

# TRIAZINE HERBICIDES and THEIR METABOLITES in URINE

8315

FORMULAS: Table 1

MW: Table 1

CAS: Table 1

RTECS: Table 1

**METHOD:** 8315, Issue 1

**EVALUATION:** PARTIAL

**Issue 1:** 15 March 2003

**BIOLOGICAL INDICATOR OF:** Exposure to triazine herbicides (1) - (4).

**SYNONYMS:** See TABLE 1

SAMPLING	MEASUREMENT
<p><b>SPECIMEN:</b> Urine</p> <p><b>VOLUME:</b> At least 15 mL of sample</p> <p><b>PRESERVATIVE:</b> None</p> <p><b>SHIPMENT:</b> Frozen</p> <p><b>SAMPLE STABILITY:</b> Not established. Appear to be quite stable frozen for long (&gt; one year) periods of time</p> <p><b>CONTROLS:</b> Urine from non-exposed persons.</p>	<p><b>TECHNIQUE:</b> GAS CHROMATOGRAPHY, MASS SELECTIVE DETECTOR</p> <p><b>ANALYTE:</b> s-Triazines (1) - (6)</p> <p><b>EXTRACTION:</b> Two liquid/liquid steps</p> <p><b>INJECTION VOLUME:</b> 1 µL</p> <p><b>TEMPERATURE</b></p> <p><b>-INJECTION:</b> 280 °C</p> <p><b>-DETECTOR:</b> 285 °C</p> <p><b>-COLUMN:</b> 50 °C hold for one minute, 50°C/min to 160 °C, 3.5 °C/min to 230 °C, 50°C/min to 280 °C, hold 2 minutes. Total run time, 26.20 min Solvent delay- 5.5 min</p> <p><b>ELECTRON MULTIPLIER</b></p> <p><b>VOLTAGE:</b> +153 mV from tune setting</p> <p><b>CARRIER GAS:</b> Helium, 1.5 mL/min</p> <p><b>COLUMN:</b> Capillary, fused silica, 30 m x 0.20-mm ID; 0.20 µm film SPB-5 or equivalent.</p> <p><b>CALIBRATION:</b> Standard solutions of analytes in ethyl acetate with internal standard.</p> <p><b>RANGE:</b> LOD to ~1900 nmol/L.</p> <p><b>ESTIMATED LOD:</b> 20 - 47 nmol/L depending on compound</p> <p><b>PRECISION (S<sub>r</sub>):</b> ~20% varies by compound</p>

**APPLICABILITY:** Triazines are common agricultural herbicides. This method measures the parent compounds and two metabolites simultaneously and specifically. It is applicable to herbicide applicators, farmers, or other occupations with triazine exposure.

**INTERFERENCES:** None identified.

**OTHER METHODS:** This method is an adaptation of one published by Catenacci, et al. [2]

**REAGENTS:**

1. Sodium bicarbonate, Certified grade.
2. Ethyl acetate, HPLC grade.\*
3. Ethyl ether, Spectranalyzed grade.\*
4. Atrazine.
5. Cyanazine.
6. Propazine.
7. Simazine.
8. Desethyl atrazine.
9. Desisopropyl atrazine.
10. Internal standard solution, Phenanthrene-d<sub>10</sub>, 100 µg/mL in methanol. Store in refrigerator.
11. Sodium sulfate, anhydrous, Certified ACS grade.
12. Sodium chloride, reagent grade.
13. Helium, zero grade.
14. Nitrogen, zero grade.
15. Methanol, HPLC grade.\*
16. Deionized water.
17. A urine pool collected, frozen until use, and then thoroughly mixed

\* See SPECIAL PRECAUTIONS

**EQUIPMENT:**

1. Gas chromatograph with mass selective detector, SPB-5 or equivalent capillary column and autosampler (page 8315-1).  
NOTE: If large batches of samples are done, chilling the autosampler tray assures that there is no evaporation of the samples
2. Guard column, 1.5 meter x 0.4 mm ID, made of the same material (or SPB-1) as the analytical column and joined by a capillary column butt connector and polyamide/graphite ferrule.  
NOTE: This guard column and the injection port liner should be changed after every 35-40 samples.
3. Analytical balance.
4. Analytical evaporator/manifold fed with nitrogen gas.
5. Centrifuge.
6. "Roto-torque" rotator.
7. Disposable screw-top culture tubes, 16x125 mm, with PTFE-lined caps.  
NOTE: Caps with rubber liners will not work.
8. Disposable volumetric pipets, 5-mL.
9. Disposable transfer pipets, both short and long.
10. Volumetric flask, 25-mL.
11. Beakers, 50-mL, 5-mL.
12. Disposable centrifuge tubes, 15-mL, with caps.
13. Autosampler vials with 100-µL polypropylene inserts and caps.
14. Syringes, 10-µL and 100-µL.
15. Polypropylene bottles, 30-mL and 250-mL.
16. Disposable filtration columns, 10-mL with 20 µm pore frit.
17. Metal scoopula.
18. Repipet dispenser bottles, 1-L.

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**SPECIAL PRECAUTIONS:** Universal precautions should always be used when dealing with bodily fluids or extracts thereof. Methanol, ethyl ether, and ethyl acetate are all highly flammable; handle with care and use in a hood. Reagents 4-10 come from the manufacturer with expiration dates on them. Be certain to heed these dates.

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**SAMPLING:**

1. Collect the urine in a polyethylene bottle and cap.
2. Ship the sample in dry ice in an insulated container, store at -80°C upon arrival at lab. A reminder: commercial shippers have special labeling requirements for packages containing dry ice.

### **SAMPLE PREPARATION:**

3. Weigh 0.7 g sodium chloride into screw-top culture tubes.
4. Weigh 0.5 g sodium bicarbonate into each of these tubes. Cap these tubes and then they can be stored for at least two weeks and most likely for as long as necessary.
5. Label two culture tubes (one empty and one containing the two salts) and one centrifuge tube with a unique number for each sample.  
NOTE: The labelling system is unimportant as long as there is no danger of the samples being mixed-up and so a record is kept of which sample corresponds to which tube number.
6. Thaw samples to room temperature. Using warm water is okay.
7. Mix thoroughly as urine becomes inhomogeneous upon freezing.
8. Transfer 5 mL of sample to its culture tube containing the salts. Leave the caps off the tubes until samples have been dispensed to every tube and any CO<sub>2</sub> evolved has dispersed.
9. Spin on Roto-torque for about one minute on setting 4 High to dissolve salt. Some salt will remain in the tube.
10. Dispense 5 mL ethyl ether into each of the urine tubes. Again leave caps off or on loosely to allow for gas to disperse.
11. Roto-torque for fifteen minutes on setting 4 High.
12. Centrifuge samples for five minutes at 3000 rpm.
13. Remove ether layer (top) with short transfer pipet to the second labelled culture tube. Remove all the ether; taking some aqueous phase does not appear to be detrimental to the analyses.
14. Dispense 5 mL ethyl acetate into each sample tube containing the remaining aqueous phase.
15. Roto-torque for fifteen minutes on setting 4 High.
16. Centrifuge samples for five minutes at 3000 rpm.
17. Remove organic layer (top) with short transfer pipet and add to the ether layer. Again, remove all the organic layer.
18. Fill the filtration columns about 3/4 full of anhydrous sodium sulfate.
19. Balance the column on top of the labelled 15-mL centrifuge tubes.
20. Using a transfer pipet, transfer the combined extract to the appropriate filtration column.
21. Wash the tube which held the organic phases with one pipetful ethyl ether and add this to the filtration column.
22. Wash the filtration column with another 2 mL ethyl ether.
23. Allow the filtration columns to drain completely.
24. Place the centrifuge tubes in the analytical evaporator and gently evaporate the solvent with a stream of nitrogen until the tube is dry.  
NOTE: A waterbath helps dissipate the cold, but it is important the bath be kept at room temperature. Raising the bath temperature to 30°C may cause marked decreases in the recoveries of analytes (5) and (6) and may well affect the other analytes as well.
25. Rinse the sides of the tubes with about 0.5 mL ethyl ether.
26. Evaporate to dryness again.
27. Add 10 µL internal standard solution to each centrifuge tube.
28. Add 90 µL ethyl acetate to each tube and mix well.
29. Let the extracts sit for at least thirty minutes.
30. Label autosampler vials and place an insert into each vial. Have the caps ready for the vials.
31. Transfer the entire extract to the correct autosampler vial with a long transfer pipet.
32. Cap the vial and place in appropriate tray position in the GC autosampler.

### **CALIBRATION AND QUALITY CONTROL:**

33. The stock solution of the six analytes is prepared from the neat solids to about 240 µmol/L by accurately weighing 1.25 mg of each analyte into a 25 mL volumetric flask (larger flasks could be used with a concomitant increase in the amount of solid weighed out.) Fill the flask to the line with ethyl acetate. It is generally necessary to sonicate the flask for complete and quick dissolution, after which the analytes stay in solution. Aliquot into autosampler vials, cap, and store this solution in the refrigerator. Calculate the exact concentration of each analyte from the amount of solid weighed out. In ethyl acetate these analytes are quite stable for extended periods of time. At no point did degradation of the standard solutions become a problem, so they are stable for at least six months under these conditions.

34. The calibration standards are made the day of the analysis. Allow the stock solution and the internal standard solution to warm to room temperature before performing any pipetting. All the "pipetting" in this step is done using a 100 µL LC syringe. Prepare a diluted standard by adding 10 µL of the stock solution to 440 µL ethyl acetate to make a secondary solution. Prepare seven standards by first pipetting 10 µL of the internal standard solution into marked autosampler inserts/vials. Then add 10, 45, and 90 µL of the diluted standard to three vials and 10, 20, 50, and 90 µL of the stock solution to four of the vials. Make each vial contain 100 µL final volume by adding the correct amounts of ethyl acetate. This will make standards that are about 0.48, 2.4, 4.8, 24, 48, 120, and 216 µmol/L. Calculate the exact concentrations of each analyte in each standard from the stock solution concentrations. Insert these standards into the autosampler batch prepared in Step 32.
35. The two levels of quality control samples were made by adding 75 µL and 250 µL, of the stock solution to 250 mL of the pooled urine. Calculate the exact concentrations of each analyte in each quality control standard from the stock solution concentrations. Run enough QC samples so that they constitute ~10% of the batch and are equally divided between the two levels. Extract 5 mL of each QC (steps 3 through 32) and run with the samples.
36. Prepare calibration curves of Area std/Area IS vs conc std/conc IS for each of the six analytes.

**MEASUREMENT:**

37. Set gas chromatograph according to manufacturer's recommendations and to conditions on page 8315-1.
38. Set mass selective detector to the following SIM Parameters:

Compounds	Dwell (ms)	Start time*	Ions
Metabolites (5) & (6)	100	5.5	173, 158, 145, 172, 187
Parents (1) - (3)	100	14.1	201, 186, 200, 215, 214, 229
Phenanthrene-d <sub>10</sub>	100	15.5	188
Cyanazine (4)	100	19.5	172, 225, 240

\* These start times may change as necessary, and should be checked regularly, especially after replacing the guard column. A full-scan chromatogram should be performed on a standard to check the retention times and SIM windows.

39. Inject 1 µL ethyl acetate extract from step 32.
40. Measure the peak areas of the samples and internal standard. Divide the peak areas of the sample by the peak area of the internal standard in the same chromatogram. The following ions are used unless the mass selective detector is capable of adding areas of more than one ion:

Desisopropylatrazine	158
Desethylatrazine	172
Simazine	201
Atrazine	200
Propazine	214
Cyanazine	225

**CALCULATIONS:**

41. Determine the concentration (C<sub>c</sub>) of the analytes in the extracts (in µmol/L) from the calibration curves.
42. The concentration (C<sub>u</sub>) of the analytes in urine is calculated from the equation:

$$C_u = \frac{C_c}{50} \times 1000, \text{ nmol} / L$$

Where 50 is the concentration factor of going from 5 mL urine to 100 µL extracts, and 1000 is the factor for converting µmol/L to nmol/L.

Note: These concentrations could be corrected for creatinine level or sample density if desired and if those values were obtained.

## GUIDES TO INTERPRETATION:

One of the limitations of this method is that different parent triazines are metabolized to the same compound. For example, de-alkylation of the isopropyl group on atrazine and cyanazine both give desisopropylatrazine, which can also be formed by the de-alkylation of one of the ethyl groups of simazine. Unless exposure is very high the parent compounds are not found in the urine, leaving the analyst with no way of knowing which parent the metabolite came from in the case of a mixed exposure which is very common in herbicide applicators. The metabolites and any parent compounds found can be summed and reported as total triazine level if desired.

There are very few biological monitoring studies of triazines and their metabolites found in the literature on which to develop a good reference range. And to our knowledge there have been no studies or reports on levels of these analytes in the general population.

1. Catenacci (1990) looking at parent atrazine in the urine found maximum excretion rates of 0.14 - 0.42 nmol/hr in workers at an atrazine manufacturing plant and very poor correlation between the concentration in the urine and the concentration in the air. These excretion rates dropped by an order of magnitude or more within twelve hours of the exposure.
2. Ikonen summed two metabolites (the desisopropyl atrazine and the bi-dealkylated atrazine, the latter of which was not investigated in this study) and found concentrations of 30-110  $\mu\text{mol/L}$  in railway sprayers.
3. Catenacci (1993) returned to the atrazine manufacturing plant with an improved method that could measure all three of the de-alkylated metabolites of atrazine and found ranges of 1.1-1.6  $\mu\text{mol/24 hr}$  for the bi-dealkylated metabolite, 0.13-0.21 for desisopropyl atrazine, 0.11-0.20 for desethyl atrazine, and 0.017-0.021 for parent atrazine. To convert to concentration it is possible to assume an average urinary output of 1.1 L/24 hr (Reference 4). Using that assumption and combining the metabolites gives average concentrations ranging from 1.2 - 1.8  $\mu\text{mol/L}$ .
4. Hines (Reference 5) provides an overview of more recent metabolism studies and results for biomonitoring of the triazines as well as showing the utility of this method (and others) in a study of herbicide applicators.
5. There have been no human studies looking at cyanazine in which to obtain reference ranges of any kind.

## EVALUATION OF METHOD:

The method was evaluated using 15 spiked urine samples ranging from 5.8 nmol/L - 1878 nmol/L and analyzing these samples on three different days. The recoveries for s-Triazines for compounds (1-5) ranged from 84-88% and for compound (6) it was 67%. Precisions and LOD were determined from this experiment and are listed on the first page of this method.

Sample stability was not exhaustively examined. There were, however, samples run after several months that had been stored at  $-20^{\circ}\text{C}$  compared to splits of those samples stored at  $-80^{\circ}\text{C}$  and no bias was found due to the different storage temperatures. This would lead one to believe that the samples would be very stable stored at  $-80^{\circ}\text{C}$  for extended periods of time. The QC samples also tell something about sample stability. The first batch of these samples was stored at  $-20^{\circ}\text{C}$  and the control chart shows no trend in these samples even when run over 18 months. There may be some loss of signal on samples that go through multiple freeze/thaw cycles, but conclusive experiments were not done and this may be a function of the amount of solids that form in the urine during this process and not because the analytes are breaking down. Because of this, it is a good idea to aliquot the QC samples out into containers that will only be thawed once or twice and then discarded.

## REFERENCES:

- [1] Catenacci G, Maroni M, Cottica D, Pozzoli L [1990]. Assessment of human exposure to atrazine through the determination of free atrazine in urine. *Bull. Environ. Contam. Toxicol.* 44: 1-7.
- [2] Catenacci G, Barbieri F, Bersani M, Ferioli A, Cottica D, Maroni M [1993]. Biological monitoring of human exposure to atrazine. *Toxicology Letters* 69: 217-222.

- [3] Ikonen R, Kangas J, Savolainen H [1988]. Urinary atrazine metabolites as indicators for rat and human exposure to atrazine. *Toxicology Letters* 44: 109-112.
- [4] Gordon Ross, Ed., [1982]. *Essentials of Human Physiology*, 2<sup>nd</sup> Edition. Chicago, IL: Year Book Medical Publishers, Inc, p. 382.
- [5] Hines C, Deddens J, Striley C, Biagini R, Shoemaker D, Brown K, MacKenzie B, Hull R [2003]. Biological monitoring for selected herbicide biomarkers in the urine of exposed custom applicators: Application of mixed-effect models. *Annals of Occupational Hygiene* 47(6): 503-517.

**METHOD WRITTEN BY:**

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**TABLE 1. STRUCTURAL FORMULAS, MOLECULAR WEIGHTS, and PROPERTIES**

Compound	Formula	Molecular Weight	CAS	RTECS	Synonyms
(1) Atrazine	C <sub>8</sub> H <sub>14</sub> ClN <sub>5</sub>	215.72	1912-24-9	XY5600000	2-Chloro-4-ethylamino-6-isopropylamino-s-triazine; 2-Chloro-4-ethylamino-6-isopropylamino-1,3,5-triazine; 6-Chloro-N-ethyl-N'-(1-methylethyl)-1,3,5-triazine-2,4-diamine
(2) Simazine	C <sub>7</sub> H <sub>12</sub> ClN <sub>5</sub>	201.69	122-34-9	XY5250000	2-Chloro-4,6-bis(ethylamino)-s-triazine
(3) Propazine	C <sub>9</sub> H <sub>16</sub> ClN <sub>5</sub>	229.75	139-40-2	XY5300000	Chloro-4,6-bis(isopropylamino)-s-triazine; 2,4-Bis(isopropylamino)-6-chloro-1,3,5-triazine
(4) Cyanazine	C <sub>9</sub> H <sub>13</sub> ClN <sub>6</sub>	240.73	21725-46-2	UG1490000	2-Chloro-4-ethylamino-6-(1-cyano-1-methyl)ethylamino-s-triazine; 2-[(4-chloro-6-ethylamino-s-triazin-2-yl)amino]-2-methylproprionitrile
(5) Desethylatrazine	C <sub>6</sub> H <sub>10</sub> ClN <sub>5</sub>	187.66	6190-65-4		2-Amino-4-chloro-6-isopropylamino-s-triazine; 6-Chloro-N-(1-methylethyl)-1,3,5-triazine-2,4-diamine
(6) Desisopropylatrazine	C <sub>5</sub> H <sub>8</sub> ClN <sub>5</sub>	173.67	1007-28-9		2-Amino-4-chloro-6-ethylamino-s-triazine; 6-Chloro-N-ethyl-1,3,5-triazine-2,4-diamine