I. SUMMARY AND INTRODUCTION

A. SCOPE

This method is to be used for the determination of *R*-metolachlor (Acetamide, 2-chloro-*N*-(2-ethyl-6-methylphenyl)-*N*-[(1*R*)-2-methoxy-1-methyl]-) in water. The limit of detection (LOD, the smallest dose that yields a response that is statistically significantly different from the response of the zero dose) is 0.10 parts per billion (ppb) of *R*-metolachlor. The limit of quantitation for *R*-metolachlor (LOQ, the lowest level of fortification that gives an acceptable recovery) is 0.20 ppb.

B. PRINCIPLE

A 0.50-mL aliquot of a water sample is added to a polystyrene culture tube coated with *R*-metolachlor antibody. The assay is carried out by sequential addition of enzyme conjugate, wash water and color reagent. The reaction is terminated by acidification. Quantification is performed spectrophotometrically at 450 nm. A flow diagram for the method is presented in Figure 1.

II. MATERIALS AND METHODS

A. APPARATUS

1.0 Beacon *R*-Metolachlor Tube Kit, enzyme immunoassay coated tube assay, Beacon cat. #CPT-021, Beacon Analytical Systems, 383 Presumpscot St., Portland, ME.

2.0 Visible wavelength spectrophotometer, Beckman-Coulter model #7400, Beckman-Coulter Instruments, Inc., 2500 N. Harbor Boulevard, P.O. Box 3100, Fullerton, CA 92834-3100 or equivalent.

3.0 Semimicro rectangular quartz cuvette, 1.0 mL capacity, Fisher cat. #14-385-914A or equivalent.

4.0 Finnpette Techpette pipetter, 500-µl fixed volume pipette, Fisher cat. #21-377-111J or equivalent.

5.0 Fisher/Wheaton adjustable digital micropipetter, dispensing range of 100 –1000 µl, Fisher cat. #13-707-5 or equivalent.

6.0 Pipette tips, 1000-µl volume, non-sterile, Consolidated Plastics Company, Inc. cat. #2162 or equivalent. Tips should be pre-packaged in a rack to allow user to fasten a tip to the pipette without touching the tip.

7.0 Flip-top polyethylene dispensing bottle, 500 mL capacity, Consolidated Plastics Company, Inc. cat. #41589LG or equivalent. The flip-top-capped bottle dispenses a heavy stream of liquid and does not lose pressure over time while dispensing. Drop dispenser bottles or squirt bottles are not suitable for this method.

8.0 No-Wire™ "Grip-rack" test tube rack for 13 mm test tubes, holds up to 90 tubes, Bel-Art cat. #F18749-0000, VWR cat. #20907-940. This rack has eight "fingers" in each tube position to hold tubes while rack is inverted and shaken.

9.0 Borosilicate disposable glass tubes, 12 mm O.D. x 75 mm in length, with labeling area. Fisher cat. #14-957-22A or equivalent.

10.0 Tube rack to accommodate 12 x 75 mm tubes, Nalgene Acetal Unwire test tube rack for 13 mm tubes, Fisher cat. #14-809-60 or equivalent.

11.0 Kimwipes EX-L, extra low-lint wiper, Fisher cat. #06-666A or equivalent.

B. REAGENTS

1.0 Distilled water (H_2O).

2.0 Enzyme conjugate, included in the *R*-metolachlor immunoassay kit, Beacon Analytical Systems.

3.0 Substrate solution, included in the *R*-metolachlor immunoassay kit, Beacon Analytical Systems. Alternatively, a commercially available horseradish peroxidase tetramethylene benzidine substrate, such as "K-Blue" (Neogen Corporation, cat. #300177) may be used.

4.0 Stop solution, included in the *R*-metolachlor immunoassay kit, Beacon Analytical Systems. Alternatively, 1.0 M HCl, prepared by the analyst, may be used.

5.0 Metolachlor analytical standard, Syngenta Crop Protection, Inc., P.O. Box 18300, Greensboro, NC 27419-8300. Storage conditions: Frozen.

C. ANALYTICAL PROCEDURE

The antibody-coated tubes, all reagents and sample and standard solutions must be warmed to room temperature, approximately 22°C, prior to use. Be certain all solutions are warmed to room temperature before running the assay because the reaction kinetics are directly proportional to temperature.

Note: The procedure described below achieves the best results when the analyst proceeds at a moderate pace. Therefore, it is important for the analyst to develop technique based upon a moderate, reproducible pace.

1.0 Enzyme Immunoassay

Label each antibody-coated tubes with appropriate identification. Arrange the labeled tubes in the No-Wire™ tube rack such that sample and standard tubes are interspersed. The first and last tubes should always be a standard with the samples and other standards intermixed.

1.1 Inhibition of Enzyme Conjugate

1.1.1 Pipette a 500-µl aliquot of sample or standard solution to previously labeled tubes. Pre-rinse each tip once with the solution to be transferred before dispensing the liquid. Hold the pipette at approximately a 45° angle downward and touch the tip to the inside of the tube about 1/2 inch down from the rim. Be sure to use a clean tip for each sample or standard.

1.1.2 Transfer 500-µl of enzyme conjugate to each sample or standard in the same order of addition and in the same manner as described in 1.1.1. Pre-rinse the tip with the enzyme conjugate solution prior to the first dispensing. After the first pipetting, do not change the pipette tip. Since the tip is not changed, pre-rinsing is not necessary.

1.1.3 After the enzyme conjugate is dispensed to all tubes, hold the tube rack horizontally at eye level and shake the rack back and forth three times. Repeat shaking while tilting the rack slightly to the left and to the right. Thus, the rack will have been shaken a total of nine times. Do not use much force while shaking. Formation of bubbles within the tubes indicates too much force has been used.

1.1.4 Set the rack on the bench top and initiate a timed twenty minute incubation.

1.2 Wash

1.2.1 Decant the contents of all tubes by holding the tube rack upside down over the stainless steel pan and shaking the rack vertically twice.

1.2.2 Fill each tube to overflowing with H_2O using the dispensing bottle. Direct the stream from the dispensing bottle towards the bottom of each tube. Maintain moderate pressure on the stream of water by squeezing the dispensing bottle. Fill each tube in the same order as previously done. When all tubes have been filled, decant the wash as described in 1.2.1 above.

1.2.3 Wash all tubes three more times in the same manner.

1.2.4 After the final wash, shake the rack upside down firmly several times. Remove most of the remaining wash by holding the rack upside down and tapping the rims of all tubes gently on a clean paper towel. Small droplets of wash may remain in the tubes but will not affect assay results.

1.3 Color Development

1.3.1 Pipette 500-µl of substrate solution to each tube in the same fashion as the previous solutions were dispensed. The pipette tip need not be changed between additions.

1.3.2 Set the rack on the bench top and initiate a timed 10 minute incubation as described above in section 1.1.4.

1.3.3 Shake the rack *gently* once every 2½ minutes during the incubation. The substrate within individual tubes will gradually turn varying shades of blue.

1.3.4 Add 500-µl of stop solution to each tube in the same manner as the Substrate solution was added. The blue color in each tube will turn various shades of yellow upon addition of stop solution.

1.3.5 The intensity of the yellow solution in the tubes should be measured within 15 minutes of addition of stop reagent.

D. INSTRUMENTATION

1.0 Description and Operating Conditions

The visible wavelength spectrophotometer should be warmed up according to the manufacturer's instructions prior to measuring the absorbance of the yellowish solutions in each tube. The Beckman-Coulter model #7400 requires only about one minute to warm up the visible wavelength lamp. Adjust the spectrophotometer to read the absorbance at 450 nm.

Rinse the cuvette with H_2O using the flip-top water bottle prior to measuring the first sample. Be sure to shake out as much liquid from the cuvette as possible. Always check to see no fingerprints are on the clear surfaces through which the measurement will be taken. Wash the cuvette as consistently as possible between samples.

2.0 Standardization

2.1 Each assay consists of standards and samples run concurrently in the same analytical set. The absorbance values obtained from sample solutions may only be compared to the absorbance of standards run in the same set. *R*-metolachlor standards range from 2.0 ng/mL to 0.10 ng/mL in addition to a blank (zero dose).

2.2 Spectrophotometric analysis of the colored reaction products obtained from standard solutions will yield absorbance values measured at 450 nm (A_{450}). With a calculator or computer, use these data to generate a log/linear regression function. This curve is in the form of y = m log(x) + b. The concentration of the *R*-metolachlor standards is plotted on a logarithmic scale on the horizontal (x) axis. The absorbance values are plotted on a linear scale on the vertical (y) axis. An example of a typical standard curve is shown in Figure 2.

E. INTERFERENCES

The cross reactivity of this method has been evaluated over a range of chloroacetanilide parent and degradate compounds (Table 4). This method has been shown to exhibit enantioselective binding to CGA-77101 over CGA-77102. Among the parent chloroacetanilides tested, dimethenamid was determined to react with the assay with less than eight percent of the reactivity of CGA-77101. The ethane sulfonic and oxanillic acid metabolites of metolachlor do not react with this method. Refer to Section III.C for a more detailed discussion of the results of the cross reactivity experiments.

F. CONFIRMATORY TECHNIQUES

None.

G. TIME REQUIRED

An analyst can analyze up to five samples and five standards plus a blank in approximately 45 minutes.

H. MODIFICATIONS AND POTENTIAL PROBLEMS

1.0 The wash step must be carried out properly to ensure removal of reagents not bound to antibody in the tubes. The analyst must maintain a constant, moderate pressure on the wash bottle to fill the tubes with a consistent stream of wash water. Inadequate washing will result in poor standard curves.

2.0 As previously stated in the introduction to the ANALYTICAL PROCEDURE, the analyst should ensure the antibody-coated tubes, reagents and sample solutions are warmed to room temperature, approximately 22°C, before use.

3.0 The analyst should ensure that all sample and standard solutions are positioned properly in the tube rack and labeled appropriately.

4.0 The analyst may observe A_{450} values less than those of the highest standard, 2.0 ng/mL. In this event, the concentration of the corresponding sample cannot be calculated since its absorbance readings do not fall within the range of the standard curve. The sample should be diluted and re-assayed to obtain absorbance readings that lie within the bounds of the standard curve. To do so, make a 10-fold dilution of the sample. The concentration of *R*-metolachlor in the original sample can be calculated by multiplying the result (concentration) of the diluted sample by ten. Should the 10-fold dilution not be sufficient to bring the sample into the range of the standard curve, make additional dilutions as needed.

5.0 The analyst may observe A_{450} values greater than those of the method LOD, 0.10 ppb. In this situation, the analyst should list the final concentration of the sample as "<0.10."

6.0 The absorbance at 450 nm of the H_2O blank should be in the range of approximately 1.600 to 2.400 absorbance units. The absorbance will vary since the turnover rate of the enzyme conjugate generating the colored signal is dependent on ambient temperature.

7.0 Transfer of solutions by pipettes requires the analyst to constantly monitor his or her technique. Pipetting errors are the major source of error in immunoassay methodology.

I. PREPARATION OF STANDARD SOLUTIONS

Analytical standards are furnished as part of the Beacon *R*-Metolachlor tube kit. These solutions should be warmed to room temperature prior to running an assay. Store these solutions at 4°C when not in use.

Alternatively, the analyst may prepare standard solutions from analytical grade racemic metolachlor. Dissolve 5.0 mg of metolachlor into 100-mL of ACN. This solution contains 50 µg/mL of metolachlor. Dilute this solution into H_2O to make 100 mL of 5.0 µg/mL of metolachlor. Serially dilute this solution into H_2O to make standards of 4.0-, 2.0-, 1.0-, 0.40- and 0.20-ng/mL of metolachlor. These solutions contain, respectively, 2.0-, 1.0-, 0.50-, 0.20- and 0.10-ng/mL of *R*-metolachlor. Label these solutions according to the concentration of *R*-metolachlor. Prepare a blank or 0 ng/mL standard consisting solely of H_2O. Store these standards at 4°C when not in use.

J. METHODS OF CALCULATION

R-metolachlor residues in sample solutions are determined by inserting the absorbance value of a given sample into the log/linear regression function generated by methods described in Section II.D.2.2. These calculations may be made on a computer or hand-held calculator.

C. SPECIFICITY

This method was extensively evaluated for cross reactivity to a variety of commercially available chloroacetanilide herbicides and their metabolites (Fig. 3). These results are summarized in Table 4. These data indicate the antibodies used in this method react primarily with CGA-77101. In particular, the antibodies demonstrate enantioselective binding to the *R* isomer of metolachlor over the *S* isomer, exhibiting approximately 100-fold greater selectivity to the *R* form. Consequently, the antibodies in this method regard CGA-77102 as an immunochemically inert test substance. Among other parent chloroacetanilides, only metolachlor, technical grade CGA-77102, and dimethenamid demonstrated reactivity. The metolachlor result is not surprising given it is a racemic mixture of the *R* and *S* forms. In like fashion, the reactivity to technical grade CGA-77102 (88:12 *S*:*R*) is restricted to the amount of *R*-metolachlor present in the formulation. Dimethenamid was

determined to have less than eight percent of the reactivity of CGA-77101. Because the concentration of dimethenamid must be greater than ten-fold that of *R*-metolachlor to obtain a similar analytical result, the presence of dimethenamid in water samples should not affect test results. The metabolite CGA-212248 was found to have less than four percent reactivity relative to *R*-metolachlor. Ethane sulfonic and oxanillic acid metabolites were demonstrated not to react with this method. Several metolachlor metabolites were found to have trace reactivity comparable to the parent *S* form. In summary, the results of the cross reactivity experiments indicate this method is specific to *R*-metolachlor with the exception of limited reactivity to dimethenamid.

D. LIMITATIONS

1.0 As noted in Section II.H, MODIFICATIONS AND POTENTIAL PROBLEMS, the analyst should constantly monitor his or her pipetting technique. Pipetting errors are the greatest source of error in immunoassays.

2.0 Because of the time involved adding each reagent to the tubes, do not analyze more than nine samples in any analytical set. This results in a total of fifteen tubes: six standards including a zero or blank standard and nine samples.

3.0 The yellow-colored reaction products generated at the end of the assay are not stable. *The absorbance of the yellow-colored products must be measured within 15 min of the end of the analysis.* Valid analytical results cannot be achieved by measuring reaction products that have aged more than 15 min.

IV. CONCLUSION

Method 1030-00 Part B is an accurate, precise and rapid method for the determination of *R*-metolachlor, CGA-77101, in water. The method has a limit of detection of 0.10 ppb and a limit of quantitation of 0.20 ppb. The method is enantioselective for CGA-77101 over CGA-77102. Among parent chloroacentanilide herbicides, only dimethenamid exhibits reactivity with the method, having less than eight percent of the reactivity of *R*-metolachlor. Within the metabolites tested, only CGA-212248 was found to have greater than one percent reactivity. The ethane sulfonic and oxanillic acid metabolites of metolachlor are not recognized by this method.

FIGURE 1. FLOW DIAGRAM FOR METHOD 1030-00 PART B

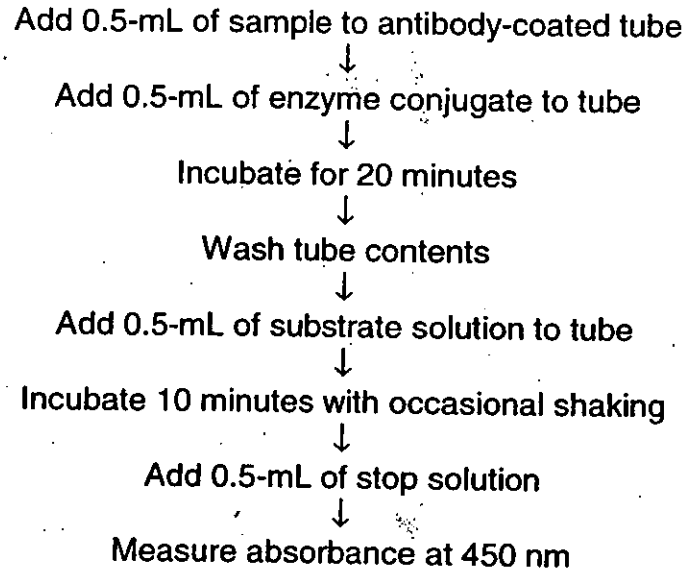
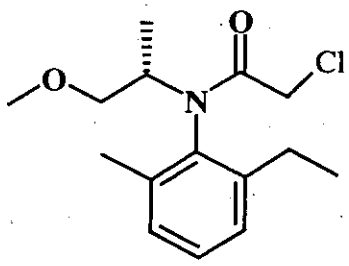
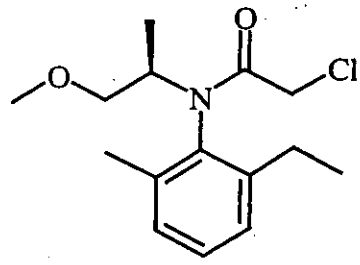


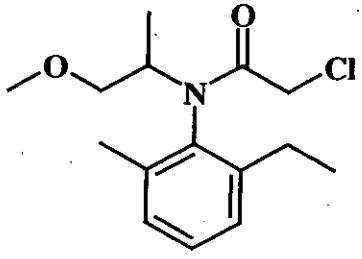
FIGURE 3. STRUCTURES OF TEST SUBSTANCES EVALUATED FOR CROSS-REACTIVITY IN THE VALIDATION OF METHOD 1030 PART B



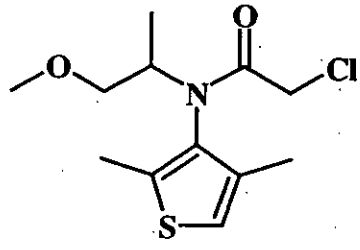
R-Metolachlor
(CGA-77101)



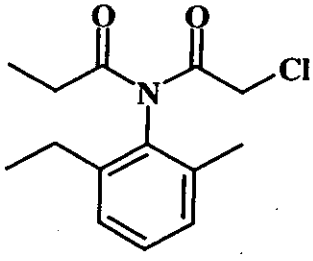
S-Metolachlor
(CGA-77102)



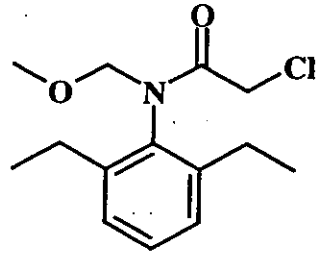
Metolachlor



Dimethenamid

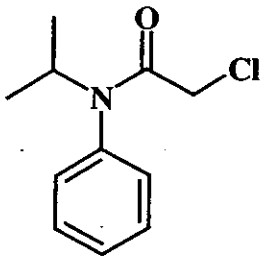


Acetochlor
CGA-212248

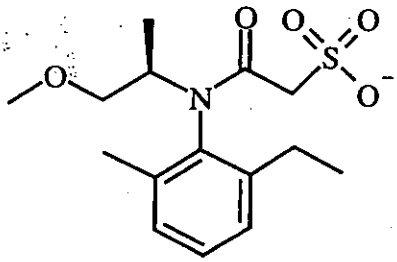


Alachlor
CGA-41507

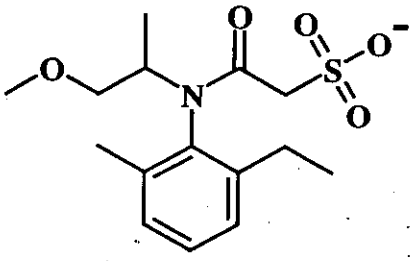
FIGURE 3. STRUCTURES OF TEST SUBSTANCES EVALUATED FOR CROSS-REACTIVITY IN THE VALIDATION OF METHOD 1030 PART B (Continued)



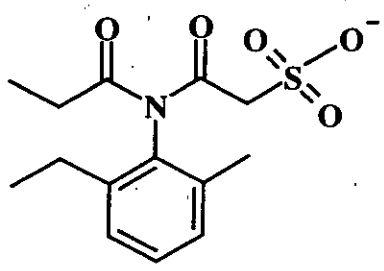
Propachlor



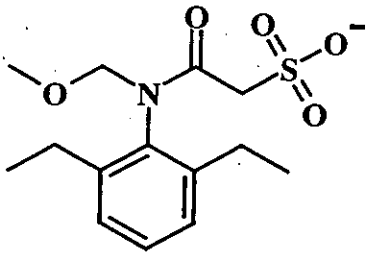
CGA-380168



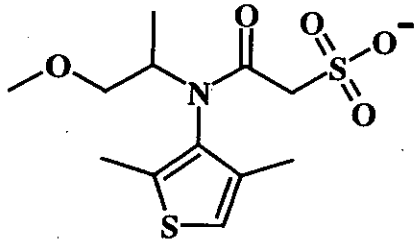
CGA-354743



Acetochlor-ESA

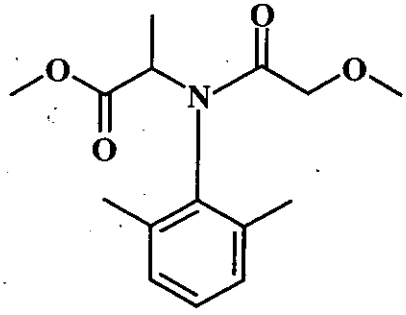


Alachlor-ESA

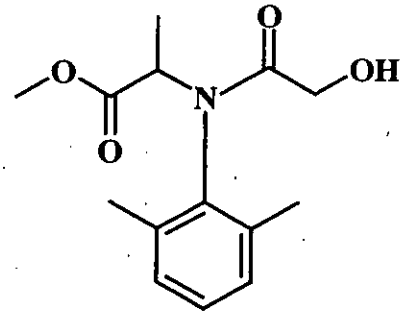


Dimethenamid-ESA

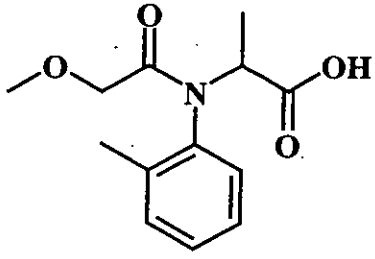
FIGURE 3. STRUCTURES OF TEST SUBSTANCES EVALUATED FOR CROSS-REACTIVITY IN THE VALIDATION OF METHOD 1030 PART B (Continued)



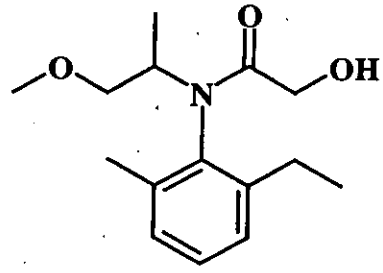
Metalaxyl



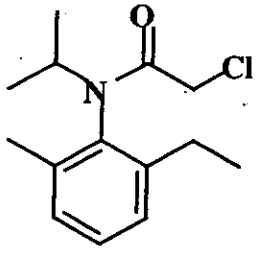
CGA-67869



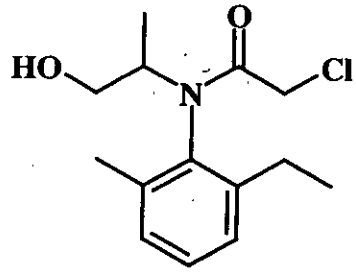
CGA-62826



CGA-40172

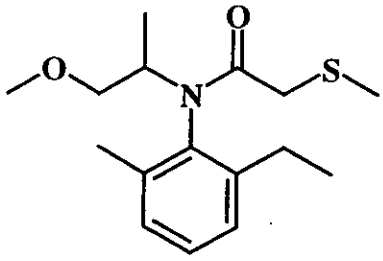


CGA-212248

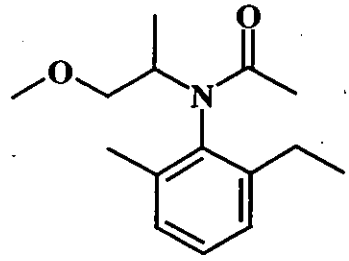


CGA-41638

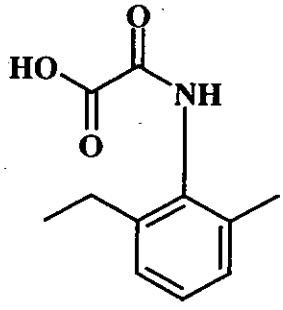
FIGURE 3. STRUCTURES OF TEST SUBSTANCES EVALUATED FOR CROSS-REACTIVITY IN THE VALIDATION OF METHOD 1030 PART B (Continued)



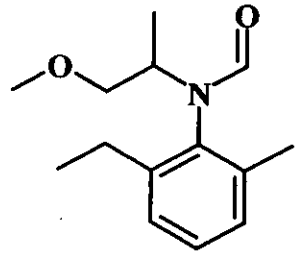
CGA-46127



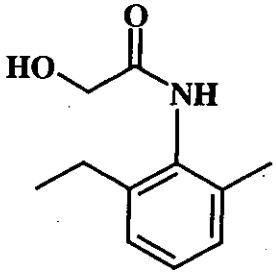
CGA-41507



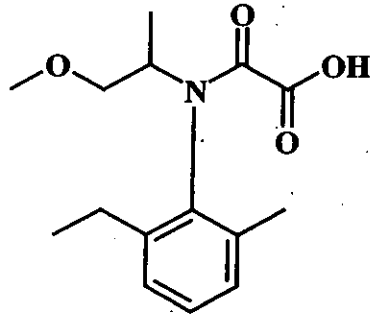
CGA-50720



CGA-67125

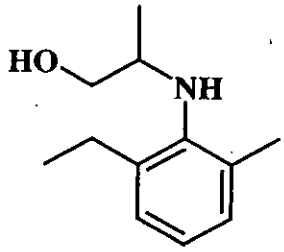


CGA-37735



CGA-51202

FIGURE 3. STRUCTURES OF TEST SUBSTANCES EVALUATED FOR CROSS-REACTIVITY IN THE VALIDATION OF METHOD 1030 PART B (Continued)



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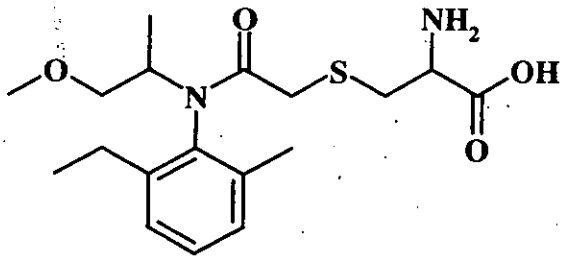
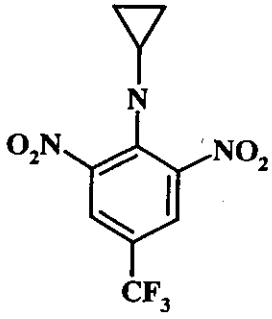
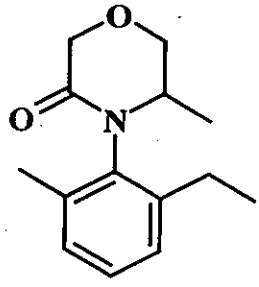


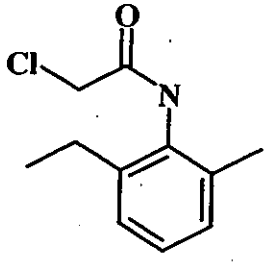
CGA-46576



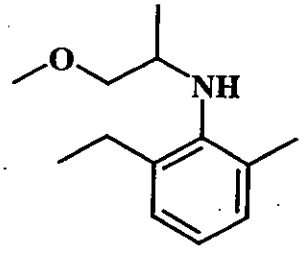
CGA-46577



CGA-40919

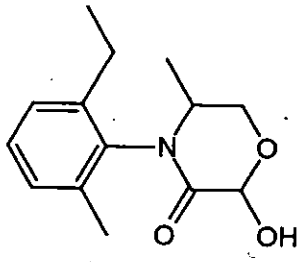


CGA-13656

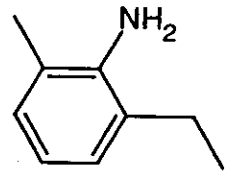


CGA-38502

FIGURE 3. STRUCTURES OF TEST SUBSTANCES EVALUATED FOR CROSS-REACTIVITY IN THE VALIDATION OF METHOD 1030 PART B (Continued)



CGA-49751



CGA-212245