Analytical Methods To Determine Residual Cypermethrin and Its Major Acid Metabolites in Bovine Milk and Tissues

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Several analytical methods were developed to determine cypermethrin and its acid metabolite residues in bovine milk, cream, kidney, liver, muscle, and fat samples. These methods used solvent extraction or acid reflux, liquid−liquid and/or solid phase extraction, with or without chemical derivatization, and quantitation by gas chromatograph with electron capture or mass selective detector. The LOQ and LOD for milk were set at 10 and 2 ppb, respectively. The average method recoveries for cypermethrin, *cis*-DCVA, *trans*-DCVA, and *m*-PBA in cow milk were 81% ($n = 39$), 96% ($n = 22$), 99% ($n = 22$), and 106% ($n = 22$), respectively. For bovine tissues and cream samples, the LOQ and LOD were 50 and 10 ppb, respectively. The overall average method recoveries for cypermethrin, *cis*-DCVA, *trans*-DCVA, and *m*-PBA in cream and tissue samples were 92% ($n = 27$), 97% ($n = 25$), 103% ($n = 25$), and 98% ($n = 25$), respectively. Satisfactory recoveries were also obtained with higher fortification levels for milk and fat samples.

Keywords: *Cypermethrin; metabolites; analysis; milk/tissues*

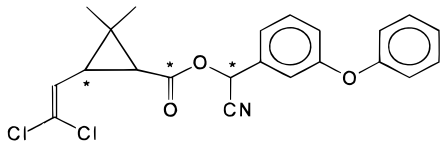
INTRODUCTION

Cypermethrin, a synthetic pyrethroid insecticide, has been used since 1972 in various products to control pests on many agricultural crops. Since cypermethrin residues may be transferred from feed crops to dairy and meat products and meat byproducts, analytical methods were developed to determine residual cypermethrin and the major animal metabolites in these commodities. The analytical methods for cypermethrin in crops, soil, water, and commercial products have been summarized (Sapiets et al., 1984; World Health Organization, 1989; Tsumura et al., 1994). The analytical procedures for residual acid metabolites, however, have not been published. Metabolism studies (unpublished) in poultry and goats using phenyl- and ring-labeled cypermethrin have identified the major metabolites as dichlorovinyl acid [DCVA; *cis*/*trans*-3-(2,2-dichloroethenyl)-2,2-dimethylcyclopropanecarboxylic acid] and *m*-phenoxybenzoic acid (*m*-PBA). Studies of dairy cow and poultry feeding with cypermethrin were conducted (Swaine and Sapiets, 1981a,b; Wallace et al., 1982); however, method recoveries for the acid metabolites in liver, kidney, and fat samples were below the normally acceptable range (70−120%). The three methods described herein were developed to determine residues of cypermethrin and its acid metabolites (shown in Figure 1) which may occur in milk and tissue samples from cows that have consumed treated crops.

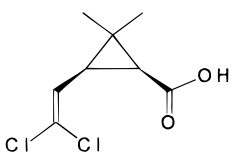
Method I concurrently analyzed cypermethrin and its acid metabolites in milk. Method II determined cypermethrin in cream and tissues. Method IIIa determined acid metabolites in kidney, liver, and pectoral muscle. Because of the high lipid content in cream, adductor muscle, and fat samples, method IIIa was modified into methods IIIb and IIIc to adequately determine the acid analytes in these matrices.

MATERIALS AND METHODS

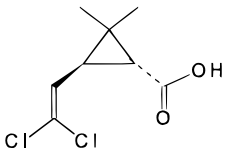
Apparatus. (*a*) A Hewlett-Packard 5890 Series II gas chromatograph equipped with an electron capture detector (ECD), an HP 7673B autosampler, and a Perkin-Elmer



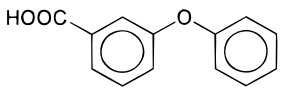
Cypermethrin (* asymmetric center)



cis-DCVA



trans-DCVA



m-PBA

Figure 1. Structures of cypermethrin and three metabolites.

Access*Chrom data collection system were used. A DB-5 megabore capillary column (15 m × 0.53 mm i.d., 1.0-μm film thickness) was used with helium carrier gas at 23 mL/min to analyze the cypermethrin residues. The injector and detector temperatures were 250 and 300 °C, respectively. The column oven temperature was increased from 230 to 280 °C at 5 °C/min and held for 4 min. A 2-μL injection in the splitless mode was used. Makeup gas to the ECD consisted of 10% CH_4/Ar at 47 mL/min.

(*b*) A Hewlett-Packard 5890A gas chromatograph in combination with an HP 5970 mass-selective detector (MSD) operating in the selective ion mode, an HP 7673A autosampler, and an HP MS data ChemStation were used. A DB-5 narrowbore capillary column (15 m × 0.25 mm i.d.) with helium carrier gas at 1 mL/min was used to separate and analyze the pentafluorobenzyl (PFB) derivatives of acid metabolites. The injector and detector temperatures were 250 and 280 °C, respectively. The column oven temperature was maintained at 120 °C for 2 min, programmed to 165 °C at 10

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°C/min, held for 1 min, programmed to 280 °C at 20 °C/min, and held for 3 min. A 2 μL injection in the splitless mode was used. The ion numbers (*m*/*z*) of 353 and 394 were monitored to quantitate *cis*/*trans*-DCVA and *m*-PBA, respectively.

(*c*) An N-Evap evaporator was used, water bath temperature ~40 °C (Organomation Associates 111, S. Berlin, MA).

(*d*) A TurboVap evaporator, water bath temperature ~45 °C (ZW640-3, Zymark Corp., Hopkinton, MA) was used.

(*e*) A TurboVap LV evaporator was used water bath temperature ~45 °C (ZW700, Zymark).

(*f*) An Ultra-Turrax tissuemizer (SDT 1810, Tekmar Co., Cincnnati, OH) was employed.

(*g*) A Visidry vacuum manifold drying attachment (Supelco, Inc., Bellefonte, PA) was used.

(*h*) A Visiprep manifold (Supelco) was used.

(*i*) A C_{18} SPE cartridge, 1 g/6 mL (Varian Sample Preparation Products, Harbor City, CA), was used.

(*j*) An SI SPE cartridge, 1 g/6 mL (J. T. Baker Inc., Phillipsburg, NJ), was used.

(*k*) An SI SPE cartridge, 2 g/10 mL (Varian Sample Preparation Products) was used.

(*l*) A platform multitube vortexer (VWR Scientific, Piscataway, NJ) was used.

Reagents. (*a*) Analytical standards included (1) *cis*-cypermethrin, (±)-α-cyano(3-phenoxyphenyl)methyl (±)-*cis*-3-(2,2-dichloroethenyl)-2,2-dimethylcyclopropanecarboxylate, 99.3%; (2) *trans*-cypermethrin, (±)-α-cyano(3-phenoxyphenyl)methyl (±)-*trans*-3-(2,2-dichloroethenyl)-2,2-dimethylcyclopropanecarboxylate, 99.2%; (3) *cis*-DCVA, (±)-*cis*-3-(2,2-dichloroethenyl)-2,2-dimethylcyclopropanecarboxylic acid, 99.3%; (4) *trans*-DCVA, (±)-*trans* 3-(2,2-dichloroethenyl)-2,2-dimethylcyclopropanecarboxylic acid, 99.2%; (5) *m*-PBA, 97.5%. These standards were synthesized and certified by FMC Analytical Sciences Department. Cypermethrin has three asymmetric centers which give rise to eight isomers. The cypermethrin standard solution used in this study contained 50:50 *cis*- and *trans*-isomers. A total amount of isomeric cypermethrin was quantitated as a single peak using the above GC/ECD parameters.

(*b*) α-Bromo-2,3,4,5,6-pentafluorotoluene (PFBBr), >99%, and tetrabutylammonium dihydrogen phosphate (TBA), 97%, were purchased from Aldrich Chemical Co.

(*c*) All organic solvents used for analysis were of resi-analyzed grade.

Samples. Control bovine milk, cream, liver, kidney, muscle, and fat samples were obtained from an in-life program. The tissue samples were finely chopped in a large cutter/mixer in liquid nitrogen before analysis.

Analytical Procedure for Cypermethrin and Acid Metabolites in Bovine Milk (Method I). A 25-g aliquot of bovine milk was weighed into a 250-mL Erlenmeyer flask. Control sample for the method recovery determination was fortified with a combined standard solution containing cypermethrin, *cis*-DCVA, *trans*-DCVA, and *m*-PBA (each at a level of 10 ppb). After 100 mL of acetone was added, the sample solution was blended using a tissuemizer for 1 min and was filtered and evaporated to remove any acetone. The solution was then transferred to a separatory funnel and was partitioned with 100 mL of hexane and 100 mL of alkaline water (pH 8−9 by indicator strips). A small amount of NaCl was added if an emulsion occurred.

Cypermethrin. The hexane layer containing parent cypermethrin was collected and concentrated to 4 mL and was partitioned with 3 × 4 mL of acetonitrile. A vortexer and a centrifuge were optionally used to help mix and accelerate the phase separation, if necessary. The combined acetonitrile solution was evaporated to near dryness (<0.1 mL, TurboVap LV), 2 mL of hexane was added, and the sample solution was added to a conditioned silica gel (SI, 2 g, Varian) solid phase extraction (SPE) cartridge for cleanup. The SI cartridge was conditioned with 10 mL of hexane. After sample loading, the cartridge was washed with 8 mL of 2.5% ethyl acetate in hexane, and the cypermethrin was eluted with 10 mL of 3.5% ethyl acetate in hexane and collected in a 13-mL test tube. The sample solution was concentrated to 1.0 mL (N-Evap) and quantitated by a gas chromatograph equipped with an electron capture detector (GC/ECD).

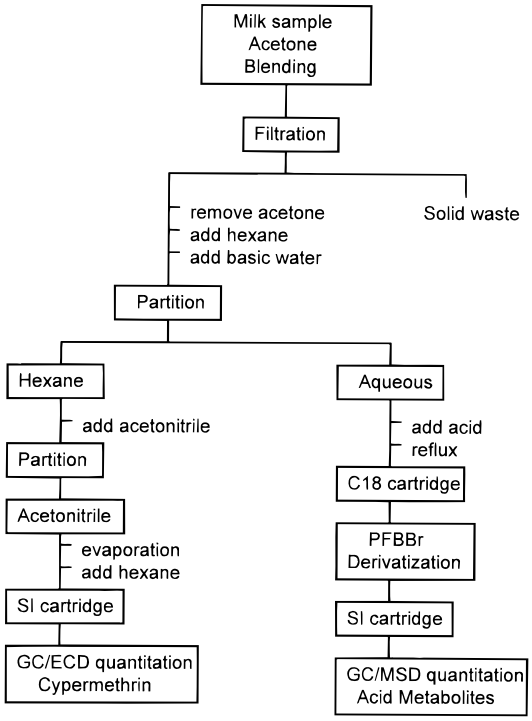

Figure 2. Method I flow scheme for cypermethrin and its acid metabolites in milk samples.

Acid Metabolites. The aqueous layer containing acid metabolites was collected in a boiling flask, acidified with 3 mL of concentrated HCl, and boiled under reflux for 1 h. The sample solution was then loaded onto a C_{18} SPE cartridge that had been conditioned with 6 mL of methanol followed by 6 mL of 0.25 N HCl. The solution flow rate through the cartridge was kept at ~5 mL/min. After sample loading, the cartridge was dried under a full vacuum (~25 psi) for 1−3 min. The acid metabolites were then eluted with 6 mL of methylene chloride in a 13-mL test tube, and the solution was concentrated to 1 mL (N-Evap). One milliliter of a 0.1 M TBA/0.25 N NaOH solution and 30 μL of PFBBr were added to the eluant. The test tube was capped and shaken vigorously on a platform multitube vortexer at room temperature for ~30 min. The sample solution was partitioned with 9 mL of hexane. The hexane layer was collected, concentrated to 2 mL (N-Evap), and loaded onto an SI SPE cartridge (1 g, Baker) for cleanup. The SI cartridge was preconditioned with 6 mL of 60% ethyl acetate in hexane and then with 6 mL of hexane. After sample loading, the cartridge was washed with 6 mL of 0.5% ethyl acetate in hexane, and the analytes of interest were eluted into a test tube with 12 mL of 2.5% ethyl acetate in hexane. This final solution was concentrated to 1.0 mL (N-Evap) and quantitated by a gas chromatograph equipped with a mass selective detector (GC/MSD). The flow scheme of method I is presented in Figure 2.

Analytical Procedure for Cypermethrin in Bovine Cream and Tissues (Method II). A 5-g homogenized matrix was weighed into a 50-mL polypropylene centrifuge tube. The untreated sample for the method recovery determination was fortified with cypermethrin at 50 ppb. About 40 mL of acetone/hexane (1:1) was added, and the sample was extracted with a shaker for 30 min. After centrifugation, the clear solution was decanted into a 250-mL separatory funnel. The extraction and centrifuge steps were repeated with another 40 mL of acetone/hexane (1:1). The combined sample solution was partitioned in a separatory funnel with 50 mL of hexane and 50 mL of deionized water (a small amount of NaCl was added if an emulsion occurred).

Kidney, Liver, and Muscle Samples. The hexane layer was collected, concentrated to 4 mL (TurboVap), and partitioned with 3 × 4 mL of acetonitrile. The sample solution in

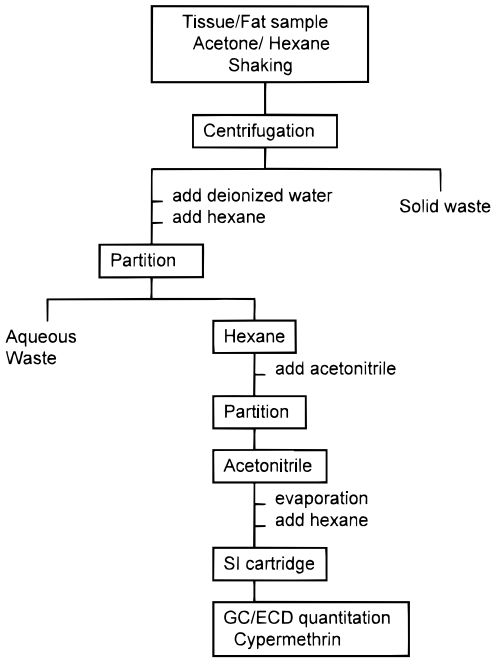

Figure 3. Method II flow scheme for cypermethrin in tissue and fat samples.

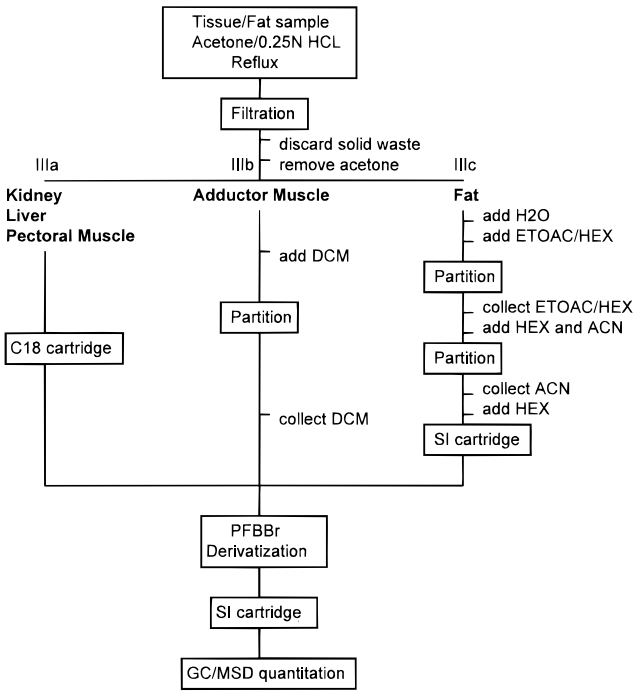

Figure 4. Method III flow scheme for acid metabolites in tissue and fat samples.

acetonitrile was then evaporated to near dryness (<0.1 mL, TurboVap LV), 2 mL of hexane was added, and the sample solution was subjected to cleanup using an SI SPE cartridge (2 g, Varian) which had been conditioned with 10 mL of 60% ethyl acetate in hexane followed by 10 mL of hexane. After sample loading, the cartridge was washed with 12 mL of 2.5% ethyl acetate in hexane, and the cypermethrin was eluted with 12 mL of 5% ethyl acetate. The final solution was concentrated to 1.0 mL (N-Evap) and quantitated by a GC/ECD.

Fat and Cream Samples. The hexane layer (50 mL) was transferred to a separatory funnel and partitioned with 2 × 50 mL of acetonitrile. The combined acetonitrile extract was evaporated to near dryness (<0.1 mL, TurboVap), 2 mL of hexane was added, and the sample solution was cleanedup by an SI SPE cartridge (2 g, Varian) that had been conditioned with 10 mL of 60% ethyl acetate in hexane and then with 10 mL of hexane. After sample loading, the cartridge was washed with 12 mL of 2.5% ethyl acetate in hexane, and the cypermethrin was eluted with 12 mL of 10% ethyl acetate. The final solution was concentrated to 1.0 mL (N-Evap) and quantitated by a GC/ECD. See Figure 3 for a flow scheme of method II.

Analytical Procedure for Acid Metabolites in Bovine Cream and Tissues (Method III). A 5-g sample was weighed into a boiling flask. The control sample for the method recovery determination was fortified with a combined standard solution containing *cis*-DCVA, *trans*-DCVA, and *m*-PBA (each at a level of 50 ppb). One hundred milliliters of acetone/0.25 N HCl (3:1 v/v) was added, and the sample solution was boiled under reflux for 1 h. After filtration, the sample solution was transferred into a 250-mL TurboVap vessel and evaporated to remove any residual acetone. Following this initial step, slightly different methods were required for specific types of tissues. These are described below. In all cases the final step prior to quantitation was the derivatization of the acid metabolites with PFBBr.

Kidney, Liver, and Pectoral Muscle Samples (Method IIIa). The aqueous sample solution was loaded onto a C_{18} SPE cartridge (1 g, Varian) that had been conditioned with 6 mL of methanol and then 6 mL of 0.25 N HCl. After sample loading, the cartridge was completely dried by vacuum with N_2 using a manifold drying attachment (N_2 pressure 30 psi, ~30 min). The analytes were then eluted with 6 mL of 10% ethyl acetate in hexane. The sample solution was evaporated to near dryness (<0.1 mL, N-Evap), and 1 mL of methylene chloride was added.

Adductor Muscle Sample (Method IIIb). The aqueous sample solution was transferred into a separatory funnel, partitioned with 50 mL of methylene chloride, and concentrated to 1 mL (TurboVap).

Cream and Peritoneal and Subcutaneous Fat Samples (Method IIIc). The aqueous sample solution was transferred into a separatory funnel and partitioned with 2 × 100 mL of 10% ethyl acetate in hexane. The solvent layer was concentrated to near dryness (TurboVap), 10 mL of hexane was added, and the sample solution was partitioned with 2 × 10 mL of acetonitrile. The combined acetonitrile solution was evaporated to near dryness (TurboVap), 2 mL of hexane was added, and the sample solution was further cleanedup with an SI SPE cartridge (1 g, Baker) that had been conditioned with 6 mL of 20% ethyl acetate in hexane followed by 6 mL of hexane. After the sample solution was loaded onto the conditioned cartridge, the cartridge was washed with 6 mL of hexane, and the analytes were eluted with 12 mL of 60% ethyl acetate. The sample solution was evaporated to near dryness (N-Evap), and 1 mL of methylene chloride was added.

PFBBr Derivatization. One milliliter of a 0.1 M TBA/0.25 N NaOH solution and 30 μL of PFBBr were added to the sample solution in methylene chloride. The test tube was capped and shaken vigorously on a platform multitube vortexer at room temperature for 30 min. The sample solution was then partitioned with 9 mL of hexane. The solvent layer was collected and concentrated to 2 mL (N-Evap), and the concentrated sample solution was loaded onto an SI SPE cartridge (1 g, Baker) that had been conditioned with 6 mL of hexane. After sample loading, the cartridge was washed with 6 mL of 0.5% ethyl acetate in hexane, and the analytes of interest were eluted with 12 mL of 2.5% ethyl acetate in hexane into a test tube. The final solution was concentrated to 1.0 mL (N-Evap) and quantitated by a GC/MSD. The flow scheme of method III is shown in Figure 4.

The PFBBr-derivatized standard solution was made by adding an appropriate amount of standard in a 13-mL test tube; 1 mL of methylene chloride, 1 mL of 0.1 M TBA in 0.25 N NaOH, and 100 μL of PFBBr were added to the test tube. The test tube was then shaken on a platform multitube vortexer for 1 h and partitioned with 9 mL of hexane. The hexane layer was transferred to a volumetric flask of proper volume and was diluted to the mark with hexane.

Calculation. The magnitude of residues of cypermethrin and the acid metabolite PFB derivatives in each sample was determined by an external standard calibration method based on the average of all standards run in a set of assays. The

Table 1. Summary of Recoveries from Fortified Cow Milk and Tissues

compd	fortifn level (ppb)	no. of analyses	recovery range (%)	mean recovery (%)	recovery SD (%)
milk					
cypermethrin	10	35	70−100	81	±7
	200	4	77−90	85	±6
cis-DCVA	10	22	71−128	96 ± 15	
trans-DCVA	10	22	77−132	99 ± 14	
m-PBA	10	22	85−128	106 12	
milk cream					
cypermethrin	50	4	89−106	100	8
cis-DCVA	50	4	114−118	116 ± 2	
trans-DCVA	50	4	96−107	101 ± 5	
m-PBA	50	4	86−98	93	±6
kidney					
cypermethrin	50	5	71−100	87	±11
cis-DCVA	50	5	90−101	94 ± 5	
trans-DCVA	50	5	96−112	106 ± 6	
m-PBA	50	5	110−129	117 7	
liver					
cypermethrin	50	4	72−91	82	±8
cis-DCVA	50	4	89−105	95 ± 7	
trans-DCVA	50	4	101−110	104 ± 4	
m-PBA	50	4	93−111	101 8	
muscle					
cypermethrin	50	8	89−114	98	±9
cis-DCVA	50	4	77−90	84	±7
trans-DCVA	50	4	79−111	96	±14
m-PBA	50	4	74−100	85 ± 11	
fat					
cypermethrin	50	4	83−103	92	±10
	500	2	83−83	83	
cis-DCVA	50	8	78−125	99 ± 17	
trans-DCVA	50	8	88−127	104	±14
m-PBA	50	8	*73*−127	94	±19

standard solution was injected at the beginning of every set and subsequently after every two sample solutions. The amount of analytes was quantitated from the detector response transmitted to the data acquisition system.

RESULTS AND DISCUSSION

Accuracy and Precision. The accuracy and precision of the present analytical methods were determined by the average recovery and standard deviation of the results from the fortified control samples. Table 1 lists the average method recoveries and standard deviations for cypermethrin and its acid metabolites in all of the bovine matrices. These methods have been used to analyze more than 300 samples from a bovine feeding study (unpublished data) and have been proven reliable and consistent compared to the earlier procedures, especially for the metabolite analysis. In addition, these methods have been validated by an independent contract laboratory. The extraction efficiency (98%) of total radio residues was also well demonstrated for method III using tissue samples from a poultry metabolism study dosed with radiolabeled cypermethrin.

Chromatograms. Selected chromatograms, showing corresponding standard, control, and fortified samples in bovine milk and fat, are presented in Figures 5−8. The large matrix background peak eluting 0.1 min before the analyte of interest in the milk was not identified. Compared to the milk or fat, GC chromatograms of other matrices have minimal coeluting peaks in the retention time windows of interest.

Modifications or Potential Problems. The previous bovine feeding study (Swaine and Sapiets, 1981a,b) had indicated that for metabolite assays any residual cypermethrin had to be removed prior to acid extraction,

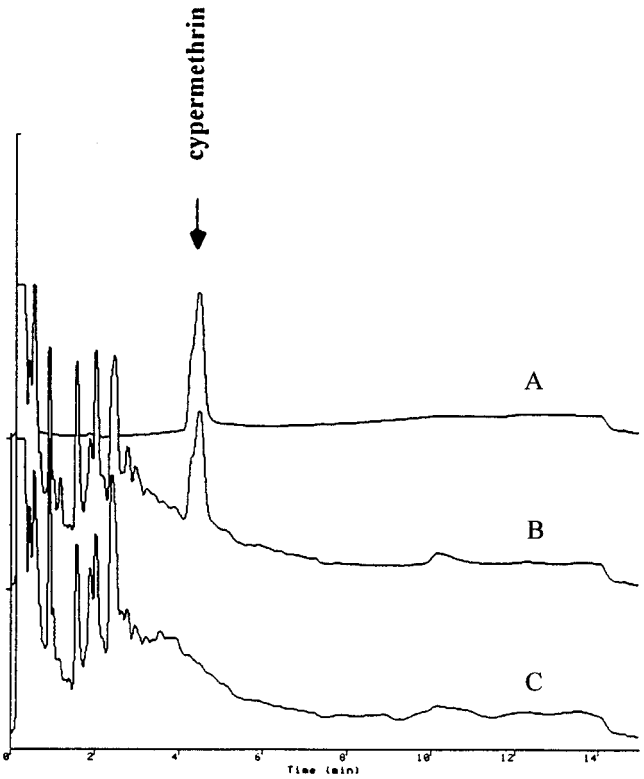

Figure 5. GC/ECD chromatograms of cypermethrin in cow milk: (A) standard solution 0.5 ng; (B) control milk fortified at 10 ppb (91%); (C) control milk.

because cypermethrin was suspected of generating false acid metabolite residues. The assumption that cypermethrin could convert to its acid metabolites during acid hydrolysis was proven wrong in our laboratory by a hexane partition with the acid hydrolysate of a milk sample that had been fortified with cypermethrin. In this experiment, all of the cypermethrin residues were recovered in the hexane after the pH was adjusted to 9, and no acid metabolites were detected in the aqueous solution.

In the milk assay (method I), it was not necessary to completely dry the C_{18} SPE cartridge prior to eluting the metabolites with methylene chloride. However, in the tissue analysis (method II), the C_{18} cartridge had to be dried completely to remove water before elution with 10% ethyl acetate in hexane. In addition, the 10% ethyl acetate in hexane was selected to minimize the elution of coextractants from the C_{18} SPE cartridge.

In method III, glass wool was used for cow tissues to prevent some particulates from going into the C_{18} packing material pores, since this would result in a longer cartridge drying time. Also, since C_{18} SPE cartridges could not provide reproducible and satisfactory recoveries of acid metabolites for adductor muscle and fat samples, the liquid−liquid extraction technique was adapted instead. For adductor muscle, one partition with aqueous and methylene chloride was feasible. However, fat and milk cream required one partition with aqueous and 10% ethyl acetate in hexane and another partition with hexane and acetonitrile. A large volume of hexane was needed to dissolve the high content of lipids in the fat samples before partitioning with acetonitrile. Further, a secondary SI SPE cartridge was needed for fat and cream to separate and eliminate as much lipid as possible in the samples before PFBBr derivatization.

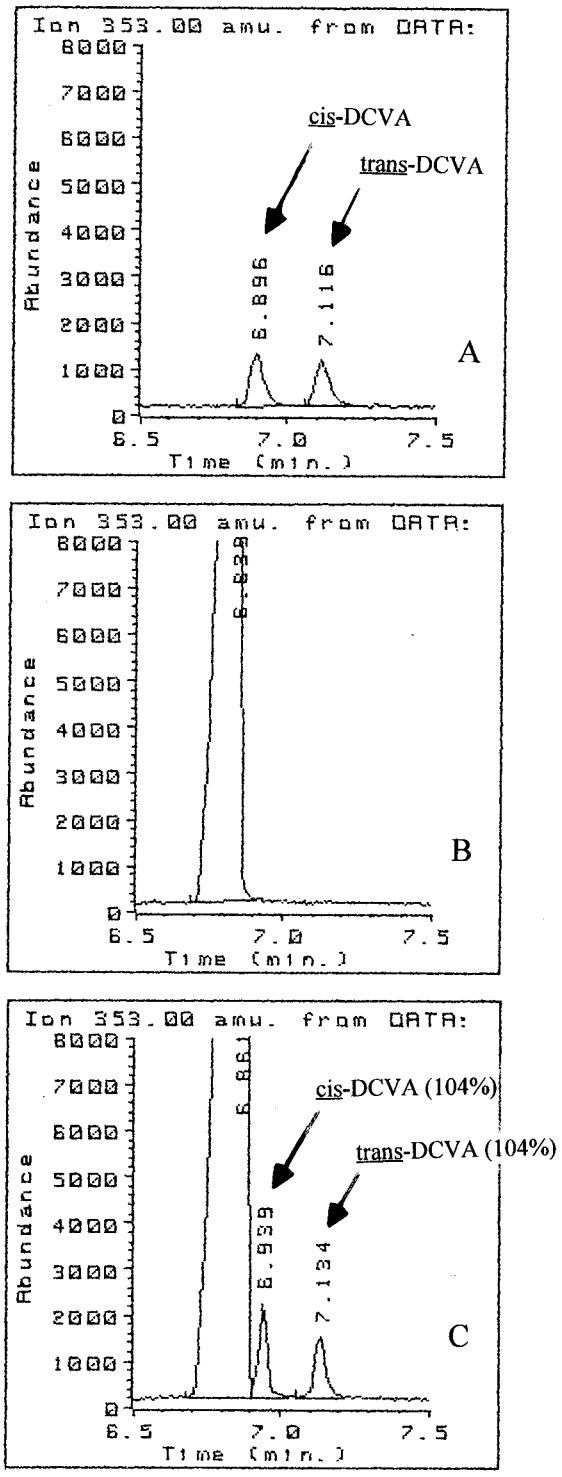

Figure 6. Selective ion chromatograms (*m/z* 353) of *cis,trans*-DCVA in cow milk: (A) standard solution 0.5 ng; (B) control milk; (C) control milk fortified at 10 ppb.

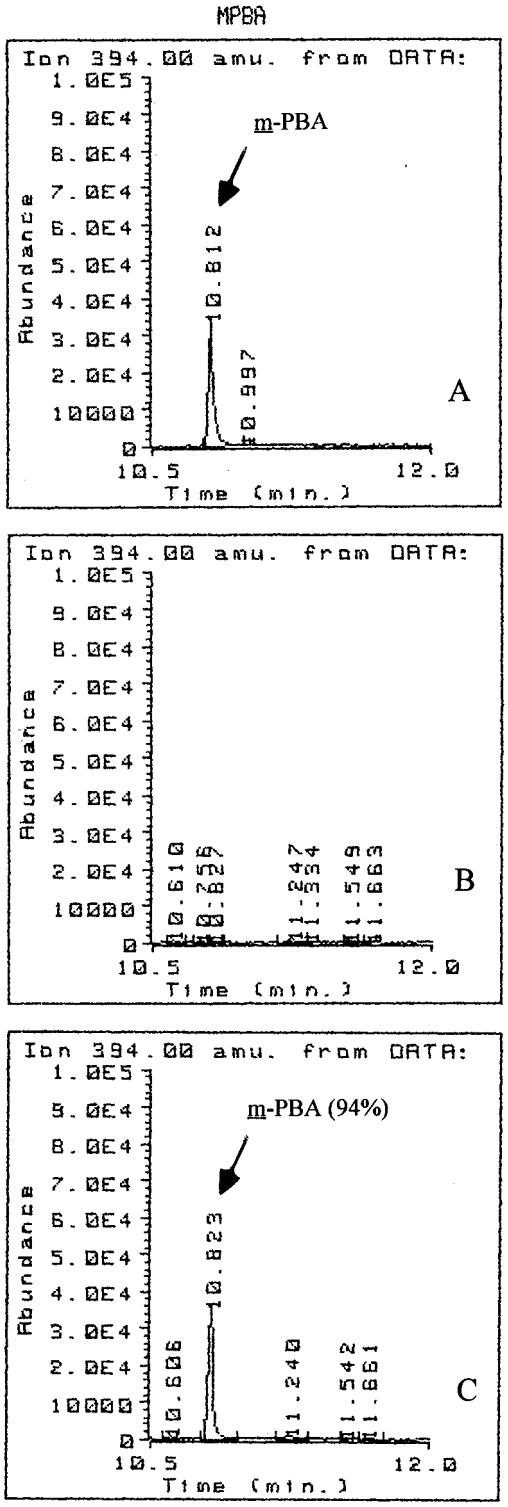

Figure 7. Selective ion chromatograms (*m/z* 394) of *m*-PBA in cow milk: (A) standard solution 0.5 ng; (B) control milk; (C) control milk fortified at 10 ppb.

The washing solvent and the volume used for the SI cleanup cartridge were critical to the method recovery. Generally, different washing solvents or volumes were used in different methods (see detailed procedures in each method). The purpose of the washing step was to reduce as much of the coextractants as possible without jeopardizing the recovery of the analytes. For instance, the acid metabolite method in cow milk required 6 mL of 0.5% ethyl acetate in hexane as washing solvent to eliminate the interference peak eluting 0.1 min earlier than the DCVA PFB derivative peak in the GC/MSD chromatogram (see Figure 6). Any additional wash would elute the analyte from the SI SPE cartridge. Other fragment mass ions for the acid metabolites were monitored with no better resolution found.

SPE cartridges from Varian and J. T. Baker were used in this study to analyze all of the cow matrices. When cartridges from other manufacturers were used, different elution patterns were observed. Therefore, the cartridge elution pattern should be evaluated prior to usage.

The success of the methods is also critically dependent on the derivatization step. Derivatization with PFBBr

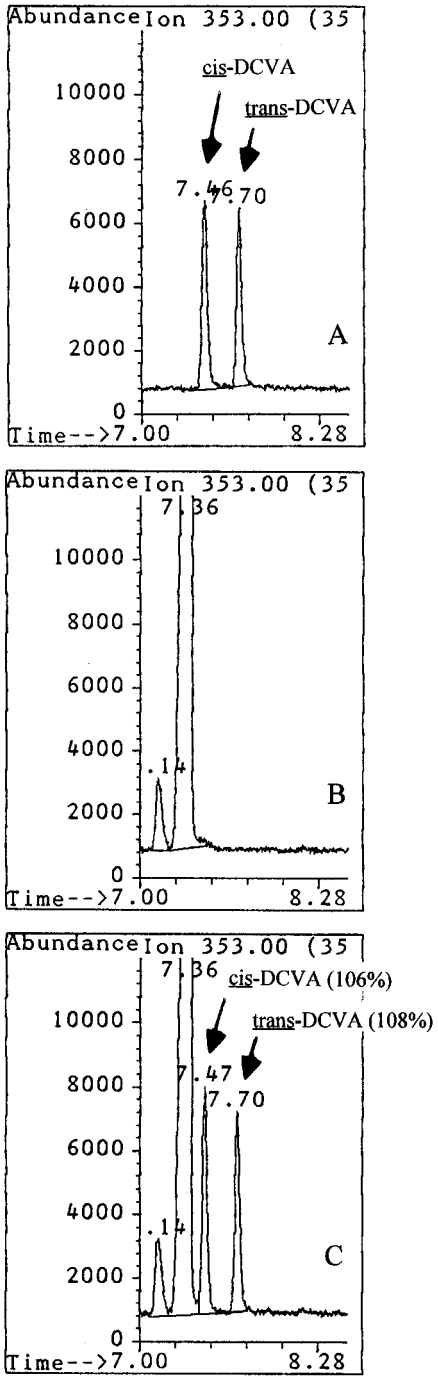

Figure 8. Selective ion chromatograms (*m/z* 353) of *cis,trans*-DCVA in cow fat: (A) standard solution 0.5 ng; (B) control fat; (C) control fat fortified at 50 ppb.

must be performed under vigorous mixing condition to enhance the chemical reaction and subsequent extraction (Schötz et al., 1995).

Conditioning the GC detection system with matrix samples before the actual run of the set is recommended to establish a stable analytical condition for the analytes. Programming the GC oven to a higher final temperature after each run is also recommended to bake out any possible late eluting fractions.

In general, the analytical procedures require approximately 8 and 16 h for cypermethrin parent and acid metabolites assays, respectively. During that time, one person can complete a set of 10 bovine matrix samples from initial weighing of samples to gas chromatographic measurement.

CONCLUSION

These methods describing the analysis of cypermethrin and its three acid metabolites in bovine milk, cream, and tissues utilize solid phase extraction techniques and are not labor- and solvent-intensive.

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