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DETERMINATION OF RESIDUES OF METSULFURON METHYL METABOLITE A AND METABOLITE A1 IN CEREAL GRAIN CROPS BY LIQUID CHROMATOGRAPHY

BY

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ABSTRACT

A method has been developed for determination of metsulfuron methyl metabolite A and metabolite A1 residues in cereal crops. The sample is extracted from crops with methanol, the solvent evaporated on a rotary evaporator, the sample redissolved in a pH 6.5 buffer and the metabolite A hydrolyzed to metabolite A1 with ß-glucosidase. The metabolite A1 is then extracted with chloroform and cleaned up on a silica cartridge. Final determination is by multidimensional normal phase liquid chromatography using a UV detector. The method is sensitive for Metabolite A1 to 0.02 ppm for grains, 0.04 ppm for green forage, and to 0.05 ppm for straw and hay based on a 10 g sample for grains, a 5 g sample for green forage, and a 4 g sample for straw and hay.

INTRODUCTION

Methyl 2-[[[[(4-methoxy-6-methyl-1,3,5-triazin-2-yl)amino] carbonyl]amino]sulfonyl]benzoate (metsulfuron methyl) is the active ingredient in Ally® Herbicide, a new Du Pont sulfonylurea herbicide. Metsulfuron methyl (formerly known as DPX-T6376) is effective in controlling a variety of weeds in grain fields.

Metsulfuron methyl has been shown by J.J. Anderson (1, 2) to be metabolized to "4-hydroxy"-metsulfuron methyl and its glucose conjugate by green wheat and barley. The procedure described here is based on determination of both "4-hydroxy"-metsulfuron methyl (metabolite A1) and its glucose conjugate (metabolite A). Both metabolites are measured and reported as metabolite A1 after hydrolysis of metabolite A to metabolite A1 with β-glucosidase. The structures of metsulfuron methyl, metabolite A, and metabolite A1 are shown in Figure 1.

Checks of the purity of metabolite A were made periodically to confirm the stability of the metabolite A standard. The purity could only be determined by enzymatically hydrolyzing the metabolite A to metabolite A1 and measuring the metabolite A1 by HPLC.

EQUIPMENT AND REAGENTS

A Du Pont Model 8800 Liquid Chromatograph consisting of a microprocessor controller, Model 870 pump, a column oven, and a data system was used. The column oven was fitted with a Valco injection valve and Model 7000 Rheodyne switching valve, Rheodyne, Inc., Cotati, California. The pneumatic actuated switching valve was controlled from the data system through a Rainin Solenoid Interface, Rainin Instruments, Inc , Woburn, Massachusetts. The detector was a Du Pont Model 850 UV absorbance detector. The HPLC columns were a Zorbax® CN (4.6 mm i.d. x 15 cm) column and a Zorbax® SIL (4.6 mm i.d. x 15 cm) column. The Du Pont equipment and Zorbax® columns were purchased from Analytical Instruments Division, E. I. du Pont de Nemours and Company, Inc., Wilmington, Delaware.

For homogenization and extraction of samples, a Tekmar Tissumizer® homogenizer Model SDT-1810, with a Model SDT-182 EN shaft and generator was employed. The Tissumizer® homogenizer was purchased from Tekmar Company, Cincinnati, Ohio. A Vortex-Genie® mixer was used for mixing of samples in centrifuge tubes. An International Equipment Company Model K centrifuge was fitted with a head to hold six 250 mL centrifuge bottles. A Precision™ Model 50 shaking water bath was used to incubate the samples during the enzymatic hydrolysis step. A Guth Universal® wash bottle was used to rinse the Tissumizer® probe between samples The mixer, centrifuge, water bath and wash bottle were purchased from Fisher Scientific Company, Pittsburgh, Pennsylvania

For concentration of samples, a vacuum rotary evaporator with a water bath set to 45°C was used with pear-shaped flasks with 24/40 ground glass joints, No. K-608700 (Kontes, Vineland, New Jersey). An N-EVAP® (Organomation Assoc., Worchester, Massachusetts) was used to concentrate samples under nitrogen.

A Millipore® all-glass filter apparatus, No. XX15 04700, with a 0.5 µm Teflon® filter, No. FHUP 04700, was used to filter solvents. Twenty-five millimeter diameter Millipore® Millex®-SR disposable 0.5 µm Teflon® filters were used to filter samples. All Millipore® equipment was purchased from Millipore Corporation, Bedford, Massachusetts.

Silica cartridges were used to clean up samples. Each cartridge was made from a Bond Elut® 75 mL reservoir fitted with two 20 µm pore size frits and packed with 10 grams of Adsorbosil® silica (200/425 Mesh). The reservoirs and frits were purchased from Analytichem International, Harbor City, California. The Absorbosil® was purchased from Alltech Associates, Inc., Deerfield, Illinois.

Buffer D was made by dissolving 5.88 g of sodium citrate in 2.0 liter of deionized water. The reference standards of metabolites A and A1 were obtained from the Agricultural Chemicals Department, E. I. du Pont de Nemours and Company, Inc., Wilmington, Delaware. The ß-glucosidase from almonds, Type II, was purchased

from Sigma Chemical Company, St. Louis, Missouri. All solvents were distilled-in-glass HPLC grade obtained from Fisher Scientific. All other chemicals were A.C.S. reagent grade obtained from Fisher Scientific.

PROCEDURE

Glassware Deactivation

All glassware was silanized to deactivate it. The silanizing reagent was prepared by mixing 30 mL of dichlorodimethyl silane and 30 mL of chlorotrimethyl silane with 1.0 liter of toluene. The glassware was dipped in the silanizing reagent for 2 minutes and then rinsed with toluene, methanol, distilled water, and methanol respectively. After drying, the glassware was ready for use. Glassware was resilanized after being used for 3 or 4 sets of samples.

Isolation

A subsample (10.0 grams for grain, 5 grams for green forage and 4.0 grams for hay and straw) was weighed into a 250 mL glass centrifuge bottle and 150 mL of methanol added. A set of samples was sequentially homogenized with the Tissumizer® homogenizer for 1.0 minute each. Between each sample, the Tissumizer® probe was rinsed with methanol from a Guth® wash

bottle and the rinse added to the sample bottle. The samples were then centrifuged at 2000 rpm for 5 minutes and the liquid decanted through a glass wool plug in a funnel into 500 mL pear-shaped flasks. After the first extraction of the sample set, the Tissumizer® probe was disassembled and cleaned with hot water, distilled water, and acetone. Before using again, it was run in a centrifuge bottle containing 80 mL of methanol to prevent cross-contamination. The extraction and centrifugation steps were repeated twice more with 100 mL of methanol each time and all methanol extracts combined.

Metabolite A Cleanup

Each sample was then concentrated on a rotary evaporator (45°C) until approximately 1/2 mL of methanol remained. Methanol (2.0 mL) was then added to the pear-shaped flask and the flask ultrasonically mixed to dissolve all the residue. Buffer D (100 mL) was then added to the flask, the solution was mixed, and the sample was then transferred to a 500 mL separatory funnel. The pear-shaped flask was rinsed with 10 mL more of Buffer D which was also transferred to the separatory funnel and 0.5 mL of 1 N NaOH added. Each sample was then washed with 3 x 100 mL of chloroform*

* Chloroform is suspected to be a weak animal carcinogen. Polyvinyl alcohol gloves should be worn, and adequate ventilation should be provided when handling chloroform.

by shaking for 1 minute. The bottom chloroform layer was discarded each time. If an emulsion formed in any sample, the sample was transferred to a 250 mL glass centrifuge bottle and centrifuged at 2000 rpm for 5 minutes to break the emulsion. The bottom chloroform layer was removed with a 50 cc glass syringe, then discarded, and the aqueous layer transferred back to the separatory funnel.

After the third wash, the remaining aqueous phase was transferred to a 250 mL glass-stoppered Erlenmeyer flask for enzymatic hydrolysis.

Enzymatic Hydrolysis

The pH of each sample was adjusted to 6.5 using a pH meter and 10% HCl. To each sample was added 500 units of ß-glucosidase and the samples were incubated overnight (>15 hrs.) at 35°C in the Precision™ shaker bath. The shaking action was adjusted to ensure good mixing of the samples during the night.

Metabolite A1 Cleanup

After at least 15 hours incubation, the samples were removed from the shaker bath and the pH of each one adjusted to 3.0 using a pH meter and 10% HCl. Each sample was then transferred to a 500 mL separatory funnel. To each Erlenmeyer flask was added 100 mL of chloroform, the flask ultrasonically mixed, and the

chloroform added to the separatory funnel. The metabolite A1 was then extracted into the chloroform from the aqueous phase. The two phases were mixed by tumbling on a rotating tumbler for 5 minutes at 35 to 50 rpm. After the phases for each sample were mixed, they were drained into a 250 mL glass centrifuge bottle. After centrifuging at 2000 rpm for 5 minutes, the bottom chloroform layer was removed with a 50 mL glass syringe and transferred to a 500 mL pear-shaped flask. Care was taken to avoid transferring any of the aqueous phase to the pear-shaped flask.

The chloroform rinse of the Erlenmeyer flask and the extraction for each sample was repeated two more times with 100 mL of chloroform each time. After all three extractions had been combined, each sample was concentrated to approximately 1.0 mL on a rotary evaporator with a water bath temperature of 45°C. The samples were saved for cleanup on a silica cartridge.

For each sample, a 20 µm pore size frit was placed in the bottom of a 75 mL Bond Elut® reservoir. Ten grams of Adsorbosil® silica was slurried with 50 mL of 2-propanol in a 150 mL beaker and then added to the reservoir. The beaker was rinsed with enough 2-propanol to completely transfer the silica to the reservoir. After the silica had settled, another frit was placed snuggly on top of the silica. Care was taken to avoid trapping air under the frit. After the 2-propanol had drained to the frit, 60 mL of solution III (see Table 1) was run through the column. The column did not run dry since the flow stopped whenever the solvent drained to the top frit.

Each sample was then transferred from the pear-shaped flask to the cartridge using 3 x 3 mL rinses with solution III. The cartridge was then rinsed with 65 mL of solution III (70 mL for straw and hay) which was discarded. Metabolite A1 was eluted with 65 mL (45 mL for straw and hay) more of solution III which was collected in a 200 mL pear-shaped flask.

Each sample was concentrated to approximately 1.0 mL on a rotary evaporator at 45°C. It was transferred from the pear-shaped flask to a 13 mL glass-stoppered centrifuge tube with 3 x 3 mL rinses of solution III. Each sample was then concentrated to dryness with the N-EVAP® and stored at <4°C until analyzed.

For HPLC analysis, each sample was redissolved in 1.0 mL of solution I (see Table 1), ultrasonically mixed for a minute, and vortex mixed for a minute. Each sample was then filtered through a Millipore® Millex®-SR filter into a clean 13 mL glass-stoppered centrifuge tube.

Standard Purity Check

The purity of the metabolite A standard was checked periodically to see if it was stable. This was done by either running several tests as a sample set or by inserting a test in a set of samples being processed.

A purity test was prepared by placing 100 mL of Buffer D in a glass-stoppered 250 mL Erlenmeyer flask. The pH was adjusted to 6.5 with 10% HCl and it was then spiked with a standard of metabolite A in methanol at 0.2 to 5.0 µg. β-glucosidase (500 units) was added and the sample incubated overnight the same as for crop samples.

The next morning the purity test sample was acidified, extracted with chloroform, and the chloroform evaporated as described in the metabolite A1 cleanup section. After the chloroform had been concentrated to about 1.0 mL the sample was transferred from the pear-shaped flask to a 13 mL glass-stoppered centrifuge tube using 3 x 3 mL rinses of solution III. The solution III was evaporated with the N-EVAP® evaporator and the sample stored at <4°C until it was analyzed by HPLC using the same conditions as for crop samples.

Liquid Chromatography

A Du Pont model 8800 HPLC fitted with a high-pressure switching valve was used for the analysis of samples. The switching valve was time programmed from a Du Pont data system. A Du Pont model 850 absorbance detector with a wavelength of 254 nm was used for the analysis. The oven temperature was 40°C and the injection volume was 100 µL. The attentuation of the detector was set at 0.01 AUFS and any further attenuation of samples or standards was on the data system.

A diagram of the columns and switching valve arrangement is shown in Figure 2. The first column, C_1, was a Zorbax® CN column and the second column, C_2, was a Zorbax® SIL column. In valve position I, the effluent from C_1 went through a 10 µL bypass loop, back to the valve, and to the detector. In the other position, II, the effluent from C_1 went to the valve, then to C_2, back to the valve, and then to the detector.

Table 2 gives a typical timing sequence for analysis of samples. At the time of injection, the valve was in position I (see Figure 2 for valve positions) and C_2 was bypassed. The mobile phase, solution I (see Table 1), was pumped at a flow rate of 1.5 mL/min. When metabolite A1 started to elute from C_1 at 4.96 min., the valve was switched to position II. After the metabolite A1 had eluted from C_1 at 5.11 min., the valve was switched back to position I. While the valve was in position II, metabolite A1 was trapped on C_2 and held there for later elution. The valve switching times at 4.96 and 5.11 min. were set at -0.10 and +0.05 min. around the retention time for metabolite A1 on C_1.

After metabolite A1 had been trapped on C_2, the mobile phase was switched to solution II (see Table 1) at 5.9 min. and the flow rate increased to 3.5 mL/min to clean off C_1 and equilibrate it to solution II. After C_1 had been equilibrated (14.5 min.), the flow rate was decreased to 1.5 mL/min. and 1.5 min. allowed for the flow to stabilize. At 16.0 min. the valve was switched to position II and metabolite A1 eluted from C_2. When elution of

metabolite A1 and other peaks from C_2 was complete at 29.9 min., the valve was switched back to position I. The mobile phase was changed to solution I and the flow rate increased to 3.5 mL/min. to quickly re-equilibrate C_1 to solution I. After 8 minutes (38.0 minutes from injection) the flow rate was decreased to 1.5 mL/min., and after it had stabilized, the next sample or standard was injected.

Standard Preparation

A stock solution of metabolite A (100 ug/mL) was made by dissolving 2.5 mg in 25 mL of methanol. A standard (10.0 ug/mL) was made by diluting 5.0 mL of the stock solution to 50 mL with methanol. A fortifing standard (1.0 µg/mL) was made by diluting 5.0 mL of the 10.0 µg/mL standard to 50 mL with methanol.

A stock solution of metabolite A1 (100 µg/mL) was made by dissolving 10.0 mg in 100 mL of chloroform. A fortifying standard (1.0 µg/mL) was made by placing 1.0 mL of the stock standard into a 100 mL volumetric flask evaporating the chloroform, and making to volume with methanol.

A stock HPLC standard (10 ug/mL) was made by placing 1.0 mL of the stock solution into a 10 mL volumetric flask and then evaporating the chloroform. The volumetric flask was made to volume with solution I. Working HPLC standards were made at concentrations

of 0.1, 0.2, 0.5, 1.0 and 2.0 µg/mL with solution I in 10 mL volumetric flasks by dilution of the stock HPLC standard. The working HPLC standards were made fresh weekly.

Calculations

The response factor for each standard, R, in (mm-mL)/µg units was calculated by the equation.

$$R = \frac{(Ps)}{C_s} \qquad (1)$$

P_s was the peak height of the standard in millimeters and C_s was the concentration in µg/mL units. Standards were injected interspersed with samples. The average response factor for the standards, R_a, was calculated and used for calculation of sample concentrations.

The sample concentration of metabolite A1, C, in µg/g units (ppm) was calculated using the equation.

$$C = \frac{(P-P_c)(X)(V)}{(R_a)\ (W)} \qquad (2)$$

P was the sample peak height in millimeters and P_c was the peak height of any interference in the unfortified control. V was the final sample volume in mL (1.0) and X was the dilution factor

from any needed dilution of the final sample extract. W was the sample weight in grams. For samples fortified with metabolite A, the concentration of metabolite A1 was converted to metabolite A by multiplying by the molecular weight ratio, M (1.41).

The recovery, H, for a standard purity test sample was calculated by the equation

$$H = \frac{(P)(V)\ (M)}{R_a\ \ (S)} \qquad (3)$$

where P, V, M, and R_a are as defined earlier. S was the amount of metabolite A, in µg units, that the sample was fortified with. A purity correction factor, F, was then calculated for a series of samples by averaging the purity test recoveries obtained while analyzing a series of samples. The purity correction factor was then used to correct all metabolite A recoveries for purity of the metabolite A standard.

The corrected concentration, C_c, was calculated by the following equation.

$$C_c = \frac{C}{F} \qquad (4)$$

RESULTS AND DISCUSSION

Results of samples analyzed were reported as metabolite A1 since it is not possible to distinquish between metabolite A and metabolite A1. Metabolite A concentrations were only calculated for samples fortified with metabolite A.

We found it was necessary to silanize the glassware used in sample processing. When unsilanized glassware was used, recoveries were low and variable.

Purity tests for the metabolite A standard were obtained periodically to insure that the standard solution being used was not breaking down. The purity results were then averaged for a series of samples and used to correct the recovery results for metabolite A fortified samples.

Recovery data for samples fortified with metabolite A are given in Table 3. The average corrected recovery for 9 grain samples fortified between 0.03 and 0.20 ppm was 93%. The average recovery for 5 straw samples fortified between 0.075 and 0.50 ppm was 82%. It was 89% for 5 wheat green forage samples fortified between 0.06 and 0.40 ppm. The limit of detection was 0.03 ppm for grain, 0.06 ppm for green forage, and 0.075 ppm for straw.

Recovery data for samples fortified with metabolite A1 are given in Table 4. The average corrected recovery for 10 grain samples fortified between 0.02 and 0.20 was 88%. It was 76% for 9

straw samples fortified between 0.05 and 0.50 ppm and 86% for 5 wheat green forage samples fortified between 0.04 and 0.40 ppm. The limit of detection was 0.02 ppm for grain, 0.04 ppm for green forage, and 0.05 ppm for straw.

Chromatograms of a control barley grain sample, the same sample fortified at 0.04 ppm with metabolite A, and a barley grain sample treated at 1/8 oz ai/A are shown in Figures 3, 4, and 5, respectively. Chromatograms of a control wheat straw sample, the same sample fortified at 0.05 ppm with metabolite A, and a sample treated at 1/4 oz ai/A is shown in Figures 6, 7, and 8 respectively. Chromatograms of a control wheat green forage sample and the same control sample fortified at 0.02 ppm with metabolite A are shown in Figures 9 and 10 respectively.

SUMMARY

The method described has been developed for the analysis of metsulfuron methyl metabolite A and metabolite A1. After extraction from the crop, metabolite A is hydrolyzed to metabolite A1 by cleaving off the glucose conjugate with ß-glucosidase. The sample is then further cleaned up by a silica cartridge and the metabolite A1 quantitated by multidimensional HPLC.

REFERENCES

(1) Anderson, J.J.; "Metabolism of [^{14}C] Metsulfuron Methyl in Field Grown Wheat", AMR-199-84, Agricultural Chemicals Department, Research Division, E.I. du Pont de Nemours and Co., Wilmington, Delaware.

(2) Anderson, J. J.; "Metabolism of [^{14}C] Metsulfuron Methyl in Field Grown Barley", AMR-211-84, Agricultural Chemicals Department, Research Division, E. I. du Pont de Nemours and Co., Wilmington, Delaware.

TABLE 1

Solution Composition

	Solution I	Solution II	Solution III
Cyclohexane	-----	-----	850 mL
Hexane	800 mL	730 mL	-----
2-propanol	-----	-----	75 mL
Methanol	-----	-----	75 mL
Tetrahydrofuran	200 mL	270 mL	-----
Glacial Acetic Acid	20 mL	20 mL	3 mL
Deionized Water	50 µL	50 µL	80 µL

Table 2
Typical Timing Sequence
HPLC

Time Range (min.)	Mobile Phase	Flow Rate (mL/min.)	Valve Position	Columns Used
0.0 to 4.96	Solution I	1.5	I	C_1
4.96 to 5.11	Solution I	1.5	II	$C_1 + C_2$
5.11 to 5.9	Solution I	1.5	I	C_1
5.9 to 14.5	Solution II	3.5	I	C_1
14.5 to 16.0	Solution II	1.5	I	C_1
16.0 to 29.9	Solution II	1.5	II	$C_1 + C_2$
29.9 to 38.0	Solution I	3.5	I	C_1

TABLE 3

Metabolite A Recovery Data

(Corrected for Metabolite A Standard Purity)

Crop	Number of Samples	Fortification Range (ppm)	Recovery Range (Percent)	Average Recovery (Percent)
Barley Grain	5	0.03-0.20	88-103	94
Barley Straw	5	0.075-0.50	73-91	82
Wheat Green Forage	5	0.06-0.40	86-95	89
Wheat Grain	4	0.03-0.20	88-96	92

TABLE 4

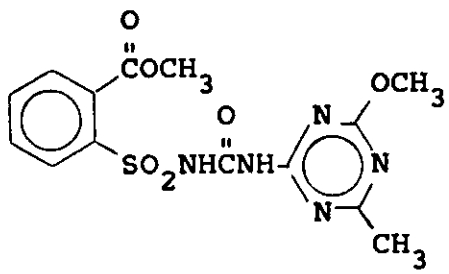
Metabolite A1 Recovery Data

(Corrected for Hydrolysis Recovery)

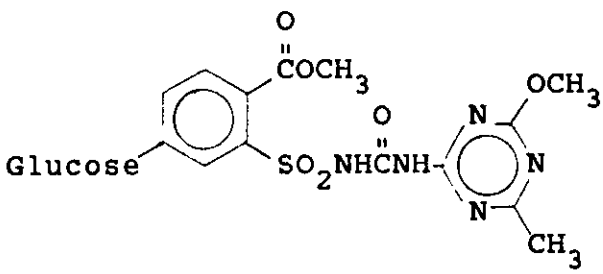
Crop	Number of Samples	Fortification Range (ppm)	Recovery Range (Percent)	Average Recovery (Percent)
Barley Grain	5	0.02-0.20	85-94	90
Barley Straw	5	0.05-0.50	69-84	79
Wheat Green Forage	5	0.04-0.40	75-95	86
Wheat Grain	5	0.02-0.20	75-91	86
Wheat Straw	4	0.05-0.50	61-78	73

Figure I

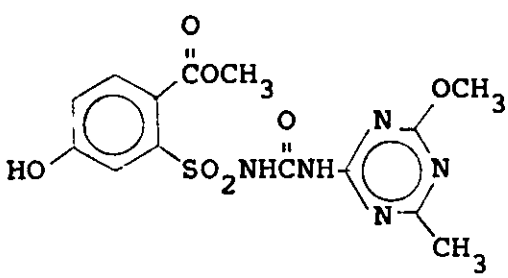
Structures



Metsulfuron Methyl



Metsulfuron Methyl Metabolite A



Metsulfuron Methyl Metabolite A1

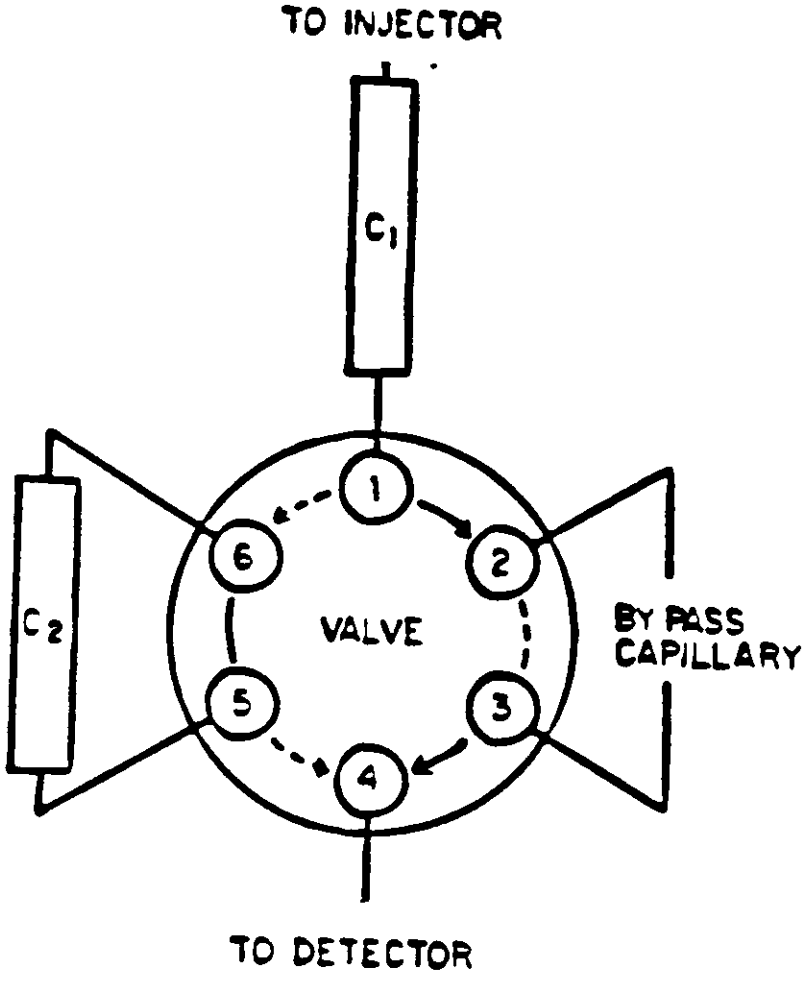


FIGURE 2

Chromatographic Column Arrangement

—— Position I

--- Position II

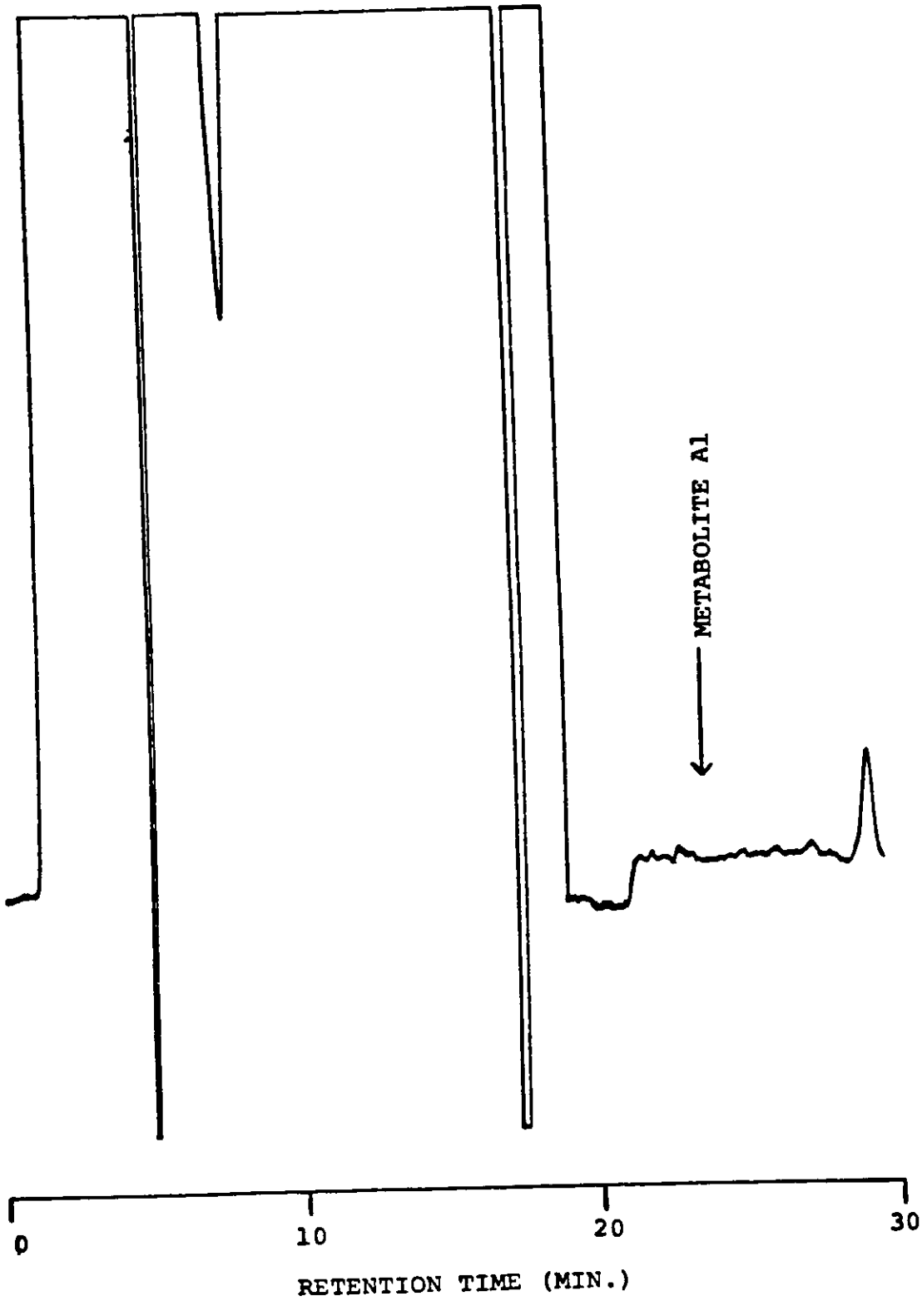


FIGURE 3. Chromatogram of a control barley grain sample from Arbon, Idaho.

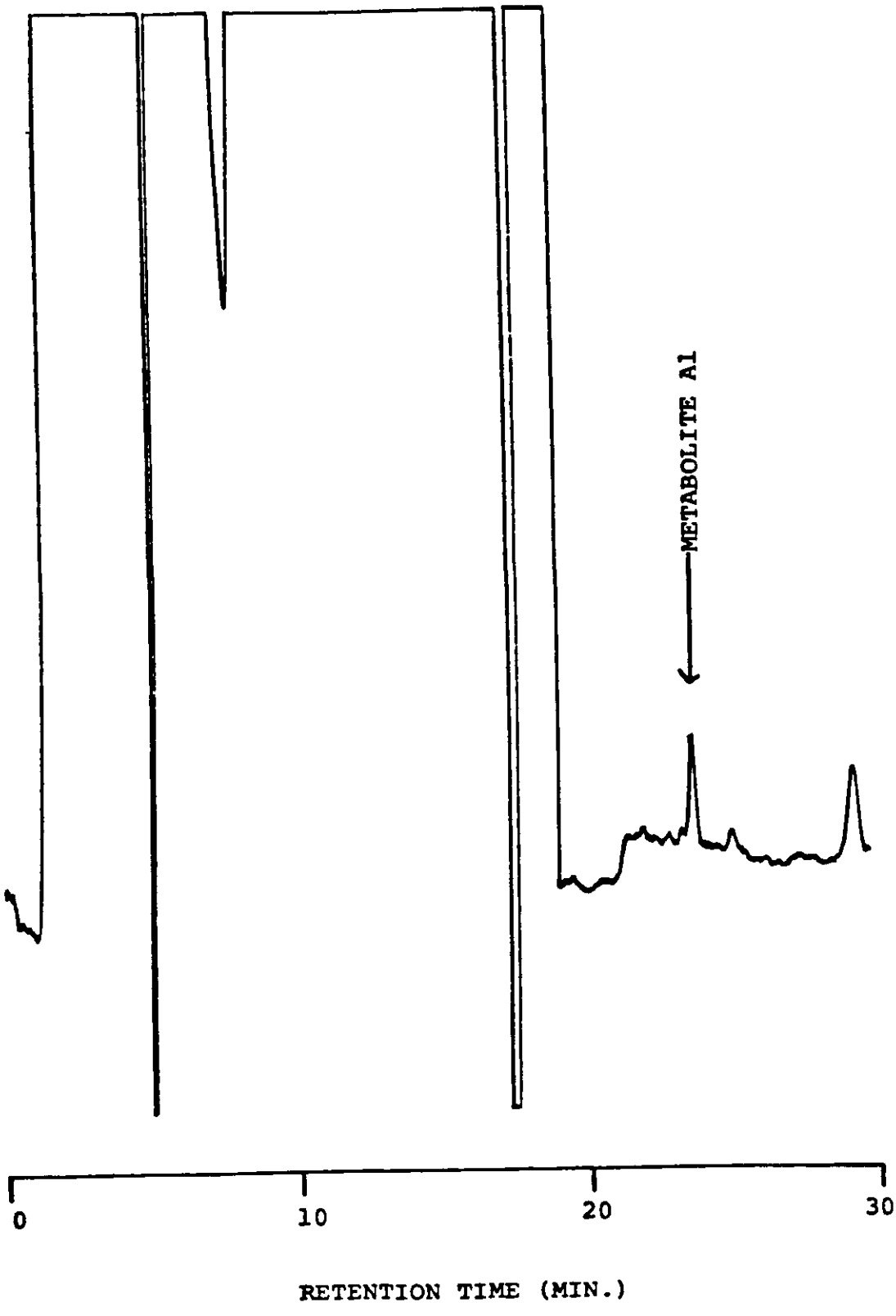


FIGURE 4. Chromatogram of the control barley grain sample in Figure 3 fortified at 0.04 ppm with metabolite A. The recovery is 80% after correction for the purity of the spiking standard.

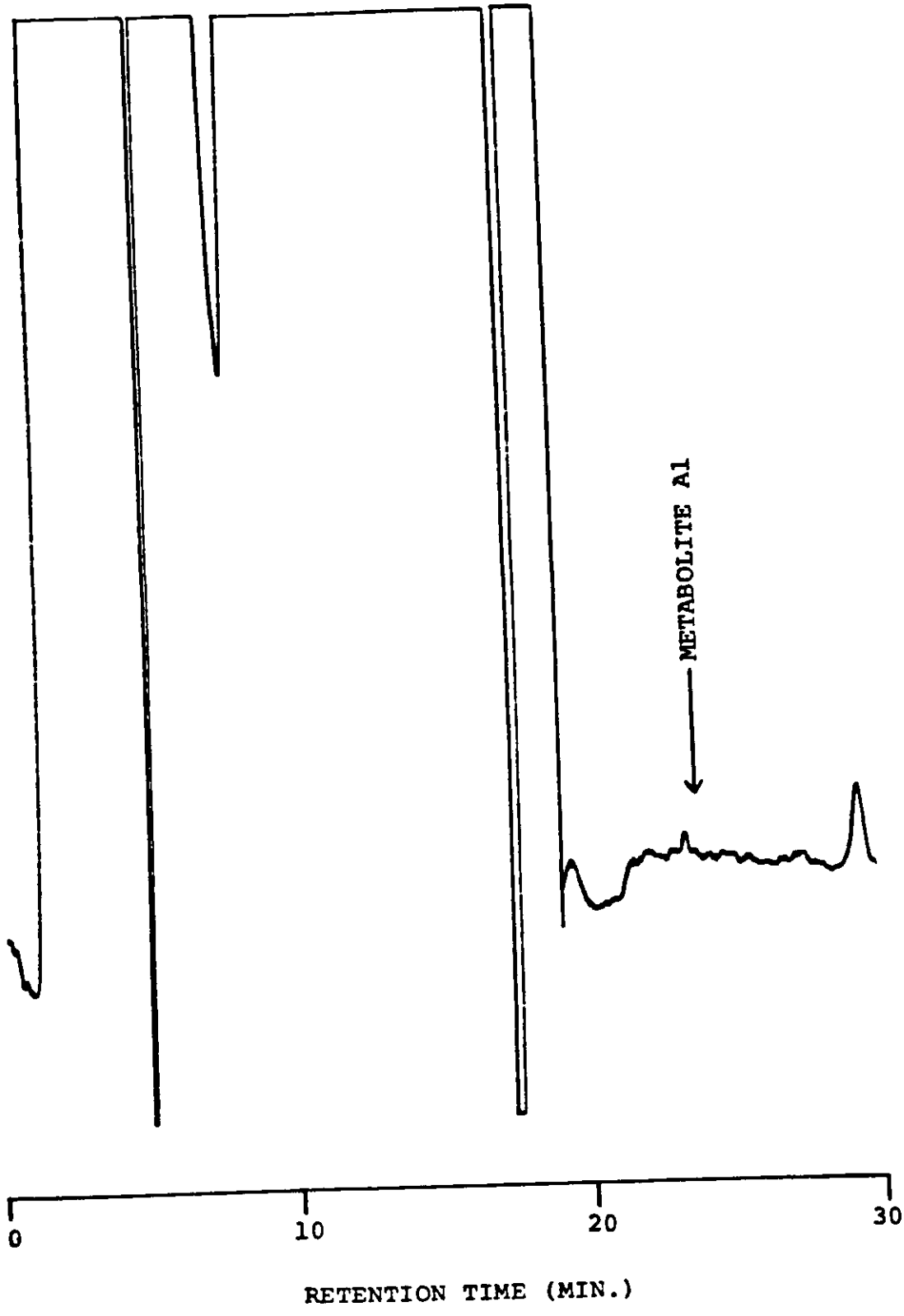


FIGURE 5. Chromatogram of a barley grain sample from Arbon, Idaho treated at 1/8 oz. ai/A.

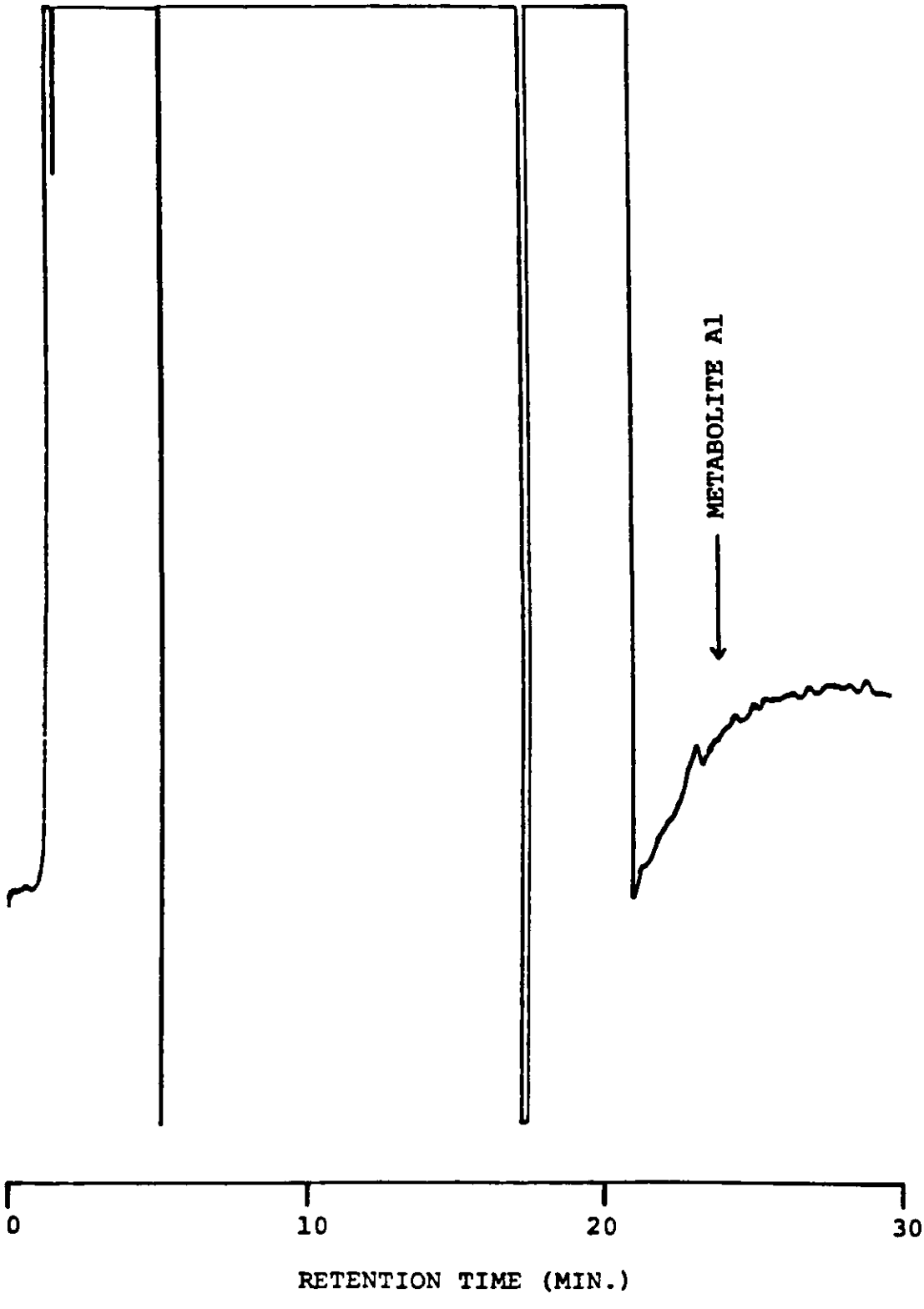


FIGURE 6. Chromatogram of a control wheat straw sample from Hays, Kansas.

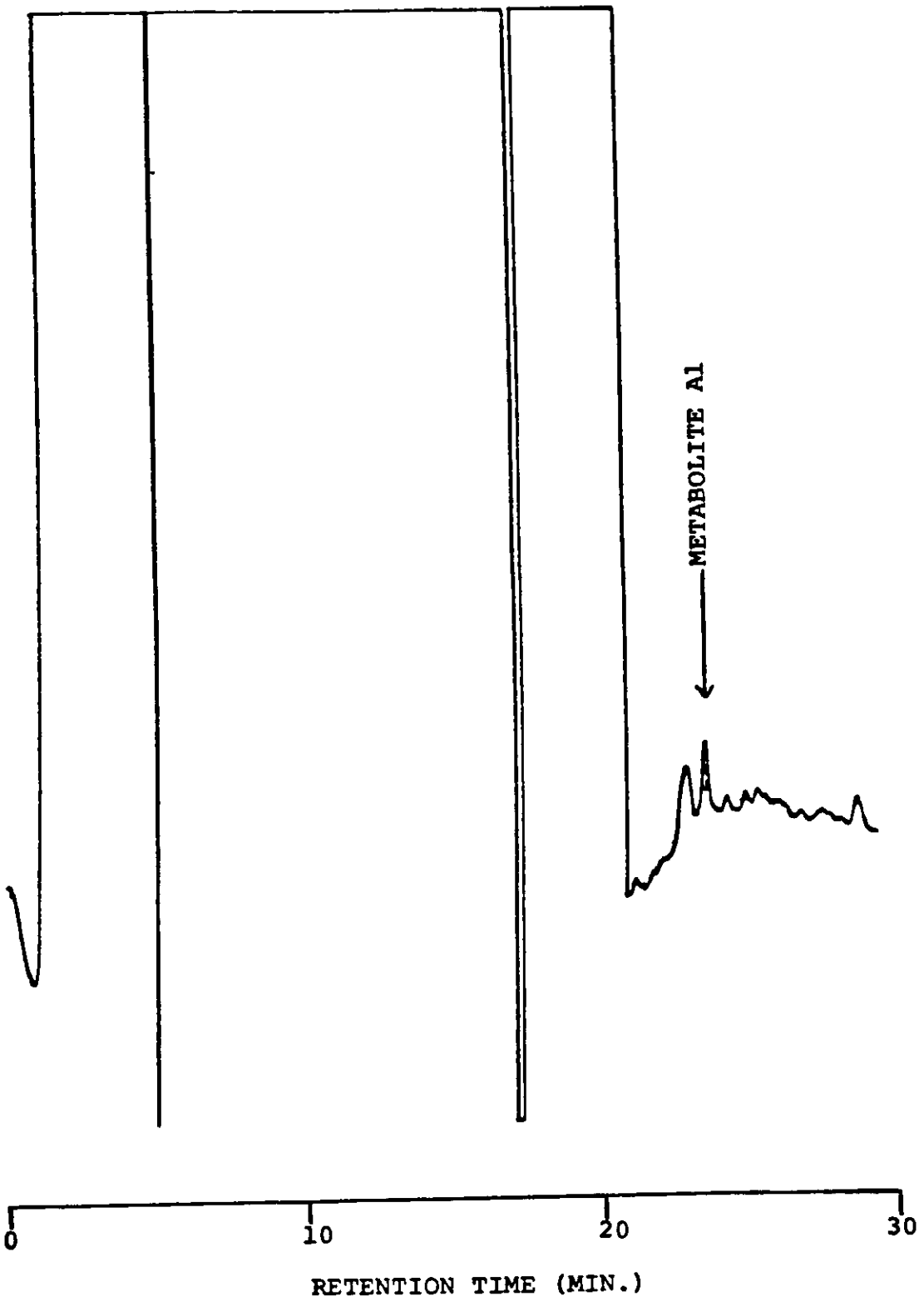


FIGURE 7. Chromatogram of the control sample in Figure 6 fortified at 0.05 ppm with metabolite A The recovery is 92% after correction for the purity of the spiking standard.

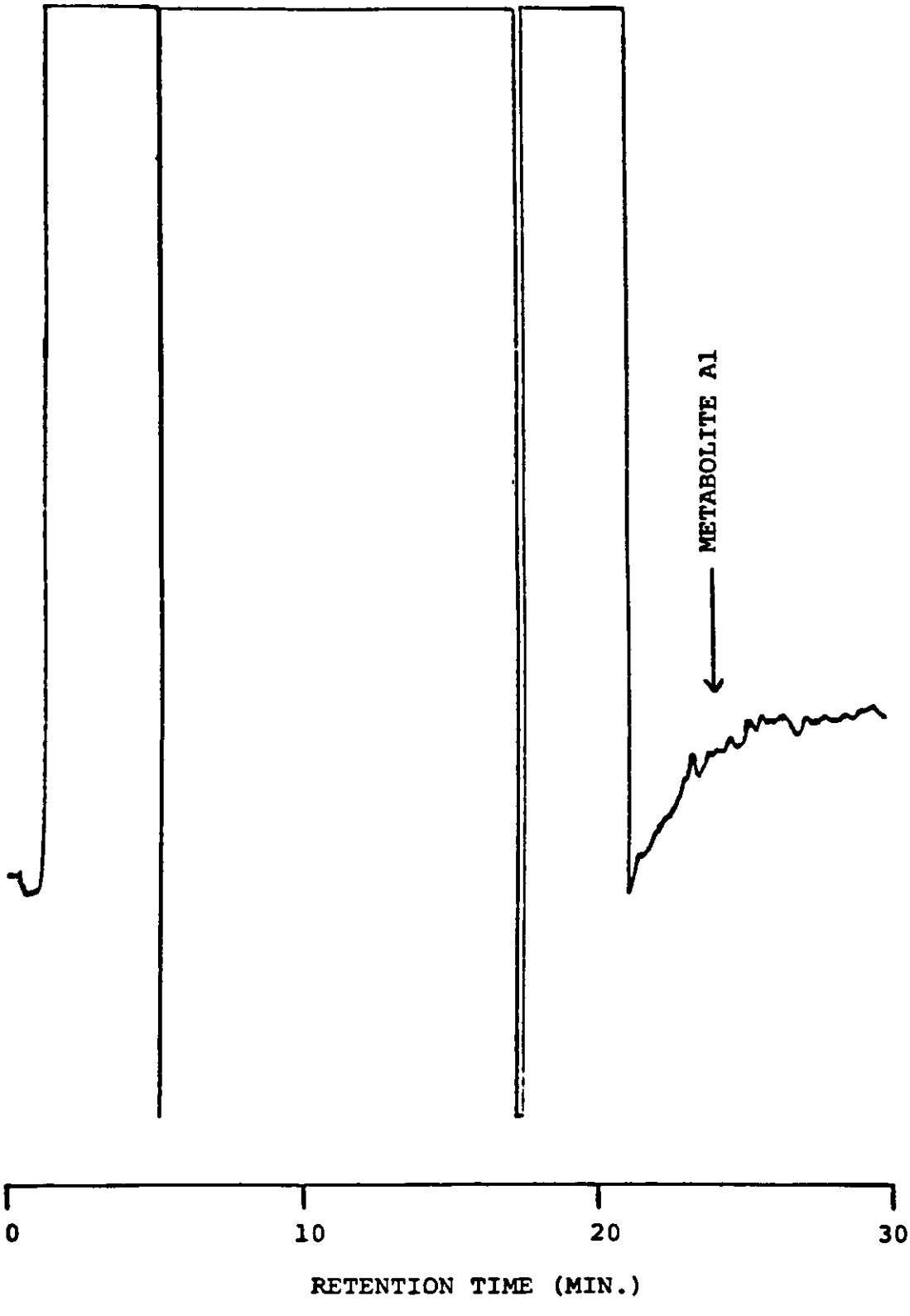


FIGURE 8. Chromatogram of a wheat straw sample from Hays, Kansas treated at 1/4 oz ai/A.

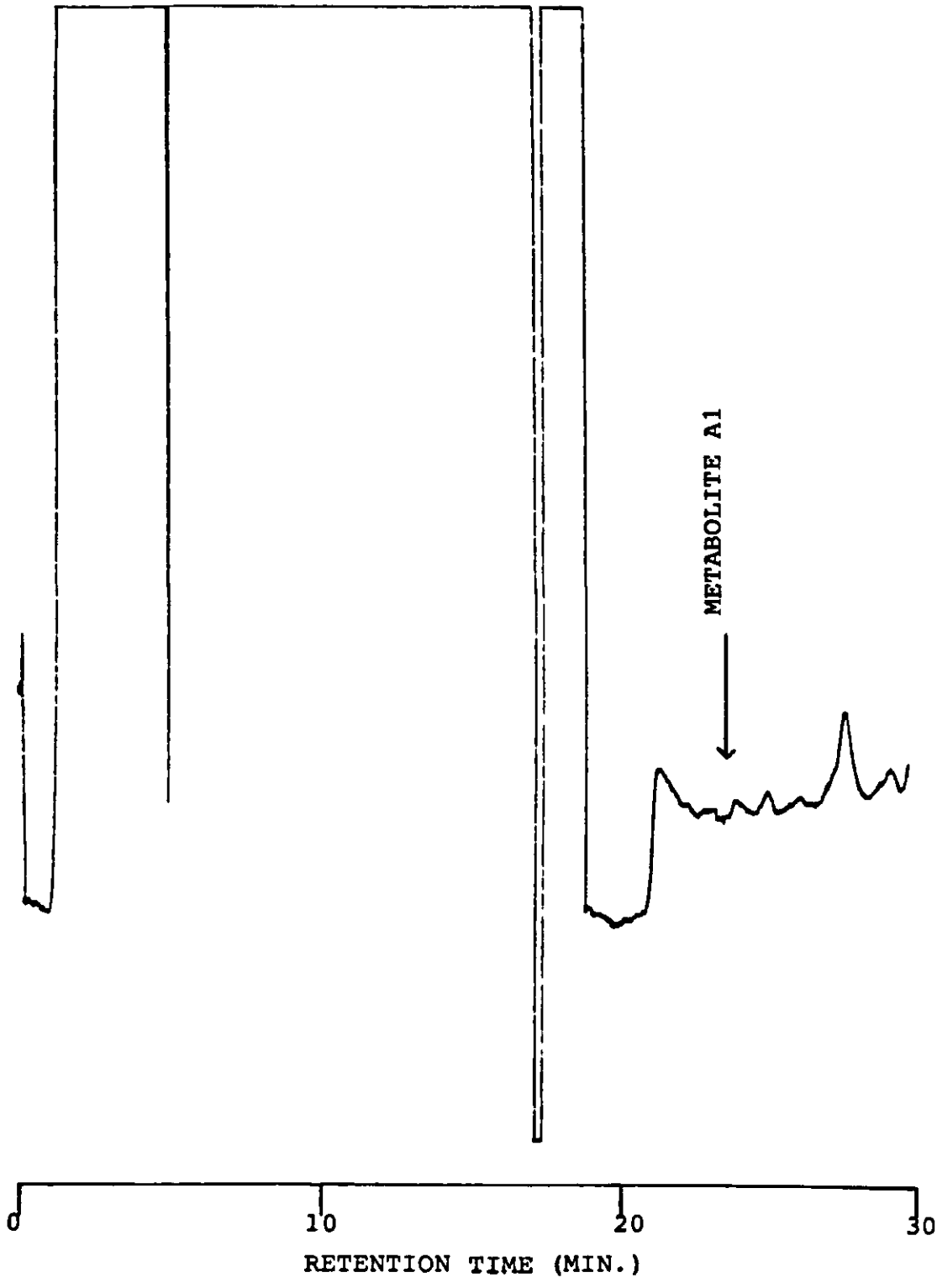


FIGURE 9. Chromatogram of a control wheat green forage sample from Newark, Delaware.

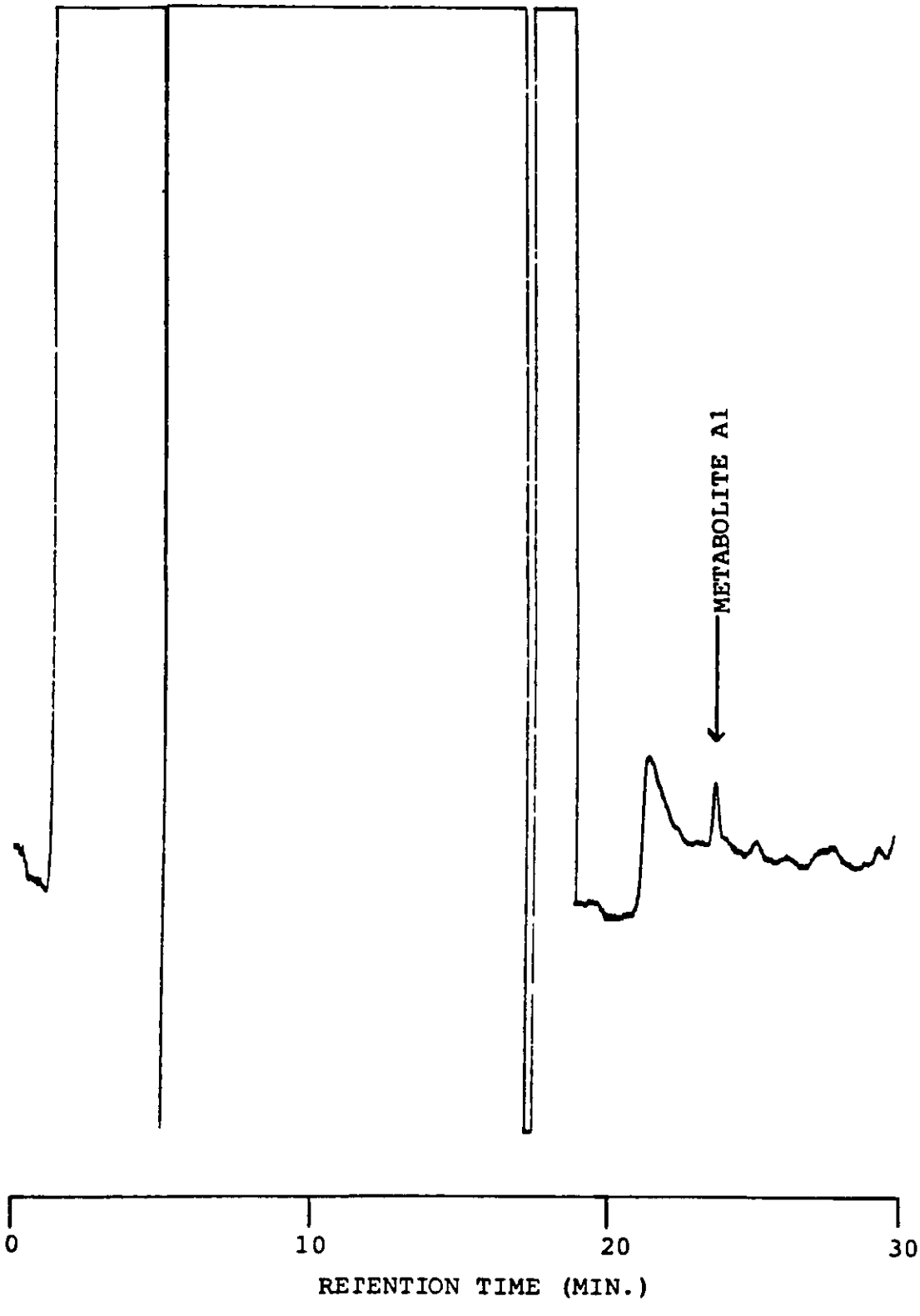


FIGURE 10. Chromatogram of the control sample in Figure 9 fortified at 0.02 ppm with metabolite A. The recovery is 87% after correction for the purity of the spiking standard