Determination of Tebuthiuron and Metabolites in Bovine Milk by GC–MSD

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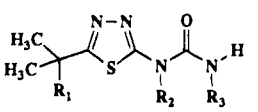
A method is described for the determination of tebuthiuron and five metabolites [metabolite 104, *N*-[5-(1,1-dimethylethyl)-1,3,4-thiadiazol-2-yl]-*N*-methylurea; metabolite 106, *N*-[5-(1,1-dimethylethyl)-1,3,4-thiadiazol-2-yl]urea; metabolite 109, *N*-[5-(1,1-dimethylethyl)-1,3,4-thiadiazol-2-yl]-*N'*-(hydroxymethyl)-*N*-methylurea; metabolite 104(OH), *N*-[5-(2-hydroxy-1,1-dimethylethyl)-1,3,4-thiadiazol-2-yl]-*N*-methylurea; and metabolite 109(OH), *N*-[5-(2-hydroxy-1,1-dimethylethyl)-1,3,4-thiadiazol-2-yl]-*N'*-(hydroxymethyl)-*N*-methylurea] in bovine milk. The compounds are extracted from acid-hydrolyzed milk with ethyl acetate or methyl *tert*-butyl ether, and samples are purified with alumina and C_{18} solid phase extractions. Following trifluoroacetylation and silylation, the analytes are quantitated by gas chromatography with mass spectrometric detection in the selected ion monitoring mode. During hydrolysis, metabolites 109 and 109(OH) are converted to 104 and 104(OH), respectively; therefore, quantitation of metabolites 104 and 104(OH) includes these two metabolites as well. Additional purification and derivatization steps are required for the isolation and determination of metabolite 106, thus necessitating separate analysis. Limits of quantitation for all of the analytes range from 0.02 to 0.09 μg/mL.

Keywords: *Tebuthiuron; urea; milk; derivatization; quantitation; GC; MSD*

INTRODUCTION

Tebuthiuron, *N*-[5-(1,1-dimethylethyl)-1,3,4-thiadiazol-2-yl]-*N,N'*-dimethylurea, is a broad spectrum herbicide used for brush and weed control in rangeland and for spot treatment in pastures and in noncropland areas to control grasses, broadleaf weeds, and woody plants (Humburg, 1989). As a result of these uses, tebuthiuron-related residues may be found in grasses growing in treated areas, where they can be consumed by grazing animals. Metabolism studies in dairy cows dosed with [^{14}C]tebuthiuron demonstrated uptake of tebuthiuron and its conversion to metabolite 104 [*N*-[5-(1,1-dimethylethyl)-1,3,4-thiadiazol-2-yl]-*N*-methylurea], metabolite 106 [*N*-[5-(1,1-dimethylethyl)-1,3,4-thiadiazol-2-yl]urea], metabolite 109 [*N*-[5-(1,1-dimethylethyl)-1,3,4-thiadiazol-2-yl]-*N'*-(hydroxymethyl)-*N*-methylurea], metabolite 104(OH) [*N*-[5-(2-hydroxy-1,1-dimethylethyl)-1,3,4-thiadiazol-2-yl]-*N*-methylurea], and metabolite 109(OH) [*N*-[5-(2-hydroxy-1,1-dimethylethyl)-1,3,4-thiadiazol-2-yl]-*N'*-(hydroxymethyl)-*N*-methylurea] in milk (Figure 1). Of the extractable radioactivity, approximately 25–30% of the residue was present as conjugates which could be released by acid hydrolysis (J. D. Magnussen and D. P. Rainey, DowElanco, unpublished results, 1989).

Tebuthiuron has been determined in soil and plant tissue by gas chromatography (GC) with detection of its thermal degradation product by flame photometry and also by high-pressure liquid chromatography (HPLC) with UV detection (Loh et al., 1980; Smith et al., 1984; Lydon et al., 1991). Tebuthiuron and its major metabolites in grass and sugarcane have been determined by GC with detection of the thermal degradation products by flame photometry or mass spectrometry using single ion monitoring (Loh et al., 1978). Analysis of tebuthiuron and metabolites 104, 106, and 109 in whole milk has been accomplished by HPLC (J. M. Rodewald, T. D. Macy, J. T. Wilson, and H. R. Yeagy, DowElanco, unpublished results, 1981). However, there are no procedures that provide for extraction of the conjugated metabolites with determination of all the major metabolites found in milk. Therefore, a new method is presented that incorporates acid hydrolysis for the release of conjugates and determines tebuthiuron and its major metabolites in milk, including 104(OH) and 109(OH).



Compound	R_1	R_2	R_3
Tebuthiuron	CH_3	CH_3	CH_3
Metabolite 104	CH_3	CH_3	H
Metabolite 106	CH_3	H	H
Metabolite 109	CH_3	CH_3	CH_2OH
Metabolite 104(OH)	CH_2OH	CH_3	H
Metabolite 109(OH)	CH_2OH	CH_3	CH_2OH

Figure 1. Structures of tebuthiuron and metabolites.

EXPERIMENTAL PROCEDURES

Apparatus. *(a) Gas Chromatograph with Mass Selective Detector (GC–MSD).* A Hewlett-Packard Model 5890A with a Model 5971A mass selective detector and Model 7673A automatic sampler in combination with a Model QS/20 Vectra PC-based ChemStation was used for measurement of peak areas. The capillary column was a fused silica DB-5, 30 m × 0.25 mm i.d., 0.25 μm film thickness (J&W Scientific). A deactivated, double-gooseneck column inlet liner (Hewlett-Packard No. 5181-3315) was used for splitless injection. The carrier gas (helium) linear velocity was approximately 30 cm/s. The injector temperature was 250 °C, and the transfer line temperature was 300 °C. The oven was temperature-programmed from 80 °C (held for 1.1 min) to 275 °C (held for 2.0 min) at 20 °C/min. The injection volume was 1 μL, and the inlet purge time was 1.0 min. Under these conditions, typical

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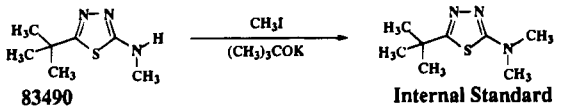

Figure 2. Synthesis of internal standard.

retention times for the derivatized analytes were 8.4, 9.5, 11.2, and 11.3 min for 104, 104(OH), tebuthiuron, and 106, respectively. A typical retention time for the internal standard was 8.9 min. Ions monitored in the electron impact mode were m/z 252, 255, 266, 267, and 170 for tebuthiuron and metabolites 104 and 104(OH) and m/z 260, 310, and 170 for metabolite 106.

(b) Extraction Column. Chem Elut columns (disposable extraction columns), 20-mL capacity, Varian, were used for liquid–liquid extraction.

(c) Solid Phase Extraction (SPE) Cartridges. Neutral alumina and C_{18} SPE cartridges, 1-g packing, Fisher Scientific, were used for sample purification. (Elution profiles were generated with standards on alumina and C_{18} SPE cartridges before the assay was attempted to ensure adequate recoveries.)

(d) 11-Dram Glass Vials. Screw-cap glass vials (28 × 108 mm) with PTFE-lined caps, Fisher Scientific, were used during sample hydrolysis and cleanup steps.

Reagents. Solvents [acetonitrile, ethyl acetate, methanol, dichloromethane (DCM), methyl *tert*-butyl ether (MTBE), tetrahydrofuran, and toluene] were all of at least HPLC grade and were purchased from EM Science, Fisher Scientific, or Mallinckrodt. Glacial acetic acid, hydrochloric acid (12 N), and sodium hydroxide pellets were obtained from Fisher Scientific or Mallinckrodt. Iodomethane and potassium *tert*-butoxide were purchased from Aldrich Chemical Co. *N*-Methylbis-(trifluoroacetamide) (MBTFA) and *N*-methyl-*N*-(*tert*-butyldimethylsilyl)trifluoroacetamide] (MTBSTFA) were purchased from Pierce Chemical Co. Analytical standards of tebuthiuron, metabolites 104, 106, 109, 104(OH), and 109(OH), and compound 83490 [5-(1,1-dimethylethyl)-2-(methylamino)-1,3,4-thiadiazole] were obtained from DowElanco, Indianapolis, IN.

Safety Precautions. Safety information on the reagents and chemicals listed may be obtained from the suppliers. All extraction and evaporation steps should be performed in a well-ventilated hood. Special precautions should be exercised in handling the derivatizing reagents. Gloves, eye protection, and protective clothing should be worn when working with these chemicals. All work with derivatizing reagents should be conducted in a well-ventilated hood.

Internal Standard Preparation. Methylated compound 83490, 5-(1,1-dimethylethyl)-2-(dimethylamino)-1,3,4-thiadiazole (Figure 2), was prepared as described by Saunders and Vanatta (1974) for use as an internal standard. Briefly, compound 83490 (1.0 mg) was dissolved in 2 mL of tetrahydrofuran. Iodomethane (100 μL) and potassium *tert*-butoxide (40 mg) were added with swirling after each addition. The mixture was kept at room temperature for 30 min, swirling occasionally. Deionized water (50 mL) was added, and the product was partitioned into DCM (2 × 25 mL). The DCM was drained through a bed of anhydrous sodium sulfate and evaporated to dryness on a rotary vacuum evaporator at approximately 40 °C. The residue was reconstituted with toluene and transferred to a 200-mL volumetric flask to give a final concentration of the methylated product of 5.4 μg/mL. The solution was further diluted with toluene to obtain a final concentration of approximately 0.25 μg/mL for use as the final sample diluent for chromatographic analysis.

Standard Preparation. Individual stock solutions of tebuthiuron and each metabolite were prepared at 1.0 mg/mL in methanol. Aliquots (10.0 mL) of each solution were combined and evaporated to dryness under vacuum. The residue was dissolved in 10 mL of methanol, transferred to a 100-mL volumetric flask, and diluted to volume with toluene to obtain a solution concentration of 100 μg/mL of each compound. Aliquots of this solution were further diluted with toluene to obtain solutions of 0.2–50 μg/mL for use in fortifying control milk for recovery samples. Calibration standards were prepared by combining aliquots of tebuthiuron and metabolites 104, 106, and 104(OH) to obtain solutions containing 0.01–1.0 μg/mL of each compound in toluene. Calibration standards (1.0 mL) were evaporated to dryness and derivatized along with the samples before analysis. Solutions of tebuthiuron standards and related materials are quite stable under normal laboratory conditions. The stock standard solutions were kept refrigerated only as a precaution against evaporation.

Hydrolysis of Milk Samples. Milk was stirred at room temperature for 5 min prior to sampling. Duplicate 10.0-mL aliquots were transferred to 11-dram glass vials. (A separate aliquot was required for analysis of metabolite 106.) Recovery samples were prepared by evaporating 1.0-mL aliquots of the fortification solutions in separate 11-dram glass vials and then adding 10.0-mL aliquots of control milk and vortex mixing. HCl (12 N) (10 mL) was added to each milk sample; each vial was sealed with a PTFE-lined cap, mixed by vortex, and placed in a water bath at approximately 95 °C for 2 h. The samples were cooled to room temperature and mixed by vortex. Centrifugation at approximately 2500 rpm for 5 min caused the milk proteins to precipitate. The vials were then placed in a freezer for approximately 20 min to solidify milk fats into a layer on top of the liquid portion. A disposable serological pipet was used to transfer 10.0 mL of the middle liquid layer to a clean 11-dram vial; the fats and precipitate were discarded. (The samples were dark brown in color.) NaOH (6 N) (10 mL) was added to the sample aliquots, which were then mixed and adjusted to pH 9 ± 0.5 by dropwise addition of 6 N NaOH or 6 N HCl. The solutions were allowed to cool, and the pH was checked again. The vial contents were mixed by vortex and centrifuged at approximately 2500 rpm for 5 min to remove additional precipitates.

Determination of Tebuthiuron and Metabolites 104, 109, 104(OH), and 109(OH) in Milk Samples. Using one each of the duplicate milk samples, the liquid contents of the vials were transferred to Chem Elut columns and allowed to adsorb for 10 min. Ethyl acetate (20 mL) was added to the Chem Elut columns, and the eluates were collected in clean 11-dram glass vials. After 5 min, a second 20-mL portion of ethyl acetate was added to each column, and the eluates were collected in the same vials. After 5 min, 15 mL of ethyl acetate was added to each column, and the eluates were collected. The combined ethyl acetate eluate from each column was evaporated to dryness under a stream of nitrogen on a water bath heated to approximately 50 °C.

Neutral alumina SPE cartridges were conditioned by passing 5 mL of methanol, followed by 5 mL of DCM, through each cartridge. The samples were transferred to the SPE cartridges using four 5-mL portions of DCM; each portion was allowed to pass into the adsorbent before the next portion was added. The eluates were discarded, and the cartridges were allowed to dry thoroughly under vacuum for 5 min. The compounds of interest were eluted with two 10-mL portions of methanol, and the eluates were collected in 11-dram glass vials. The methanol was evaporated to dryness using nitrogen and heat as before. The residues were dissolved in 5.0 mL of methanol using sonication and vortex mixing and then centrifuged to remove alumina particles. Sample aliquots (1.0 mL) were transferred to 3-mL screw-cap vials and evaporated to dryness using nitrogen and heat as before.

MBTFA (100 μL) was added to each sample, and the vials were sealed securely with PTFE-lined caps. The samples were heated in a heat block at 90 °C for 2 h. After cooling, 1.0 mL of toluene containing internal standard was added, and the contents were mixed well. A portion of each derivatized sample was then transferred to a GC vial for injection into the GC–MSD.

Determination of Metabolite 106 in Milk Samples. The second hydrolyzed milk sample from each duplicate set was extracted from a Chem Elut column with MTBE instead of ethyl acetate, using the same procedure as described for the ethyl acetate. The MTBE was evaporated to dryness under a stream of nitrogen on a water bath heated to approximately 50 °C.

Reversed phase C_{18} SPE cartridges were conditioned by passing 5 mL of acetonitrile, followed by 5 mL of deionized water, through each cartridge. Sample residues were dissolved

in 1.0 mL of acetonitrile using sonication and diluted with 9.0 mL of water. After vortex mixing, the samples were transferred to the SPE cartridges. The vials were washed with 5 mL of water, which was also transferred to the SPE cartridges. The eluates were discarded and the cartridges dried under vacuum for at least 30 min. The compound of interest was eluted with two 10-mL portions of acetonitrile, which was evaporated to dryness using nitrogen and heat as before.

Neutral alumina SPE cartridges were conditioned by passing 5 mL of methanol, followed by 5 mL of DCM, through each one. The samples were transferred to the SPE cartridges using two 5-mL portions of DCM, followed by 10 mL of DCM. The eluates were discarded, and the cartridges were dried under vacuum for 5 min. The compound was eluted with two 10-mL portions of methanol containing 1% acetic acid. The solvent was evaporated under nitrogen on a water bath as before. The residue was dissolved in 5.0 mL of methanol, sonicated and vortexed, and then centrifuged to remove alumina particles. Sample aliquots (1.0 mL) were transferred to 3-mL screw-cap vials and evaporated to dryness using nitrogen and heat.

MBTFA (100 μL) was added, and the vials were sealed securely with a PTFE-lined caps and then heated in a heat block at 90 °C for 2 h. After cooling, MTBSTFA (150 μL) was added, and the vials were heated for 1 h at 90 °C. After cooling, 1.0 mL of toluene containing internal standard was added, and the contents were mixed well. A portion of each derivatized sample was then transferred to a GC vial for injection.

Chromatography. Sixteen samples, including a system blank, three controls, three fortified recovery samples, and nine treated samples, constituted a typical analysis set. Calibration standards, derivatized along with the appropriate sample set, were injected before and after the sample set. Some precautions were necessary to preserve adequate chromatographic performance. The GC inlet liner was replaced periodically and the column clipped to avoid buildup of degradation materials at the column inlet. Samples for analysis of metabolite 106 were not injected directly following injection of samples for analysis of tebuthiuron and the other metabolites, as the latter contained partially derivatized 106 which appeared as carryover peaks. Several injections of a solution containing both MBTFA and MTBSTFA were required to remove underivatized 106 from the injection port and column after injection of solutions containing tebuthiuron and metabolites plus MBTFA. When reanalysis was necessary, freshly derivatized solutions were prepared as the injection solutions degraded upon exposure to air and moisture.

Calculation of Results. Integration of peak areas was performed by the GC–MSD data station. The area of each analyte peak (m/z 252 for tebuthiuron and metabolites 104 and 104(OH); m/z 310 for metabolite 106) divided by the area of the internal standard peak (m/z 170) in each injection gave quantitation ratios for each of the analyte peaks. Log linear regression analysis of the quantitation ratios for the standard curve solutions was performed for each analyte. Recoveries of the fortified control samples were calculated from the standard curve which was obtained for each analyte.

$$x = (y/A)^{1/B}$$

where x is the analyte concentration (μg/mL), y is the quantitation ratio, A is a constant, and B is an exponent.

$$\% \text{ recovery} = \frac{\mu\text{g/mL (from standard curve)} \times 1.0 \text{ mL} \times 100}{\mu\text{g/mL compound added*}}$$

*For metabolites 104 and 109, this value must be the sum of the two compounds expressed as 104 equivalents; 104(OH) + 109(OH) must be expressed as 104(OH) equivalents.

The following calculation was used for actual sample residues:

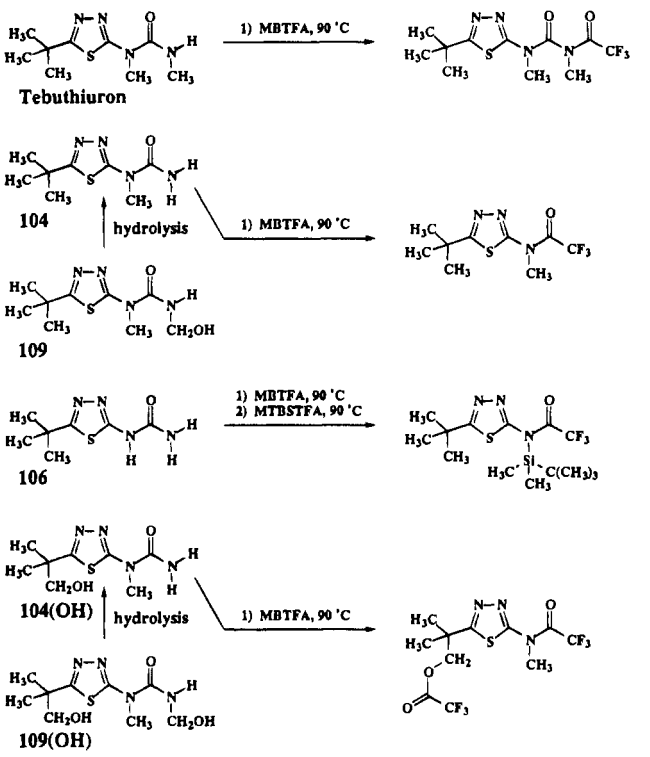

Figure 3. Derivatization reactions of tebuthiuron and metabolites.

$$\mu\text{g/mL (corrected for recovery)} = \frac{\mu\text{g/mL (from standard curve)} \times 1.0 \text{ mL} \times 100}{1.0 \text{ mL} \times \text{av \% recovery}}$$

$$\text{total tebuthiuron-related material} = \text{sum of } \mu\text{g/mL of each analyte}$$

Confirmation of Residues. The peak area ratio of two analyte ions [m/z 255 ÷ m/z 252 for tebuthiuron, m/z 267 ÷ m/z 252 for 104, m/z 266 ÷ m/z 252 for 104(OH), and m/z 260 ÷ m/z 310 for 106] was calculated for each analyte in each injection. Analyte identity was confirmed if the ratio in an unknown sample was within ±20% of the average for that analyte peak in the calibration standards.

RESULTS AND DISCUSSION

Acid hydrolysis releases the conjugated milk metabolites so that they can be partitioned into ethyl acetate or MTBE. Metabolites 109 and 109(OH) are converted to 104 and 104(OH), respectively, during hydrolysis. Physical separation of the milk fat and proteins by chilling and centrifugation, respectively, allows increased recovery of all compounds. Tebuthiuron and metabolites 104 and 104(OH) are partitioned into ethyl acetate, while metabolite 106 is partitioned into MTBE. The use of MTBE allows increased recovery of metabolite 106 from the hydrolyzed milk sample but does not provide good recovery of the more polar hydroxy metabolite 104(OH). Therefore, two separate cleanup procedures are required following the hydrolysis step. The C_{18} SPE provides additional purification necessary for metabolite 106 determination.

Because tebuthiuron and its metabolites are thermally unstable at temperatures required for gas chromatographic analysis, they are reacted with MBTFA to form trifluoroacetyl derivatives. Tebuthiuron is derivatized at the terminal nitrogen, whereas the metabolites all undergo thermal degradation to the thiadiazole amine moiety prior to reaction with MBTFA as shown

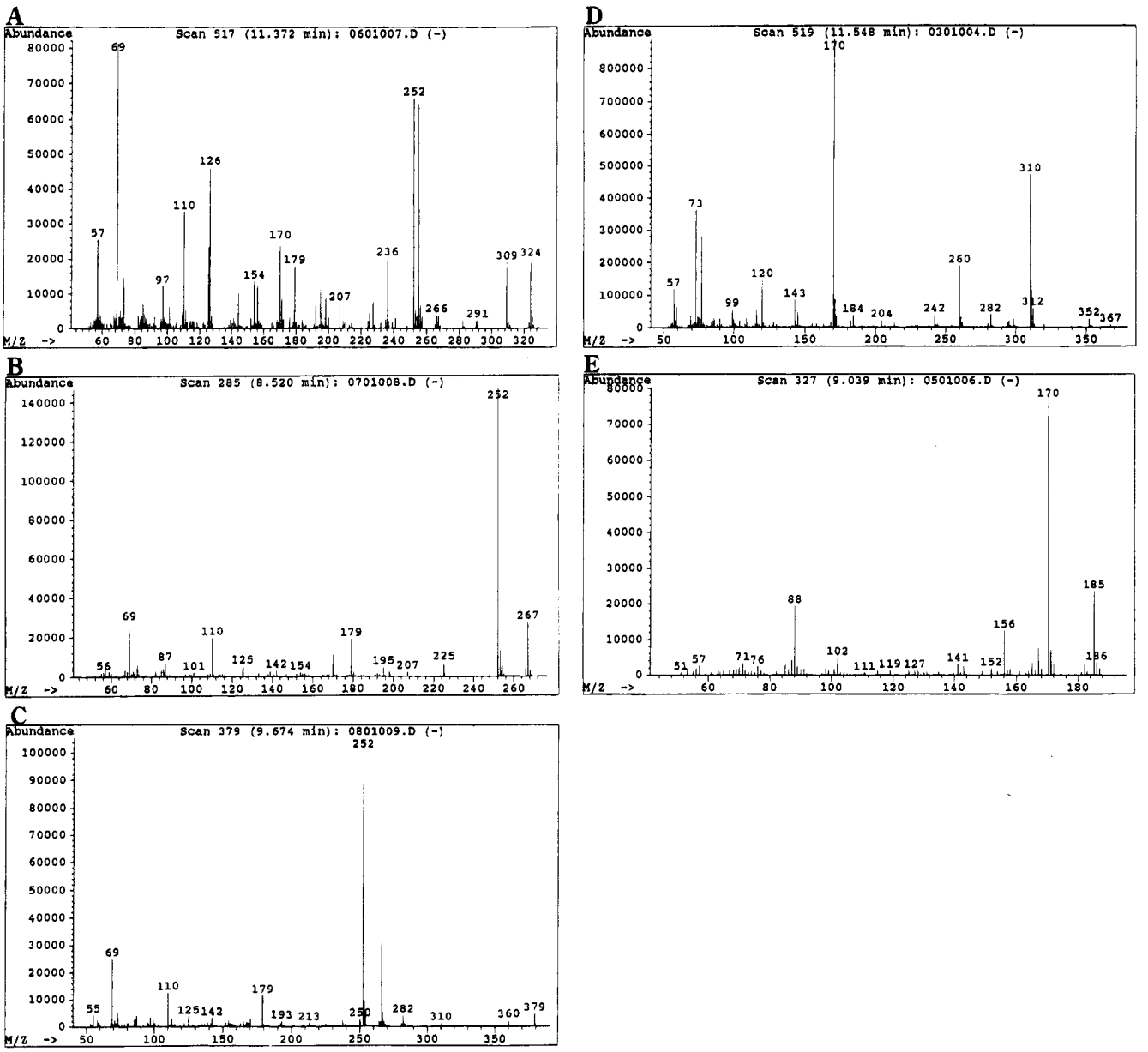

Figure 4. Mass spectra of analytes and internal standard: (A) tebuthiuron derivative (MW 324); (B) metabolite 104 derivative (MW 267); (C) metabolite 104(OH) derivative (MW 379); (D) metabolite 106 derivative (MW 367); (E) internal standard (MW 185).

in Figure 3. The hydroxyl group on the *tert*-butyl side chain of metabolite 104(OH) also reacts with MBTFA. Metabolite 106 reacts with MBTFA to add only one trifluoroacetyl group to the primary amine formed by thermal degradation, but reacts further with MTBSTFA to add a *tert*-butyldimethylsilyl group to the monoacetylated thiadiazole amine. The derivatives formed by these reactions are thermally stable under the GC conditions given in the method. Mass spectra of the products are depicted in Figure 4. The expected molecular ion for each product is apparent, and the quantitation and confirmation ions for each analyte are generally the largest ions depicted.

The internal standard, which is similar in structure to the compounds being determined, adds precision to the quantitation procedure by reducing variability during injection. The confirmation ratios are used to determine whether or not peaks detected at the analyte retention times are in fact the correct molecule. If the ratio is outside the established limits, the identity of the detected peak is not confirmed as an analyte. The recovery samples are fortified with all six compounds, but the assay determines only four analytes because of the conversion of metabolites 109 and 109(OH) to metabolites 104 and 104(OH).

Ion chromatograms shown in Figures 5–8 depict typical standard, control, and fortified milk samples analyzed for tebuthiuron and metabolites 104, 104(OH), and 106. Each chromatogram depicts the selected ion peaks corresponding to the quantitation and confirmation ions for the analyte and the internal standard associated with that injection. Integration of the resulting chromatograms may be achieved by the ChemStation software. The assay procedure requires two full working days.

Control milk fortified with tebuthiuron and metabolites 104, 106, 109, 104(OH), and 109(OH) at levels ranging from 0.02 to 18.8 μg/mL was used to validate the procedure. Recovery and precision data for each fortification level are given in Table 1. Overall recoveries averaged 85 ± 15% for tebuthiuron, 91 ± 15% for metabolites 104 and 109, 80 ± 9% for metabolites 104-(OH) and 109(OH), and 103 ± 18% for metabolite 106. (Metabolite 106 recovery was reduced if the milk sample

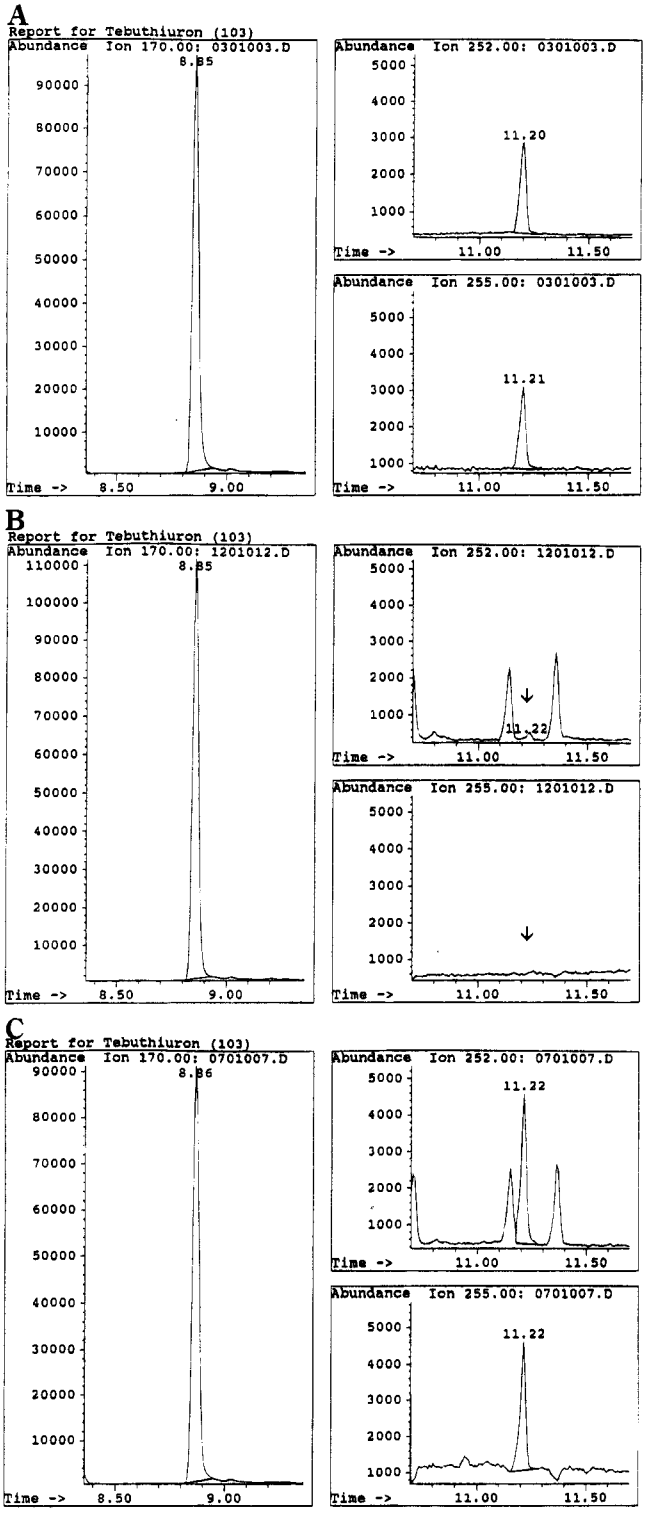

Figure 5. Representative chromatograms of tebuthiuron: (A) standard, 0.1 μg/mL of tebuthiuron; (B) control milk; (C) control milk fortified with 0.1 μg/mL tebuthiuron. Retention time internal standard = 8.9 min; retention time tebuthiuron = 11.2 min.

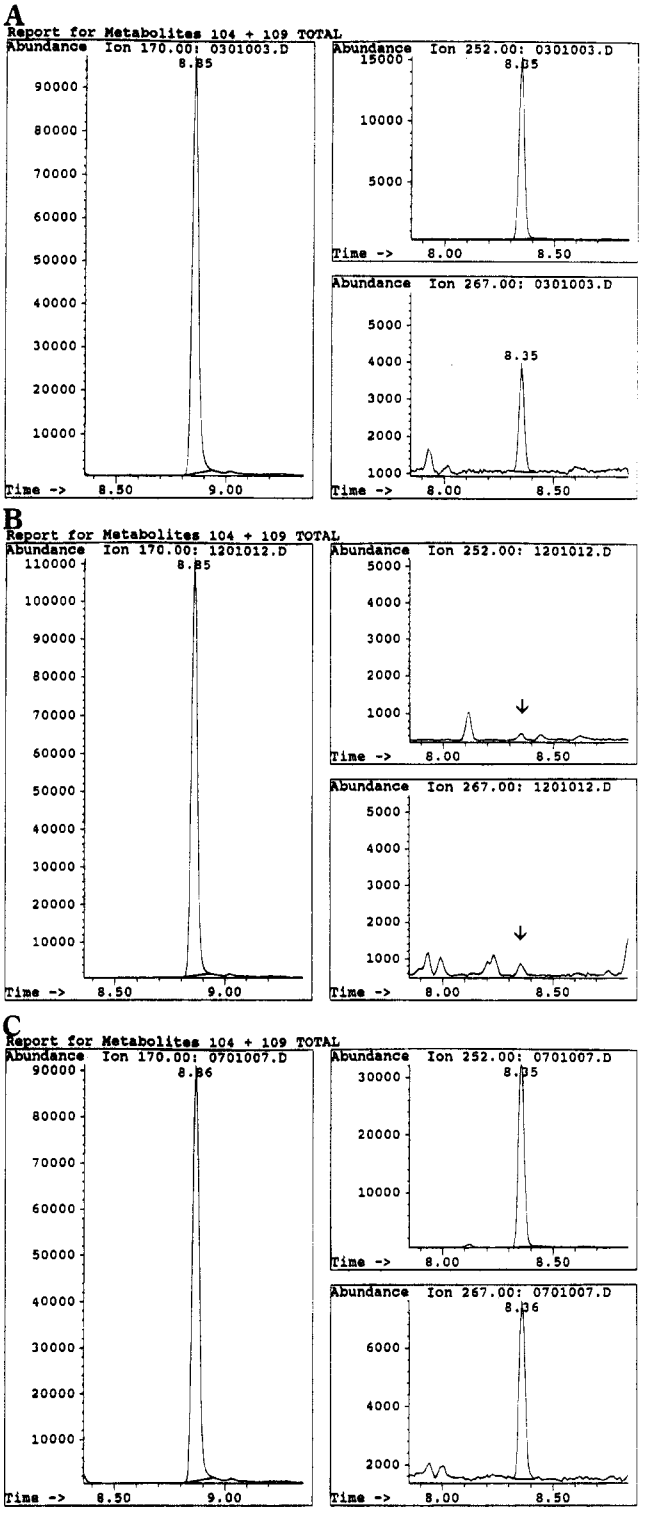

Figure 6. Representative chromatograms of metabolite 104: (A) standard, 0.1 μg/mL of metabolite 104; (B) control milk; (C) control milk fortified with 0.1 μg/mL each metabolite 104 + 109. Retention time internal standard = 8.9 min; retention time metabolite 104 = 8.4 min.

had been frozen and thawed more than once before fortification.) Control milk from 12 different cows was used in the recovery experiments.

Milk samples from a ^{14}C metabolism study (Magnussen and Rainey, unpublished results, 1989) were analyzed using this method. GC–MSD results agreed closely with concurrent ^{14}C radiographic analysis and demonstrated the stability of tebuthiuron-related metabolites in milk that had been frozen for approximately 6 years.

The limits of detection (LOD) and quantitation (LOQ) were calculated using the standard deviation calculated from the lowest recovery level. Following a published technique (Keith et al., 1983), the LOD was calculated as 3 times the standard deviation, and the LOQ was calculated as 10 times the standard deviation. The calculated LOD and LOQ for tebuthiuron are 0.03 and 0.09 μg/mL, for metabolite 104 are 0.02 and 0.05 μg/mL, for metabolite 104(OH) are 0.01 and 0.02 μg/mL, and for metabolite 106 are 0.002 and 0.01 μg/mL,

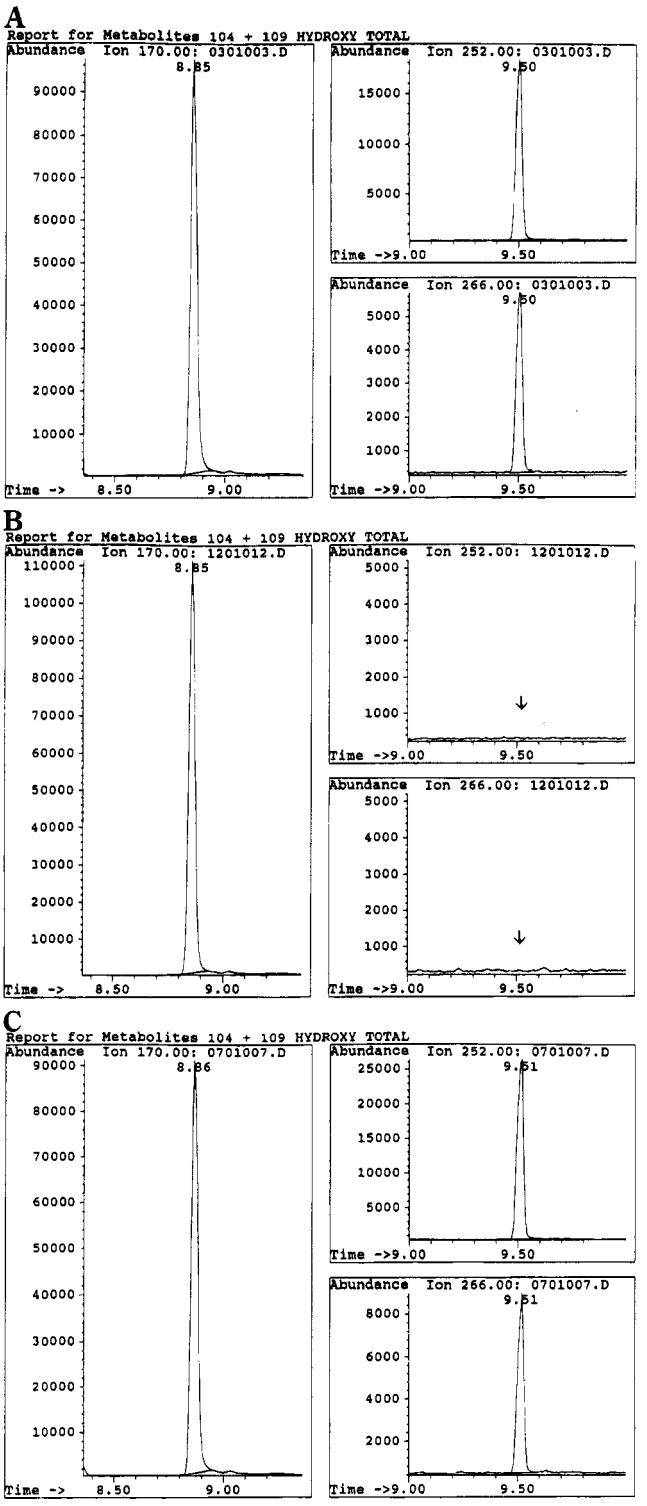

Figure 7. Representative chromatograms of metabolite 104(OH): (A) standard, 0.1 μg/mL of metabolite 104(OH); (B) control milk; (C) control milk fortified with 0.1 μg/mL each metabolite 104(OH) + 109(OH). Retention time internal standard = 8.9 min; retention time metabolite 104(OH) = 9.5 min.

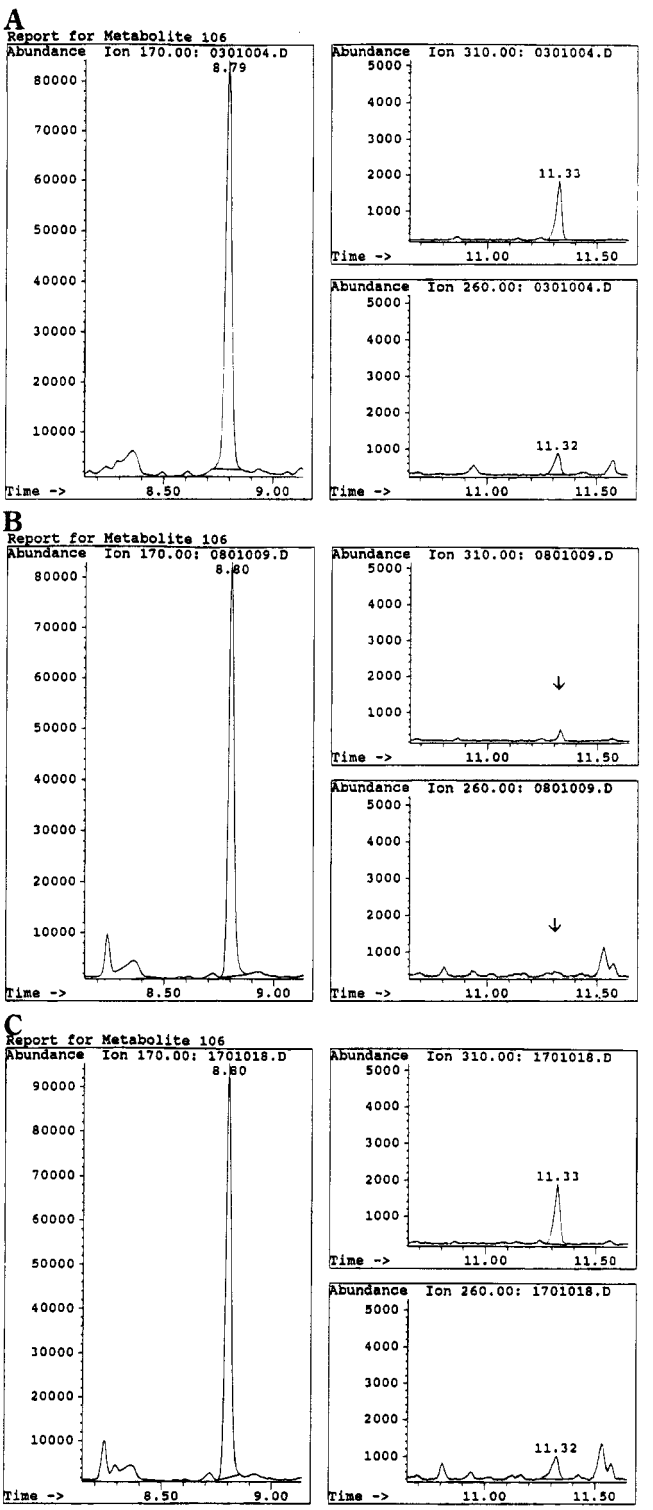

Figure 8. Representative chromatograms of metabolite 106: (A) standard, 0.1 μg/mL metabolite 106; (B) control milk; (C) control milk fortified with 0.1 μg/mL metabolite 106. Retention time internal standard = 8.8 min; retention time metabolite 106 = 11.3 min.

respectively. Since the calculated values for 106 are lower than the fortified recovery levels, the validated LOQ for 106 is reported as 0.02 μg/mL and the LOD at half that level, or 0.01 μg/mL.

This method is suitable for the determination of tebuthiuron and its major metabolites in bovine milk. Extraction of conjugated metabolites is accomplished by acid hydrolysis, and purification of the resulting complex matrix is achieved by physical and chemical separation steps. Derivatization produces thermally stable products which are quantitated by GC–MSD. The specificity and sensitivity are sufficient to measure tebuthiuron-related residues at or below the established tolerance of 0.3 ppm in milk.

ACKNOWLEDGMENT

D. O. Duebelbeis provided technical advice and assistance.

Table 1. Recovery of Tebuthiuron and Metabolites from Whole Milk

compd added		fortification level, μg/mL	n	recovery, % mean	recovery, % SD	calcd LOD	calcd LOQ, μg/mL
tebuthiuron		0.05	8	98	17	0.03	0.09
		0.10	2	89	19		
		0.50	5	81	12		
		1.0	5	81	13		
		5.0	5	75	12		
		10.0	5	82	8		
	overall		30	85	15		
metabolites 104 + 109 (expressed as 104 equivalents)[a]		0.038	8	82	13	0.02	0.05
		0.188	2	81	13		
		0.939	5	92	13		
		1.88	5	95	17		
		9.39	5	89	11		
		18.8	5	96	11		
metabolite 104		1.0	5	95	21		
metabolite 109		0.877	5	98	18		
	overall		40	91	15		
metabolites 104(OH) + 109(OH) (expressed as 104(OH) equivalents)[b]		0.038	8	79	5	0.01	0.02
		0.188	2	76	7		
		0.942	5	81	9		
		1.89	5	82	15		
		9.42	5	76	6		
		18.9	5	81	6		
metabolite 104(OH)		1.0	5	84	14		
metabolite 109(OH)		0.885	5	82	11		
	overall		40	80	9		
metabolite 106		0.02	8	99	4	0.002	0.01
		0.10	2	96	6		
		0.50	1	82			
		1.0	2	88	11		
		5.0	2	131	29		
		10.0	2	126	25		
	overall		17	103	18		

[a] Fortified with equal weights of metabolites 104 and 109 which have been converted to the 104 equivalent concentration. [b] Fortified with equal weights of metabolites 104(OH) and 109(OH) which have been converted to the 104(OH) equivalent concentration.

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