Laboratory Procedure Manual

Analytes: Atrazine, Phenylurea, and Sulfonylurea Herbicide Metabolites

Matrix: Urine

Method: High-performance Liquid Chromatography- Tandem Mass Spectrometry (HPLC-MS/MS)

as performed by: *Toxicology Branch*
Division of Laboratory Sciences
National Center for Environmental Health, CDC

Contact: *Dr. Dana Barr*
1-770-488-7886

Important Information for Users
CDC periodically refines these laboratory methods. It is the responsibility of the user to contact the person listed on the title page of each write-up before using the analytical method to find out whether any changes have been made and what revisions, if any, have been incorporated.

Public Release Data Set Information

This document details the Lab Protocol for testing the items listed in the following table:

Data file name	Variable name	SAS Label
L26UPP_C	URXAAZ	Atrazine(ng/mL)
	URXDAM	Desethyl atrazine mercapturate (ng/mL)
	URXDCZ	Diaminochloroatrazine (ng/mL)
	URXDTZ	Desethyl atrazine (ng/mL)
	URXSIS	Desisopropyl atrazine(ng/mL)
	URXUCR	Creatinine, urine (mg/mL)

; ;

Atrazine, Phenylurea, amd Sulfonylurea Herbicide Metabolites in Urine
NHANES 2003-2004

1. SUMMARY OF TEST PRINCIPLE AND CLINICAL RELEVANCE

A high-performance liquid chromatography- tandem mass spectrometry (HPLC-MS/MS) method measures metabolites of atrazine, phenylurea, and sulfonylurea herbicides in human urine. The metabolites were extracted from urine by simple solid-phase extraction using a mixed-bed cartridge and were analyzed by HPLC-MS/MS.

Quantification of the atrazine metabolites was achieved using isotope-dilution calibration. Urea metabolites were quantified using similarly structured chemicals as internal standards. Extraction recoveries ranged from 88% to 106%. Limits of detection for the entire method ranged from 0.125 ng/mL to 1 ng/mL, and the average relative standard deviation of repeat measurements was approximately 13%.

The metabolites measured in the method include atrazine mercapturate, desethyl atrazine, and desisopropyl atrazine as markers of atrazine exposure; dichlorophenyl urea, dichlorophenylmethyl urea, diuron, and linuron as markers of phenylurea herbicide exposure; and dimethoxypyrimidine, dimethylpyrimidine, and methoxymethyl triazine as markers for sulfonylurea herbicide exposure.

Atrazine (ATZ) is an herbicide belonging to the triazine family. It is used to control annual grasses and broad-leaf weeds, with 70-80 million pounds applied annually in the United States[1]. Atrazine is used in agricultural, commercial and residential applications and is the second most abundantly applied pesticide in the United States[1].

Phenylurea herbicides are used for pre- and post-emergence control of broad-leaf weeds and grasses. They do not readily evaporate from the soil, and their herbicidal properties persist up to a year for some specific herbicides[2]. The relative stability of phenylurea herbicides in the environment allows for less frequent application, which is
one reason they are popular for agriculture use.

Sulfonylurea herbicides are a relatively new class of herbicides, consisting of 23 commercially produced variants. Also used for pre- and post-emergence weed control, their widespread use is due to their effectiveness at relatively low doses, crop selectivity, environmental friendliness, and relative safety for applicators.[2] They are divided into two groups, based on their basic chemical structure: triazinyl- and pyrimidinyl sulfonylureas.

2. SAFETY PRECAUTIONS

A. Reagent Toxicity or Carcinogenicity: The reagents used can be both toxic and carcinogenic. Special care should be taken to avoid inhalation or dermal exposure to the acids and solvents necessary to carry out the procedure.

B. Radioactive Hazards: None

C. Microbiological Hazards: The possibility of being exposed to various microbiological hazards exists since human urine is the matrix in which the pesticide metabolites are found. Measures should be taken to avoid any direct contact with the specimen. A Hepatitis B vaccination series is usually recommended for health care and laboratory workers who are exposed to human fluids and tissues.

D. Mechanical Hazards: There is minimal hazard when carrying out this procedure.

E. Protective Equipment: Lab coat, safety glasses, durable gloves, fume hood, face mask (optional).

F. Training: Training and experience in the use of a triple quadrupole mass spectrometer should be obtained by anyone using this procedure. Formal training is not necessary; however, personnel should be trained appropriately by an experienced operator of the instrument and are required to read the operation manuals.

G. Personal Hygiene: Care should be taken in handling urine samples. Use gloves and wash hands thoroughly after sample handling.

H. Disposal of Wastes: Solvents and reagents should always be put to waste in an appropriate container clearly marked for waste products and temporarily stored under a fume hood. Containers, glassware, etc., that come in direct contact with the specimens should be autoclaved and disposed of in a routine manner. Urine may also be decontaminated with 50% bleach.

3. COMPUTERIZATION; DATA SYSTEM MANAGEMENT

A. Software and Knowledge Requirements. A database named NPP2 has been developed on the DLS-PC Network using R:Base 4.5+ (Microrim Inc., Redmond, WA). This database is used for storage, retrieval, and analysis of data from the pesticide residue analyses. Statistical analyses of data are performed using Statistical Analysis System (SAS) software (SAS Institute, Cary, NC). Knowledge of and experience with these

software packages (or their equivalent) are required to utilize and maintain the data management structure.

B. Sample Information. Information pertaining to particular specimens is transferred electronically into the database or manually entered. Data that are manually entered include the sample identification number, the notebook number associated with the sample preparation, the sample type, standard number, and any other information not associated with the mass spectral analysis. The analytical information obtained from the sample is electronically transferred from a UNIX-based system to a PC via an ethernet connection. The data are then transferred electronically into the database.

C. Data Maintenance. All sample and analytical data are checked after being entered into the database for transcription errors and overall validity. The database is routinely (at least once weekly) backed up onto a computer hard drive and onto a network magnetic tape.

4. SPECIMEN COLLECTION, STORAGE, AND HANDLING PROCEDURES; CRITERIA FOR SPECIMEN REJECTION

A. Sample Collection. Urine specimens are collected from subjects in standard urine collection cups. Samples should be refrigerated as soon as possible and transferred to specimen vials within 4 hours of collection. A minimum of 10 milliliters of urine is collected, and poured into sterile 14 mL vials with snap cap tops. The specimens are then labeled, stored immediately at: -30 °C, and shipped on dry ice. Special care must be taken in packing to protect vials from breakage during shipment. All samples should be stored at -30 °C until analysis.

B. Sample Handling. Some problems with breakage due to freezing and subsequent thawing have occurred. Samples are thawed, aliquoted, and the residual specimen is again stored at -70 °C until needed.

5. PROCEDURES FOR MICROSCOPIC EXAMINATIONS; CRITERIA FOR REJECTION OF INADEQUATELY PREPARED SLIDES

Not applicable for this procedure.

6. PREPARATION OF REAGENTS, CALIBRATORS (STANDARDS), CONTROLS, AND ALL OTHER MATERIALS; EQUIPMENT AND INSTRUMENTATION

Atrazine, Phenylurea, amd Sulfonylurea Herbicide Metabolites in Urine
NHANES 2003-2004

A. Chemicals

(1) All solvents used were of analytical grade.

(2) Acetonitrile was purchased from Tedia (Fairfield, OH).

(3) Deionized water was organically and biologically purified with a NANOpure Infinity ultrapure from Barnstead International (Dubuque, IA).

(4) Nitrogen was purchased from Airgas Inc. (Radnor, PA).

(5) The DEA, DIA, DMeP, diuron, and linuron standards were purchased from ChemService (West Chester, PA).

(7) The DCP urea, DCPM urea, and DMeOP standards were purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany).

(8) The MMT standard was purchased from Absolute Standards, Inc. (Hamden, CT).

(9) The AM, ring-$^{13}C_3$-AM; dimethyl-d_6-2-diethylamino-6-methyl-4 (1H)-pyrimidone-(d_6-DEAMPY); dimethyl-d_6-N,N- diethyl-M-toluamide (d_6-DEET); and methyl-4,5,6-$^{13}C_4$-2-isopropyl-4-methyl-6-hydroxypyrimidine ($^{13}C_4$-IMPY) standards were purchased from Cambridge Isotope Laboratories (Andover, MA).

(10) Isotopically labeled ethyl-d_5-DIA and isopropyl-d_6-DEA used for isotope-dilution quantification was purchased from EQ Laboratories (Atlanta, GA).

(11) Methanol was purchased from Tedia (Fairfield, OH, USA). .

(12) Glacial Acetic acid was purchased from J.T. Baker (Phillipsburg, NJ).

B. QC Materials

Pooled, filtered urine was used to prepare QC pools at three concentration levels by fortifying with the appropriate native standard and mixing well. The low-level QC pool (QCL) was spiked at 1 ng/mL urine for all analytes, the medium-level QC pool (QCM) was spiked at 5 ng/mL, and the high-level QC pool (QCH) was spiked at 15 ng/mL. All QC materials were stored at -40°C until analyzed.

C. Spiking Standard Preparation

Ten different calibrant spiking standards prepared in ACN contained all target analytes at concentrations ranging from 10 ng/µL to 20,000 ng/µL so that a 25 µL aliquot spiked into 2 mL of urine would yield the desired concentration. Similarly, an internal standard solution (ISTD) was prepared at 1 ng/µL, which contained 3 labeled atrazine metabolite analogs, and 3 labeled surrogate standards. Into each sample, 25 µL of ISTD were added to yield a final ISTD concentration of 12.5 ng/mL urine.

D. Equipment

(1) Microbalance - Sartorius Ultramicro, Westbury, NY

(2) Rotator - Glas-Col, RD-230

(3) TurboVap LV evaporator (Zymark, Farmingham, MA)

(4) Agilent 1100 HPLC system (Agilent Technologies, Waldbronn, Germany)
(a) binary pump
(b) degasser
(c) thermostattable column compartment

(5) Finnigan TSQ 7000 triple quadrupole mass spectrometer (ThermoFinnigan,San Jose, CA)
(a) Atmospheric pressure chemical ionization (APCI) application),

(6) SPE cartridge (3 capacity, Waters Corporation, Milford, MA)

(7) Polycap 75 TC filter device (0.6/.45 µm pore size, Whatman, NJ

E. Other Materials

(1) Conical bottom 15-mL screw capped tubes (Pyrex or Kimax)

(2) Phenolic screw caps with Teflon inner seals for both sizes of tubes (Corning, Scientific Services, CDC)

(3) Micro/pettor and glass tips (SMI Liquid Handling Products)

(4) Vortex Genie (Scientific Industries Inc., Springfield, MA)

(5) Round bottom 50-mL screw capped tubes (Pyrex or Kimax)

(6) Micro autosampler vials with resealing septa (Fisher Scientific, Norcross, GA)

(7) Compressed nitrogen, helium, and argon, liquid nitrogen (Holox Ltd, Atlanta, GA)

(8) Prodigy column (5µ, phenyl-3, 100 x 4.6 mm) was purchased from Phenomenex (Torrance, CA).

F. Instrumentation

Analytes were separated on a Prodigy column (5µ, phenyl-3, 100 x 4.6 mm). The column was connected to an Agilent 1100 high performance liquid chromatography (HPLC) system, consisting of an autosampler, a binary pump, a degasser, and a thermostattable column compartment . The HPLC system was interfaced to a Finnigan TSQ 7000 triple quadrupole mass spectrometer, with an atmospheric pressure chemical ionization (APCI) source.

Current throughput of the method is 36 unknown samples (48 samples including calibration standards, blanks, and QC materials) per day per laboratory analyst, with the analyst performing the entire procedure. Sample preparation requires approximately 4 hours of analyst time. Mass spectral analysis time is 12 min per sample; thus samples are being analyzed on the instrument for about 11 hours for one run. Final processing of the data requires about 2 hours of analyst time. Factoring in a 20% downtime, the predicted yearly throughput of this method is approximately 8,000 samples per laboratory analyst, which is suitable for large-scale studies.

This method provides a simple, fast, and specific tool for the analysis of several herbicides and some metabolites thereof. Sample clean-up requires only a basic SPE method and requires no pretreatment of samples (e.g., buffering, deconjugation, derivatization, or centrifugation). Inclusion of stable isotope analogues for some of the analytes and use of MS/MS analysis provides specificity and accuracy in their detection and quantification.

G. Method Validation.

(1) Ion suppression
Possible matrix effects on ions were evaluated by performing an infusion ion suppression test[3]. Under normal method-operating conditions, a standard mixture was infused into the mass spectrometer via a syringe pump, which generated a flat baseline.

Concurrently, a blank urine extract sample was injected by the HPLC into the mass spectrometer. The normal acquisition method was applied.

(2) Stability
A stability experiment was performed to assess any degradation of the analytes in solution. Vials of the standard mixture in ACN were stored at -70ºC, -25ºC, 4ºC, 25ºC, and 37ºC for 36 days. Each analyte's MS signal

responses after storage at the respective temperatures for 1, 2, 3, 7, 10, and 36 days were compared with their initial MS signal responses stored at -70ºC at day 0. . Stability in urine was evaluated only over the course of the validation process of approximately one month. Evaluation of this parameter will continue. Evaluation of this parameter will continue.

(3) Limits of Detection.
The limits of detection (LODs) were calculated as $3s_0$ where s_0 is the standard deviation at zero concentration[4]. The calculated LODs were verified by visually inspecting spiked samples at that range to determine if the concentration could be easily detected. In instances where the calculated values could not be verified visually, the LOD concentration was set to be the lowest standard where the analyte could be easily detected with a signal-to-noise ratio of 3 or greater.

(4) Extraction Efficiency.
The recovery experiment was performed at two concentrations: 5 ng/mL and 50 ng/mL. For each concentration level, 25 µL of the appropriate native standard mixture were spiked into 5 of 10 blank urine samples, which yielded a final native standard concentration of 5 ng/mL or 50 ng/mL urine, depending upon the standard used.

All samples were extracted according to the method. The 5 samples not spiked before extraction for each concentration were then spiked with the same amount of native standard as those spiked before extraction. All extracts for each concentration were then spiked with 25 µL of the labeled ISTD mixture. The rest of the clean up procedure was completed according to the method. To determine recovery, the samples spiked before extraction were compared to those spiked after extraction.

The term “recovery” is used to indicate the percentage of the amount of the chemical that is recovered in the extraction process. Using the isotopically labeled analogues and structurally-related surrogates, the values spiked into samples within a few percent of 100% are accurately quantified (Table 1). This is often called a relative recovery. Overall, the extraction recoveries of the analytes ranged from 88% to 106% and were similar regardless of the analyte concentration. Isotopically labeled analogs were available for only the three atrazine metabolites.

To provide some reasonable recovery correction for analytes that lacked analogous labeled standards, we quantified the metabolites using structurally similar, isotopically labeled compounds that eluted close to the compound of interest. AM was used as a surrogate for DCPM urea; DEAMPY was used as a surrogate for DMeOP; DEET was used as a

surrogate for diuron and linuron; and IMPY was used as a surrogate for DMeP and MMT.

(5) Precision

The precision of the method was determined from 30 replicate analyses of spiked QC materials over 5 different days (Table 2). RSDs ranged from about 3% to 19%. Most RSDs were at or below 13%. As would be expected but with some exceptions, the RSDs at higher concentrations were typically lower than those at concentrations near the LOD.

The chemicals appeared stable in both urine and solvent over the course of method development and validation.

(6) Matrix Effects.

Clear separation of analyte by mass or time was achieved.

During development of the mass-spectral procedures, considerable solvent adduct formation with DCP urea was observed. Instead of the expected ion at m/z 205, the major ion was at m/z 237, indicative of association adduct with MeOH (32 amu), a major component of the mobile phase. Analyte-solvent adducts are commonly observed in HPLC, so this was not a surprise. The major product ion obtained from this precursor adduct species had a molecular weight of m/z 127, which is an expected fragment of DCP urea representing chloroaniline. Thus, the MeOH adduct of DCP as a precurson ion was used. DCP urea was the only anlyte for which we encountered any MeOH adduct formation.

7. CALIBRATION AND CALIBRATION VERIFICATION PROCEDURES

A. Mass Spectrometer

The TSQ7000 mass spectrometer is calibrated and tuned using 2-methoxyethanol-ammonia clusters according to the instructions in the operator's manual. The "OPT" ICL program can be modified and executed to determine the optimum for each parameter. After the instrument is calibrated with unit resolution and maximum sensitivity, the instrument is prepared for analysis of the pesticide metabolites as described in the Procedure Operating Instructions.

B. Calibration Curve

The calibration plots were linear throughout the entire standard range (LOD to 250 ng/mL) (Table 1). A best-fit line of the data showed good correlation coefficients. Errors about the slope were less than 1%.

Current throughput of the method is 36 unknown samples (48 samples including calibration standards, blanks, and QC materials) per day per laboratory analyst, with the analyst performing the entire procedure.

Sample preparation requires approximately 4 hours of analyst time. Mass spectral analysis time is 12 minutes per sample; thus samples are being analyzed on the instrument for about 11 hours for one run. Final processing of the data requires about 2 hours of analyst time. Factoring in a 20% downtime, the predicted yearly throughput of this method is approximately 8,000 samples per laboratory analyst, which is suitable for large-scale studies.

(1) The lowest point on the calibration curve is at or below the measurable detection limits and the highest point is above the expected range of results.

(2) The slope and intercept of this curve is determined by linear least squares fit using SAS software.

(3) R-squared values for the curve must be greater than 0.95. Linearity of standard curves should extend over the entire standard range. Intercepts, calculated from the least squares fit of the data, should not be significantly different from 0; if it is, the source of this bias should be identified.

(4) The standard curve should be recalculated periodically to incorporate the newest data points. Whenever a new combined labelled isotope solution is prepared, the standard curve must be re-established.

C. Calibration Verification

(1) Calibration verification materials are analyzed, using the same procedure used with the unknown samples, after any substantive change in the method or instrumentation to verify the continuation of integrity of the calibration curve slope, linearity, and dynamic range. For example, CV materials should be analyzed before samples are analyzed, if the instrument has been used in another method, after a new column is installed, after preventative maintenance is performed, or after the source of the mass spectrometer is cleaned.

Calibration plots were generated by plotting the area-of-the-analyte/area-of-the internal standard against the calibrant concentration. A best-fit line was generated that provided a slope and intercept from which unknown analyte concentrations could be calculated. A full calibrant set was analyzed with each analytic run.

(2) Calibration verification should be performed a minimum of once every 6 months while the method is in use.

(3) Three Quality Control (QC) Materials; The low-level QC pool (QCL) was spiked at 1 ng/mL urine for all analytes, the medium-level QC pool (QCM) was spiked at 5 ng/mL, and the high-level QC pool (QCH) was spiked at 15 ng/mL. The slope, intercept, and linearity of a regression

analysis of the CV materials should not differ significantly from that of the calibration curve.

(4) If there is a significant difference, analyses using this method should be halted until corrective actions are taken and QC materials are consistent with the calibration curve.

(5) All calibration verification runs and results shall be appropriately documented.

8. PROCEDURE OPERATING INSTRUCTIONS; CALCULATIONS; INTERPRETATION OF RESULTS

A. Sample Preparation

Urine samples were frozen within 1 hour of collection and stored at -20 °C before analysis. For each analytical run, calibration samples, two fortified urine samples (one high and one low dose), one blank urine sample, and one solvent blank were prepared, extracted, and analyzed in parallel with the unknown samples.

B. Sample Cleanup

A 2-mL urine sample was spiked with 25 µL isotopically labeled internal standard solution and mixed. The sample was prepared using OASIS HLB solid-phase extraction (SPE) cartridges (3 cc capacity). The cartridge was conditioned with 1 methanol (MEOH) and then equilabrated with 1 mL deionized water. The urine sample was loaded onto the cartridge. The cartridge was rinsed with 1 mL 5% MeOH to wash away any contaminants from the urine matrix that possibly adhered to the sorbent. Analytes were eluted from the column with 2 mL MeOH. The MeOH was evaporated to dryness in a TurboVap LV Evaporator set at 40°C using 10 psi of nitrogen. MeOH (350 µL) was added into the tube and mixed to wash residues from sides of the tube. Again, the sample was taken to dryness. The residue was reconstituted in 50 µL MeOH:ACN (3:17).

C. Instrumentation

Analytes were separated on a Prodigy column (5µ, phenyl-3, 100 x 4.6 mm.. The column was connected to an Agilent 1100 high performance liquid chromatography (HPLC) system, consisting of an autosampler, a binary pump, a degasser, and a thermostattable column compartment. The HPLC system was interfaced to a

Finnigan TSQ 7000 triple quadrupole mass spectrometer, with an atmospheric pressure chemical ionization (APCI) source.

D. HPLC Operating Conditions.

The analytes were separated using a gradient elution at a constant flow rate of 1 mL/min. Initial mobile phase conditions were 95% A (water with 0.1% acetic acid) and 5% B (methanol with 0.1% acetic acid). From 0 to 8 minutes, mobile phase B was increased linearly to 95%. From 8 to 8.15 minutes, the initial mobile phase conditions were restored and held for 3.85 minutes to allow reequilibration of the column. The total run time was 12 minutes, and the injection volume was 10 µL. The column compartment was set at 25°C. The spray voltage was 4.0 kV, the vaporizer temperature was 450°C, and the heated capillary was set to 250°C. The sheath gas (N_2) was set to 50 psi, and the collision gas (Ar) pressure was set to 2 mT.

The acquisition method contained 5 time segments. All segments were operated in positive ion mode and used multiple reaction monitoring (MRM) to detect the quantification and confirmation ions listed in Table 2. The first (0 – 4 min) and fifth (8.86 – 12 min) segments did not contain any peaks of interest and were set up to detect only an improbable precursor/product ion transition (999@12 [999]), to produce an aesthetically enhanced chromatographic baseline during those periods. The other timed segments acquired data from 4.01 – 5.8 min, 5.81 – 7.0 min, and 7.01 – 8.85 min.

F. Processing of data

(1) Quantification

For method development, validation, and quality control, urine was collected from volunteers anonymously. Urine was collected and stored at 4°C until approximately one liter was obtained. The urine was then pooled and pressure filtered through a Polycap 75 TC filter device (0.6/.45 µm pore size), which was attached to a stainless steel filtration tank. The pooled, filtered urine was further mixed for 5 minutes at 4°C to produce a homogenous urine matrix to make blank samples, calibration standards and quality control (QC) materials.

Pooled, filtered urine was used as the matrix material for calibrants. For each analytic run, 10 blank urine samples (2 mL each) were spiked with the 10 different samples standard solutions previously described to give calibration samples that

ranged in concentration from 0.125 ng/mL to 250 ng/mL urine. Each calibrant was spiked with 25 µL ISTD before extraction. An additional 2 mL blank urine sample was also spiked with 25 µL of the labeled ISTD mixture. The blank urine and calibration standards were extracted according to the SPE method described previously.

Calibration plots were generated by plotting the area-of-the-analyte/area-of-the internal standard against the calibrant concentration. A best-fit line was generated that provided a slope and intercept from which unknown analyte concentrations could be calculated. A full calibrant set was analyzed with each analytic run.

9. REPORTABLE RANGE OF RESULTS

The linear range of the standard calibration curves determines the highest and lowest analytical values of an analyte that are reportable. The calibration verification of the method encompasses this reportable range. However, urine samples with analytical data values exceeding the highest reportable limit may be diluted and reanalyzed so that the result will be in the reportable range.

10. QUALITY CONTROL (QC) PROCEDURES

A. Quality Control Material. The control materials used for each unknown run were urine pools enriched with known amounts of pesticide residues.

B. Collection of Urine for QC Pools. Three quality control pools were prepared and are used in each run of unknown samples. The urine for each pool was collected from volunteers and was screened to ensure that the endogenous levels of pesticide residues were low or nondetectable. The urine samples were combined and homogenized to form a base pool.

C. Urine Enrichment. Pooled, filtered urine was used to prepare QC pools at three concentration levels by fortifying with the appropriate native standard and mixing well. The low-level QC pool (QCL) was spiked at 1 ng/mL, urine for all analytes, the medium-level QC pool (QCM) was spiked at 5 ng/mL, and the high-level QC pool (QCH) was spiked at 15 ng/mL. All QC materials were stored at -40°C until analyzed.

D. Filtration and Dispensing. Each pool was clean filtered to 0.2 µ. The urine was dispensed in 12-mL aliquots into 25-mL sterile screw-capped vials. The vials labeled appropriately and the QC materials were then frozen at -20 °C until needed.

E. Characterization of QC Materials. QC materials were characterized by 6 replicates on each of 5 days, thus in total 30 replicates, to determine the mean and relative standard deviation (RSD). The QC characterization results were used to determine the within and between day variation, as well as the accuracy of the method. The accuracy was defined as the mean of estimated value divided by the theoretical value.

F. Use of Quality Control Samples. For a given analytic run of 36 unknown samples, three QC materials were prepared along with the urine blank sample and the full set of calibration samples. .

G. Out of Control Runs. The run was considered "out-of-control" if the calculated values of the QC samples violated the Westgard multirules[5].

11. REMEDIAL ACTION IF CALIBRATION OR QC SYSTEMS FAIL TO MEET ACCEPTABLE CRITERIA

If the calibration or QC systems, all operations are suspended until the source or cause of failure is identified and corrected. If the source of failure is easily identifiable, for instance, failure of the mass spectrometer or a pipetting error, the problem is immediately corrected. Otherwise, fresh reagents are prepared and the mass spectrometer system is cleaned. Before beginning another analytical run, several QC materials (in the case of QC failure) or calibration verification samples (in the case of calibration failure) are reanalyzed. After calibration or quality control has been reestablished, analytical runs may be resumed.

12. LIMITATIONS OF METHOD; INTERFERING SUBSTANCES AND CONDITIONS

The chromatography of the first eluting peaks is not good and the possibility for improvement is substantial. Stable isotope analogues are still not available for some of the analytes, thereby affecting the quantification of these analytes (DCP urea, diuron, linuron, DMeP, and MMT).

Although the method has been tested on in-house QC material, it has not yet been applied to real-life samples.

13. REFERENCE RANGES (NORMAL VALUES)

Reference range values for most of these analytes do not exist. However, in the time that atrazine mercapturate has been measured by this lab, only a few samples have had detectable amounts, resulting from incidental exposures.

14. CRITICAL CALL RESULTS ("PANIC VALUES")

There are no panic values for these analytes.

15. SPECIMEN STORAGE AND HANDLING DURING TESTING

Urine samples may be stored overnight in refrigeration to expedite thawing prior to aliquoting the sample. The urine extracts are stored in autosampler vials in a -20 °C freezer after analysis. Stability in urine was evaluated only over the course of the validation process of approximately one month. Evaluation of this parameter will continue.

16. ALTERNATIVE METHODS FOR PERFORMING TEST OR STORING SPECIMENS IF TEST SYSTEM FAILS

If testing cannot be performed, the specimens are stored at -70°C.

17. TEST RESULT REPORTING SYSTEM; PROTOCOL FOR REPORTING CRITICAL CALLS (IF APPLICABLE)

Once the validity of the data has been established by the QC/QA system outlined above and has been verified by a DLS statistician, one hardcopy and one electronic copy of the data will be generated. This data, a cover letter, and a table of method specifications and reference range values will be routed through the appropriate channels for approval (i.e. supervisor, branch chief, division director). Once approved at the division level, they will be sent to the contact person who requested the analyses.

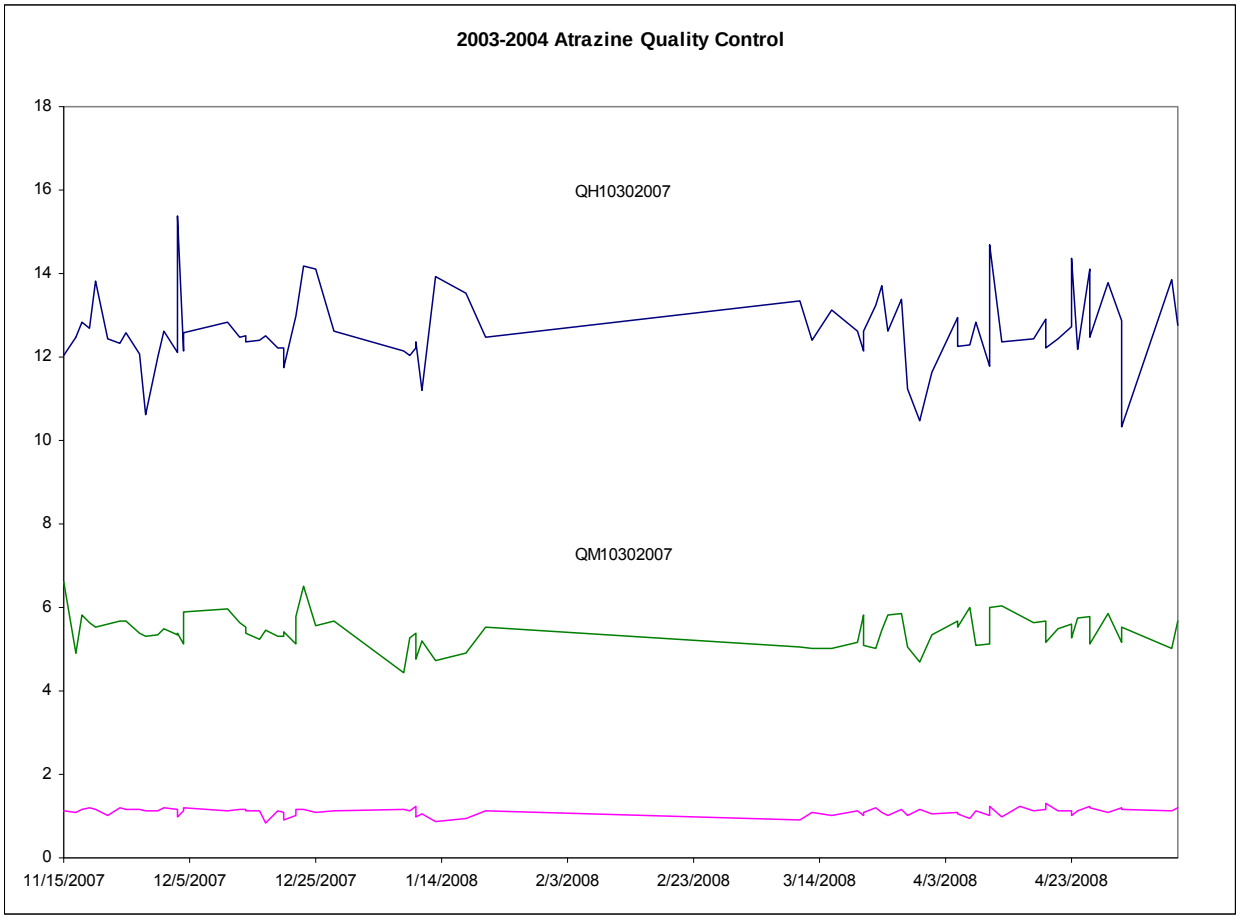
18. TRANSFER OR REFERRAL OF SPECIMENS; PROCEDURES FOR SPECIMEN ACCOUNTABILITY AND TRACKING

Standard record keeping systems (i.e. notebooks, sample logs, data files, creatinine logs, demographic logs) should be employed to keep track of all specimens. Specimens will only be transferred or referred to CLIA certified laboratories.

19. SUMMARY STATISTICS AND GRAPHS

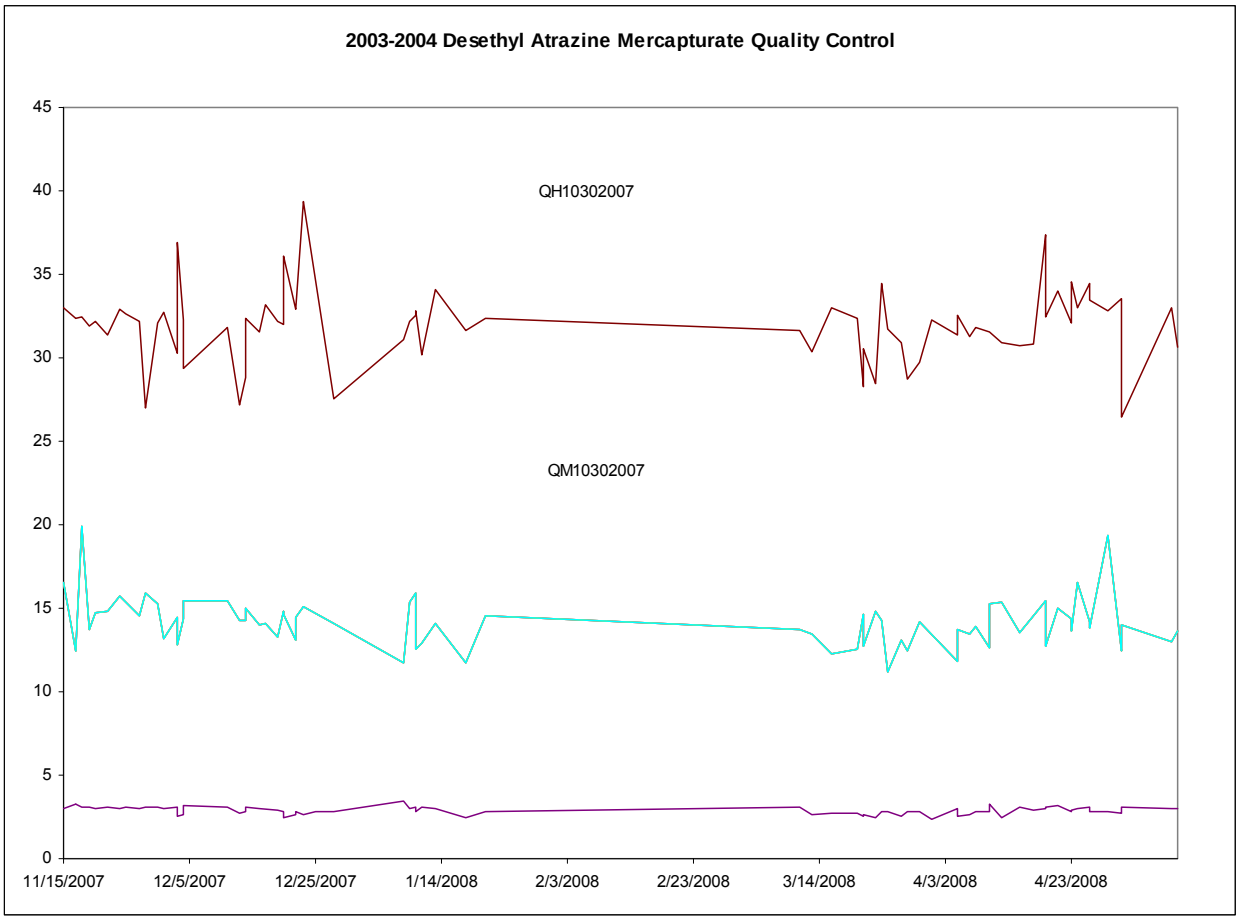
Summary Statistics for Atrazine by Lot

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
QL10302007	73	11/15/2007	5/10/2008	1.1064	0.0925	8.4
QM10302007	72	11/15/2007	5/10/2008	5.436	0.3897	7.2
QH10302007	71	11/15/2007	5/10/2008	12.6344	0.898	7.1



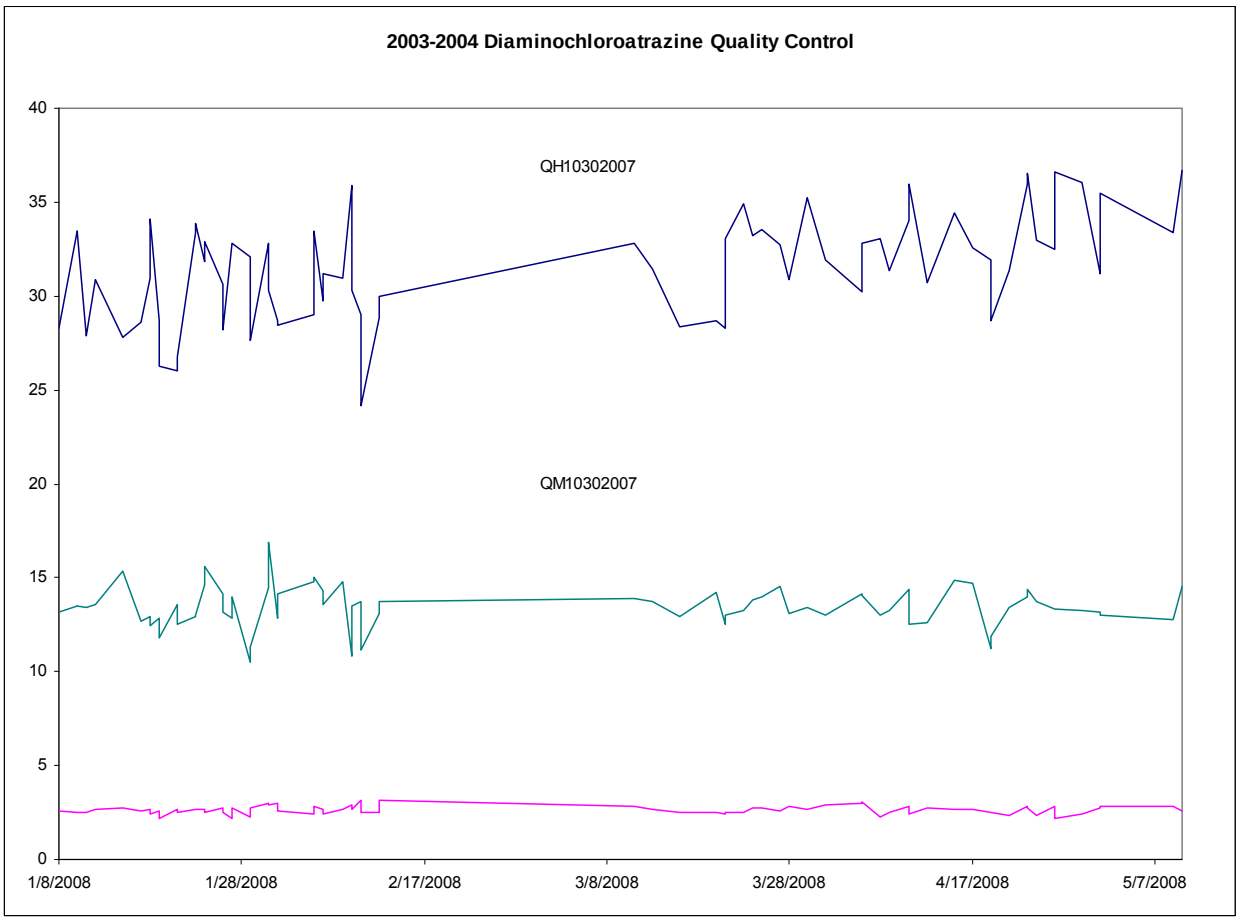
Summary Statistics for Desethyl Atrazine Mercapturate by Lot

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
QL10302007	72	11/15/2007	5/10/2008	2.8871	0.2271	7.9
QM10302007	71	11/15/2007	5/10/2008	14.168	1.502	10.6
QH10302007	70	11/15/2007	5/10/2008	31.9232	2.2401	7.0



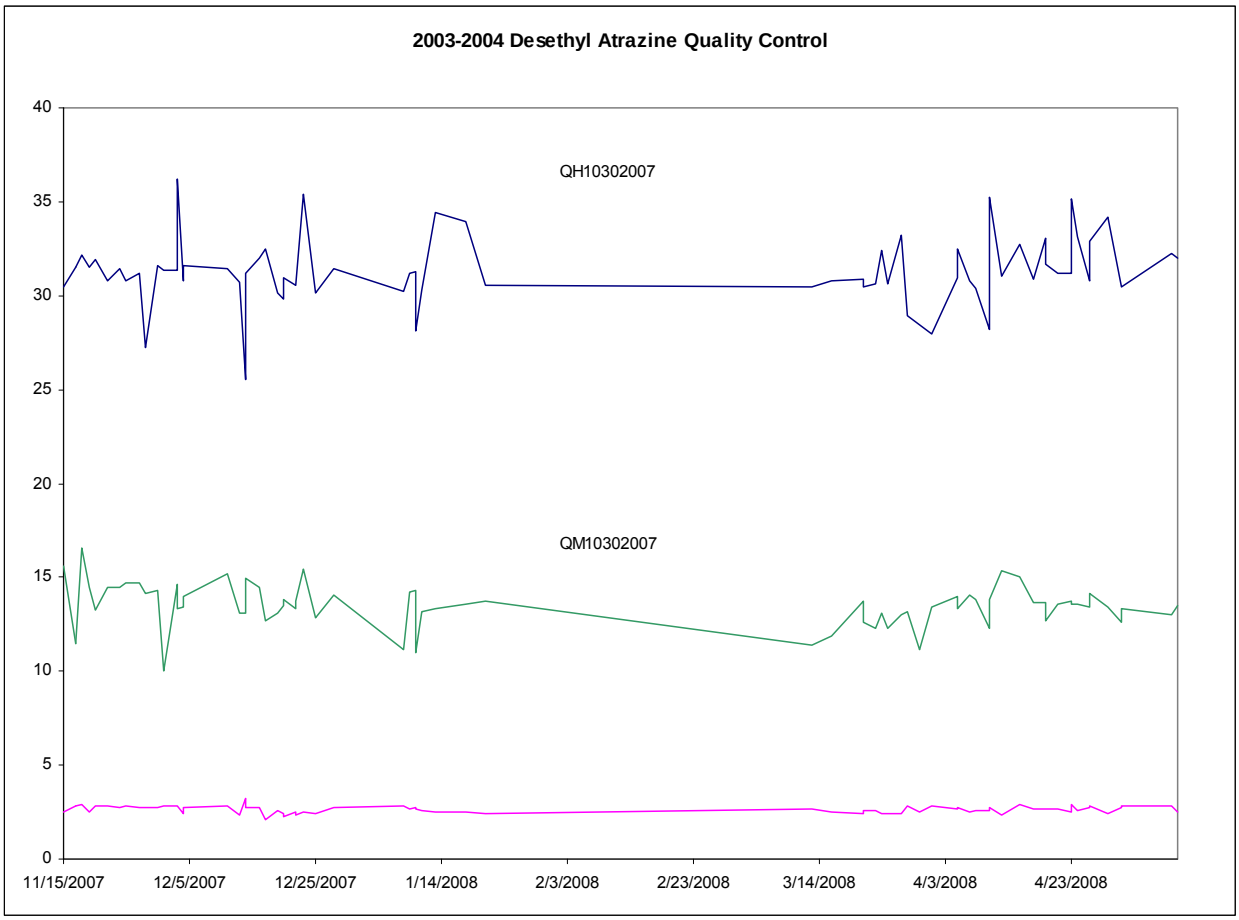
Summary Statistics for Diaminochloroatrazine by Lot

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
QL10302007	72	1/8/2008	5/10/2008	2.6432	0.2189	8.3
QM10302007	72	1/8/2008	5/10/2008	13.4399	1.0901	8.1
QH10302007	71	1/8/2008	5/10/2008	31.473	2.8521	9.1



Summary Statistics for Desethyl Atrazine by Lot

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
QL10302007	71	11/15/2007	5/10/2008	2.6397	0.1866	7.1
QM10302007	71	11/15/2007	5/10/2008	13.5058	1.135	8.4
QH10302007	68	11/15/2007	5/10/2008	31.3348	1.8109	5.8



Summary Statistics for Desisopropyl Atrazine by Lot

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
QL10302007	71	11/15/2007	5/10/2008	2.7299	0.2832	10.4
QM10302007	71	11/15/2007	5/10/2008	14.2538	1.4639	10.3
QH10302007	72	11/15/2007	5/10/2008	32.1942	3.307	10.3

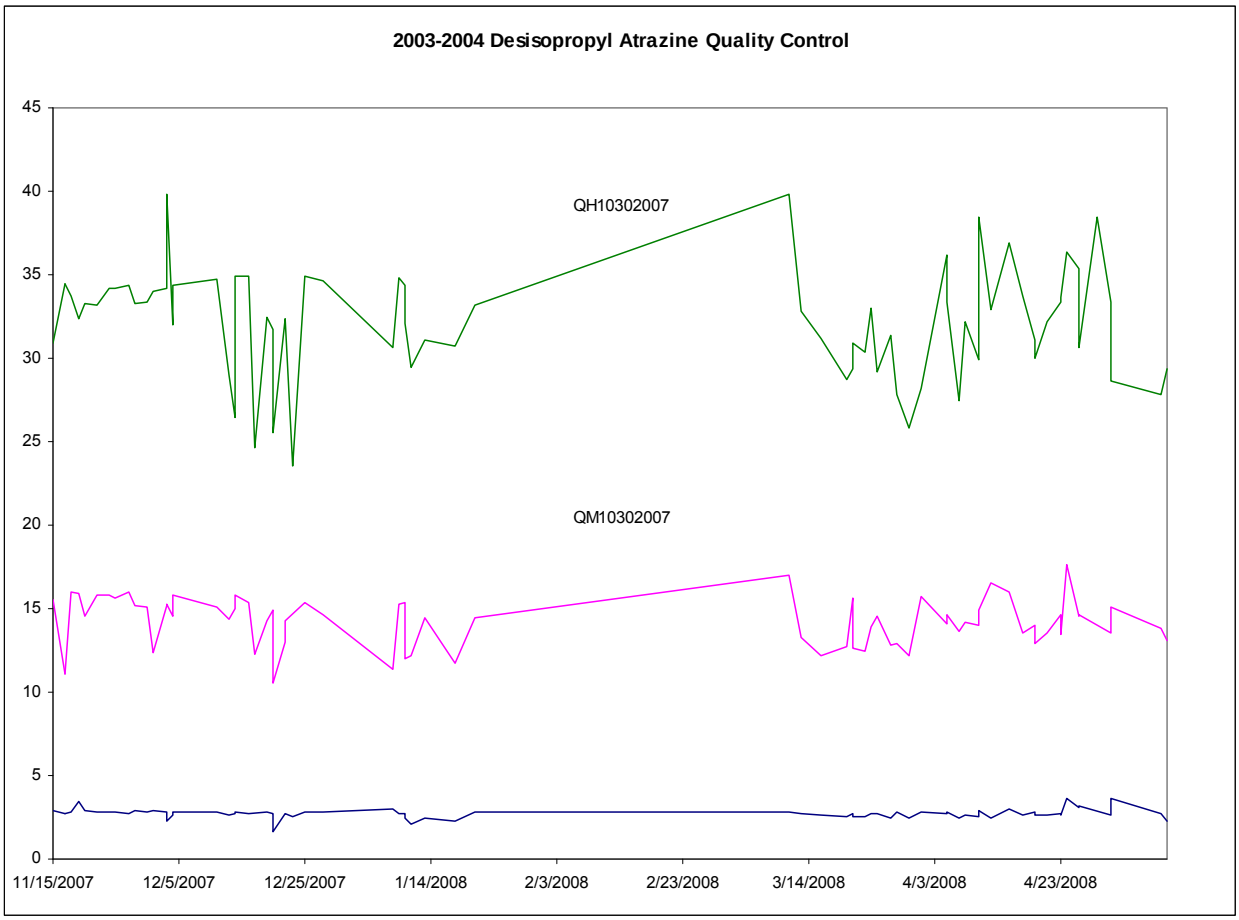


Table 1. R^2, errors about the slope for calibration data, limits of detection(LOD), and relative recovery percentage for each analyte.

Analyte	R^2	Average error about slope, %	LOD ng/mL	Relaltive Recovery, %
DEA	0.9998	0.16	0.250	100
DIA	0.9993	0.44	1.00	100
AM	0.9997	0.32	0.125	100
DCP urea	0.9963	0.98	0.125	100
DCPM urea	0.9997	0.24	0.500	100
Diuron	0.9996	0.20	0.125	100
Linuron	0.9996	0.16	0.125	100
DMeP	0.9996	0.20	1.00	100
DMeOP	0.9991	0,49	0.250	100
MMT	0.9997	0.30	1.00	100

DEA=desethyl atrazine; DIA=desisopropyl atrazine; AM=atrazine mercapturate; DCP urea=1-(3,4-dichlorophenyl) urea; DCPM urea=1-(3,4-dichlorophenyl)-3-methyl urea; DMeP=2-amino-4,6- dimethylpyrimidine; DMeOP=2-amino-4,6-dimethoxypyrimidine; andMMT=2-amino-4-methoxy-6-methyl-1,3,5-triazine

Relative recovery is determined from the slope of the line formed by plotting the calculated concentration of the analyte against its spiked concentration in the sample.

Table 2. Average concentration and relative standard deviation of each analyte at each quality control material concentration level (n=30)..

Analyte	QCL		QCM		QCH	
	Mean ng/mL	RSD %	Mean ng/mL	RSD %	Mean ng/mL	RSD %
DEA	1.00	10.9	5.19	13.3	15.7	8.60
DIA	0.995	9.28	4.78	11.3	15.2	4.28
AM	1.09	4.30	4.79	12.3	15.4	2.72
DCP urea	0.967	12.7	4.67	13.0	13.3	17.4
DCPM urea	0.976	14.9	4.46	12.6	12.8	19.3
Diuron	0.994	7.76	4.60	12.1	13.7	4.04
Linuron	0.946	14.6	4.35	14.3	13.1	4.13
DMeP	1.05	8.48	4.76	14.7	16.4	7.48
DMeOP	1.01	13.8	4.60	13.9	15.2	3.80
MMT	1.32	13.1	6.59	15.5	21.2	4.51

QCL=low level quality control material;QCM=medium level quality control material;QCH=high level quality control material; RSD=Relative standard deviation; DEA=desethyl atrazine; DIA=desisopropyl atrazine; AM=atrazine mercapturate; DCP urea=1-(3,4-dichlorophenyl) urea; DCPM urea=1-(3,4-dichlorophenyl)-3-methyl urea; DMeP=2-amino-4,6-dimethylpyrimidine;DMeOP=2-amino-4,6-dimethoxypyrimidine;andMMT=2-amino-4-methoxy-6-methyl-1,3,5-triazine

REFERENCES

(1) T. Kiely, D.Donaldson, A.Grube. Pesticide industry sales and usage: 2000 and 2001 market estimates. (2004) Washington, DC, U.S. EPA.
(2) M.A. Kamrin. Pesticide Profiles: toxicity, environmental impact, and fate. Lewis Publishers, New York, 1997.
(3) R. Bonfiglio, R.C. King, T.V. Olah, K. Merkle. Rapid Commun. Mass Spectrom. 13 (1999) 1175.
(4) J.K. Taylor. Quality assurance of chemical measurements. CRC Press, Boca Raton, FL, 1987.
(5) J.O. Westgard. Basic QC Practices: Training in Statistical Quality Control for Health Care Laboratories. Westgard QC, Inc., Madison, WI, 2002.

Other References

(1) B.A. Buchholz, E. Fultz, 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.
(2) 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.
(3) M.D. Beeson, W.J. Driskell, D.B. Barr. Anal Chem 71 (1999) 3526.
(4) 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.