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<tr>
<td>DMDTP</td>
<td>Dimethyldithiophosphate</td>
</tr>
<tr>
<td>FT-ICR</td>
<td>Fourier transform ion cyclotron resonance</td>
</tr>
<tr>
<td>GC</td>
<td>Gas chromatography</td>
</tr>
<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
</tr>
<tr>
<td>LCT</td>
<td>Lambda-cyhalothrin</td>
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<tr>
<td>MOA</td>
<td>Mode of action</td>
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<tr>
<td>MS</td>
<td>Mass spectrometry</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance spectroscopy</td>
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<tr>
<td>TOF</td>
<td>Time of flight</td>
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<tr>
<td>TEB</td>
<td>Tebuconazole</td>
</tr>
<tr>
<td>UPLC</td>
<td>Ultra performance liquid chromatography</td>
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CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

ACKNOWLEDGEMENTS

The authors’ work is currently supported partly by grants from National Natural Science Foundation of China (Nos. 31272365, 31301927, 31472007), National Basic Research Program of China (No. 2012CB114103), and CAS Strategic Priority Research Program (No. XDB14040203).

REFERENCES

the joint toxic action of long-term low-level exposure to a mixture of chemicals using mass spectrometry-based metabonomic techniques to analyze the coelomic fluid: a complement to an environmentally relevant model


Yuk, J.; Simpson, M. J.; Simpson, A. J. 1-D and 2-D NMR-based metabolomics of earthworms exposed to endosulfan and endosulfan sulfide in soil. Environ. Pollut., 2013, 175, 35–44.


