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Temporal variability of urinary levels of nonpersistent insecticides in adult men

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Widespread application of contemporary-use insecticides results in low-level exposure for a majority of the population through a variety of pathways. Urinary insecticide biomarkers account for all exposure pathways, but failure to account for temporal within-subject variability of urinary levels can lead to exposure misclassification. To examine temporal variability in urinary markers of contemporary-use insecticides, nine repeated urine samples were collected over 3 months from 10 men participating in an ongoing study of male reproductive health. These 90 samples were analyzed for urinary metabolites of chlorpyrifos (3,5,6-trichloro-2-pyridinol (TCPY)) and carbaryl (1-naphthol (1N)). Volume- based (unadjusted), as well as creatinine (CRE)- and specific gravity (SG)-adjusted concentrations were measured. TCPY had low reliability with an intraclass correlation coefficient between 0.15 and 0.21, while 1N was moderately reliable with an intraclass correlation coefficient between 0.55 and 0.61. When the 10 men were divided into tertiles based on 3-month geometric mean TCPY and 1N levels, a single urine sample performed adequately in classifying a subject into the highest or lowest exposure tertiles. Sensitivity and specificity ranged from 0.44 to 0.84 for TCPY and from 0.56 to 0.89 for 1N. Some differences in the results between unadjusted metabolite concentrations and concentrations adjusted for CRE and SG were observed. Questionnaires were used to assess diet in the 24 h preceding the collection of each urine sample. In mixed-effects models, TCPY was significantly associated with season as well as with consuming grapes and cheese, while 1N levels were associated with consuming strawberries. In conclusion, although a single sample adequately predicted longer-term average exposure, a second sample collected at least 1 month following the first sample would reduce exposure measurement error.
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Introduction

The annual domestic use of insecticides in the US is an estimated 129 million pounds of active ingredients (EPA, 1999). The widespread application of insecticides in both agricultural and residential settings makes low-level environmental exposures to these compounds unavoidable for most people (Hill et al., 1995). As a result of regulatory action over the past few decades, nonpersistent (also known as contemporary-use) insecticides are now the chemicals most frequently applied to crops, lawns, gardens, and inside homes for pest control. The majority of contemporary-use insecticides fall into the general classes of organophosphates, carbamates, and pyrethroids. Since the effects of widespread, low-dose exposure are unclear for most of these compounds, they are currently the focus of epidemiology research. Accurately measuring human exposure to nonpersistent insecticides is central in any study investigating environmental exposures and their association with potential adverse health effects.

Insecticides can enter the human body through inhalation of air and dust, ingestion of foods and liquids, nondietary ingestion, or dermal contact (Barr et al., 1999). Since it is difficult to accurately assess external exposure and uptake through all routes, biomonitoring is a useful indicator of internal dose integrating the various routes through which the contaminant enters the body. Contemporary-use insecticides do not circulate in the bloodstream for extended periods of time nor do they generally accumulate in tissues. They are metabolized rapidly (Nolan et al., 1984; Fenske and Elkner, 1990; Griffin et al., 1999; Krieger et al., 2001) and the more

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Abbreviations: CDC, Centers for Disease Control and Prevention; CRE, creatinine; EPA, US Environment Protection Agency; FDA, Food and Drug Administration; ICC, intraclass correlation coefficient; NHANES, National Health and Nutrition Examination Survey; NHEXAS, National Human Exposure Assessment Study; NWS, National Weather Service; RSD, relative standard deviation; SG, specific gravity; TCPY, 3,5,6-trichloro-2-pyridinol; USDA, U.S. Department of Agriculture; 1N, 1-naphthol
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polar metabolites are excreted in the urine (Barr et al., 1999). Urine is often the preferred matrix for pesticide measurements because its collection is noninvasive and the excreted metabolite concentrations can be easily measured. These qualities also make biomonitoring in urine a simple and useful way to assess individual exposures in epidemiology studies. Metabolites of specific organophosphorus and carbamate insecticides have been measured throughout the US population in reference-range studies (Kutz et al., 1992; Hill et al., 1995; MacIntosh et al., 1999; Adgate et al., 2001; Fenske et al., 2002; Berkowitz et al., 2003; CDC, 2003). Most recently, CDC (2003) reported measurable levels of urinary 3,5,6-trichloro-2-pyridinol (TCPY), a metabolite of chlorpyrifos and chlorpyrifos-methyl, and 1-naphthol (1N), a metabolite of carbaryl and naphthalene, in greater than 90% and 75% of people in the US, respectively, indicating environmental exposure to these compounds is common among the general population.

Since contemporary-use insecticides are rapidly metabolized and excreted by the human body, metabolite levels in urine reflect recent exposure from the past 1 to 2 days. It has been suggested that a single measure of a specific urinary insecticide metabolite may not be sufficient to characterize accurately the relative magnitude of a person's typical acute or chronic exposure to the respective parent compound (MacIntosh et al., 1999). The ability to accurately characterize the relative magnitude of exposure for subjects within a study cohort is dependent upon the between-subject variability relative to the amount of within-subject variability. If within-subject exposure variability is high compared to between-subject variability, it is likely that a single measure would not adequately reflect a subject's average exposure over time. The failure to characterize accurately relative magnitudes of exposure leads to increased exposure measurement error and misclassification, which can limit the ability to detect an association between exposure and outcome in an epidemiology study. Characterizing the extent and nature of within- and between-subject variability may also lead to the identification of significant exposure sources, which could direct or enhance exposure controls and risk management.

Variability in a person's exposure to insecticides over time can result from specific insecticide application events, alterations in dietary intake, changes in activity patterns, and/or changes in insecticide levels within relevant microenvironments. These variables may be associated with region, climate, season, or day of the week and may give rise to exposure misclassification. In most epidemiology studies, cost, subject participation, and other issues may limit the investigator's ability to measure temporal variability of nonpersistent compounds. Therefore, the present study was designed to assess the temporal variability of contemporary-use insecticides and to determine how well a single urine sample, which is to be used in a subsequent study of male reproductive health, predicts a subject's 3-month average exposure to insecticides. A secondary goal was to utilize repeated urine and questionnaire data to investigate activities or foods associated with increased insecticide metabolite levels.

Methods

A convenience sample of 10 men from an ongoing study of the relationship between environmental agents and male reproductive health agreed to participate in the variability study. Participant recruitment into the male reproductive health study has been previously described (Hauser et al., 2003). Briefly, the study consisted of 370 men aged 20–52 years who are partners in couples seeking fertility evaluation for inability to conceive, and each man provided a single spot urine and semen sample. The study site is the Massachusetts General Hospital Andrology Laboratory and therefore most men reside in the New England area. For the variability study, men were approached and selected independently of study factors (insecticide exposure, smoking status, age, season, semen quality, etc.) from December 2001 to June 2003. All 10 men were nonsmokers and reported no occupational exposure to pesticides.

Each of the 10 variability study subjects collected an additional nine spot (first morning void) urine samples over a 3-month period (93 days). Therefore, these 10 men contributed a total of 10 urine samples (nine for the variability study and one for the male reproductive health study). The time period chosen was 3 months over which urine samples were collected because it is the approximate duration of spermatogenesis. The nine variability samples were taken over three cycles, each cycle consisted of a spot urine sample from each of 3 consecutive days. The first cycle began at enrollment into the study (days 1–3), the second cycle 30 days after enrollment (days 31–33 after enrollment), and the third cycle 90 days following enrollment (days 91–93 after enrollment). Most cycles (87% of the 90 samples) fell on a weekday as opposed to a Saturday or Sunday.

The 10 variability subjects also completed questionnaires on residential use of insecticides and dietary intake of specific foods for the 24 h preceding each of the nine urine samples. Participants were asked about insecticide storage in their home as well as application of insecticides indoors or outdoors on lawns or gardens. Foods appearing on the questionnaire were chosen from national data and represent specific produce items that potentially contain significant levels of insecticide residues. These food items include wine, apples, grapes, strawberries, grape juice, peanuts, peanut butter, tomatoes, spinach, cherries, raisins, sprouts, collards, and celery. Other meat and dairy food items were included in the questionnaire from a concurrent investigation of phthalate exposure. Subjects were asked, yes or no, if they had eaten each of these items in the past 24 h.

Subjects were instructed to freeze urine samples immediately following collection. Frozen urine samples were sent to the Center for Disease Control and Prevention (CDC) where TCPY and 1N were measured. Analytical methods for measuring these metabolites in urine have been developed by CDC and used in previous studies (Hill et al., 1995; CDC, 2003; Bravo et al., 2004). Briefly, samples were fortified with stable isotope analogues of the target analytes and glucuronide or sulfate-bound metabolites were liberated using an enzyme hydrolysis. TCPY and 1N were isolated using liquid–liquid extraction, chemically derivatized, and measured using gas chromatography–chemical ionization–tandem mass spectrometry. Recoveries determined at 6, 20 and 100 μg/l for 1N ranged from 88% to 93% with a relative standard deviation (RSD) of 2–4%. Recoveries determined at the same concentrations for TCPY ranged from 94% to 95% with RSDs of 2–3%. The overall precision of the method as determined by the RSDs of two fortification levels of quality control materials (i.e., fortified and characterized matrix samples) over a minimum 3-month period were 10.5% and 8.5% for 1N and TCPY, respectively.

Urine metabolite data were expressed as volume-based concentrations (micrograms of analyte per liter of urine), creatinine (CRE)-adjusted concentrations (micrograms of analyte per gram of creatinine), as well as concentrations adjusted for specific gravity (SG). CRE and SG are commonly used to account for variability in urine dilution of spot samples (Teass et al., 1998). CRE was measured photometrically using kinetic colorimetric assay technology with a Hitachi 911 automated chemistry analyzer (Roche Diagnostics, Indianapolis, IN, USA). Specific gravity was measured using a handheld refractometer (National Instrument Company, Inc., Baltimore, MD, USA), which was calibrated with deionized water before each measurement.

Statistical Analysis

Volume-based (unadjusted), CRE-adjusted, and SG-adjusted metabolite concentration data were used in statistical analyses. For values below the limit of detection (LOD), corresponding to 0.25 μg/l for TCPY and 0.40 μg/l for 1N, an imputed value equal to one-half the LOD was used. Descriptive statistics and distributions of individual insecticide metabolites were tabulated. Graphs were constructed to compare metabolite levels within and between subjects. Spearman correlation coefficients were calculated to investigate correlations among the target analytes. Spearman correlation coefficients were also calculated between sample times across all subjects for each metabolite to determine if samples collected closer together in time (i.e., days) are more strongly correlated than samples collected over longer intervals (i.e., months).

To assess between- and within-person variability of metabolite levels, intraclass correlation coefficients (ICC) were calculated for each metabolite. Calculations were based on output from a random effects model fit using PROC MIXED in the SAS version 8 (Rosner, 1999). ICC is a measure of reliability of repeated measures over time, defined as the ratio of between-subject variance to total variance. ICC ranges from 0 to 1, with values near one indicating high reliability and values near zero indicating poor reliability. ICC can also be used in an internal validation study to account for measurement error in epidemiology effect estimates (Rosner et al., 1992; Carroll et al., 1995).

To investigate sources of within-subject variability, a hierarchical model was fit (using PROC MIXED in SAS) to apportion Cycle variance (variability between cycles 30, 60, and 90 days apart) and Day variance (variability between samples 1 or 2 days apart regardless of cycle) in addition to between-subject variability (Box et al., 1978). Nested within each of the three cycles there are three urine samples, collected on day 1, 2, and 3 of each cycle. Day variance is the variance in insecticide levels between samples 1 or 2 days apart, regardless of whether they were collected in cycle 1, 2, or 3. Cycle variance is the variance between the three cycles, also described as the variance due to cycle after accounting for Day variances. Replicate laboratory analyses were performed on 15 samples, and results were included in the statistical analysis to assess analytical variability. For a more robust estimate of between-subject variability, the 370 single urine samples from the ongoing male reproductive health study were also used in the analysis of variance apportionment. Since the 10 men in the variability study were recruited from the semen quality study cohort, 10 of the 370 additional samples served as a 10th urine sample for the variability subjects and contributed additional variability information.

While ICC is an indicator of reliability for continuous measures, it does not quantify how much exposure misclassification may occur if subjects are categorized into exposure groups (i.e., tertiles of low, medium, and high exposure). For categorical data analysis, tertiles were created using the repeated samples from the 10 men. Creating tertiles using data from the 370 single urine samples from the male reproductive health study was considered, but produced an unbalanced and unstable design since some of these tertiles contained zero men with repeated urine samples and noncalculable results. Therefore, analyses using tertiles based on the 370 single urine samples were not presented.

Geometric mean values for surrogate categories were calculated to show the quantitative differences in metabolite levels that correspond to the relative categories defined by a single urine sample (Willett, 1998). Variability subjects were first grouped into tertiles using a single spot urine sample (the surrogate method). The ''true value'' for the same subjects based on their 3-month geometric mean metabolite levels (using the nine repeated samples) was then assigned to the tertiles defined by the single (surrogate) sample. Each of the nine samples was used as the surrogate sample in separate calculations to check for consistency. Each subject's 10th

sample from the male reproductive health study was not used in this analysis since it was collected up to 12 months earlier.

Sensitivity and specificity of a single urine sample as a predictor of high and low tertiles of 3-month average insecticide metabolite levels were also evaluated by comparing the distribution of predicted and observed levels for agreement. For observed or ''true'' exposure, 3-month average metabolite levels (using the nine repeated samples) were calculated for each subject and the subjects were divided into tertiles. The distribution of variability samples (10 subjects with nine repeated samples $=90$) was also then divided into tertiles, with each sample representing a predicted value based on a single spot urine sample. For each sample time (days 1–93), agreement between predicted and observed ''true'' tertile categorization was recorded across all subjects, resulting in nine separate contingency tables. All nine tables were then combined into a single table, where overall sensitivity and specificity was calculated (Peck et al., 2003). The same method was used to assess the amount of agreement if two or three samples were collected for each subject at least 1 month apart within a 3-month time period. When evaluating the sensitivity of two and three samples all possible combinations of sample pairings from the nine repeated samples, excluding samples from the same cycle, were used in the analysis. The goal was to simulate and compare the ability of exposure assessments that involve 1, 2, or 3 urine samples to predict a subject's ''true'' 3-month average exposure tertile classification.

A mixed-effects model was fit for each insecticide to determine which season, pesticide usage, and diet factors contributed to increased urinary metabolite levels while taking into account random (subject-specific) effects. Due to the large number of items on the questionnaire, variables that were marginally significant ($P<0.1$) in preliminary univariate mixed-effects models were retained in the multivariate model. Biological and statistical considerations were used to exclude nonpredictive variables from the final models.

Results

A total of 460 urine samples, consisting of 10 samples from each of the 10 men in the variability study and 360 single samples from men in the male reproductive health study, were analyzed for TCPY and 1N. Urine TCPY and 1N levels were above the limit of detection for greater than 93% and 99% of samples, respectively. For the 370 single samples, unadjusted TCPY levels ranged from less than the LOD of 0.25 to 32.2 μg/l, while 1N ranged from less than the LOD of 0.40 to 140 μg/l. Geometric means and distributions for unadjusted, CRE-adjusted, and SG-adjusted TCPY and 1N are presented in Table 1. TCPY and 1N concentrations were moderately correlated, with a Spearman correlation coefficient of 0.40 for unadjusted metabolite levels. Spearman correlation coefficients for CRE- and SG-adjusted data were 0.35 and 0.36, respectively. Spearman correlations between sample times across all 10 subjects demonstrated that for 1N, samples collected 1 to 2 days apart were more highly correlated (Spearman coefficients ranged from 0.32 to 0.81) than samples collected 1–3 months apart (Spearman coefficient ranged from -0.07 to 0.55). The pattern was not as clear for TCPY, where Spearman correlation coefficients ranged from 0.39 to 0.79 for samples collected 1 or 2 days apart compared to coefficients of -0.27 to 0.82 for samples collected at least 1 month apart.

Results of the variance apportionment analyses are shown in Table 2. Between-subject variability makes up more than half of the total variability for unadjusted and adjusted 1N (which is equal to an ICC of 0.61, 0.57, and 0.55 for unadjusted, CRE-adjusted, and SG-adjusted 1N, respectively), while between-subject variability only accounts for about one-fifth of TCPY variance (ICC = 0.15, 0.21, 0.18, respectively). A high level of precision in the analytical technique for both metabolites is reflected by the extremely small Replicate variances (replicate samples had a calculated mean coefficient of variation of 5.6% for TCPY and 2.0%

Table 1. Distribution of insecticide metabolites measured in urine among 370 men[a].

Insecticide metabolite[b,c]	Geometric mean	Selected percentiles						
		10th	25th	50th	75th	90th	95th	Max
Unadjusted (μg/l)								
TCPY	2.26	0.51	1.44	2.67	4.53	7.35	9.94	32.2
1N	2.79	0.94	1.61	2.87	4.50	7.54	12.7	140
Creatinine (μg/g)								
TCPY	1.94	0.51	1.18	2.28	3.63	5.62	7.30	35.1
1N	2.40	0.73	1.30	2.26	4.77	8.58	12.9	151
Specific gravity (μg/l)								
TCPY	2.74	0.73	1.70	3.32	5.15	8.30	10.7	40.7
1N	3.38	0.96	1.97	3.43	5.85	11.4	16.6	160

[a]370 single samples from male reproductive health study.
[b]TCPY = 3,5,6-trichloro-2-pyridinol; 1N = 1-naphthol.
[c]LOD = limit of detection; TCPY = 0.25 μg/l; 1N = 0.40 μg/l. 93% of samples > LOD for TCPY, and 99.7% of samples > LOD for 1N.

Table 2. Variance apportionment for between-subject and within-subject, which consists of Cycle, Day, and Replicate samples ($N = 475$)[a].

	Unadjusted (μg/l)			Creatinine-Adjusted (μg/g)			Specific Gravity (μg/l)		
	Variance estimate	Std. error of variance	% of total variance	Variance estimate	Std. error of variance	% of total variance	Variance estimate	Std. error of variance	% of total variance
ln(TCPY)									
Between-subject	0.20	0.12	15	0.21	0.12	21	0.21	0.14	18
Within-subject			85			79			82
Cycle[b]	0.58	0.15		0.50	0.13		0.59	0.15	
Day[c]	0.53	0.09		0.31	0.05		0.36	0.06	
Replicate[d]	0.006	0.002		0.005	0.002		0.005	0.002	
ln(1N)									
Between-subject	0.47	0.08	61	0.56	0.11	57	0.48	0.12	55
Within-subject			39			43			45
Cycle	0.12	0.06		0.08	0.07		0.15	0.09	
Day	0.18	0.03		0.34	0.07		0.24	0.04	
Replicate	0.0006	0.0002		0.0007	0.0003		0.0007	0.0003	

[a]N = number of samples; 10 subjects with 10 samples each; 360 subjects with a single sample, and 15 replicate samples.
[b]Cycle = variance between three cycles after accounting for nested day-to-day and replicate variances.
[c]Day = variance in insecticide levels between samples 1 or 2 days apart, regardless of whether they were collected in cycle 1, 2 or 3, after accounting for nested replicate sample variance.
[d]Replicate = variance of replicate samples (i.e., laboratory analysis repeated on a single urine sample).

for 1N). Thus, the laboratory analysis is not a significant source of within-subject variability. For both TCPY and 1N, Cycle and Day variances are on the same order of magnitude, which shows that both sources contribute to within-subject variability. For example, within-subject variability for unadjusted 1N consists of 60% Day variance (0.18/(0.18 + 0.12 + 0.0006)] and 40% Cycle variance. The variance apportionment analysis for CRE- and SG-adjusted data was repeated after removing samples that were highly concentrated or very dilute (31 of 460 samples with CRE less than 30 or greater than 300 mg/dl; 76 of 460 samples with SG less than 1.01 or greater than 1.03) (Teass et al., 1998). The results were similar as ICC estimates remained stable (all were within 3% of ICC estimates when values were included).

To further test the robustness of the variability data, the variance apportionment analysis was repeated for 1N after removing a single extreme value (the only 1N value in the variability data that was more than 10-fold above or below that subject's 3-month geometric mean value). There was a considerable increase in both between-subject variability and Cycle variability. For example, after removing the extreme value the unadjusted 1N between-subject variability accounted for 70% of total variability (ICC = 0.7) compared to 61% before the extreme value was removed. Cycle made up 61% of total within-subject variability compared to only 40% before the extreme value was removed.

To determine how well a single sample predicts categorical exposure (i.e., tertiles), geometric mean values for surrogate categories were calculated. The values presented in Table 3 showed that when a single sample was used to group subjects into low, medium, and high exposure groups, the true geometric mean metabolite levels increased across the exposure groups. For example, when using results from Day 1 as a surrogate to divide men into exposure tertiles, 3-month geometric means of unadjusted metabolite levels increased from 0.59 μg/l among the group designated as low exposure to 2.14 μg/l in the high exposure group for TCPY, and from 1.91 to 4.23 μg/l for 1N. Similar results were obtained when other single sample times were used to assign exposure groups for unadjusted metabolite levels. However, not all surrogate sample times resulted in an increasing trend in geometric means. Therefore, exposure misclassification may occur when only one urine sample is used to predict 3-month metabolite level. As compared to unadjusted and SG-adjusted TCPY and 1N values, the pattern of increasing geometric means across surrogate tertiles was not as consistent for CRE-adjusted TCPY and 1N values. However, the geometric mean for the highest CRE-adjusted TCPY and 1N tertile was usually greater than that of the lowest tertile.

For a more quantitative assessment of the predictive ability of a single urine sample, a sensitivity and specificity analysis was conducted. The proportion of men that truly had the highest 3-month average metabolite levels (top 33%) that would be identified as such using a single urine sample anytime throughout that 3-month period (i.e., sensitivity) was 0.63 for unadjusted TCPY and 0.56 for unadjusted 1N. The proportion of men with truly lower exposure (tertiles 2 and 3) that were classified correctly (i.e. specificity) was 0.84

Table 3. Values for surrogate exposure categories (low, medium, and high) comparing a single urine sample with 3-month geometric mean levels based on nine repeated samples from 10 subjects.

Surrogate sample day	Unadjusted (μg/l) geometric means			Creatinine-adjusted (μg/g) geometric means			Specific gravity (μg/l) geometric means		
	Low	Med	High	Low	Med	High	Low	Med	High
TCPY									
1	0.59	1.69	2.14	0.69	2.16	1.30	0.69	1.49	2.14
2	0.59	1.52	2.46	0.69	2.05	1.40	0.66	1.74	1.83
3	0.69	1.66	1.86	1.54	1.53	0.93	1.26	1.84	0.89
31	0.92	1.48	1.63	1.24	1.52	1.16	0.92	1.68	1.37
32	0.69	1.46	2.20	0.96	1.17	2.13	0.63	1.57	2.20
33	0.97	1.38	1.68	1.01	1.34	1.68	0.92	1.44	1.68
91	0.59	1.32	2.96	0.76	1.08	2.96	0.59	1.32	2.96
92	0.66	1.18	3.05	0.78	1.36	2.14	0.78	1.36	2.14
93	0.78	1.25	2.39	0.73	1.32	2.39	0.98	1.06	2.39
1N									
1	1.91	2.94	4.23	2.66	3.00	2.95	2.17	3.71	2.73
2	1.97	2.92	4.13	2.48	3.85	2.27	2.30	3.79	2.51
3	2.22	2.94	3.65	2.81	2.68	3.26	2.81	2.38	3.81
31	2.40	2.29	4.69	2.50	2.13	4.96	2.40	2.29	4.69
32	2.49	2.23	4.69	2.43	2.39	4.40	2.49	2.23	4.69
33	2.36	2.23	4.96	2.10	3.59	2.95	1.96	3.59	3.15
91	2.07	2.57	4.66	2.56	2.27	4.45	2.07	2.46	4.96
92	2.34	2.28	4.85	2.62	2.51	3.82	2.69	2.19	4.45
93	2.07	2.91	3.97	2.97	2.62	3.17	2.78	2.46	3.70

Table 4. Sensitivity for predicting men with highest 3-month geometric mean TCPY and 1N levels (top 33%) and specificity for predicting men with lowest (bottom 66%) 3-month geometric mean TCPY and 1N.

	Unadjusted (μg/l)		Creatinine-adjusted (μg/g)		Specific gravity (μg/l)	
	Sensitivity	Specificity	Sensitivity	Specificity	Sensitivity	Specificity
TCPY						
One sample	0.63	0.84	0.44	0.79	0.52	0.83
Two samples (at least 1month apart)	0.75	0.89	0.53	0.81	0.63	0.86
Three samples (each at least 1 month apart)	0.79	0.91	0.70	0.88	0.78	0.90
Three samples (3 consecutive days)	0.62	0.86	0.33	0.76	0.67	0.90
1N						
One sample	0.56	0.81	0.67	0.89	0.59	0.86
Two samples (at least 1month apart)	0.68	0.86	0.75	0.90	0.85	0.97
Three samples (each at least 1 month apart)	0.79	0.91	0.81	0.92	0.86	0.94
Three samples (3 consecutive days)	0.67	0.86	0.78	0.90	0.78	0.95

for unadjusted TCPY and 0.81 for unadjusted 1N. Alternatively, the sensitivity and specificity of single urine samples as an indicator of low exposure (bottom 33%) were 0.59 and 0.83, respectively, for unadjusted TCPY and 0.56 and 0.81, respectively, for unadjusted 1N. Comparisons from the sensitivity analyses for taking 1–3 samples are shown in Table 4. Unadjusted and adjusted TCPY and 1N all show a marked increase when going from one to two samples, which may reflect valuable information being added to measure the substantial Cycle variability described earlier in Table 2. Table 4 also shows that if three samples are collected on consecutive days they do not provide as much information on 3-month average metabolite levels as three, or possibly only two, samples that are collected at least 1 month apart.

Samples obtained from repeated days, coupled with data on explanatory variables that can change with time within an

individual allows for each subject to serve as his own comparison when investigating external factors that may influence urinary insecticide metabolite levels. Season was significantly associated with unadjusted and adjusted TCPY, but was not associated with 1N levels (Table 5). However, a subsequent analysis was conducted where samples collected during the six warmest months of the year in the Northeast were compared to samples collected during the six coldest months (NWS 2003). Results approached significance with higher 1N concentrations in the warm half of the year (*P*-values <0.1). Other variables that approached significance for increased metabolite levels in the single variable mixed-effects analysis included grapes and cheese for TCPY, compared to strawberries, tomatoes, and cheese for 1N. The lack of association between reported metabolite levels and insecticide storage or use may be due to storage and use of insecticides other than chlorpyrifos or carbaryl. This information was unavailable because the questionnaire did not ask about specific types of insecticides stored or used. In the multivariate analysis for unadjusted TCPY, season remained significant (*P*-value $= 0.02$) with elevated concentrations in the fall. Eating grapes (*P*-value $= 0.01$) and cheese (*P*-value $= 0.03$) remained significant dietary factors for increased TCPY levels. Samples taken in the warmest 6 months of the year remained a borderline significant predictor (*P*-value $= 0.08$) for increased unadjusted 1N concentrations, as did consumption of strawberries (*P*-value $= 0.07$) and cheese (*P*-value $= 0.05$). The consumption

Table 5. Geometric mean TCPY and 1N concentrations stratified by season pesticide use, or diet questionnaire data.

Variable	N[a]	N[b]	TCPY						1N					
			Unadjusted (μg/l)		Creatinine (μg/g)		Spec. gravity (μg/l)		Unadjusted (μg/l)		Creatinine (μg/g)		Spec. gravity (μg/l)	
			GMean	*P*-value[c]	GMean	*P*-value[c]	GMean	*P*-value[c]	GMean	*P*-value[c]	GMean	*P*-value[c]	GMean	*P*-value[c]
Overall	90	10	1.29	—	0.99	—	1.47	—	2.88	—	2.17	—	3.2	—
Season[d]														
Spring	42	9	1.29		0.91		1.38		2.97		2.1		3.19	
Summer	18	5	1.38		0.97		1.45		2.67		1.88		2.82	
Fall	6	2	1.74		1.33		2.17		3.26		2.48		4.06	
Winter	24	5	1.24	<0.01[e]	1.09	<0.01[e]	1.48	<0.01[e]	2.8	0.3	2.47	0.3	3.35	0.5
Pest. storage[f]														
No	64	7	1.09		0.93		1.28		2.98		2.53		3.5	
Yes	26	3	2.11	0.2	1.17	0.6	2.04	0.3	2.66	0.3	1.48	0.03	2.57	0.2
Pest. home[g]														
No	78	10	1.29		0.99		1.43		2.93		2.25		3.24	
Yes	9	2	1.29		0.82		1.46		2.48		1.59		2.81	
Not sure	3	1	2.51	0.5	1.88	0.4	2.92	0.5	2.93	0.9	2.2	0.9	3.42	0.8
Pest. lawn[h]														
No	71	9	1.12		0.89		1.29		2.81		2.24		3.23	
Yes	10	2	2.73		1.45		2.4		3.02		1.6		2.65	
Not sure	9	3	2.15	0.6	1.55	0.4	2.35	0.4	3.33	1.0	2.39	1.0	3.64	0.9
Strawberry														
No	81	10	1.22		0.95		1.39		2.7		2.09		3.06	
Yes	9	4	2.65	0.6	1.54	0.5	2.41	0.6	5.23	0.05	3.03	0.1	4.75	0.09
Grape														
No	78	10	1.18		0.9		1.32		2.94		2.26		3.32	
Yes	12	6	2.81	0.02	1.82	0.03	2.83	0.02	2.53	0.7	1.64	0.1	2.55	0.3
Tomato														
No	45	8	1.28		0.93		1.36		2.77		2		2.95	
Yes	45	8	1.36	0.9	1.07	1.0	1.58	0.9	3	0.04	2.35	0.2	3.48	0.07
Cheese														
No	45	8	0.82		0.75		0.97		2.68		2.42		3.14	
Yes	45	8	2.11	<0.01	1.32	0.01	2.22	<0.01	3.1	0.04	1.94	0.4	3.26	0.2

[a] n = Total number of samples in each questionnaire category.
[b] N = Number of subjects with at least one response (day) in that category.
[c] Test for fixed effects using log-transformed data in mixed models with random subject effects.
[d] Spring: March 21–June 20; Summer: June 21–Sept 20; Fall: Sept 21–Dec 20; Winter: Dec 21–March 20.
[e] Fall Significantly higher than winter, spring and summer.
[f] Question, "Do you currently have pesticides stored in your home?".
[g] Question, "Have you recently applied pesticides inside of your home?".
[h] Question, "Have you applied pesticides to your lawn, garden, or other outdoor location?".

of tomatoes was no longer associated with 1N levels (P-value >0.1) and the variable was removed from the final model.

For CRE and SG-adjusted TCPY levels, the final models included season (P-values <0.05), grapes (P-values <0.05), and cheese (P-values <0.05). The final models for CRE- and SG-adjusted 1N included only strawberries (P-values <0.05) and grapes. Although grapes were not associated with adjusted 1N levels, they were confounders and thus remained in both of the models. In these 1N models, the warmest 6 months of the year, tomatoes, and cheese became non-significant (P-values >0.1) and were removed from the final models.

Discussion

The present study was designed to assess between- and within-subject variability of urinary insecticide metabolite levels in men. We also explored the relationship between a single spot urine sample and the 3-month average insecticide metabolite level calculated from nine urine samples. The exposure duration of interest was chosen because these data are part of an ongoing study of environmental chemicals and male reproductive health, and spermatogenesis is a cyclical process taking approximately 3 months. Several different techniques were used to assess temporal variability in exposure as both a continuous and categorical variable. Results from the present study will be used in the ongoing male reproductive health study to correct for measurement error in the effect estimates of environmental exposures to contemporary-use insecticides. The findings from this variability study may also be pertinent to other end points with relevant exposure periods of months, such as pregnancy.

Urinary metabolite levels found in this study were similar to reference ranges measured in representative subsets of the US population (CDC, 2003) and were detected in a similar percentage of samples. Comparing the present data with concentrations measured in males for the National Health and Nutrition Examination Survey (NHANES) during 1999–2000 (CDC, 2003), the median of unadjusted TCPY levels was slightly higher in this study with a value of 2.7 *versus* 1.9 μg/l from NHANES 1999–2000, while the 90th and 95th percentile values were nearly identical (7.4 and 9.9 μg/l, respectively, compared to 7.3 and 9.9 μg/l from NHANES 1999–2000). A similar pattern was seen when comparing CRE-adjusted TCPY distributions. For 1N, both unadjusted and CRE-adjusted distributions were higher in this study. The median and 95th percentile unadjusted 1N levels were 2.9 and 12.7 μg/l, respectively, compared to 1.4 and 11.0 μg/l, respectively, from NHANES 1999–2000.

MacIntosh et al. (1999) reported longitudinal data collected in Maryland as part of the U.S. Environmental Protection Agency's National Human Exposure Assessment Study (NHEXAS Maryland) and concluded that a single measure of urinary TCPY and 1N was not sufficient to accurately characterize the relative magnitude of a person's typical acute or chronic exposure to the respective parent compounds. Our results partially support this observation. Within-subject TCPY variability was large (ICC ranged from 0.15 to 0.21 for unadjusted and adjusted results). However, the intraclass correlation coefficient for 1N ranged from 0.55 to 0.61 for unadjusted and adjusted samples, showing a moderate level of reliability. Although within-subject variability was large for TCPY and moderate for 1N, our data showed that a single urine sample performed adequately in classifying a subject into the highest or lowest exposure tertiles. Sensitivity and specificity ranged from 0.44 to 0.84 for TCPY and from 0.56 to 0.89 for 1N.

It is possible that the sensitivities and specificities calculated may be overestimated to a small degree because predicted values were included in the calculation of the observed values. Therefore, the errors of the predicted and observed values are not independent, which can lead to an overestimation of sensitivity and specificity (Willett, 1998). Furthermore, a portion of the increased sensitivity and specificity when two or three samples per subject were used instead of a single sample may be partly due to the increased dependence between the errors of the predicted and observed values.

Apportioning the sources of variability in urine metabolite levels can be used to design more valid and efficient exposure assessments. Both Cycle and Day variances were shown to contribute substantially to overall within-subject variability. Differences between the degree of Cycle and Day variability may result from absorption and metabolic properties of the insecticides, seasonal effects such as alterations in diet, pesticide use or activity patterns, or other environmental or biological factors. Daily and monthly variability patterns have been found in other studies. High within-subject variability for TCPY and 1N between weekly cycles over the course of a year was demonstrated in NHEXAS Maryland (MacIntosh et al., 1999). On the other hand, Adgate et al. (2001) reported between-subject variability was significantly greater than within-subject variability for TCPY and 1N in Minnesota children based upon spot samples collected on days 3, 5, and 7 within 1 week. In our study, the large contribution of Cycle variability to total within-subject variability suggests that a second urine sample taken at least a month after the first sample may substantially reduce the potential for random exposure misclassification. Using the earlier example from Table 2, if an investigator collected two samples on each subject to estimate unadjusted 1N levels, collecting them 1 day apart would only measure 60% [0.18/(0.18 + 0.12 + 0.0006)] of total within-subject variability, whereas two samples taken at least a month apart would account for both Day and Cycle variability, or 100% of within-subject variability. The lack of information gained from obtaining multiple samples close together in time was shown by the lower sensitivity when three samples were

collected on consecutive days compared to either two or three samples collected at least a month apart (Table 4). For 1N, the sizeable contribution of between-subject variability suggests that it may be more important to recruit more subjects with a single urine sample rather than applying resources toward obtaining repeated samples from the same subjects.

Apart from direct exposure to an insecticide application event, the majority of environmental exposure to insecticides occurs through inhalation of indoor air and/or ingestion of residues in food (Buck et al., 2001; Pang et al., 2002). Although chlorpyrifos and carbaryl are metabolized rapidly, the high sensitivity for a single urine sample to predict 3-month average exposure may reflect consistent activity and dietary patterns within an individual over time relative to other individuals. A person's exposure level from sources other than diet may be stable if they are in the same microenvironments for the same amount of time each day over the course of months and if concentrations of the contaminant of interest in those microenvironments remain consistent over that same period. Although chlorpyrifos and carbaryl are defined as nonpersistent, they have been shown to persist for longer periods of time indoors where they are protected from moisture, sunlight, temperature extremes, and most microbial activity (Wright et al., 1994; Berger-Preiss et al., 1997; Schenk et al., 1997; Lewis, 2000; Camann et al., 2002). Thus, concentrations of common household insecticides are generally higher indoors than outdoors (Whitmore et al., 1994; Gordon et al., 1999; Mukerjee et al., 1997) and may remain relatively stable indoors over extended periods of time. Most men spend a majority of their time indoors at work or at home, although it may vary by season, day of week, and by individual (Echols et al., 1999). A diet consisting of similar foods over time within an individual could also explain the high sensitivity seen in this study. In the US and other industrialized countries, day-to-day variability in diet may contribute substantially to total variability of nutrient intake (Beaton et al., 1979) but month-to-month or seasonal variation in diet may not be large (Van Staveren et al., 1986; Willett, 1998). In one study, the correlations between 1-week diet records did not vary appreciably when they were collected at intervals of 3, 6, 9, or 12 months (Willett et al., 1985).

A secondary aim of this variability study was to assess factors that may influence an individual's urinary metabolite levels. The seasonal variability in TCPY levels demonstrated here was not unexpected. Inhalation of indoor air contributed to a substantial portion of overall chlorpyrifos exposure among the general US population (Buckley et al., 1997; Buck et al., 2001; Pang et al., 2002). Concentrations of chlorpyrifos in indoor air have been shown to be higher in summer than in winter (Whitmore et al., 1994; Mukerjee et al., 1997), likely due to an increased need for residential and agricultural pest control, and thus increased insecticide application, in warmer months. For semivolatile insecticides such as chlorpyrifos, higher temperatures in warm months also allow for more volatilization, increasing the potential for inhalation exposure in the vapor phase even if an application event has not occurred recently. A recent study of pregnant women in an urban environment did not find any seasonal variation of TCPY based on single urine samples from 386 women (Berkowitz et al., 2003). However, with repeated samples within subjects in six cycles over the course of a year, seasonal differences in urinary TCPY were found in NHEXAS Maryland (MacIntosh et al., 1999). A community exposure study in Texas also found significantly higher TCPY levels in summer than in spring (Buckley et al., 1997). In addition, higher levels of chlorpyrifos residues were found on solid food samples in spring and summer compared to other times of the year (MacIntosh et al., 2001). With the US Environmental Protection Agency's recent ban of residential chlorpyrifos use in 2000, diet may contribute more to total chlorpyrifos exposure, and thus urinary TCPY levels, compared to inhalation exposure shown in previous studies. In the present study, TCPY levels were highest in autumn and not in summer as expected. However, because only six of 90 variability samples (two subjects had one cycle each during these months) were obtained in the fall these results may not represent typical TCPY levels found in those months relative to other times of the year.

Neither NHEXAS Maryland nor the Texas study found seasonal variation in 1N levels (Buckley et al., 1997; MacIntosh et al., 1999). The seasonal differences (warmest 6 months *versus* coldest 6 months) in 1N in our variability study may suggest carbaryl is more likely than naphthalene to be the major parent compound of 1N among men in this cohort. Carbaryl is often applied residentially to lawns and gardens in the form of Sevin®, which would tend to be used more abundantly in warmer months in the New England area and lead to higher 1N concentrations in late spring and summer. Carbaryl is often not detectable in air (Whitmore et al., 1994; Whyatt et al., 2002; Mukerjee et al., 1997), so dietary ingestion is likely the primary exposure route unless a specific application event occurs in or around the home or other microenvironment. There are currently no known studies on seasonal variation of carbaryl residue on foods, but intake of some fruits and vegetables may vary substantially by time of year (Ziegler et al., 1987). In our study, it was difficult to definitively determine if carbaryl exposure was associated with specific application events in or around the home because the questionnaire did not address the use of specific insecticides. Within-subject 1N levels remained relatively stable over time, whereas more extensive variability would be expected from residential exposures resulting from application events. For example, insecticide concentrations within a residence can increase abruptly and by as much as 10-fold following application (Fenske and Elkner, 1990; Byrne et al., 1998; Lu and Fenske, 1998).

However, exposures stemming from adjacent carbaryl applications (i.e., a neighbor's lawn or garden) would likely result in more moderate seasonal differences in 1N concentrations.

More data are needed to more completely investigate the impact of dietary intake of insecticides on urinary metabolite concentrations. In addition to the parent compounds, TCPY and 1N could be ingested directly due to their presence as degradation products on foods (Wilson et al., 2003). In this study, ingestion of grapes was found to potentially influence chlorpyrifos metabolite levels in urine, and strawberries for carbaryl. Based on residue screening data from the US Department of Agriculture (USDA, 2000), chlorpyrifos was detected on 67 of 741 grape samples (9%) screened with values ranging up to 0.31 ppm. Carbaryl was detected in 17% and 27% of fresh and frozen strawberry samples screened, respectively, with concentrations on fresh strawberries as high as 4.4 ppm. The association between TCPY and cheese found in this study is not clear, but may warrant further exploration. In the Total Diet Study conducted by the US Food and Drug Administration, chlorpyrifos and carbaryl were not among the pesticides examined in cheese (FDA, 2003). Documentation of the presence of these compounds in cheese was not found elsewhere.

Using national residue data, a recent study demonstrated that a relatively small number of food items may contribute substantively to total ingestion exposure to various chemicals, including chlorpyrifos (Moschandreas et al., 2002). However, no relationship was observed between self-reported food consumption and TCPY and 1N levels among 978 adults in the US (Kieszak et al., 2002), possibly because the subjects were asked to recall food intake for the previous month rather than the previous 1–2 days, which would be more appropriate based on the rapid metabolism of the parent insecticides. Results from these two studies, along with findings in our variability study, suggest that associating diet with insecticide metabolite levels may be possible with an appropriate dietary exposure assessment method, a large enough cohort, and repeat measures.

For pesticides, the most common method of accounting for variation in dilution between spot urine samples has been to adjust metabolite levels using creatinine concentration. However, CRE levels vary by gender, age, muscle mass, race, diet, activity, and time of day. Furthermore, dilution adjustment with creatinine is not appropriate for compounds that undergo active tubular secretion, which includes organic compounds like TCPY and 1N that can be conjugated by the liver in the form of glucuronides or sulfates (Boeniger et al., 1993). Adjusting urine metabolite concentrations to specific gravity is susceptible to some of the same factors as creatinine, especially size, diet and sweating, but may decrease some variability associated with urine flow (Boeniger et al., 1993; Elkins et al., 1974). Results from the present study suggest temporal reliability of urine samples remain similar between unadjusted, CRE-adjusted, and SG-adjusted TCPY and 1N concentrations. Some minor differences of note are that for 1N, unadjusted levels displayed lower within-subject variability than CRE or SG-adjusted 1N, and also seemed to display more defined gradients between exposure groups in the surrogate category analysis where the geometric mean of the group designated as highest exposed was higher than each of the lower two groups when any of the nine sample days were used as the surrogate. The same assessment of the surrogate category analysis holds true for unadjusted TCPY compared to adjusted, though CRE-adjusted TCPY displayed the lowest amount of within-subject variability in Table 2.

In conclusion, insecticide metabolite levels from a single urine sample taken from each subject to reflect that subject's exposure over several months may be more predictive than originally believed. Although nondifferential exposure misclassification is likely to occur to some extent, a single measure may adequately designate relative exposure groups that represent true average exposure over a longer period of time. The degree of between- and within-subject variability, and thus potential for random misclassification, differs between specific insecticide metabolites signifying that the most efficient exposure assessment strategy for a particular study may depend on the insecticides of interest.

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