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Reversed-phase high-performance liquid chromatographic method for the determination of soil-bound [^{14}C]atrazine and its radiolabeled metabolites in a soil metabolism study

ABU M. RUSTUM*[a], STEVE ASH and ADESH SAXENA
Department of Environmental Fate, Hazleton Laboratories America, Inc., Madison, WI (U.S.A.)
and
K. BALU
Ciba-Geigy Corporation, Greensboro, NC (U.S.A.)
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ABSTRACT

A rapid and simple reversed-phase high-performance liquid chromatography (RP-HPLC) method has been developed to determine [^{14}C]atrazine and its radiolabeled degradation products using radioisotope detection and liquid scintillation counting quantitation. A 25 cm × 4.6 mm, 5-μm octadecylsilane (C_{18}) column was used with gradient elution of acetonitrile and 0.01 *M* monobasic potassium phosphate (pH 2.0). The mobile phase was delivered at 1.0 ml/min. Fractions of the HPLC eluate were collected and the radioactive peak were quantitated as percent of the total radioactivity injected into the chromatographic system. A histogram of radioactivity (dpm) *versus* fraction number was constructed. Identification of individual radioactive peaks was conducted by comparing the retention time of the peaks in the histogram with the retention time of the peaks of non-radiolabeled standards obtained by ultraviolet detection at 230 nm.

This method was applied to determine the degradation products of [^{14}C]atrazine in a soil metabolism study. Four degradation products in the soil metabolism study were positively identified by co-chromatography technique. The limit of detection of the method for [^{14}C]atrazine and its degradation products was 5 ng/ml for a 100-μl injection volume and when [^{14}C]atrazine had a specific activity of $7.62 \cdot 10^5$ Bq/mg.

INTRODUCTION

Atrazine (2-chloro-4-ethylamino-6-isopropylamino-*s*-1,3,5-triazine) is a triazine herbicide used worldwide for weed control in various agricultural crops. Multiple

[a] Present address: Abbott Laboratories, Division of Pharmaceutical Products Development, Department No. 417, 14th Sheridan Road, North Chicago, IL 60064, U.S.A.

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analytical problems are usually encountered when soil metabolism and other environmental fate studies are conducted using a non-radiolabeled test compound. Most of these analytical problems can be solved by using the test material labeled with ^{14}C, ^{3}H or other appropriate radioactive isotopes.

Paper chromatography has been used to determine several triazine herbicides[1–2]. Gas chromatography (GC) has been used to determine the triazines in different sample matrices[3–6]. Most of the GC methods in the literature to determine the hydroxy-*s*-triazines use a pre-chromatography derivatization step.

Recently, high-performance liquid chromatography (HPLC) has been used extensively to determine atrazine, its residues and its metabolites in soil and other sample matrices[7–16].

Most of the reported procedures for the isolation of atrazine and its degradation products from soil and other sample matrices use complex and multiple-step extraction for sample preparation before HPLC analysis. These HPLC procedures use ultraviolet (UV) detection (typically in the 230- to 220-nm range) to monitor atrazine and its degradation products.

UV detection is usually a non-selective mode of detection. Interference from the sample matrix peak(s) is often a common problem. Hence, long procedures for sample cleanup are usually followed to eliminate the interference peak(s) of the sample matrices.

The use of radioisotopes and liquid scintillation counting quantitation in HPLC eliminates the interference problems involved in UV detection, is usually more sensitive than UV detection, and is an absolute method of quantitation. By knowing the specific activity of the test material, one can directly quantitate the analyte simply by measuring the total disintegrations per minute (dpm) of the chromatographic peak. Therefore, a calibration curve is not required for quantitation of the analyte in an unknown sample. The linear dynamic range of the analytical signal for radioisotope detection is typically six orders of magnitude.

In this report, a simple and sensitive HPLC method has been described to quantitate atrazine and its radiolabeled degradation products in a soil matrix. The use of radioisotopes detection made the method more sensitive than most of the methods reported in the literature. The one-step extraction procedure resulted in a simpler, reproducible, and more accurate method than those reported previously.

EXPERIMENTAL

The HPLC system consisted of a Perkin-Elmer (Norwalk, CT, U.S.A.) Series 410B solvent delivery unit, equipped with a Rheodyne (Cotati, CA, U.S.A.) 7275 sample injector with a 200-μl sample loop. An Applied Biosystem (Ramsey, NJ, U.S.A.) UV–visible detector (Model No. Spectroflow 783) with a flow cell of 8-mm path length was used. The chromatograms were recorded on a Houston Instrument (Austin, TX, U.S.A.) D-5237-2 strip chart recorder. Deionized water was collected from a Milli-Q System (Millipore, Bedford, MA, U.S.A.). A Model 2200 Branson sonicator was used to degas the mobile phase (Branson Cleaning Equipment, Shelton, CT, U.S.A.). The samples were centrifuged using an IEC centrifuge, Model-K (Damon, IEC Division, Needham Heights, MA, U.S.A.). A fraction collector (Model 203, Gilson, Middleton, WI, U.S.A.) was used to collect the fractions from the

chromatographic system. A Model 3801 liquid scintillation system was used to determine the radioactivity in the HPLC fraction (Beckman, Fullerton, CA, U.S.A.). A 25 cm × 4.6 mm C_{18} (ODS), 5-μm Econosphere column (Alltech, Deerfield, IL, U.S.A.) was used to test the developed method.

Materials

Acetonitrile and methanol (HPLC grade) were purchased from EM Science (Cherry Hill, NJ, U.S.A.). Atrazine (radiolabeled and non-radiolabeled) and the non-radiolabeled degradation products (GS-17792, GS-28273, GS-17794, G-28279, G-30033 and G-34048) were obtained from Ciba-Geigy (Greensboro, NC, U.S.A.). All standards were used as received without further purification. The standards used in this experiment were at least 98% pure. Potassium phosphate monobasic (KH_2PO_4) and hydrochloric acid were purchased from Fisher Scientific (Fairlawn, NJ, U.S.A.). Liquid scintillation cocktail was purchased from Packard (Downers Grove, IL, U.S.A.).

Chromatographic conditions

The mobile phase consisted of a gradient elution of acetonitrile and 0.01 *M* KH_2PO_4 (pH adjusted to approximately 2.0 with phosphoric acid). The rate of the mobile phase delivery through the analytical column was 1.0 ml/min. The non-radiolabeled standards of atrazine and its degradation products were monitored by a UV visible absorbance detector at a wavelength of 230 nm and from 0.20 to 0.01 absorbance units full scale (a.u.f.s.). The mobile phase gradient is listed in Table I.

Prechromatography isolation of atrazine and its degradation products from soil

Isolation of atrazine and its degradation products from the soil samples was performed as follows. Approximately 10 g of a soil sample (from a soil metabolism study of atrazine) containing soil-bound atrazine and its degradation products was transferred into a container. This soil was heated at reflux with 1 *M* HCl in methanol (solvent–soil ratio approximately 10:1) and stirred with a magnetic stir bar for 2 h. The refluxed sample was allowed to equilibrate to ambient temperature, and was then vacuum filtered through a 0.45-μm nylon-66 membrane. The solids in the filter were washed with an aliquot of the extraction solvent. The filtered extract was concentrated to 1–2 ml by rotary evaporation at approximately 60°C in vacuum. The concentrated extract was diluted with 0.01 *M* KH_2PO_4 to a total volume of 10.0 ml. A 100- to 200-μl aliquot of this solution was injected directly into the chromatographic system. A flow chart for the extraction of the soil sample for a metabolism study is in Fig. 1.

TABLE I

MOBILE PHASE GRADIENT OF THE HPLC SYSTEM

Step time (min)	*Acetonitrile (%)*	*0.01 M KH_2PO_4 (pH ca. 2.0) (%)*
0 to 10	5	95
10 to 15	20	80
15 to 20	30	70
20 to 35	70	30

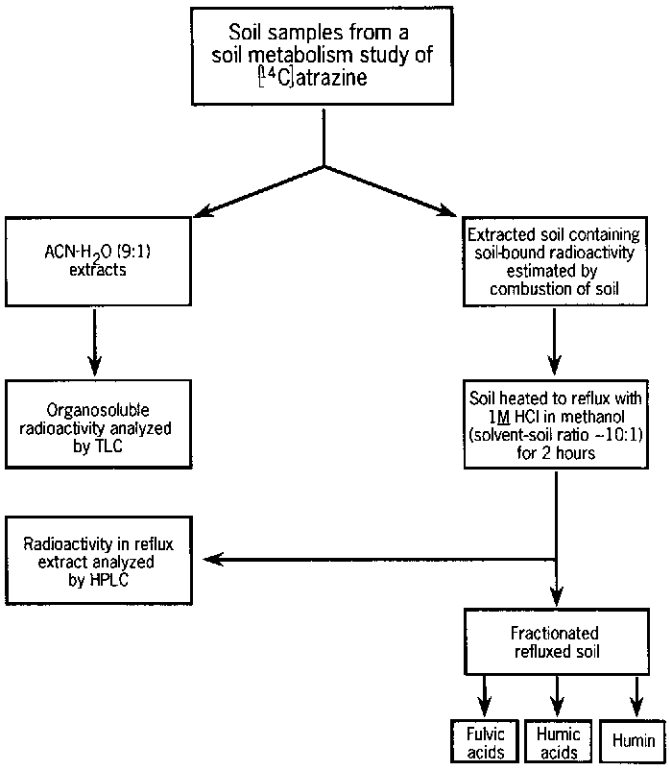


Fig. 1. Flow chart for extraction of [^{14}C]atrazine and its metabolites from soil samples of the soil metabolism study. ACN = Acetonitrile; TLC = thin-layer chromatography.

Fraction collection and histogram of the injected sample radioactivity

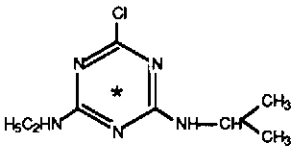
For samples containing radioactivity, the eluate from the column was collected in 0.5-ml fractions, which were then analyzed by liquid scintillation counting. A histogram was generated from each HPLC assay by plotting fraction number *versus* radioactivity (dpm).

Preparation of the stock solution

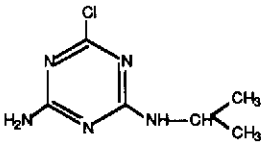
Stock solutions of [^{14}C]atrazine, non-radiolabeled atrazine, and non-radiolabeled degradation products of atrazine were prepared in neat acetonitrile. The chemical structures of the standards used in this experiment are shown in Fig. 2. The retention times of the non-radiolabeled standards (by UV detection) with those of the [^{14}C]atrazine and degradation products (as determined by HPLC–liquid scintillation counting histograms) were compared.

Calculations

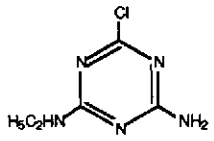
The percentage of [^{14}C]atrazine, its individual degradation products in soil, and radioactivity recovered from the HPLC system (after injection) was calculated with respect to total radioactivity injected into the HPLC system.



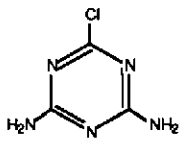
Atrazine
(2-chloro-4-ethylamino-6-isopropylamino-s-triazine)
* Position of radiolabel.



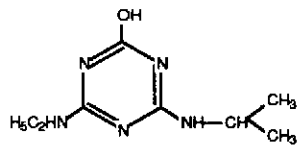
G-30033
(2-amino-4-chloro-6-isopropylamino-s-triazine)



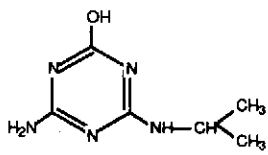
G-28279
(2-amino-4-chloro-6-ethylamino-s-triazine)



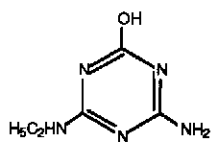
G-28273
(2,4-diamino-6-chloro-s-triazine)



G-34048 (Hyroxy Atrazine)
(2-ethylamino-4-hydroxy-6-isopropylamino-s-triazine)



GS-17794
(2-amino-4-hydroxy-6-isopropylamino-s-triazine)



GS-17792
(2-amino-4-ethylamino-6-hydroxy-s-triazine)

Fig. 2. Structure of atrazine and its major degradation products.

$$\% \text{ of } [^{14}\text{C}]\text{atrazine (or degradation product)} = \frac{\text{Radioactivity (dpm) in the fractions of the atrazine peak (or degradation products)}}{\text{Total radioactivity (dpm) injected into the HPLC system}} \times 100$$

$$\% \text{ Radioactivity (dpm) recovered from the HPLC system} = \frac{\text{Sum of the radioactivity (dpm) in all the fractions of the histogram}}{\text{Total radioactivity (dpm) injected into the HPLC system}}$$

The total radioactivity (dpm) injected into the HPLC system was determined directly by liquid scintillation counting using duplicate aliquots of an aliquot (same volume as injected into the HPLC system) of the extract.

Counts per minute (cpm) were converted to disintegrations per minute (dpm) using the external standard method. The background radioactivity of the HPLC fractions was determined by collecting the HPLC eluate (in duplicate) before the

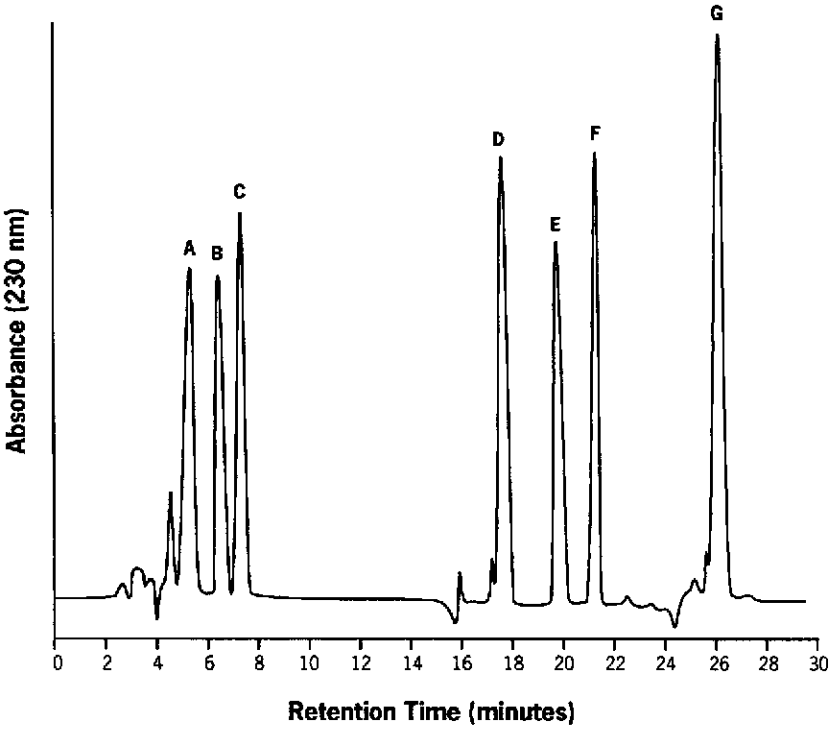


Fig. 3. Representative chromatogram of non-radiolabeled atrazine and its major degradation products. Peaks: A = GS-17792; B = G-28273; C = GS-17794; D = G-28279; E = G-34048; F = G-30033; G = atrazine. The detector was at 230 nm and 0.200 a.u.f.s.

radioactive sample extract was injected. The mean radioactivity of the two blanks was then subtracted from each of the HPLC fractions. Similarly, the background radioactivity for the extract of the soil samples was subtracted to eliminate the matrix effect on chemiluminescence and quenching.

RESULTS AND DISCUSSION

The HPLC method described in this report is capable of separating (baseline) atrazine and its six degradation products in less than 30 min. Fig. 3 shows a chromatogram of the nonradiolabeled standards of the degradation products and atrazine (at 230 nm). The chromatographic conditions used in this experiment were optimum in terms of resolution and sensitivity. The pH of the mobile phase is very critical for the separation of the first three peaks in Fig. 3. When the pH of the mobile phase was greater than 3.0, peaks 2 and 3 coeluted with identical retention times.

Fig. 4 is a representative histogram of a soil reflux extract from a soil metabolism study of atrazine. The atrazine was incubated for 181 days under aerobic conditions. Four degradation products of atrazine were identified in the histogram of the day-181 soil metabolism sample. One unknown degradation product (trace) was also observed in this sample which is not shown in Fig. 4. The peaks in the histogram were identified

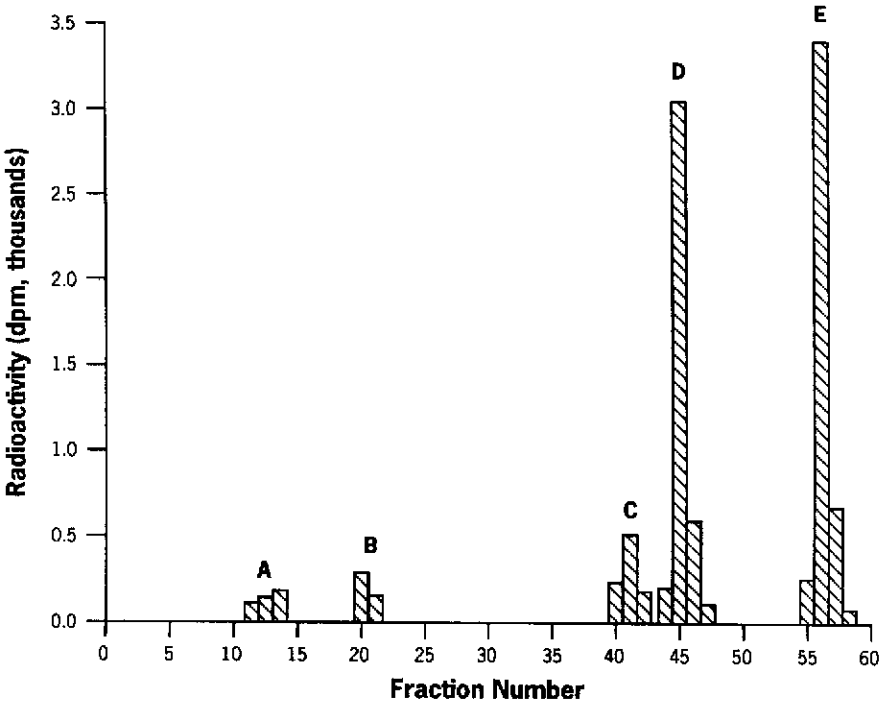


Fig. 4. Representative HPLC histogram of 1 *M* HCl in methanol reflux extract of day-181 sample of soil metabolism of atrazine. Fractions: 12–14 = GS-17792; 20 and 21 = GS-17794; 40–42 = G-28279; 44–47 = G-34048; 55–58 = atrazine.

TABLE II

REPRESENTATIVE HPLC RETENTION TIMES OF NON-RADIOLABELED STANDARDS OF ATRAZINE AND ITS DEGRADATION PRODUCTS

Retention times are representative values. Actual retention times varied slightly in day-to-day analyses with new batches of mobile phase.

Standard identification	*Retention time (min)*
Atrazine	27.0
G-30033	22.0
G-28279	18.5
G-28273	6.5
G-34048	20.5
GS-17792	5.0
GS-17794	8.0

by comparing the retention times of the non-radiolabeled standards (Fig. 3). The retention times of atrazine and its degradation products are listed in Table II.

The extract of a control soil sample was also injected into the HPLC system to determine the retention behavior of the UV-absorbing components extracted from the soil background. It was found that most of these components (at 230 nm) eluted at the void volume of the chromatographic system. Therefore, most of the degradation products of non-radiolabeled atrazine may be quantitated by UV detection in a soil metabolism study.

Recovery of [^{14}C]atrazine from soil by extraction was approximately 93% at day

TABLE III

DISTRIBUTION OF ATRAZINE AND METABOLITES IN BIOLOGICALLY ACTIVE AEROBIC SAMPLES

ND = Not detected.

Incubation time (days)	*Distribution (%)*[a]						
	Atrazine	*G-30033*	*G-28279*	*G-28273*	*G-34048*	*GS-17794*	*GS-17792*
0	90.7	0.8	1.3	0.1	ND	ND	ND
3	85.6	2.1	0.6	0.7	ND	ND	ND
7	80.8	2.2	0.6	0.4	ND	ND	ND
14	77.8	2.6	0.8	0.2	ND	ND	ND
32	67.0	2.1	0.7	0.3	ND	ND	ND
62	59.1	2.7	0.9	ND	0.7	ND	ND
94	56.5	3.0	1.7	ND	0.7	ND	ND
181	33.1	3.5	1.6	ND	0.5	ND	ND
244	26.5	4.3	2.0	0.2	0.6	ND	ND
300	21.2	4.6	1.2	0.1	0.2	ND	ND

[a] The data are mean values (n = 4) based on the percentage of the radioactivity applied to the test system. The R.S.D. for one-step extraction was 10% or less for atrazine and its metabolites.

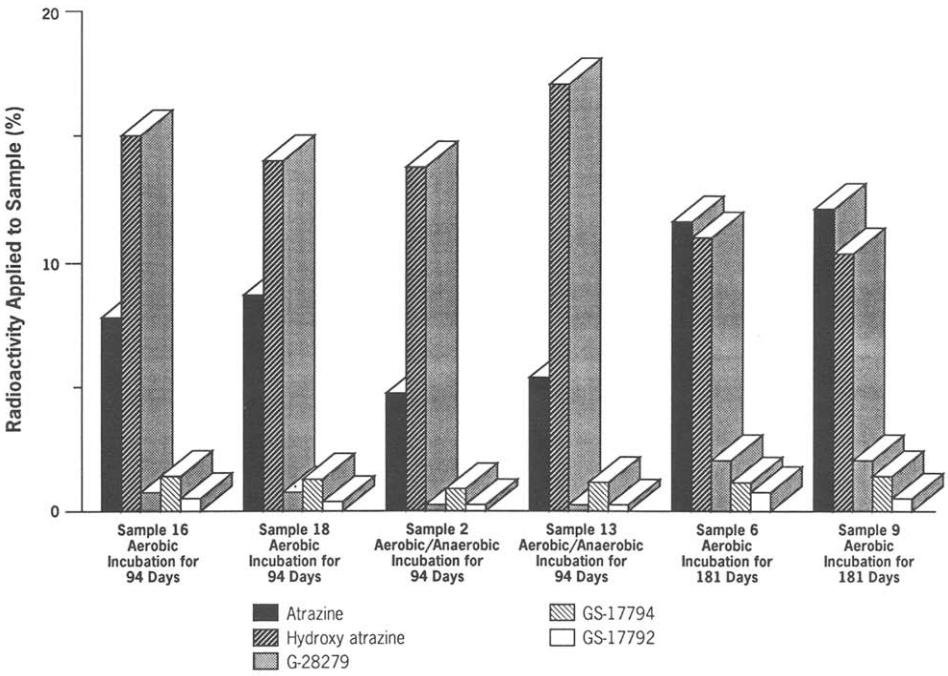


Fig. 5. Recovery of [^{14}C]atrazine and its degradation products under aerobic and anaerobic incubation conditions for days 94 and 181.

0. The total ^{14}C recovery from the soil decreases with time due to binding with soil and soil materials. Approximately 78% of the total ^{14}C material extracted by organic solvent after the [^{14}C]atrazine in soil was incubated for 300 days under aerobic conditions.

The distribution of atrazine and its degradation products during 300 days of incubation is shown in Table III. The relative standard deviation (R.S.D.) for atrazine and its metabolites was 10% or less for four extractions. This indicates that the reproducibility of the single-step extraction procedure used in this experiment is good. No systematic study was conducted to determine the recovery of the hydroxylated atrazine products by the single-step extraction procedure. However, the recovery of the more polar hydroxylated products decreased with the increase of incubation period. The recovery of the parent also decreased with time, but to a lesser extent than the degradation products. The total radioactivity recovered during the study was also calculated. The total radioactivity recovered from day 0 to day 300 was approximately 98.6% $\pm$ 5%. The recovery of atrazine and its metabolites incubated under aerobic and anaerobic conditions for different days is in Fig. 5.

Radiolabeled materials are frequently used in soil metabolism and animal metabolism studies. Most of the reported methods for conducting soil and animal metabolism studies use TLC. TLC methods[17] usually provide poor resolution and are very time consuming compared with HPLC methods. The sample extract for radiolabeled compounds can be injected into a reversed-phase chromatography

system without any preconcentration because large volumes of the extract (up to 500 μl) can be injected into an HPLC system. Therefore, for metabolism studies, an HPLC method is usually more simple, reproducible, and accurate than a TLC method. This method may also be used in animal metabolism studies of [^{14}C]atrazine. The lower limit of quantitation for [^{14}C]atrazine and its metabolites was 20 ng/ml for a 100-μl injection volume and a R.S.D. of less than 10%. Instead of fraction collection and quantitation by liquid scintillation counting, a radioactivity detector for HPLC can be used to conduct on-line quantitation of the analytes.

CONCLUSION

This HPLC method for determining atrazine and its degradation products is rapid, sensitive and more accurate than most of the methods reported in the literature. This method was used to determine atrazine and its metabolites from a soil metabolism study. All of the metabolites of atrazine that were formed in a 300-day soil metabolism study were separated and quantitated by the described HPLC method. No interference peak was observed in any of the sample extracts, obtained at different time points. Because of good selectivity, this method may also be used in animal metabolism studies of atrazine.

REFERENCES

1 L. Fishbein, *Chromatogr. Rev.*, 12 (1970) 167.
2 L. Fishbein, *Chromatography of Environmental Hazards*, Vol. 3, Elsevier, Amsterdam, 1975, Ch. 17.
3 P. G. Stokes and A. W. Schwartz, *J. Chromatogr.*, 168 (1979) 455.
4 D. C. Muir and B. E. Baker, *J. Agric. Food Chem.*, 26 (1978) 420.
5 W. R. Lusby and P. C. Kearney, *J. Agric. Food Chem.*, 26 (1978) 635.
6 L. Stadler and W. Pestemer, *Weed Res.*, 20 (1980) 341.
7 B. A. Demian, V. Borbeby, F. Kerek, B. Bader, M. Cirstea and E. Dragusin, *Rev. Chem.*, 30 (1979) 281.
8 E. Smolkova and V. Pacakova, *Chromatographia*, 11 (1978) 698.
9 J. F. Lawrence and R. Leduc, *Anal. Chem.*, 50 (1978) 1, 161.
10 K. A. Ramsteiner and W. D. Horman, *J. Agric. Food Chem.*, 27 (1979) 934.
11 J. F. Lawrence and D. Turton, *J. Chromatogr.*, 159 (1978) 207.
12 P. H. Plaested and M. L. Thronton, *Contrib. Boyce Thompson Inst.*, 22 (1974) 399.
13 D. C. Muir, *J. Agric. Food Chem.*, 28 (1980) 714.
14 H. M. J. Vermeulen, Z. Apostolides, D. J. J. Potgiester, P. C. Nel and N. S. H. Smit, *J. Chromatogr.*, 240 (1982) 247.
15 M. T. Giardi, M. C. Giardina and E. Brancaleoni, in A. Frigerio (Editor), Chromatography in Biochemistry, Medicine and Environmental Research, 1 (*Analytical Chemistry Symposia Series*, Vol. 13), Elsevier, Amsterdam, 1983, p. 53.
16 M. T. Giardi, M. C. Giardina and G. Filacchioni, *Agric. Biol. Chem.*, 49 (1988) 1551.
17 P. Capriel and A. Haisch, *Z. Pflanzenernaehr. Bodenk.*, 146 (1983) 474.