Available online at www.sciencedirect.com
SCIENCE DIRECT
Analytica Chimica Acta 572 (2006) 180–189
ANALYTICA CHIMICA ACTA
www.elsevier.com/locate/aca

Improved methods for urinary atrazine mercapturate analysis—Assessment of an enzyme-linked immunosorbent assay (ELISA) and a novel liquid chromatography–mass spectrometry (LC–MS) method utilizing online solid phase extraction (SPE)

Marja E. Koivunen[a,1], Katja Dettmer[a,2], Roel Vermeulen[b], Berit Bakke[b,c], Shirley J. Gee[a], Bruce D. Hammock[a,*]

[a] *Department of Entomology and the UC Davis Cancer Center, University of California, Davis, United States*
[b] *Division of Cancer Epidemiology and Genetics, National Cancer Institute, NIH, DHHS, Rockville, MD, United States*
[c] *National Institute of Occupational Health, Oslo, Norway*

Received 5 February 2006; received in revised form 8 May 2006; accepted 10 May 2006
Available online 16 May 2006

Abstract

Elimination of interfering substances in urine by solid phase extraction (SPE) prior to analysis resulted in 10-fold improvement in the sensitivity of atrazine mercapturate (AM) enzyme-linked immunosorbent assay (ELISA) compared to previous reports. Of the two tested SPE systems, Oasis® HLB and MCX, the mixed-mode MCX gave good recoveries (82%) of AM in spiked samples measured by ELISA, whereas the reverse-phase HLB phase was not compatible with the immunochemical method. At relatively high concentrations of urinary AM (>20 ng mL^{-1}), sample dilution was effective enough for the elimination of interfering substances. The new liquid chromatography–mass spectrometry (LC–MS) method developed for AM utilizes online-SPE with Oasis® HLB, column switching and a stable-isotope internal standard. The limit of quantification (0.05 ng mL^{-1}) indicates improved sensitivity compared with most previously published LC–MS methods for AM. Validation of all three methods, LC–MS, ELISA + SPE and ELISA + dilution with spiked urine samples showed good correlation between the known and measured concentrations with R^2 values of 0.996, 0.957 and 0.961, respectively. When a set ($n = 70$ plus 12 blind duplicates) of urine samples from farmers exposed to atrazine was analyzed, there was a good agreement ($R^2 = 0.917$) between the log normalized data obtained by ELISA + SPE and LC–MS. High correlation among the data obtained by the two tested methods and the LC–MS method by the Center of Disease Control and Prevention (CDC), together with low variability among the blind duplicates, suggests that both methods reported here would be suitable for the analysis of urinary AM as a biomarker for human exposure of atrazine.

Keywords: Atrazine mercapturate; Enzyme-linked immunosorbent assay (ELISA); Solid phase extraction (SPE); Online solid phase extraction; Liquid chromatography–mass spectrometry (LC–MS); Human biomonitoring

1. Introduction

Atrazine is one of the most widely used herbicides in the United States. Due to its fairly good mobility in soil, it is one of the main surface water contaminants in the Midwestern United States [1,2]. Atrazine has a low acute toxicity to humans but it has been implicated as a clastogen, an agent that causes chromosomal damage [3] and quite recently, atrazine at environmental concentrations has been reported to have adverse effects on the endocrine system of several animal models [4–7]. Despite its low acute toxicity to humans, atrazine may pose a health risk to humans, especially to agricultural workers through occupational exposure.

Due to its lipophilic nature and rapid metabolism, atrazine metabolites are more likely to be found in urine and feces than the parent compound [8,9]. A major urinary metabolite, atrazine mercapturate (AM; *N*-acetyl cysteine derivative of atrazine) [10]

* Corresponding author. Tel.: +1 530 752 7519; fax: +1 530 752 1537.
E-mail address: bdhammock@ucdavis.edu (B.D. Hammock).
[1] Present address: Antibodies Incorporated, P.O. Box 1560, Davis CA 95617, United States.
[2] Present address: Institute of Functional Genomics, University of Regensburg, Josef-Engert-Str. 9, 93053 Regensburg, Germany.

0003-2670/$ – see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.aca.2006.05.037

is quite stable and hence, has been suggested as a relevant biomarker for atrazine exposure in humans [11]. In epidemiological studies, low levels of atrazine metabolites are often detected using mass spectrometry coupled to gas chromatography [10,12,13] or liquid chromatography [10,11,14–18]. Although these methods can give excellent quantitative analytical results, they often require expensive instrumentation, extensive sample clean-up using organic solvents or derivatization. Immunochemical methods, on the other hand, are powerful tools for exposure analysis and assessment offering low-cost screening with minimal sample pretreatment requirements combined with high sensitivity and selectivity. An enzyme-linked immunosorbent assay (ELISA) for atrazine mercapturate was first developed in our laboratory by Lucas et al. [10]. The assay was based on a monoclonal antibody, and was able to detect AM down to 0.5 ng mL^{-1} in crude urine diluted to 25% with buffer. Similarly, an ELISA based on a polyclonal anti-AM antibody offered a limit of quantification of 0.3 ng mL^{-1} after a simple (1:4) sample dilution [19].

Attempts to analyze AM in urine by classical GC-MS techniques illustrate the exceptional difficulty of such an approach [10]. The molecule contains a variety of polar functional groups that normally require derivatization for successful GC analysis. Alternate strategies, such as HPLC combined with MS suffer from numerous endogenous biological substances present in urine, and sample clean-up or extraction is usually required before analysis. Similarly, endogenous compounds with structural similarities to AM can interfere with the immunochemical detection of AM by ELISA. In some cases, a simple sample dilution is enough to eliminate interfering substances for ELISA analysis [10,19]. However, this might in turn decrease the assay sensitivity and increase the limit of detection (LOD).

Solid phase extraction (SPE) has gradually replaced more tedious sample clean-up methods in the human exposure analysis. So far, only a few attempts have been made to extract urine with SPE for AM-ELISA. Lucas et al. [10] tested an acidic (phenyl) column to extract AM from urine with recoveries of spiked AM ranging from 77 to 110%. They also used affinity purification with a polyclonal antiserum against 2-hydroxy-4-(ethylamino)-s-triazine for the elimination of interfering substances. Jaeger et al. [19] tested three SPE systems for urine clean-up prior to ELISA analysis—C18, phenyl and a mixed-mode column, Certify II. Even with the best system, C18, structurally similar compounds were co-eluting with AM, and the authors concluded that SPE clean-up would only be worthwhile if a lower limit of quantification (LOQ) than one obtained by dilution (0.3 ng mL^{-1}) could be achieved. For epidemiological studies, in particular in the general population with trace amounts of urinary metabolites, high assay sensitivity is often required and hence, the study presented here aimed at improved sensitivity of AM-ELISA using different SPE pretreatment methods and comparing them with sample dilution. For data validation, a new HPLC–MS method using on-line SPE and column switching was developed for the analysis of urinary AM. Methods were further assessed and validated using a set of field urine samples collected in the National Cancer Institute (NCI) corn farming study [20], and the results obtained were compared with the ones from an liquid chromatography–mass spectroscopy (LC–MS) analysis performed by the Centers for Disease Control and Prevention (CDC).

2. Experimental

2.1. Chemicals and reagents

Atrazine mercapturic acid [*N*-acetyl-S-{4-(ethylamino)-6-[(1-methylethylamino]-1,3,5-triazin-2-yl}-L-cysteine] was prepared in the laboratory previously [10]. Ovalbumin, goat anti-rabbit IgG horseradish peroxidase conjugate, 3,3′,5,5′-tetramethylbenzidine (TMB) sodium salt and Tween 20 were from Sigma Chemical Co. (St. Louis, MO). All other chemicals were of reagent grade or better from Fisher Scientific (Pittsburgh, PA). The polyclonal sheep anti-AM antiserum (226) was prepared in the laboratory previously [19] and stored in a freezer in 1 mL aliquots. The coating antigen (7-BSA) was freshly synthesized using previously reported methods [19]. Ring-labeled ^{14}C (93%) atrazine mercapturate (25 μCi mg^{-1}) was a kind gift from Ciba Geigy (Basel, Switzerland), and the stable-isotope-labeled LC–MS internal standard, atrazine mercapturate-$^{13}C_3$-ring (99%) was purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA).

2.2. SPE cartridges and columns

Oasis® HLB cartridges 3 mL (60 mg) containing [poly-(divinylbenzene-co-*N*-vinylpyrrolidone)] and Oasis® MCX cartridges (3 mL 60 mg^{-1}) containing strong cation-exchange sulfonic groups on the surface of the HLB polymeric resin were purchased from Waters Corporation (Milford, MA).

2.3. Urine samples

2.3.1. Storage and preparation

Urine samples from non-exposed male volunteers were stored in polypropylene tubes in a refrigerator (+4 °C). The samples from NCI were received blind and numerically coded, and stored at −80 °C until the analysis. Frozen urine samples were thawed for 16 h at room temperature after which they were vortexed and centrifuged at 15 °C for 1.5 min at 2500 g, and the supernatant was transferred to an Eppendorf tube. The samples were then diluted using a Tecan Miniprep liquid handling system (Tecan Systems Inc., San Jose, CA) or extracted using the SPE protocols below.

2.3.2. SPE-clean-up

2.3.2.1. Reverse-phase sorbent—Oasis® HLB. The 3 mL (60 mg) cartridges were first equilibrated with 1 mL additions of MeOH containing 1% acetic acid. The sample (0.5 mL) was then mixed with 0.5 mL of 200 mM acetate buffer (pH 3.0) and extracted by vacuum aspiration through the SPE cartridge at a flow rate of approximately 2 mL min^{-1}. After a 1 mL addition of acetate buffer the cartridge was sequentially washed with 1 mL of 30% MeOH in 1% acetic acid and 1 mL of 40% MeOH. The cartridge was dried under vacuum for 5 min after

which AM was eluted with 1 mL 70% MeOH. The samples were evaporated to dryness using a Heto VR-1 centrifugal concentrator (Appropriate Technical Resources, Laurel, MD) and reconstituted in 0.5 mL phosphate-buffered saline (PBS, pH 7.4) before analysis.

2.3.2.2. Mixed-mode cation exchange sorbent—Oasis® MCX. For the urine clean-up, 3 mL (60 mg) Oasis® MCX sorbent cartridges (Waters, MA, USA) were equilibrated with 1 mL of MeOH then 1 mL of 100 mM sodium carbonate/bicarbonate buffer (SCB, pH 9.5). A 0.5 mL urine sample mixed with 0.5 mL of SCB buffer was extracted by vacuum aspiration through the SPE cartridge at a flow rate of approx. 2 mL min^{-1} and followed by another 1 mL of SCB. For removal of interfering substances the cartridge was then rinsed with 1 mL of 40% (v/v) methanol in 1% acetic acid in water after which the cartridge was dried under vacuum. Atrazine mercapturate was eluted from the cartridge using 1 mL of ice-cold 2% (v/v) NH_4OH in 100% MeOH. Fresh 2% NH_4OH in MeOH was prepared daily. Samples were collected in Eppendorf tubes and evaporated to dryness after which they were reconstituted with 1.0 mL of PBS. This 1:1 dilution was chosen in order to neutralize the sample.

2.4. ELISA

A solid phase indirect competitive ELISA [19] was used for measurement of AM in prepared samples. Briefly, Nunc Maxisorp 96-well plates (Nunc, Roskilde, Denmark) were coated overnight at 4 °C with the coating antigen 7-BSA prepared after Lucas et al., [10] (1/160,000 dilution, 0.07 μg mL^{-1}) in a 0.05 M sodium carbonate/bicarbonate buffer (pH 9.6). The following day, the coating antigen was washed off the plate with a phosphate-buffered saline solution containing 0.05% Tween 20 (PBST), pH 7.5 and the wells were blocked for 1 h with 100 μL 0.5% ovalbumin in phosphate buffered saline (PBS). After washing with an automatic plate washer (Bio-Tek Instruments, Inc., Winooski, VT), each sample or standard in triplicate were pipetted into each well (50 μL/well). The standard curve for AM was prepared from the frozen stock standard (10 μg mL^{-1} AM in MeOH) and contained 0–250 ng of AM in 1 mL PBST. Prior to loading onto the plate, samples were either diluted with PBST so that the concentration of AM would fit into the linear range of the standard curve or they were extracted using SPE clean-up procedures described previously. To each sample well 50 μL of the AM polyclonal antibody 226 [19] was added in 1/33,000 dilution, and the mixture was incubated at room temperature for 60 min. The sample matrix was washed away leaving only the antibodies bound to the coating antigen. Then, 100 μL of the anti-rabbit IgG-horseradish peroxidase conjugate (IgG-HRP, dilution 1:2750) was added into each well, and the IgG-HRP was allowed to bind to IgG sites on the bound antibodies for 60 min. Unbound IgG-HRP was washed away to leave an amount of HRP enzyme that was inversely proportional to the AM concentration in the samples or standards. Finally, a colorless substrate, 1% H_2O_2, and a chromogen, 3, 3′, 5, 5′ tetramethylbenzidine were incubated with the bound enzyme to produce a blue color. A 50 μL aliquot of 2 M H_2SO_4 was added as a stop solution to change the color to a stable yellow. ELISA absorbances at 450 nm were measured with a microplate reader (Molecular Devices, Menlo Park, CA). The content of AM in the unknown samples was calculated based on the standard curves in each plate. The software package Softmax (Molecular Devices) was used for fitting the 11-point sigmoidal standard curve based on a four-parameter logistic method of Rodbard [21].

2.5. LC–MS method

2.5.1. Internal standard

Ten calibration solutions containing AM in a range of 0.01–500 ng mL^{-1} in phosphate-buffered saline were prepared fresh from a stock AM standard of 10 μg mL^{-1} MeOH before each run. The concentration of the stable-isotope-labeled internal standard was kept constant at 10 ng mL^{-1}. Urine aliquots (95 μL) were spiked correspondingly with 5 μL internal standard (200 ng mL^{-1}) resulting in a concentration of 10 ng mL^{-1}. Acetonitrile of HPLC grade was purchased from Fisher Scientific (Pittsburgh, PA).

2.5.2. Online-SPE–HPLC–MS conditions

LC–MS analysis was performed using a Shimadzu ASP10 HPLC system equipped with a 10-port valve, a two-channel UV detector and a Quattro Premier tandem quadrupole mass spectrometer (Waters/Micromass, UK), HPLC–tandem-MS for short. The HPLC system consisted of three pumps. Pump A and B were used for binary high pressure gradient mixing and pump C was delivering an independent eluate solvent flow. Urine extraction was performed using an Oasis® HLB pre-column (20 mm × 2.1 mm i.d., Waters). Analytes were separated on a reversed phase HPLC column (Atlantis dC18, 3 μm C18-BD, 150 mm × 2.1 mm; Waters, Milford, MA) using gradient elution with a water–0.1% formic acid (solvent A)/acetonitrile–0.1% formic acid (solvent B) solvent system. A scheme of the analytical instrumentation with the online-SPE set up is shown in Fig. 1. In load position the sample was injected on the pre-column (Oasis® HLB) followed by a wash step to remove salts and other matrix components. The load and wash steps were performed using a flow rate of 0.495 mL min^{-1} to increase the speed of the sample clean-up step. The wash fluid was directed to waste while pump C delivers solvent (5 μL min^{-1} acetonitrile–0.1% formic acid) independently to the mass spectrometer to maintain a stable spray in the electrospray source during the sample extraction step. Subsequently, the valve was switched to elute position and the pre-column was eluted in reverse direction using solvent C (100% acetonitrile, 0.1% formic acid). Before entering the analytical reversed-phase column, the elution solvent was diluted with aqueous mobile phase to reduce the mobile-phase-strength and facilitate retention on the stationary phase. Valve switching events as well as the gradient and solvent flow program are summarized in Table 1. Using these parameters, a retention time of 10.6 min was obtained for AM. The samples were kept at 5 °C in the autosampler, and the injection volume was 10 μL.

Analytes were detected by electrospray ionization in positive mode—tandem quadrupole mass spectrometry in multiple

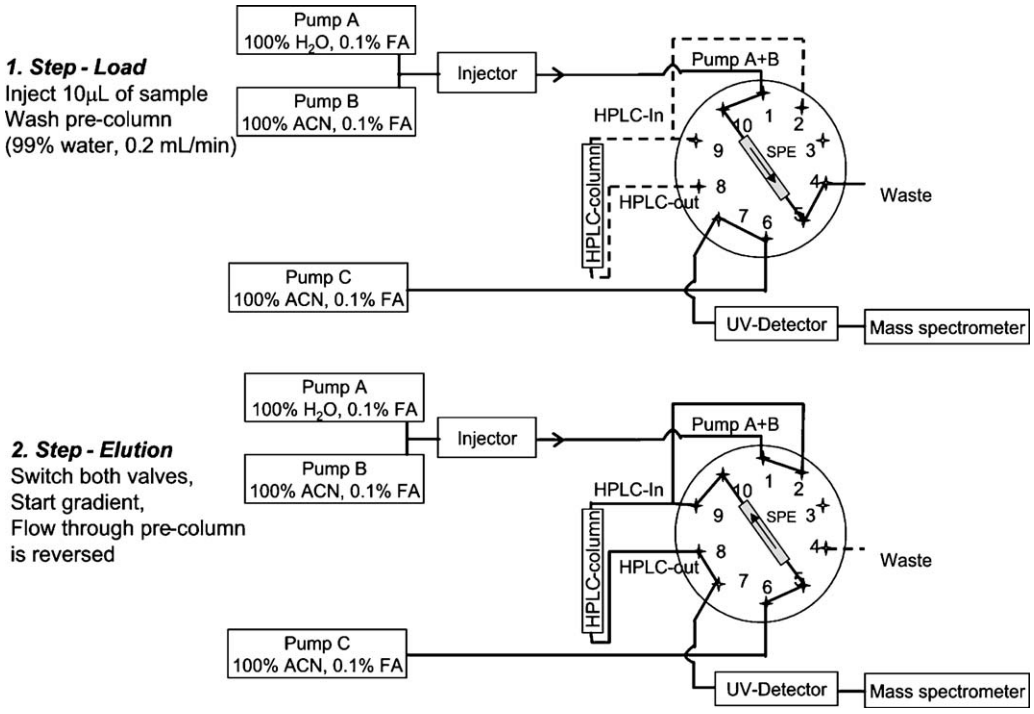

Fig. 1. Online-SPE–HPLC–MS set up used for the analysis of urinary atrazine mercapturate.

reaction-monitoring mode (MRM) using the HPLC–tandem-MS described earlier. Nitrogen gas flow rates were fixed with a cone gas flow of 50 L h^{-1} and a desolvation gas flow of 750 L h^{-1}. A source temperature of 125 °C and a desolvation temperature of 300 °C were applied. Electrospray ionization was performed in positive mode with a capillary voltage fixed at 2.00 kV. Tune parameters including capillary voltage, cone voltage and extraction cone were optimized in an infusion experiment (data not shown), and an extraction cone voltage of 5 V was selected. Offset values of 0.6 and 2.0 were used for the ion energies applied to quadrupole 1 and 2, respectively. Quadrupole resolution in MRM mode was set at an offset value of 13 resulting in a peak width at half-height of 1 Da. A multiplier voltage of 650 V was applied. Argon was used as collision gas with a flow rate of 0.23 mL min^{-1}. The MRM conditions used for detection of AM and the internal standard were optimized in an infusion experiment. A transition of 343.4 > 214.4 *m/z* for AM and 346.4 > 217.4 *m/z* for the internal standard was monitored using a cone voltage of 30 V and a collision voltage of 21 V.

2.6. Method validation using spiked and field samples

For method validation, a set ($n = 38$) of pooled urine samples spiked with known amounts of AM (0–70 ng mL^{-1}) were analyzed in a blind fashion using LC–MS with online-SPE and the results were compared with those obtained by ELISA after either sample dilution or SPE clean-up with Oasis® MCX. The unknown field urine samples from NCI ($n = 70$ plus 12 blind duplicates inserted for QC purposes) were analyzed by both the SPE + ELISA and the newly developed LC–MS method, and the results were compared with LC–MS analysis run by CDC using the following method by Olsson et al. [16].

Briefly, a 2 mL aliquot of urine was spiked with isotopically labeled standards, and then diluted with 1.5 mL of 0.2 M acetate buffer to which 800 activity units of β-glucuronidase/sulfatase had been added. The solution was allowed to incubate at 37 °C overnight to liberate glucuronide- and sulfate bound conjugates, and the hydrolysate was applied to an Oasis® HLB SPE cartridge. The SPE cartridge was washed with 1.5 mL methanol,

Table 1
HPLC conditions

Time (min)	Solvent A H_2O (%), 0.1% formic acid	Solvent B ACN (%), 0.1% formic acid	Solvent C ACN (%), 0.1% formic acid	Flow (mL min^{-1})	Valve position
0	98	1	1	0.50	A, Load sample
2.4	98	1	1	0.50	A, Wash
2.5	69	1	30	0.25	B, Elution
10.0	60	35	5	0.25	B, Elution
12.0–15.0	0	95	5	0.25	B, Elution
16.0–19.0	98	1	1		B, Equilibrate column
19.1–20.0	1	98	1	0.50	A, Wash pre-column
20.1–21.0	98	1	1	0.50	A, Equilibrate precolumn

the eluent was then diluted with 2 mL of acetonitrile, and evaporated to dryness. The residue was reconstituted in 50 μL acetonitrile. AM was then measured in the sample extract using HPLC–tandem-MS with atmospheric pressure chemical ionization. A multiple reaction monitoring experiment was used to isolate specific precursor and product ion pair. Calibration standards, quality control materials and blank samples were prepared and analyzed concurrently with unknown samples, and the concentrations of AM were calculated using isotope dilution quantification.

Based on probability plots, the results from the field sample set were best described by log normal distributions and therefore, data from this validation study was first ln-transformed before further analysis.

3. Results and discussion

3.1. Method development

3.1.1. Optimization of MS parameters

Selective reaction monitoring was chosen as the operating mode for the tandem mass spectrometer in order to develop a specific method for AM with low detection limits. First, the optimum cone voltage for the formation of the precursor ion during electrospray ionization was determined experimentally by infusing AM into the mass spectrometer. Being a zwitterion, AM ionizes in both positive and negative mode forming $[M+H]^+$ and $[M-H]^-$ ions, respectively. Further experiments were performed to determine the fragmentation behavior of these precursor ions and the corresponding optimum collision energy. Representative product ion spectra of the pseudo-molecular ions in positive and negative mode presented in Fig. 2, show one major fragment ion. Fragmentation occurs at the sulfur forming a dominant ion of the atrazine moiety while the mercapturate group is predominantly leaving as a neutral product. The dominant fragment ions seen in the product ion spectra were used to set up the MRM transition parameters, and a series of calibration solutions were analyzed with positive/negative mode switching to compare ionization with regards to instrument detection limits. Positive mode ionization was chosen for all further experiments because it provided about 50-fold lower detection limit than negative mode ionization (data not shown).

3.1.2. LC–MS calibration

Calibration curves were obtained based on chromatographic separation of the AM and peak calibration using the labeled ^{13}C-AM as internal standard. Calibration curves were generated using the peak area response. The instrumental detection limit at a signal to noise ratio of 3:1 was determined as 0.01 ng mL^{-1} and the limit of quantification as 0.05 ng mL^{-1}. The instrument response was linear in the tested range of 0.05–500 ng mL^{-1}, which was used as working range.

The calibration solutions were prepared in PBS buffer to mimic the urine matrix. Matrix calibration using blank urine was not performed because the urine matrix can be variable from subject to subject and a well-chosen internal standard can correct for potential matrix effects. The use of an isotopically (^{13}C)-labeled atrazine mercapturate as an internal standard for the new LC–MS method was preferable because of a similar mechanism of chromatography for both unlabeled and labeled compound allowing optimal correction for matrix effects caused by co-eluting components. Similar results were recently demonstrated for S-phenylmercapturic acid by Lin et al. [22]. Other internal standards tested in this study – benzylmercapturate, 2,5,- dinitrophenylmercapturate and hexylmercapturate – did not provide an efficient correction for matrix effects caused by interfering compounds.

3.1.3. Online-SPE extraction

Evaluation of the Oasis® HLB pre-column for urinary AM extraction was performed using breakthrough experiments with elution chromatography. Five microliters of an AM standard were injected on the pre-column and eluted directly in the MS using isocratic solvent systems with different percentages of acetonitrile. This method is based on the assumption that the analyte is present at infinite dilution and the breakthrough is only caused

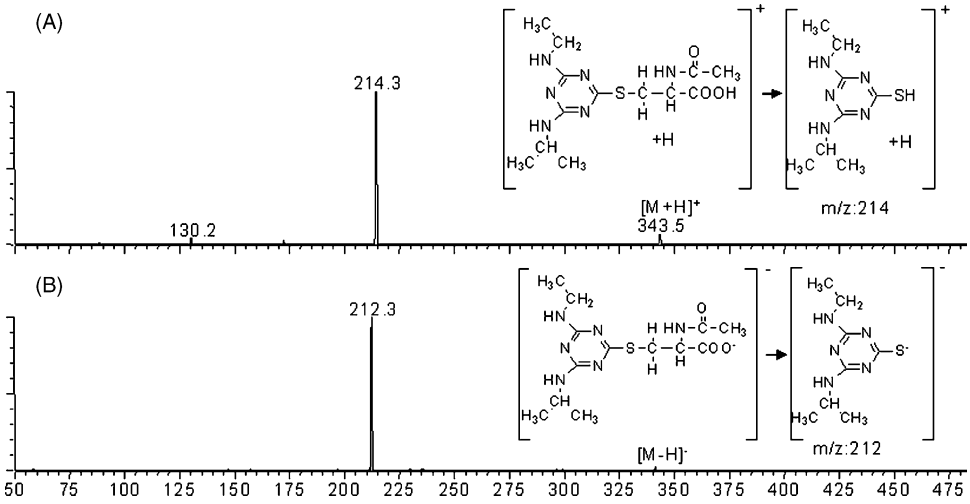

Fig. 2. Product ion spectra of atrazine mercapturate in positive (A) and negative mode (B) using a cone voltage of 30 V and a collision energy of 21 V in a positive mode and 17 V in a negative mode, respectively.

by a migration of the analyte through the sorbent bed analogous to an isocratic HPLC separation. The retention volume of AM on the selected Oasis® HLB extraction column was calculated using the reduced retention time based on three replicate measurements. The log transformed reduced retention volume was then plotted over the percent organic solvent providing a linear relationship which allows the calculation of the specific retention volume in milliliter at any given solvent composition ($\ln V'_R = -0.141 \times (\%\ \text{acetonitrile}) + 1.798$, $R^2 = 99.6\%$). For example, at 1% acetonitrile used for sample loading, a retention volume of 5.2 mL was calculated. Since a total of 1.25 mL of aqueous solvent containing 1% acetonitrile is passed through the pre-column during sample loading and column wash before the valve is switched, a breakthrough of AM based on migration is not expected. Also, it was estimated that the column volume was exchanged at least 20 times during the wash step, which was considered sufficient to remove salts.

Although only one target analyte was measured with the described method, analysis was performed using a HPLC separation. In order to increase sample throughput it is possible to elute the extraction column in reverse direction directly into the MS without HPLC separation, using 100% organic solvent. However, with direct elution, significant signal suppression was observed, and the peak width was increased by a factor of 2.5 compared to HPLC separation. The better peak shape with the HPLC separation is probably due to a focusing effect at the head of the analytical HPLC column supported by the dilution of the eluate from the pre-column with aqueous solvent. Although the use of a stable-isotope-labeled internal standard can correct for ion suppression during quantification, the method detection limit is nevertheless increased due to signal suppression and wide peaks. Since AM was expected at low concentration in the urine samples in this study, the use of an HPLC separation was chosen to minimize ion suppression and achieve narrow peaks at the expense of analytical speed. A retention time of 10.6 min was still considered reasonable.

3.1.4. Matrix effects

As discussed above, signal suppression by the matrix can be minimized with HPLC separation. No significant ion suppression was observed with a sample volume of 5 μL. However, in some cases it would be beneficial to increase the injection volume to reduce the method detection limits. To test maximum injection volumes, three different urine samples were spiked at a concentration of 5 ng mL^{-1} and analyzed using injection volumes of 5, 10, 20, 30, 40 and 50 μL. The correlation between AM signal response and injection volume is shown in Fig. 3. A linear relationship between signal response and injection volume was observed for sample 1. However, for sample 2 and 3 with volumes greater than 20 μL, the response started to plateau. This indicates possible ion suppression caused by co-eluting matrix components. However, the same trend in signal response was observed for the ^{13}C-labeled internal standard, which consequently will correct for the ion suppression. Quantification of AM in the injection experiments using internal standard correction resulted in an average concentration of 4.97 ng mL^{-1} ($n = 18$) with a CV of 5.7%. This demonstrates that injection volumes of up to 50 μL can be used to further decrease the detection limit if a stable-isotope-labeled AM is used as an internal standard.

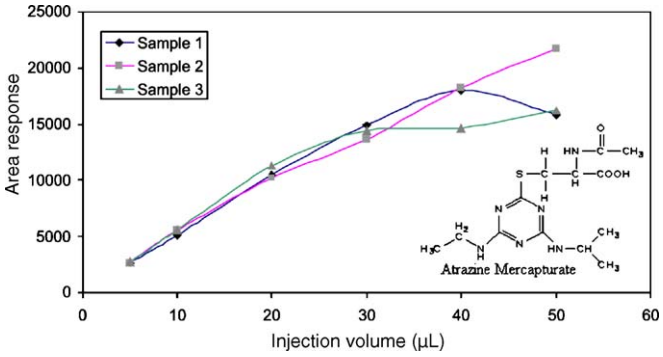

Fig. 3. Relationship between signal response and injection volume in three different urine samples spiked with AM at a concentration of 5 ng mL^{-1}. Points represent mean values of three replicate measurements (CV % less than 6).

3.1.5. Optimization of manual SPE clean-up

Optimization of SPE clean-up was performed using urine samples from non-exposed male individuals spiked with ^{14}C-AM to give a final AM concentration of 30 ng mL^{-1}. For the optimized protocols, the recovery of ^{14}C-AM in five replicated 0.5 mL samples was 98 ± 1.2% for both cartridges. According to a separate loading study, approximately 70 ng of AM in urine could be retained by one 3 mL (60 mg) sorbent cartridge, and the amount of urine used for the clean-up was adjusted accordingly (0.25–1.0 mL).

In order to evaluate if interfering substances were eluting simultaneously with AM, four different urine samples were spiked at 0.5 ng mL^{-1} of AM, passed through the SPE cartridges, and the eluents were further tested using ELISA. For both sorbents (HLB and MCX), increasing the eluent MeOH concentration generally improved the recovery of AM (Table 2), but for the reverse-phase HLB sorbent, concentrations above 70% resulted in a huge increase in recoveries up to 276% measured by ELISA rendering these high-organic-solvent HLB-eluents incompatible with ELISA. This may be an indication of a simultaneous, non-specific elution of interfering substances with AM. The manufacturer's recommendation of urine SPE for basic drugs using Oasis® HLB or MCX columns includes sample loading and washing steps at an acidic pH (3.0). However, our ^{14}C-AM loading studies showed that AM is retained by the MCX sorbent also at basic conditions (up to pH 10.5), probably due to its amphoteric nature, and loading at high pH helped to wash out interfering substances leading to better recovery in ELISA. Interestingly, loading at high pH did not improve recoveries with the HLB sorbent. The mixed-mode cation exchange sorbent, MCX, was chosen for the manual SPE clean-up for ELISA because of the consistently good recoveries of AM (70–95%) over a wide concentration range (Table 3). On the other hand, the HLB column was chosen for the online-SPE clean-up because the MCX protocol was not compatible with the LC–MS instrument due to a high concentration of NaOH in the final eluent.

Table 2
Recovery of spiked AM from four different urine samples (spiked at 0.5 ng mL^{-1}) using sample dilution and two different SPE methods

Urine spiked at 0.5 ng mL^{-1}	Original sample		SPE-Oasis® HLB % MeOH in eluent		SPE-Oasis® MCX % MeOH in eluent	
	Non-diluted	Diluted 1:4	70%	100%	25%	100%
Recovery (%) by ELISA						
A	85	79	72	187	60	80
B	86	84	58	244	70	85
C	61	52	68	255	64	72
D	58	77	50	276	50	62
Average ± S.D.	72 ± 15	73 ± 14	62 ± 10	240 ± 38	61 ± 8	75 ± 10

3.2. Method validation

3.2.1. ELISA with sample dilution

For elimination of interfering substances, dilution (1:4) suggested by previous investigators [10,19] proved to be insufficient for some urine samples leading to low recovery of AM in the spiked urine (Table 2). For a few samples, a dilution of 1:100 was required in order to eliminate the background absorbance in the non-spiked sample (data not shown). Taking into account the 1:4 sample dilution, the limit of detection for ELISA calculated as the value for a non-spiked sample + 3 × S.D. [23,24] increased from 0.04 to 0.16 ng mL^{-1} making the assay substantially less sensitive. In the validation experiment with 38 spiked urine samples, the ability of ELISA to measure urinary AM throughout the whole concentration range (0–70 ng mL^{-1}) was quite good (Fig. 4a; $R^2 = 0.961$) with slope = 0.84 and *y*-intercept = 1.55 but there was less correlation between the known and the measured concentrations at the lower (<20 ng mL^{-1}) concentration range (Fig. 4b). These results indicate that ELISA with simple sample dilution would be suitable for analysis of samples from occupationally exposed populations, but it is less suitable than ELISA + SPE or LC–MS for the general population in which AM levels are expected to be in the low ng mL^{-1} level [10,17–19,25].

3.2.2. ELISA with SPE using Oasis® MCX

After an SPE clean-up with a MCX column, ELISA was able to detect 0.08 ng AM per milliliter of urine, and the assay sensitivity could be doubled (LOD = 0.04 ng mL) by increasing the sample volume to 1 mL. The intra-assay (intraplate) precision of ELISA, expressed as a coefficient of variation (CV %) between three replicates was always <15%, and in most cases <5%. The between-day precision measured for the same sample solutions during 4 different days did not exceed 12%. The precision and accuracy of the whole system for the spiked samples, including the SPE clean-up and ELISA, is presented in Table 3. For quality assessment, pooled urine samples were spiked at four different concentrations (0.5–500 ng mL^{-1}), and the samples were analyzed on multiple days. The average recovery efficiency for all tested concentrations was 82.5%.

With the spiked samples, ELISA with dilution and SPE pretreatments showed a tendency for under-prediction of AM (Fig. 4a; slope = 0.84–0.87). This might be due to incomplete

Table 3
Recovery (%) of spiked AM and the precision of ELISA results using an SPE clean-up with Oasis®-MCX

AM added (ng mL^{-1})	No. of samples	Recovery (%)	CV (%)
0.5	6	70.2	12.8
5.0	6	72.4	13.9
50.0	6	95.3	12.6
500.0	4	92.0	9.8

Number of different urine samples used for each concentration and the variability among urine samples is indicated in the table.

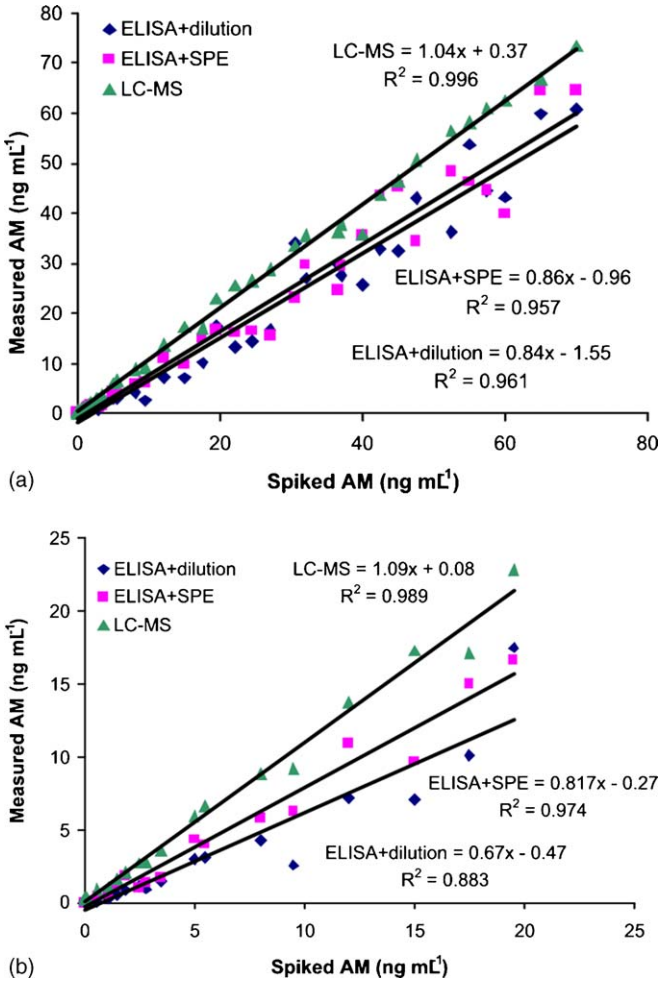

Fig. 4. Correlation between the known and measured concentrations of AM for ELISA + dilution, ELISA + SPE and LC–MS methods; (a) range 0–80 ng mL^{-1}; (b) range 0–25 ng mL^{-1}.

Table 4
Recovery (%) of AM in spiked urine samples using LC–MS with online-SPE

AM added (ng mL^{-1})	Recovery ± S.D. (%)			
	Sample 1	Sample 2	Sample 3	Average
0.5	81 ± 7	91 ± 7	91 ± 18	87 ± 7
5.0	88 ± 8	88 ± 7	88 ± 8	88 ± 6
50	103 ± 10	101 ± 5	88 ± 4	97 ± 8

The creatinine content in samples 1–3 varied from low–high.

recovery of AM in the sample pretreatment step. Overall, the correlation between the known and measured concentrations was quite similar for both sample pretreatment methods but as Fig. 4b shows, at the lower concentration range (<20 ng mL^{-1}), the performance of the assay with SPE clean-up was superior to the one with sample dilution making it more suitable for samples with low levels of AM.

3.2.3. LC–MS with online-SPE

Recovery experiments were performed using three blank urine samples with distinctly different creatinine concentrations that were spiked with AM at concentrations of 0.5, 5 and 50 ng mL^{-1}. Each sample was analyzed in triplicate, and the values presented in Table 4 show an average recovery rate of at least 87% at all three spike levels with CVs % ranging from 6 to 8%. Validation of the new LC–MS method with the blind spiked samples ($n = 38$) revealed an excellent correlation ($R^2 = 99.6\%$, slope = 1.036 and y-intercept = 0.36) between the known and measured urinary AM concentrations, the association between known and measured values being similar throughout the whole concentration range (Fig. 4a and b).

3.3. Method validation using field urine samples

For method validation and to test the applicability of the improved methods in real samples, a set of urine samples from a study among corn farmers [20] was analyzed using both LC–MS and ELISA + SPE. The two methods were chosen based on the assumption that levels of urinary AM in the samples would be at the low nanogram per milliliter range. Previously, Lucas et al. [10] had measured urinary AM concentrations ranging from trace to 1756 ng mL^{-1} with a median of 46 ng mL^{-1}, and according to Perry et al. [13] mean concentrations of urinary AM in samples from atrazine applicators was 6.4 ng mL^{-1} with a range of 1.5 to >40 ng mL^{-1}, while the corresponding value in control samples was 2.9 ng mL^{-1} with a range of 1.5–3.6 ng mL^{-1}. A study among a probability-based sample set of 102 children found urinary AM levels ranging from non-detectable to 16 ng mL^{-1} with a mean of 0.55 ng mL^{-1} [17]. The geometric mean urinary AM measured in a study by Curwin et al. was 0.015 and 0.043 ng mL^{-1}, for non-farmers and farmers not involved in pesticide application, respectively. The levels found among farmers involved in the spraying were much higher with a geometric mean of 1.2 ng mL^{-1} [18].

The set of samples ($n = 70 + 12$ blind duplicates) for the method validation study were selected according to the LC–MS results obtained by CDC: <ND $n = 10$; 0.1 to <1 ng mL^{-1} $n = 20$; 1 to <3 ng mL^{-1} $n = 20$; 3 to <80 ng mL^{-1} $n = 20$. In our study, the levels of urinary AM measured in these samples ranged from 0 to 74 ng mL^{-1} (LC–MS) and 0 to 66 ng mL^{-1} (SPE + ELISA). Median CV % based on the blind duplicates was 1.4% (mean 6.4%) and 8.4% (mean 24%) for LC–MS and ELISA, respectively, indicating good reproducibility for both methods over a wide concentration range.

Instrumental quantitative analysis of AM utilizing online-SPE–LC–MS followed a standard quality control protocol. Calibration using a full set of calibration solutions was performed before and after analysis of the field samples. Samples were analyzed in random order to minimize systematic errors due to instrumental response shifts. Analysis of method blanks did not show significant carry-over effects. Replicate analysis ($n = 5$) of a spiked urine sample was performed throughout the sample batch and met performance criteria with a recovery of 91% and a CV of 3.8%. In addition, calibration check solutions were analyzed every 20 samples to ensure stability of the analytical calibration throughout a given analysis. Quantification of the calibration check solution resulted in a recovery of 94% with a CV of 2% ($n = 4$). Quantification of the calibration checks was within 10% of the theoretical value with a CV less than 15% and therefore met performance criteria.

Paired sample t-test and linear regression analysis of the ln-transformed data showed statistically significant ($p < 0.001$) correlation between results obtained by all three methods (Fig. 5) with a tendency of CDC-LC–MS results being consistently lower than the ones obtained with LC–MS with online-SPE or ELISA + SPE (slope = 0.79). This might be due to analyte losses during the manual SPE clean-up process. Both methods tested in this study have similar limits of detection (ELISA + SPE 0.04 ng mL^{-1} with a 1 mL sample, LC–MS with online-SPE 0.05 ng mL^{-1} with a 10 μL sample) which are lower than in most reported studies on AM: ELISA

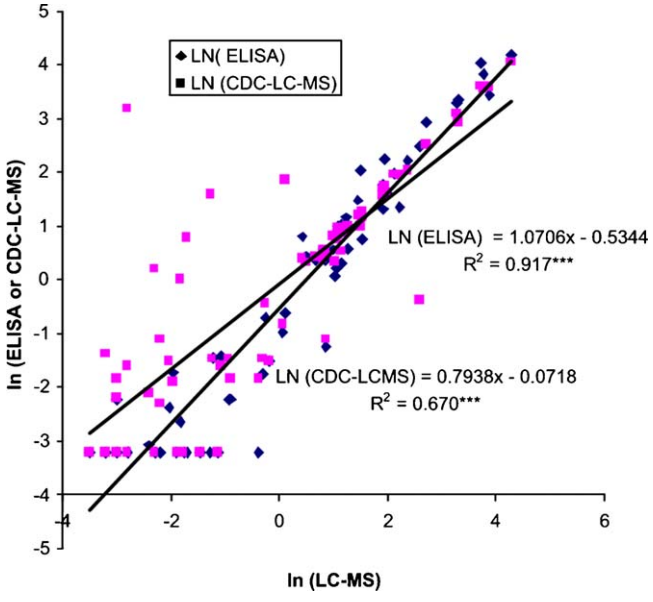

Fig. 5. Linear regression equations derived using log normally transformed data for the samples obtained from the corn farming study analyzed for AM using ELISA + SPE and two different LC–MS methods.

0.3–0.5 ng mL^{-1} [10,19], LC–MS 0.29 [14], 0.53–1.0 [17], 0.3 [16] and 0.026 ng mL^{-1}[18].

For both ELISA and LC–MS, SPE clean-up (either manual or online) dramatically improved the assay sensitivity which made them feasible methods for studies on human exposure to atrazine. For screening purposes, ELISA combined with SPE would be an ideal low-cost yet easy, specific and sensitive method for urinary AM analysis [26]. As an example, 36 duplicated samples can be quantified in one ELISA plate within 2.5 h, whereas the LC–MS analysis for the same number of samples is much more expensive and takes a total of 25 h. On the other hand, a novel LC–MS method with online-SPE and internal stable-isotope standard would be more suitable for studies requiring a quantitative analytical approach and good accuracy over a wide concentration range even though its sensitivity still might need improvement to facilitate the measurement of urinary AM among the general population as indicated by Curwin et al. [18]. The LC–MS method also facilitates simultaneous analysis of a multiple metabolism pathways or multiple urinary biomarkers of exposure as demonstrated recently by Lin et al. [27]. There are potential advantages for monitoring multiple pathways of atrazine metabolism in the same sample. For example, by monitoring both *N*-dealkylation products and mercapturates one could estimate the relative contribution of cytochrome P450 and glutathionine transferase-dominated pathways in human population. It seems obvious that such multi-analyte assays can be carried out more easily using LC–MS than ELISA-based methods.

One of the most interesting findings in this study was that for best results, SPE clean-up protocols should be developed specifically not only for each analyte but also for each analysis method. The work reported here indicates that for analytes like AM, a sorbent suitable for online-SPE coupled to LC–MS does not offer the best separation for ELISA, which often requires more complete elimination of structurally related, interfering substances.

4. Conclusions

In this study, the sensitivity of the atrazine mercapturate ELISA was improved 10-fold compared to previous reports by eliminating interfering substances with a solid phase extraction prior to analysis. Of the two tested SPE systems, Oasis® HLB and MCX, the mixed-mode MCX resulted in good recoveries (82%) of AM in spiked samples measured by ELISA whereas the reverse-phase HLB was not compatible with the immunochemical method under the conditions used. At relatively high concentrations of urinary AM (>20 ng mL^{-1}), sample dilution was effective enough for elimination of interfering substances, but dilution as a sample pretreatment method decreases assay sensitivity making it unsuitable for analysis of urinary AM at very low concentrations expected in studies involving the general population.

The new LC–MS method developed for AM utilizes online-SPE with Oasis® HLB sorbent, column switching and a stable-isotope internal standard. The limit of quantification (0.05 ng mL^{-1}) for this method is lower than in most previously reported AM methods for LC–MS. Validation of all three methods, LC–MS, ELISA + SPE and ELISA + dilution with spiked urine samples showed good correlation between the known and measured concentrations with R^2 values of 0.996, 0.957 and 0.961, respectively. With spiked samples, both ELISA methods tended to underestimate the urinary AM concentration (slopes = 0.84–0.86), which is probably an indication of an incomplete recovery of AM during the SPE clean-up. However, further validation of the methods using field samples containing incurred residues and lognormalized data did not show significant underprediction by ELISA. There was a good agreement (R^2 = 0.917) between the ln-transformed values obtained by ELISA + SPE and LC–MS suggesting that both methods would be suitable for the analysis of urinary AM used as a biomarker for human exposure to atrazine. In addition, the field study sample results obtained using methods developed in this study correlated well to the ones from the LC–MS analysis conducted by the CDC.

Acknowledgements

This study was supported in part by the NIEHS Superfund Basic Research Program 5P42ES04699; NIEHS Center for Environmental Health Sciences P30ES05707 and the NIOSH Western Center for Agricultural Health Research 1 U50OH07550. Additional support was provided by the Intramural Research Program of the NIH, NCI (for RV and BB). The authors also wish to thank Dr. Dana Barr of the CDC for the LC–MS analysis.

References

[1] D.A. Belluck, S.L. Benjamin, T. Dawson, ACS Symp. Ser. 459 (1991) 254.
[2] E.A. Scribner, W.A. Battaglin, D.A. Goolsby, E.M. Thurman, Sci. Total Environ. 248 (2000) 255.
[3] J. Yoder, M. Watson, W.W. Benson, Mutat. Res. 21 (1973) 335.
[4] J.L. Freeman, A.L. Rayburn, Environ. Toxicol. Chem. 24 (2005) 1648.
[5] G. Giusi, R.M. Facciolo, M. Canonaco, A.E.V. Belloni, F. Dessi'-Fulgheri, D. Santucci, Toxicol. Sci. 89 (2006) 257.
[6] K.W. Wilhelms, S.A. Cutler, J.A. Proudman, L.L. Anderson, C.G. Scanes, Toxicol. Sci. 86 (2005) 152.
[7] J.R. Rohr, T. Sager, M. Sesterhenn, B.D. Palmer, Environ. Health Perspect. 114 (2006) 46.
[8] R. Ikonen, J. Kangas, H. Savolainen, Toxicol. Lett. 44 (1988) 109.
[9] G. Catenacci, M. Maroni, D. Cottica, L. Pozzoli, Bull. Environ. Contam. Toxicol. 44 (1990) 1.
[10] A.D. Lucas, A.D. Jones, M.H. Goodrow, S.G. Saiz, C. Blewett, J.N. Seiber, B.D. Hammock, Chem. Res. Toxicol. 6 (1993) 107.
[11] B.S. Buchholz, E. GFultz, K.W. Haack, J.S. Vogel, S.D. Gilman, S.J. Gee, B.D. Hammock, X. Hui, R.C. Wester, H.I. Maibach, Anal. Chem. 71 (1999) 3519.
[12] C.J. Hines, J.A. Deddens, C.A.F. Striley, R.E. Biagini, D.A. Shoemaker, K.K. Brown, B.A. Mackenzie, R.D. Hull, Ann. Occup. Hyg. 47 (2003) 503.
[13] M.J. Perry, D.C. Christiani, J. Mathew, D. Degenhardt, J. Tortorelli, J. Strauss, W.C. Sonzogni, Toxicol. Ind. Health 16 (2000) 285.
[14] S.E. Baker, D.B. Barr, W.J. Driskell, M.D. Beeson, L.L. Needham, J. Exp. Anal. Environ. Epidemiol. 10 (2000) 789.
[15] J.M. Pozzebon, W. Vilegas, I.C.S.F. Jardim, J. Chromatogr. A 987 (2003) 375.

[16] A.O. Olsson, S.E. Baker, J.V. Nguyen, L.C. Romanoff, S.O. Udunka, R.D. Walker, K.L. Flemmen, D.B. Barr, Anal. Chem. 76 (2004) 2453.
[17] J.L. Adgate, D.B. Barr, C.A. Clayton, L.E. Eberly, N.C.G. Freeman, P.J. Lioy, L.L. Needham, E.D. Pellizzari, J.J. Quackenboss, A. Roy, K. Sexton, Environ. Health Perspect. 109 (2001) 583.
[18] B.D. Curwin, M.J. Hein, W.T. Sanderson, D.A. Barr, D. Heederik, S.J. Reynolds, E.M. Ward, M.C. Alavanja, J. Exp. Anal. Environ. Epidemiol. 15 (2005) 500.
[19] L.L. Jaeger, A.D. Jones, B.D. Hammock, Chem. Res. Toxicol. 11 (1998) 342.
[20] R. Vermeulen, A.J. De Roos, B. Bakke, A. Blair, A. Hildesheim, L. Pinto, P.P. Gillette, C.F. Lynch, R.H. Allen, M.C. Alavanja, J. Biochem. Mol. Toxicol. 19 (2005) 172.
[21] D. Rodbard, in: J. Langan, J.J. Clapp (Eds.), Ligand Assay, Massin Publishing, New York, 1981, p. 45.
[22] L.C. Lin, J.F. Shih, T.S. Shih, Y.J. Li, P.-C. Liao, Rapid Commun. Mass Spectrom. 18 (2004) 2743.
[23] T. Chard, Characteristics of immunoassays—sensitivity, in: P.C. van der Vliet (Ed.), Introduction to Radioimmunoassay and Related Techniques, Elsevier, Amsterdam, 1995, pp. 169–184.
[24] H.E. Grotjan, B.A. Keel, Data interpretation and quality control, in: E. Diamandis, T. Christopoulos (Eds.), Immunoassay, Academic Press, San Diego, 1996, pp. 51–93.
[25] D.L. Macintosh, L.L. Needham, K.A. Hammerstrom, P.B. Ryan, J. Exp. Anal. Environ. Epidemiol. 9 (1999) 494.
[26] S.J. Gee, A.D. Lucas, B.D. Hammock, in: P.B. Curry, et al. (Eds.), Methods of Pesticide Exposure Assessment, Plenum Press, New York, 1995, p. 143.
[27] L.-C. Lin, Y.-M. Chiung, J.-F. Shih, T.-S. Shih, P.-C. Liao, Anal. Chim. Acta 555 (2006) 34.