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Preformed Biomarkers Including Dialkylphosphates (DAPs) in Produce May Confound Biomonitoring in Pesticide Exposure and Risk Assessment

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Supporting Information

ABSTRACT: Low levels of pesticides and their metabolites/degradates occur in produce when pesticides are used in conventional or organic crop protection. Human dietary and nonoccupational urine biomonitoring studies may be confounded by preformed pesticide biomarkers in the diet. The extent of formation of putative urine biomarkers, including malathion specific (MMA, MDA; malathion mono- and diacids), organophosphorus generic (DMP, DMTP, DMDTP; dimethyl-, dimethylthio-, and dimethydithiophosphate), pyrethroid generic (3-PBA; 3-phenoxybenzoic acid), and captan-specific metabolites (THPI; tetrahydrophthalimide), was measured in produce samples containing the parent pesticide. Every produce sample of 19 types of fruits and vegetables contained biomarkers of potential human exposure. A total of 134 of 157 (85%) samples contained more molar equivalent biomarkers than parent pesticide. Malathion and fenpropathrin were sprayed (1 lb/A), and the time-dependent formation of pesticide biomarkers in strawberries was investigated under field conditions typical of commercial production in California. Malathion and fenpropathrin residues were always below established residue tolerances. Malathion, MMA, and MDA dissipated, while DMP, DMTP, and DMDTP increased, during a 20 day study period following the preharvest interval. The mole ratios of biomarkers/(malathion + malaoxon) were always greater than 1 and increased from day 4 to day 23 postapplication. Fenpropathrin and 3-PBA also dissipated in strawberries during each monitoring period. The mole ratios of 3-PBA/fenpropathrin were always less than 1 and decreased from day 4 to day 14. The absorption of pesticide biomarkers in produce and excretion in urine would falsely indicate consumer pesticide exposure if used to reconstruct dose for risk characterization.

KEYWORDS: *biomarkers, dialkylphosphates, malathion dicarboxylic acid, 3-phenoxybenzoic acid, captan, tetrahydrophthalimide, malathion*

INTRODUCTION

Over 1.1 billion pounds of insecticides, herbicides, and fungicides as well as other pesticides are used in crop protection annually in the United States.[1] As a result, fresh fruits and vegetables may contain trace levels of these products. The Federal Food Drug and Cosmetics Act (FFDCA) authorizes the Environmental Protection Agency (EPA) to establish a tolerance for the maximum amount of a pesticide residue that may be legally present in or on produce. Tolerances are established when EPA grants registration under the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) for the use of a pesticide for crop protection. The Food Quality Protection Act of 1996 (FQPA) amended FIFRA and the FFDCA so that in setting tolerances EPA must make a safety finding that the pesticide can be used with "reasonable certainty of no harm" with an emphasis on protecting the health of infants and children. Accordingly, EPA includes aggregate and cumulative risk assessment among its refined methods of risk characterization. Aggregate risk assessments illustrate the importance of diet as a source of trace level human pesticide exposures.

The United States Department of Agriculture (USDA) monitors the food supply for the occurrence of pesticide residues. The program is the primary source of pesticide residue data that supports the dietary exposure component of aggregate risk assessments performed by the EPA. The standard operating procedures of the Pesticide Data Program (PDP)[2] obtain a representative nationwide produce sample. Crops are selected for residue sampling based upon consumption patterns of the general population. The pesticide residue data produced by PDP are reported annually in a summary available at http://www.ams.usda.gov/science/pdp. The USDA PDP reveals that about 65% of fresh fruits and vegetables contained detectable levels of one or more pesticides.[2]

The need for aggregate pesticide exposure assessments for comprehensive risk characterization has resulted in increased reliance upon biomonitoring as a means to collect human exposure data.[3−5] The assessment of human exposure may involve the use of questionnaires and environmental sampling, but the most reliable procedures available are believed to include biomonitoring.[5] Biomonitoring estimates of internal

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dose using a chemical measure of the amount of pesticide or its metabolites in human tissues most commonly utilize metabolites excreted in urine.

Abiotic (hydrolysis and photolysis) and biotic metabolic processes (e.g., microbial, animal, and human) result in the formation of biomarkers during the dissipation of pesticide residues in the environment and in living things. Use of urine biomarkers as indicators of OP exposure requires an appreciation of their fate and disposition in animals, people, and the target environment (e.g., plants to which they are applied for crop protection). Biomarkers are typically chosen based on their abundance in urine following dosing of mammals with the parent compound. Analytical methods and techniques are available for quantitative analysis of biomarkers of OP exposure in blood and urine in amounts well below toxic levels, signaled by high levels of cholinesterase inhibition.[6]

When foods containing pesticide residues are consumed, their metabolites excreted in urine may be used to estimate exposure to the parent pesticide. For example, following consumption of foods containing OP residues, their relatively rapid absorption and metabolism results in excretion of hydrophilic metabolites in animals and humans. The metabolites that are excreted in urine are convenient biomarkers of exposure and internal or absorbed dose.[7] However, if the produce contains the same biomarkers that are also bioavailable, the reliability of the biomarkers to reconstruct OP exposure is compromised, since the polar degradates are not toxic anticholinesterases like the parent insecticides.

The dialkylphosphates (DAPs), including dimethylphosphate (DMP), diethylphosphate (DEP), dimethylthiophosphate (DMTP), diethylthiophosphate (DETP), dimethyldithiophosphate (DMDTP), and diethyldithiophosphate (DEDTP), were first used as biomarkers of occupational exposure to OPs.[8] Their measurement in urine was usually coupled with knowledge of the OP used and assessment of the cholinesterase status of exposed workers.[9] Malathion metabolism is broadly similar in plants and animals.[10] Malaoxon is the desulfurated derivative responsible for the acetylcholinesterase inhibition and neurotoxicity of malathion, and is produced by plants and animals. DAPs such as DMP, DMDTP, and DMTP, which are dissipation products of dimethylphosphorothionate insecticides, have long been used as general urinary biomarkers for this class of OP insecticides.[3,11−18] The DAPs are generic, being produced by many OPs, and they differ from the alcohol moiety "leaving groups", which tend to be pesticide-specific.

The determination of the extent of OP exposure relies on the assumption that urinary measurements of biomarkers reflect exposure to OPs as opposed to preformed biomarkers. However, few studies have concurrently measured both pesticides and their derivatives, that are potential biomarkers of exposure in environmental media. The possible impact of preformed biomarkers in produce is especially important given the role of diet in human aggregate pesticide exposure.[9,19−21] Surface contamination with pesticides and their biomarkers may also contribute to uncertainty in establishing nondietary sources of OP and biomarker exposure.[22,23]

Publication of 2002−2004 residue data from 153 samples of California produce representing 44 crops treated with OP insecticides[19] revealed use of 12 insecticides, including mevinphos, naled, acephate, methamidophos, oxidemeton-methyl, azinphos-methyl, dimethoate, malathion, methidathion, phosmet, chlorpyrifos, and diazinon. All parent OPs were below regulatory tolerances, and 91 of the 153 samples contained more DAPs than the parent insecticide. The average over all 153 samples was 6-fold more DAPs than OPs on a molar basis. When OP insecticides were used in crop protection, the trace residues of parent insecticide and their corresponding nontoxic biomarkers are in produce. Lu et al.[21] measured DAPs in conventional and organic orange and apple juices. Parent insecticide levels were not reported. Pesticide spiked juice samples also yielded dimethyl- and diethylphosphates after 72 h at 4 °C.[21] The resulting occurrence of biomarkers in human urine may represent metabolites of residual insecticide or preformed biomarkers.[19,21]

In addition to OPs, strawberries are also frequently treated with a pyrethroid and a fungicide such as captan. Pyrethroid insecticides widely used in agricultural, forestry, industrial, and residential applications have potential for human exposure.[24] 3-Phenoxybenzoic acid (3-PBA) is a urinary metabolite and potential exposure biomarker of six pyrethroid pesticides, including cyhalothrin, cypermethrin, deltamethrin, fenpropathrin, permethrin, and tralomethrin.[3] Use of 3-PBA as a urine biomarker without knowledge of parent pesticide exposure carries uncertainties analogous to those surrounding the use of DAPs in exposure and risk assessment.

Captan is widely used in different crops to treat fungal diseases. In mammals, captan is primarily metabolized to tetrahydrophthalimide (THPI) and thiophosgene. THPI excreted in urine was suggested as a possible biomarker of captan exposure by the EPA.[25] Thiophosgene is conjugated with glutathione (GSH) or cysteine and excreted as 2-thiazolidinethione-4-carboxylic acid (TTCA), after enzyme degradation and ring closure.[26,27] TTCA may also appear as a potential biomarker of captan exposure, but it is not specific to this fungicide.

This study was intended to measure preformed biomarkers in produce and to evaluate their possible influence on pesticide exposure assessment.

MATERIALS AND METHODS

Chemicals and Solvents. Malathion (CAS No. 121-75-5, 99.1% purity), fenpropathrin (CAS No. 64257-84-7, 98% purity), and captan (CAS No. 133-06-2, 98% purity) were purchased from Chem Sources International, Inc. Malathion monocarboxylic acid (MMA, CAS No. 35884-76-5, 89.4% purity), malathion dicarboxylic acid (MDA, CAS No. 1190-28-9, 99.6% purity), *O*,*O*-dimethylthiophosphatedicyclohexylammonium salt (DMTP-Q, CAS No. 13941-61-2, 97.9% purity), and *O*,*O*-dimethyldithiophosphate potassium salt (DMDTP-K, CAS No. 16001-68-6, 98.7% purity) were kindly provided by Cheminova, Inc. *O*,*O*-Dimethylphosphate (DMP, CAS No. 813-78-5, 98% purity), 3-phenoxybenzoic acid (3-PBA, CAS No. 3739-38-6, 99% purity), *cis*-1,2,3,6-tetrahydrophthalimide (THPI, CAS No. 27813-21−4, 96% purity), and 2,3,4,5,6-pentafluorobenzyl bromide (PFBBr, CAS No. 1765-40-8, 97% purity) were purchased from Acros Organics. *N*,*N*-Diisopropylethylamine (DIPEA, CAS No. 7087-68-5, 99% purity) and boron trifluoride−methanol solution (CAS No. 373-57-9, ∼10%) were purchased from Sigma-Aldrich. Primary secondary amine (PSA) reagent was purchased from Agela Technologies, Inc. Acetonitrile, hexane, ethyl acetate, 6 N hydrochloric acid (HCl) solution, sodium chloride, and anhydrous magnesium sulfate were purchased from Fisher.

Individual pesticide and metabolite standard stock solutions (1000 mg/L) were prepared by dissolving each pesticide standard in organic solvent and stored at −20 °C. Pesticide working solutions were prepared by diluting the stock solutions. For quantification, matrix matched standard curves were prepared alongside each set of samples by spiking the matrix with working solutions.

Produce Samples. Produce samples (N = 157) representing 19 different kinds of fruits and vegetables were obtained from primuslabs.com, Santa Maria, CA. All samples had been previously tested for pesticide residues by growers or shippers prior to entering the channels of trade. The primuslabs.com multiresidue screen included OP pesticides and revealed only malathion (LOQ 0.01 ppm). The analytical data derived from these samples represent premarket produce pesticide residue positive for malathion (with no other OPs reported) and corresponding preformed biomarker levels.

Field Study. Fresh strawberries were collected in Santa Maria, CA, in both August 2010 and June 2011. The 2010 samples were harvested at intervals of 4 (after the 3 day preharvest interval when berries may first be picked for consumption), 8, 10, and 14 days after a routine malathion and fenpropathrin application (1 lb of active ingredient/acre), and the 2011 samples were collected at 4, 7, 11, 15, 20, and 23 days after an identical treatment. All samples were stored at −20 °C until analyzed. Each collection consisted of 8 1-lb samples picked by separate harvesters as part of their normal daily production. The berries were transported the same day to primuslabs.com, where a homogenized sample was prepared for analysis of malathion and malaoxon. A portion of the chopped berries were stored frozen and transported to UC Riverside, where they were stored at −20 °C until analyzed for the malathion biomarkers.

Sample Preparation. *Malathion, Malaoxon, and Fenpropathrin in Strawberries.* Ten grams of previously homogenized sample was taken into a 50 mL Teflon centrifuge tube and mixed thoroughly by vortexing for 1 min with 10 mL of acetonitrile containing 0.5% acetic acid (HOAc). After addition of 4 g of anhydrous magnesium sulfate and 1 g of sodium chloride, the sample was vortexed for 1 min and centrifuged for 5 min at 5000 rpm. Then, 3 mL of the acetonitrile layer was transferred into a 7 mL tube containing 200 mg of PSA sorbent and 300 mg of anhydrous magnesium sulfate. The sample was vortexed for 1 min and centrifuged for 5 min at 5000 rpm. A portion of the acetonitrile layer (2 mL) was transferred into a vial and evaporated to dryness under nitrogen. Then the sample was redissolved in 0.4 mL of acetonitrile and transferred into an autosampler vial for GC-MS analysis.

MMA, MDA, and 3-PBA in Strawberries. Strawberry samples (10 g portions of 454 g samples chopped in a blender) were acidified with 6 N hydrochloric acid to pH 1. Sodium chloride sufficient to saturate the aqueous phase and 10 mL of acetonitrile were added. The sample was vortexed for 1 min and then centrifuged for 15 min at 5000 rpm. After centrifugation, the supernatant was transferred to a round-bottom flask and the sample was re-extracted with 5 mL of acetonitrile and centrifuged. The combined extract was evaporated to about 1 mL at 40 °C by rotary evaporator, transferred to a 7 mL vial, and evaporated to dryness under a stream of nitrogen. To ensure a completely dry residue, 1 mL of acetonitrile was added and the drying step was repeated. The next step in the derivatization procedure was a 2 h incubation at 65 °C with 2 mL of 10% boron trifluoride−methanol solution. After derivatization, 2 mL of ultrapure water was added and the esters formed were extracted by 1 mL of hexane. Of the upper layer, 0.4 mL was transferred to a vial and evaporated to dryness under a stream of nitrogen and then redissolved in 0.2 mL of ethyl acetate.

DMP, DMTP, and DMDTP in Strawberries. Samples were processed prior to derivatization as described in the previous section. The extracts were derivatized for 4 h at 50 °C with 0.5 mL of 6% PFBBr acetone solution (v/v) and 0.5 mL of 3% DIPEA acetone solution (v/v). After derivatization, 2 mL of ultrapure water was added and the esters formed were extracted with 1 mL of hexane. The upper layer (0.4 mL) was transferred to a vial and evaporated to dryness under a stream of nitrogen and then redissolved in 0.2 mL of ethyl acetate.

Captan in Strawberries. Previously homogenized sample (10 g) was transferred to a 50 mL Teflon centrifuge tube and mixed thoroughly by vortexing for 1 min with 10 mL of acetonitrile. The centrifuge tube was then stored in a freezer for 30 min. When the temperature of the sample reached 4 °C, 4 g of anhydrous magnesium sulfate and 1 g of sodium chloride were added and the sample was vortexed immediately for 1 min and centrifuged for 5 min at 5000 rpm. An aliquot of acetonitrile layer (1 mL) was transferred into a 2 mL microcentrifuge tube containing 100 mg of PSA sorbent and 150 mg of anhydrous magnesium sulfate. The sample was vortexed for 1 min and centrifuged for 5 min at 5000 rpm. A portion (0.6 mL) of acetonitrile layer was transferred into a vial and evaporated to dryness under nitrogen. Then the sample was redissolved in 0.3 mL of ethyl acetate and transferred into an autosampler vial for GC-ECD analysis.

THPI in Strawberries. Ten grams of previously homogenized sample was transferred to a 50 mL Teflon centrifuge tube and mixed thoroughly by vortexing for 1 min with 10 mL of ethyl acetate. After addition of 4 g of anhydrous magnesium sulfate and 1 g of sodium chloride, the sample was vortexed for 1 min and centrifuged for 5 min at 5000 rpm. Then, 3 mL of ethyl acetate layer was transferred into a 7 mL tube containing 200 mg of PSA sorbent and 300 mg of anhydrous magnesium sulfate. The sample was vortexed for 1 min and centrifuged for 5 min at 5000 rpm. An aliquot (2 mL) of ethyl acetate layer was transferred into a vial and evaporated to dryness under nitrogen. Then the sample was redissolved in 0.4 mL of ethyl acetate and transferred into an autosampler vial for GC-NPD analysis.

Dissipation Rate Constant and Half-Life of Pesticides and Selected Biomarkers. The degradation rate constant and half-life were calculated using the first order rate equation: $C_t = C_0 e^{-kt}$, where C_t represents the concentration of the pesticide residue at the time t of harvest in days, C_0 represents the initial residue after the preharvest interval (PHI) following the application, and k is the dissipation rate constant (days^{-1}). The dissipation half-life ($t_{1/2}$) was calculated from the k value for each analyte, as $t_{1/2} = \ln 2/k$.

Instrumental Analysis. *GC-MS Analysis.* The extracts were analyzed with a 6890N gas chromatograph (Agilent Technologies) equipped with a 5973 mass spectrometric detector (Agilent Technologies). The GC-MS system included a DB-1701 capillary column (30 m length × 0.25 mm i.d × 0.25 μm film thickness), and helium was used as carrier gas at a flow rate of 1.0 mL/min. Analysis was performed with the injection temperature set at 250 °C. The oven temperature for MDA and MMA analysis was programmed at 50 °C for 1 min, raised at 10 °C/min to 180 °C, then raised at 3 °C/min to 240 °C, and then raised at 15 °C/min to 260 °C in a splitless mode. The oven temperature for DMP, DMTP, and DMDTP analysis was programmed at 50 °C for 1 min, raised at 10 °C/min to 180 °C, then raised at 3 °C/min to 210 °C, then raised at 15 °C/min to 260 °C, and finally maintained for 8 min at that temperature in a splitless mode. The solvent delay was 6.00 min, and the injection volume was 1 μL. The mass spectrometric detector was equipped with a quadrupole analyzer operating in electron impact ionization (EI) mode with an ionizing energy of 70 eV. The ion source temperature was 230 °C, the MS Quad temperature was 150 °C, and the transfer line temperature was 280 °C. Identification/quantification of target analytes was performed in selected ion monitoring (SIM) mode. Monitored ions (m/z) of the various analytes are shown in Table 1.

GC-ECD Analysis. Captan was analyzed with a 5890 gas chromatograph (Agilent Technologies) equipped with an electron capture detector (ECD). The GC-ECD system utilized a HP-5MS capillary column (30 m length × 0.25 mm i.d × 0.25 μm film thickness). Analysis was carried with injection temperature and

Table 1. Monitored Ions of Malathion, Fenpropathrin, the Methyl Esters of MAs and 3-PBA, and the Pentafluorobenzyl Esters of DMPs

compd	quantification ion (m/z)	identification ions (m/z)
malathion	173	125, 127
fenpropathrin	181	125, 265
3-PBA	228	141, 197
MDA	145	125, 158
MMA	159	125, 158
DMP	306	110, 194
DMTP	322	110, 211
DMDTP	338	125, 157

detector temperature set at 220 and 280 °C, respectively. The oven temperature was programmed at 50 °C for 1 min, raised at 15 °C/min to 190 °C, then raised at 3 °C/min to 220 °C, and then raised at 10 °C/min to 280 °C in a splitless mode. The injection volume was 1 μL.

GC-NPD Analysis. THPI was analyzed with a 5890 gas chromatograph (Agilent Technologies) equipped with a nitrogen phosphorus detector. The GC-NPD system was equipped with a HP-5MS capillary column (30 m length × 0.25 mm i.d. × 0.25 μm film thickness). Analysis was performed with injection temperature and detector temperature set at 220 and 300 °C. The oven temperature was programmed at 50 °C for 1 min, raised at 15 °C/min to 190 °C, then raised at 10 °C/min to 220 °C, then raised at 20 °C/min to 280 °C, and finally maintained for 13 min in a splitless mode. The injection volume was 1 μL. The flow rates of air, hydrogen, nitrogen, and carrier gases were 110, 4, 35, and 2 mL/min, respectively.

Biomarker Derivatization Optimization. To ensure derivatization completeness, two derivatization procedures, BF_3/methanol and PFBBr/DIPEA esterification, were optimized. The influence of different parameters and their interactions on each derivatization process were determined. The studied parameters included the temperature, the reaction time, the amount of catalyst (BF_3), and the derivatization solvent.

RESULTS AND DISCUSSION

Optimization of Derivatization of Biomarkers. *BF_3–Methanol Derivatization of MMA and MDA.* Three factors that affect the derivatization efficiency of the malathion acids were studied, including temperature, catalyst amount (BF_3), and reaction time. The best result was obtained for a 2 h derivatization at 65 °C with 10% BF_3 in methanol. In order to increase the partition coefficient during hexane extraction of the esters, water was added to the derivatization mixture. The factorial design and results are included in Supporting Information Table A.

PFBBr and DIPEA Derivatization of DMP, DMTP, and DMDTP. The samples were allowed to derivatize at 30, 40, and 50 °C for different time intervals. The following factors were studied: derivatization solvent, temperature, and reaction time. The factorial design is described in Tables B and C of the Supporting Information. DMP did not react with PFBBr and DIPEA in methanol at 20 and 40 °C, and the reaction went poorly at 50 and 60 °C. The reaction of DMP occurred in acetonitrile, but not as effectively as in acetone. The reaction of DMTP was more effective in acetone than in acetonitrile and methanol. The derivatization efficiency of DMP increased with the temperature. However, temperature could not be further increased above the boiling point of acetone. The temperature interaction is not significant for DMTP and DMDTP. Longer derivatization time at 50 °C in acetone leads to degradation of the DMTP and DMDTP pentafluorobenzyl esters. Accordingly, the derivatization of the DAPs was most efficient in acetone at 50 °C for 4 h.

Method Validation. Methods were developed and validated for the analysis of parent compounds and biomarkers in strawberries. For quantification, matrix standard curves were prepared alongside each set of samples by spiking the matrix with authentic compounds. Average recoveries of all of the compounds in strawberries were in the range of 72.3–119% with relative standard deviations (RSD) of 0.45–13.3% at three fortification levels. The limits of detection (LODs) of malathion, MMA, MDA, DMP, DMTP, DMDTP, fenpropathrin, 3-PBA, captan, and THPI in strawberries were 10, 2, 2, 1, 0.3, 0.3, 10, 0.3, 10, and 10 μg/kg, respectively. The limits of quantification (LOQs) of malathion, MMA, MDA, DMP, DMTP, DMDTP, fenpropathrin, 3-PBA, captan, and THPI in strawberries were 33, 5, 5, 3, 1, 1, 33, 1, 33, and 33 μg/kg, respectively. These values for the analysis of malathion, fenpropathrin, captan, and THPI are within the acceptable range for trace residue analysis for demonstration of compliance with MRLs. Recovery data are presented in Table D of the Supporting Information.

Parent Compounds and Preformed Biomarkers in Produce. The results of produce analysis for malathion and its preformed biomarkers are summarized in Table 2. The arithmetic mean, geometric mean, and median of the 129 samples were calculated. In order to examine how the mole fraction of preformed biomarkers and the mole ratio of preformed biomarkers to malathion distribute in each category of produce, the samples were categorized based on the type of produce. As indicated in Tables 3 and 4, the mole fraction of preformed biomarkers and mole ratio of preformed biomarkers to malathion seemed to exhibit differences among the types of produce. Some produce (broccoli, green beans, and rutabaga) consistently had larger mole fractions and mole ratios than others. As shown in Table 4, malathion was detectable in beet tops but not detectable in beet bottoms (root) while malathion derivatives (MMA, MDA, DMP, DMTP, and DMDTP) were detected in both beet tops and bottoms (root), indicating a possible phloem movement of malathion derivatives in produce.

Table 2. Malathion and Its Preformed Biomarkers from Individual Measures in 129 Produce Samples

	range	arithm mean	geom mean	median
malathion (nmol/g)	ND[a]–41.8	0.60	0.12	0.09
preformed biomkrs (nmol/g)	0.09–34.6	3.29	2.05	2.40
mole frac of preformed biomkrs[b]	0.41–1.00	0.91	0.90	0.94
mole ratio of biomkrs/malathion[c]	0.70–333	32.6	16.5	16.4

[a]ND = nondetected. Half of LOQ of 0.05 μg/g was used in the calculation if residue was not detected but not measurable. [b]Mole fraction of preformed biomarkers = moles of (MMA + MDA + DMP + DMTP + DMDTP)/moles of (malathion + MMA + MDA + DMP + DMTP + DMDT). [c]Mole ratio of biomarkers/malathion = moles of (MMA + MDA + DMP + DMTP +DMDTP)/moles of malathion.

The malathion mole ratios listed in Tables 2–4 and the corresponding ratios in subsequent tables represent measured residues in produce as the commodity entered the channels of trade. Cold storage, transport, marketing, and food processing will be associated with further time-dependent dissipation of pesticide residues and relative increased levels of biomarkers (Table 4). The resulting urine biomarker levels of consumers resulting from dietary exposure will become progressively less representative of parent pesticide exposure. This research as well as previous reports of others, e.g. Chuang and Wilson, 2010,[28] demonstrate the feasibility of concurrently measuring parent insecticide and biomarkers in foods. Such measurements are particularly critical to avoid misclassification of source in low level epidemiology research.

Shown in Table 5 are the fenpropathrin and 3-PBA residue levels. A total of 1 of 14 produce samples (7%) contained more biomarker than parent compound (mole ratio of 3-PBA/fenpropathrin > 1).

Table 3. Mole Ratio of Biomarkers/Malathion and Mole Fraction of Biomarkers in the Produce That Contained Malathion[a]

		mole frac preformed biomkrs[b]				mole ratio preformed biomkrs[c]			
produce	*N*	range	arithm mean	geom mean	median	range	arithm mean	geom mean	median
blackberries	35	0.70−0.98	0.93	0.92	0.94	2.31−55.5	19.1	15.4	16.5
blueberries	12	0.75−0.99	0.95	0.94	0.97	2.94−94.8	35.7	26.3	32.5
broccoli	6	0.97−1.00	0.99	0.99	0.99	28.6−313	150	114	131
cherries	11	0.58−0.99	0.86	0.85	0.89	1.38−101	18.8	9.31	8.05
cilantro	4	0.66−0.85	0.73	0.73	0.70	1.97−5.78	3.12	2.82	2.37
green onions	15	0.45−0.98	0.88	0.87	0.92	0.83−48.3	15.2	10.6	11.7
raspberries	8	0.94−0.99	0.98	0.98	0.98	16.7−88.3	52.1	46.6	51.1
strawberries	25	0.76−0.99	0.92	0.92	0.94	3.19−97.4	28.0	17.3	16.4

[a]Only those categories with 3 or more samples are listed (see also Table 4). [b]Mole fraction of preformed biomarkers = moles of (MMA + MDA + DMP + DMTP + DMDTP) ÷moles of (malathion + MMA + MDA + DMP + DMTP + DMDTP). [c]Mole ratio of biomarkers/malathion = moles of (MMA + MDA + DMP + DMTP + DMDTP) ÷ moles of malathion.

Table 4. Mole Ratio of Biomarkers/Malathion and Mole Fraction of Biomarkers in the Produce with Less than Three Samples That Contained Malathion[a,b,c]

produce	malathion (μg/g)	malathion (nmol/g)	monoacid (nmol/g)	diacid (nmol/g)	DMP (nmol/g)	DMTP (nmol/g)	DMDTP (nmol/g)	sum biomkrs (nmol/g)[d]	mole frac preformed biomkrs[e]	mole ratio biomkrs to malathion[f]
beets bottoms-1	ND	ND	0.06	0.13	0.06	0.02	0.01	0.29	0.95	18.9
beets tops-1	0.03	0.09	0.41	0.26	0.29	0.50	0.03	1.49	0.94	16.4
beets bottoms-2	ND	ND	0.15	0.26	0.05	0.02	0.01	0.49	0.97	32.1
beets tops-2	0.18	0.55	1.39	0.64	0.25	0.46	0.19	2.94	0.84	5.39
bell peppers	0.03	0.09	0.24	0.25	0.39	0.24	0.06	1.17	0.93	12.9
red bell pepper	0.03	0.09	0.12	0.12	0.15	0.09	0.03	0.51	0.85	5.66
chili	0.05	0.15	0.12	0.15	0.40	0.14	0.01	0.82	0.84	5.43
green beans	0.01	0.03	1.68	3.75	1.70	2.85	0.14	10.12	1.00	334
lemons	0.07	0.21	0.31	0.00	0.01	0.07	0.00	0.39	0.65	1.85
oranges	0.38	1.15	0.50	0.08	0.03	0.19	0.00	0.81	0.41	0.70
rutabaga	ND	ND	1.45	1.50	0.05	0.00	0.00	3.00	0.99	198
tomatoes	0.02	0.06	0.43	0.15	0.03	0.05	0.01	0.67	0.92	11.1
roma tomatoes	0.60	1.82	4.05	1.60	0.64	0.38	0.02	6.69	0.79	3.68

[a]Those categories with less than three samples are listed. [b]ND = nondetected, but analyzed due to history of malathion application. [c]Half of the LOD value was used in the calculation if residue was not detected. [d]Sum of biomarkers (nmol/g) = MMA + MDA + DMP + DMTP + DMDTP. [e]Mole fraction of preformed biomarkers = moles of (MMA + MDA + DMP + DMTP + DMDTP) ÷ moles of (malathion + MMA + MDA + DMP + DMTP + DMDTP). [f]Mole ratio of biomarkers/malathion = moles of (MMA + MDA + DMP + DMTP + DMDTP)/mole of malathion.

Table 5. Fenpropathrin and 3-PBA in Produce That Contained the Pyrethroid

produce	fenpropathrin (μg/g)	fenpropathrin (nmol/g)	3-PBA (nmol/g)	mole frac 3-PBA[a]	mole ratio 3-PBA/fenpropathrin[b]
cherries	1.02	2.92	0.047	0.02	0.02
cherries	0.72	2.06	0.045	0.02	0.02
cherries	1.72	4.93	0.049	0.01	0.01
strawberries	0.71	2.03	0.022	0.01	0.01
strawberries	0.27	0.77	0.017	0.02	0.02
strawberries	0.32	0.92	0.024	0.03	0.03
strawberries	0.80	2.29	0.031	0.01	0.01
strawberries	0.72	2.06	0.036	0.02	0.02
strawberries	0.75	2.15	0.026	0.01	0.01
strawberries	0.82	2.35	0.032	0.01	0.01
strawberries	0.86	2.46	0.023	0.01	0.01
strawberries	0.49	1.40	0.022	0.02	0.02
strawberries	0.25	0.72	0.015	0.02	0.02
strawberries	0.02	0.06	0.074	0.56	1.30

[a]Mole fraction of 3-PBA = mole of 3-PBA/moles of (3-PBA + fenpropathrin). [b]Mole ratio of 3-PBA/fenpropathrin = mole of 3-PBA/mole of fenpropathrin.

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Table 6. Pyrethroids, Excluding Fenpropathrin, and 3-PBA in Selected Produce That Contained One or More Pyrethroids

produce	pyrethroids	pyrethroids (μg/g)	sum of pyrethroids (nmol/g)	3-PBA (nmol/g)	mole frac 3-PBA[a]	mole ratio 3-PBA/pyrethroids[b]
blackberries	fenvalerate	0.02	0.05	0.075	0.61	1.58
blackberries	esFenvalerate	0.07	0.17	0.078	0.32	0.47
blackberries	cypermethrin	0.04	0.10	0.097	0.50	1.01
blueberries	ζ-cypermethrin	0.05	0.19	0.046	0.19	0.24
	fenvalerate	0.03				
cherries	λ-cyhalothrin	0.08	0.18	0.040	0.19	0.23
cherries	λ-cyhalothrin	0.06	0.13	0.040	0.23	0.30
cherries	λ-cyhalothrin	0.07	0.16	0.040	0.21	0.26
cherries	fenvalerate	0.13	0.39	0.041	0.10	0.11
	permethrin	0.03				
green onions	deltamethrin	0.18	0.55	0.090	0.14	0.16
	ζ-cypermethrin	0.08				

[a]Mole fraction of 3-PBA = mole of 3-PBA/moles of (3-PBA + pyrethroids). [b]Mole ratio of 3-PBA/pyrethroid(s) = mole of 3-PBA/mole of pyrethroid(s).

The results of produce analysis for trace residual pyrethroid pesticides other than fenpropathrin and 3-PBA are presented in Table 6. Seven pyrethroid pesticides were identified from nine produce samples. The pyrethroid pesticides included fenvalerate, esfenvalerate, cypermethrin, ζ-cypermethrin, λ-cyhalothrin, permethrin, and deltamethrin. The arithmetic mean, geometric mean, and median of the nine samples were calculated. Two of nine produce samples (22%) contained more biomarker than parent compound (mole ratio of 3-PBA/pyrethroids > 1). The pyrethroids listed in Table 6 had higher mole fractions and mole ratios (0.28 and 0.48, respectively) than fenpropathrin (0.06 and 0.11, respectively).

The results of produce analysis for captan and THPI are shown in Table 7. Four of five produce samples (80%) contained more biomarker than parent compound (mole ratio of THPI/captan > 1).

Table 7. Captan and a Potential Biomarker, THPI, in Selected Produce

produce	captan (μg/g)	captan (nmol/g)	THPI (nmol/g)	mole frac THPI[a]	mole ratio THPI/captan[b]
blackberries	0.24	0.80	0.79	0.50	0.99
raspberries	0.04	0.13	1.13	0.89	8.41
strawberries	0.40	1.34	1.99	0.60	1.48
strawberries	0.77	2.58	3.71	0.59	1.44
strawberries	0.32	1.07	1.99	0.65	1.86

[a]Mole fraction of THPI = mole of THPI/moles of (THPI + captan). [b]Mole ratio of THPI/captan = mole of THPI/mole of captan.

Parent Insecticides and Preformed Biomarkers in Fresh Market Strawberries. The formation of potential human urine biomarkers in strawberries under normal field conditions in Santa Maria, CA, was measured in 2010 and 2011. Malathion and fenpropathrin were applied as tank mixes by the cooperator during the June through August time frame. Those insecticides were the only known potential sources of DAPs and 3-PBA, respectively, in the vicinity of the study site.

The result of the 2011 prospective study of the transformation of malathion to biomarkers is presented in Table 8. The results for fenpropathrin and 3-PBA are shown in Tables 9 and 10 and Figures 1 and 2. The tolerances established by EPA for malathion and fenpropathrin in strawberries of 8 and 2 μg/g, respectively, were never exceeded. Potential human urine biomarkers (MMA, MDA, DMP, DMTP, DMDTP, and 3-PBA) of pesticide exposure were present in every sample of fresh strawberries.

The concentration (μg/g) of malathion and each of the five analytes (Table 8) plotted as a function of days postapplication yielded an exponential dissipation curve. Malathion, MMA, and MDA dissipated with $t_{1/2}$ values of 7.3, 10.3, and 6.4 days, respectively, while DMP, DMTP, and DMDTP increased during a 23 day study period postmalathion application (Table 8). Malaoxon was either low or unquantifiable in strawberries at each sampling interval. MDA was the most abundant biomarker at the first three sampling days, and DMTP was the most abundant one at the last three sampling days. The mole ratios of biomarkers/(malathion + malaoxon) were always greater than 1 and increased from 8.0 on day 4 to 47.0 on day 23 (Table 8).

Malathion residues decreased from 0.39 nmol/g on day 4 to 0.03 nmol/g on day 23. Importantly, the molar sum of malathion and its metabolites (malaoxon, MMA, MDA, DMP, DMTP, and DMDTP) at 23 days post-treatment was still 53% of the value at 4 days, indicating the total mass of circulating metabolites declined much more slowly than the parent OP. Biomarkers were present in every sample and were more persistent than malathion.

At the first permitted harvest of strawberries following the commercial malathion application on day 4 after the PHI of 3 days, malathion and malaoxon residues were 0.13 and 0.005 μg/g, respectively. The malathion residues continued to dissipate with an apparent $t_{1/2}$ of 7.3 days. The $t_{1/2}$ was greater than the 4.3 day $t_{1/2}$ recorded in 2010 based upon four harvests of berries (data not shown). In large measure the longer $t_{1/2}$ resulted from inconsistently low malathion levels reported for the second harvest on postapplication day 7 (Table 8). When day 7 residues were removed as apparent outliers (i.e., the actual residue of malathion cannot be higher 4 to 8 days after day 7 unless day 7 values were not representative or there was significant malathion contamination of the entire field between day 7 and 11), the malathion $t_{1/2}$ was 5.2 days, more consistent with previous experience and the overall dissipation curve. Critical examination of the residue profile including MDA, MMA, DMP, DMTP, and DMDTP reveals important insight concerning the potential impact of these degradates as putative pesticide biomarkers of dietary malathion exposure when ingested, absorbed, and excreted in urine for the reconstruction of human exposure. The five biomarker/detoxification products in the berries increased markedly relative to the parent OP

Table 8. Malathion and Preformed Biomarkers in Strawberries from Santa Maria, CA (Mean ± SD, n = 8), 2011[a]

time interval (D)	malathion (μg/g)	malathion (nmol/g)	malaoxon (nmol/g)	MMA (nmol/g)	MDA (nmol/g)	DMP (nmol/g)	DMTP (nmol/g)	DMDTP (nmol/g)	mole frac preformed biomkrs[b]	mole ratio biomkrs to (malathion + malaoxon)[c]
4	0.13 ± 0.03	0.39 ± 0.09	0.02 ± 0.00[c]	0.81 ± 0.09	2.00 ± 0.27	0.05 ± 0.01	0.21 ± 0.03	0.03 ± 0.01	0.89 ± 0.02	8.00 ± 1.91
7	0.03 ± 0.02	0.09 ± 0.07	0.02 ± 0.00[c]	0.33 ± 0.23	0.72 ± 0.54	0.08 ± 0.05	0.21 ± 0.13	0.06 ± 0.05	0.92 ± 0.03	12.0 ± 4.49
11	0.06 ± 0.01	0.17 ± 0.04	0.03 ± 0.01	0.42 ± 0.09	0.50 ± 0.07	0.08 ± 0.06	0.35 ± 0.35	0.07 ± 0.08	0.87 ± 0.03	7.11 ± 2.06
15	0.06 ± 0.04	0.17 ± 0.11	0.02 ± 0.00[c]	0.39 ± 0.26	0.44 ± 0.23	0.25 ± 0.08	0.88 ± 0.83	0.15 ± 0.16	0.91 ± 0.02	10.9 ± 2.78
20	0.03 ± 0.01	0.08 ± 0.03	0.02 ± 0.00[c]	0.17 ± 0.04	0.19 ± 0.02	0.24 ± 0.05	0.62 ± 0.29	0.06 ± 0.03	0.93 ± 0.02	14.7 ± 3.79
23	0.01 ± 0.01	0.03 ± 0.02	0.02 ± 0.00[d]	0.16 ± 0.08	0.17 ± 0.05	0.19 ± 0.05	1.15 ± 0.35	0.13 ± 0.01	0.98 ± 0.01	47.0 ± 18.1

[a]Eight 1 lb boxes of strawberries picked by separate harvesters were randomly selected at each time interval. [b]Mole fraction of preformed biomarkers = moles of (MMA + MDA + DMP + DMTP + DMDTP) ÷ moles of (malathion + malaoxon + MMA + MDA + DMP + DMTP + DMDTP). [c]Mole ratio of preformed biomarkers = moles of (MMA + MDA + DMP + DMTP + DMDTP) ÷ moles of (malathion + malaoxon). [d]Half of LOD value was used in the calculation if residue was not detected. LOD of malaoxon was 0.01 μg/g.

Table 9. Fenpropathrin and 3-PBA in Strawberries from Santa Maria, CA (Mean ± SD, n = 8), 2010

time interval (D)	fenpropathrin (μg/g)	fenpropathrin (nmol/g)	3-PBA (nmol/g)	mole frac 3-PBA[a]	mole ratio 3-PBA/fenpropathrin[b]
4	0.46 ± 0.15	1.33 ± 0.44	0.41 ± 0.25	0.22 ± 0.07	0.29 ± 0.10
8	0.28 ± 0.05	0.79 ± 0.14	0.15 ± 0.07	0.16 ± 0.05	0.20 ± 0.07
10	0.23 ± 0.07	0.66 ± 0.20	0.10 ± 0.09	0.12 ± 0.08	0.15 ± 0.12
14	0.18 ± 0.02	0.52 ± 0.06	0.01 ± 0.00	0.02 ± 0.00	0.02 ± 0.00

[a]Mole fraction of 3-PBA = mole of 3-PBA/moles of (3-PBA + fenpropathrin). [b]Mole ratio of 3-PBA/fenpropathrin = mole of 3-PBA ÷ mole of fenpropathrin.

Table 10. Fenpropathrin and 3-PBA in Strawberries from Santa Maria, CA (Mean ± SD, n = 8), 2011

time interval (D)	fenpropathrin (μg/g)	fenpropathrin (nmol/g)	3-PBA (nmol/g)	mole frac 3-PBA[a]	mole ratio 3-PBA/fenpropathrin[b]
4	0.80 ± 0.11	2.30 ± 0.30	0.034 ± 0.005	0.01 ± 0.00	0.01 ± 0.00
7	0.34 ± 0.29	0.96 ± 0.84	0.027 ± 0.005	0.13 ± 0.15	0.18 ± 0.23
11	0.45 ± 0.06	1.30 ± 0.18	0.013 ± 0.003	0.01 ± 0.00	0.01 ± 0.00
15	0.20 ± 0.09	0.56 ± 0.25	0.013 ± 0.006	0.02 ± 0.00	0.02 ± 0.00
20	0.03 ± 0.01	0.09 ± 0.03	0.003 ± 0.001	0.03 ± 0.01	0.03 ± 0.01
23	0.04 ± 0.01	0.11 ± 0.04	0.003 ± 0.002	0.03 ± 0.02	0.03 ± 0.02

[a]Mole fraction of 3-PBA = mole of 3-PBA/moles of (3-PBA + fenpropathrin). [b]Mole ratio of 3-PBA/fenpropathrin = mole of 3-PBA/mole of fenpropathrin.

insecticide during the 23 day study period. As time progressed, DMTP and DMP became the most prominent malathion metabolites in the sprayed produce. The biomarkers accounted for 89% of the total molar residue at 4 days and 98% after 23 days. At each sampling interval, MDA, DMTP, and DMP were more prominent than MMA and DMDTP among the five derivatives detected.

The results of the sequential harvest of strawberries reported here are consistent with large-scale sampling and analysis of the Pesticide Data Program of the USDA.[2] The 2009 national fresh produce sample included 744 samples of strawberries. Malathion was detected in 160 samples with a mean of 0.059 μg/g (21.5% detections; range 0.004−0.35 μg/g), and malaoxon was detected in 77 with a mean of 0.006 μg/g (10.3% detections; range 0.003−0.027 μg/g). The residues in berries obtained immediately after harvest (at the "farm gate" in the present investigation) ranged from 0.13 μg/g at the PHI to 0.01 μg/g at the sixth harvest 23 days after the malathion application (Table 8). Although not measured in the national sample, based upon the findings reported in Table 8, berries that contained a malathion residue would carry substantially more nontoxic biomarkers than parent insecticide itself, since the biomarkers are more persistent than the parent insecticide. The data presented in Table 8 are derived from a produce matrix that provides an opportunity to measure the levels of the parent insecticide and its derivatives under very favorable natural conditions with respect to time and setting.

Fenpropathrin and 3-PBA residues on strawberries decreased from 1.33 and 0.41 nmol/g on day 4 to from 0.52 to 0.01 nmol/g on day 14 with $t_{1/2}$ values of 7.56 and 1.95 days, respectively, in 2010 (Table 9; Figure 1). The mole ratios of 3-PBA/fenpropathrin were always less than 1 and decreased from day 4 to day 14. Fenpropathrin and 3-PBA residues on strawberries were 2.30 and 0.034 nmol/g on day 4 in 2011 (Table 10; Figure 2), which declined to 0.11 and 0.003 nmol/g, respectively, by the 23rd day, and the corresponding half-lives were observed to be 4.49 and 4.95 days. The mole ratios of 3-PBA/fenpropathrin were always less than 1. There were no apparent differences in the farming operations during the two consecutive years, but different 3-PBA/fenpropathrin mole ratios were measured in the produce. Growth dilution might have influenced the dissipation of fenpropathrin in strawberries during those two years in addition to physical and chemical factors such as light, heat, pH, and moisture.

Implications of Preformed Biomarkers for Biomonitoring. Our produce study revealed that potential human urine biomarkers of pesticide exposure that may be used to reconstruct pesticide dose were present in every produce sample. A total of 134 of 157 (85%) samples that contained trace residues of malathion included more potential human urine biomarkers than parent pesticide. As indicated in Tables

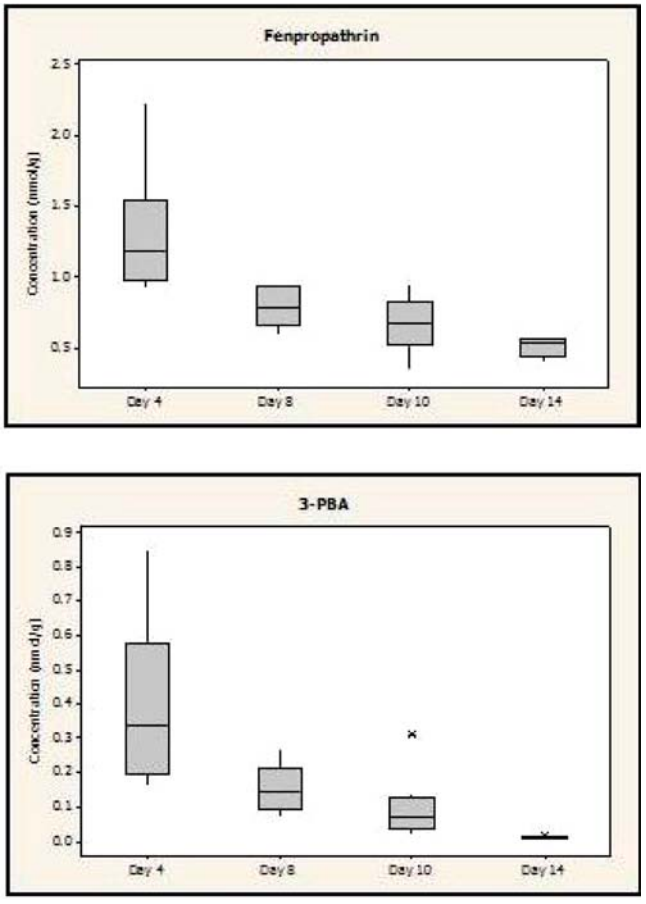

Figure 1. Fenpropathrin and 3-PBA in strawberries from day 4 to day 14 under field conditions, 2010.

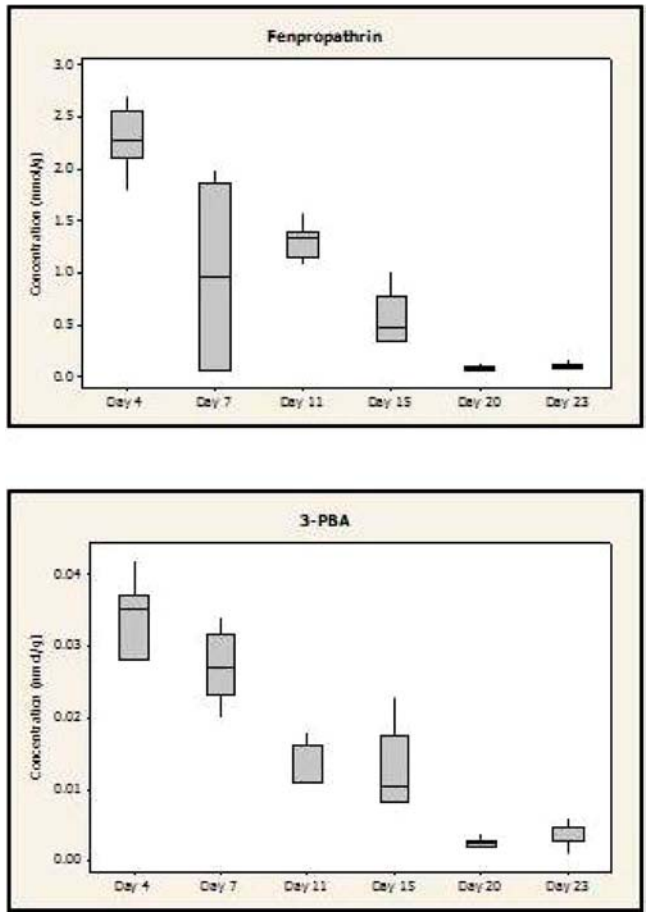

Figure 2. Fenpropathrin and 3-PBA in strawberries from day 4 to day 23 under field conditions, 2011.

2, 5, 6, and 7, both the mole fraction of preformed biomarkers and the mole ratio of preformed biomarkers to parent compound were greater for malathion than for captan (Table 7) and the pyrethroids (Tables 5 and 6). As shown in Tables 3 and 4, the mole fractions of preformed biomarkers in various produce were more similar to each other than were their respective mole ratios of preformed biomarkers. Caution must be exercised in assigning significance to these differences based upon the potential influence of sampling time on estimation of mole fraction (Tables 5 and 6) and mole ratio (Table 8) of biomarkers to pesticide.

Both malathion and fenpropathrin field studies conducted under commercial production conditions clearly demonstrate important time-dependent changes of biomarkers in fruit that could confound human exposure biomonitoring. This report primarily concerns the fate of malathion in strawberries under field conditions, the occurrence of its detoxification products in selected fruits and vegetables from the channels of trade in California, and the potential of those residues to confound human exposure assessment. Competing abiotic and biotic processes, particularly hydrolysis, are indicative of the time-dependent complexity of malathion dissipation in plants. Compound-specific dissipation of malathion yields MMA and MDA, initially the most abundant residues in strawberries. However, both malathion acids form DMP, DMTP, and DMDTP in both strawberries and in humans. These transformations have been studied in detail in strawberries under field conditions, and additional results are included for 157 other produce samples representing 19 types of fruits and vegetables where similar patterns of malathion dissipation have been observed. An overarching conclusion of these studies is that the exposure potential of malathion biomarkers is much greater than the exposure potential of malathion itself due to the persistence of the degradates in strawberries. Accordingly, use of the malathion acids or the dialkylphosphates as quantitative biomarkers of malathion exposure requires specific knowledge of the relative abundance of parent OP insecticide in any environmental or food matrix considered a source of exposure for risk assessment. Other environmental matrices, including dust, indoor surfaces, surface water used for drinking, and worker clothing, that do not present the opportunity for temporal studies such as those reported here must be regarded as potential sources of both insecticide and potential human urine biomarkers exposure.

CDC[3] reported that general population exposure to OP insecticides may occur by ingesting contaminated food and from hand-to-mouth contact with surfaces containing OP insecticides and the general population may be exposed to pyrethroid insecticides primarily from the ingestion of food or from residential use. However, pesticides can be metabolized by plants or degrade in the environment, leading to the presence of preformed derivatives in food and environmental media. Thus, urinary biomarker levels may represent exposure to parent pesticides and to preformed derivatives from food and the environment. Direct back-calculation of parent pesticide exposure from urinary biomarkers will lead to overestimation

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and misclassification of exposure for risk assessments and epidemiologic studies, particularly in low level exposures in nonoccupational settings. Morgan et al.[22] suggested that surface contamination by the specific chlorpyrifos biomarker TCPy (3,5,6-trichloro-2-pyridinol) may have made unmeasured contributions to children's exposure following previous residential or day care center insecticide use. Later observational studies in a cohort of Ohio children resulted in the conclusion that TCPy and 2-isopropyl-6-methyl-4-pyrimidinol, the specific biomarker of diazinon, may have been directly absorbed and excreted.[23] We suspect that when similar conditions occur in agriculture, surface residues of biomarkers may contribute to the extent of apparent pesticide exposure of hand harvesters.

Thus, in human exposure, MMA, MDA, and DAPs may come from different sources after malathion or other OP application: they can be from the metabolism of OPs in the human body, from the metabolites in plants used for food, and from the decomposition process of parent compounds in the environment. The presence of 3-PBA in urine not only reflects the dissipation of any of the six pyrethroid pesticides (cyhalothrin, cypermethrin, deltamethrin, fenpropathrin, permethrin, and tralomethrin) but also can reflect direct exposure to 3-PBA formed in produce from the degradation of these pesticides.[29−32] Similarly, THPI will be ingested with captan in food and eliminated from the human body in the urine along with the THPI produced from captan metabolism in humans.

In conclusion, the presence of biomarkers in urine could result from parent compound metabolism in humans or from preformed biomarkers in the diet or other sources. All preformed biomarkers represent false positives for exposure to the parent compound. When MMA, MDA, DMP, DMTP, DMDTP, 3-PBA, and THPI are used as the biomarkers of pesticide exposure, caution should be taken.

■ ASSOCIATED CONTENT

Supporting Information

Design and results of experimental studies to optimize the analyses of biomarkers. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Notes

The authors declare no competing financial interest.

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