

# Determination of Melengestrol Acetate in Supercritical Fluid–Solid Phase Extracts of Bovine Fat Tissue by HPLC-UV and GC–MS

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## Abstract

A method is developed for the determination of melengestrol acetate in bovine fat tissue at or less than the established tolerance level of 25 ppb. The procedure uses a combination of supercritical fluid extraction (SFE) and solid-phase extraction (SPE) techniques to produce an extract suitable for analysis with either high-performance liquid chromatography with ultraviolet detection or gas chromatography–mass spectrometry. Overall recovery of the analyte from bovine fat tissue is 99.4% with a coefficient of variation of 4.14%. The SFE–SPE procedure uses a total of 12 mL of organic solvent per fat tissue sample versus more than 1.7 L consumed in current extraction procedures.

## Introduction

Melengestrol acetate (17 $\alpha$ -hydroxy-6-methyl-16-methylene pregna-4,6,-diene-3,20-dione acetate) (MGA) is a Food and Drug Administration (FDA) approved synthetic steroidal hormone added to the feed of heifers to suppress estrus, thereby leading to improved feed efficiency and rate of weight gain. Recent action by the FDA has revised the tolerance levels for residues of MGA in edible tissues from 0 to 25 ppb based on evidence that residues at or less than this concentration do not elicit a hormonal response (1).

Several extraction procedures are available for detecting MGA at or less than the tolerance level. Chichila and co-workers (2) devised an automated coupled-column normal-phase high-performance liquid chromatographic (HPLC) procedure that is capable of detecting less than 5 ppb MGA in bovine liver and muscle tissue. However, the procedure is not adaptable to fat tissue, which is the target tissue for residues of the drug in beef animals (C. Henry, Food Safety and Inspection Service [FSIS] Midwestern Laboratory, St. Louis, MO, personal communication, 1995). Other procedures, capable of detecting MGA in both fat and nonfat tissues at less than 25 ppb, consume voluminous amounts of organic solvents in their extraction procedures (3–5). In light of the Environmental Protection Agency

(EPA) regulations regarding solvent consumption (6), new approaches for isolating MGA from fat tissues are needed.

Supercritical fluid extraction (SFE) has been used as an analytical tool to extract steroids from biological matrices including androsterone from boar fat (7) and nortestosterone, testosterone, and methyl testosterone from bovine liver (8) and urine (A.A.M. Stolker, L.A. van Ginkel, R.W. Stephany, et al. Multi residue supercritical fluid extraction of nortestosterone, testosterone, and methyl testosterone at low ppb levels from fortified bovine urine. Submitted for publication.). In the first study, in which off-line collection of the analyte was used, 0.05  $\mu$ g androsterone per gram of fat was detected by gas chromatography–mass spectrometry (GC–MS). However, although the SFE procedure extracted 77% of the steroid, it also extracted 10% of the fat, which increased the potential for regular fouling of the GC column or the MS ion source or both. In the later procedures, an in-line adsorbent was used to trap the extracted analyte from the supercritical carbon dioxide (SCCO<sub>2</sub>) while allowing nonadsorbable extractables, such as fat, to pass to an off-line trap. That approach appeared ideal for the extraction of MGA from bovine fat, and a method that involved both SFE and solid-phase extraction (SPE) was developed.

## Experimental

### Materials

Hydromatrix (Celite 566) (part no. 0019-8003) was obtained from Varian Sample Preparation Products (Harbor City, CA). Aluminum oxide (Al<sub>2</sub>O<sub>3</sub>)-activated, neutral, Brockmann I (catalog no. 19,997.4) was purchased from Aldrich Chemical (Milwaukee, WI). Methanol (MeOH), acetone, ethyl acetate (EtOAc), isooctane, and acetonitrile (CH<sub>3</sub>CN), which are Burdick and Jackson high-purity solvents, were products of Baxter Health Care (Muskegon, MI). Supercritical fluid chromatography (SFC) grade CO<sub>2</sub> was purchased from Scott Specialty Gas (Plumsteadville, PA). Polypropylene wool (Aldrich Chemical) was extracted with SCCO<sub>2</sub> for 20 min at 10,000 psi, 50°C, and a flow rate of 3 L/min (expanded gas). Polyethylene frits (20  $\mu$ m) (part no. 7956) were obtained from Applied Separations (Allentown,

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PA). Heptafluorobutyric acid anhydride (HFBA) (catalog no. 63164) was a product of Pierce (Rockford, IL). MGA was a control reference standard of the Upjohn Company (Kalamazoo, MI). Standard solutions containing 34, 17, and 8.5 ng MGA per microliter of MeOH were prepared and used to fortify tissue samples.

### Procedure

For sample preparation, 1.0 g of a rectangular slice of control perirenal fat tissue was fortified with 3  $\mu$ L of the MGA standard by depositing the solution on the surface of the tissue. The tissue was held at room temperature for 10 min to allow for permeation into the tissue and evaporation of the solvent. The fortified tissue was then added to 4.0 g hydromatrix, which was contained in a 50-mL beaker, and 0.75 mL distilled H<sub>2</sub>O was added dropwise. The tissue was ground thoroughly into the "wetted" hydromatrix with a metal spatula.

### Packing of the SFE vessel

The extraction vessel (Applied Separations) (15 cm  $\times$  14-mm i.d.), which had a 24-mL capacity, was packed tightly with a tamping rod in the following sequence relative to the exit of the vessel: a plug of polypropylene wool; two polyethylene frits; 2 g Al<sub>2</sub>O<sub>3</sub> (analyte trap); a polyethylene frit; tissue-hydromatrix mixture; a polyethylene frit; 3 g Al<sub>2</sub>O<sub>3</sub> (presample trap); and a polyethylene frit.

### SFE

Two extractions were carried out simultaneously with use of the Spe-ed SFE Model 680 bar (Applied Separations) extraction system. The air-driven Haskel pump (Haskel; Burbank, CA) was equipped with a laboratory-assembled chiller cooled by a refrigerated circulating bath set at  $-15^{\circ}\text{C}$ . The use of this device obviated helium-pressurized CO<sub>2</sub>, which is required for standard operation with the noncooled pump. The extracted fat was collected off-line in a 9-mL vial fitted with a septum. The vial was vented to a Fline SFE-51 flow meter/gas totalizer purchased from Scott Specialty Gas. The extractions were performed at  $50^{\circ}\text{C}$  and 10,000 psi ( $d = 1.021$ ) and, after a 5-min static period, at 2 L of expanded gas per minute for 20 min.

### Procedure after SFE

After decompression of the extraction vessel, the in-line Al<sub>2</sub>O<sub>3</sub> analyte trap was removed from the vessel by pouring the contents into an empty 6-mL SPE column that contained a frit. The

**Table I. SFE Recovery of Melengestrol Acetate from Fortified Perirenal Fat Tissue\***

Fortification level (ppb*)	No. of determinations	Mean (% $\pm$ SD)
100	5	101.1 $\pm$ 3.8
50	5	99.0 $\pm$ 4.0
25	6	98.4 $\pm$ 4.5

\* SFE conditions used were the following: temperature,  $50^{\circ}\text{C}$ ; pressure, 10,000 psi (density, 1.021); flow rate, 5 min (static) then 2 L/min (expanded gas) for 20 min.

Al<sub>2</sub>O<sub>3</sub> was compacted by tapping the sides and top of the SPE column with a spatula and layered with 0.25 cm of sand. The Al<sub>2</sub>O<sub>3</sub> column was eluted with MeOH-H<sub>2</sub>O (6.5:3.5, v/v), and the first 2 mL of eluant were passed directly over a SPE column (Applied Separations) that contained 1.0 g 18% C<sub>18</sub> packing. The C<sub>18</sub> column was washed with two 1-mL portions of MeOH-H<sub>2</sub>O (6.5:3.5, v/v) and two 2-mL portions of deionized H<sub>2</sub>O. The C<sub>18</sub> column was dried by vacuum and eluted with MeOH. The MeOH eluant (2 mL) was evaporated to dryness in a 5-mL screw-capped specimen vial. To the residue was added 250  $\mu$ L of the HPLC mobile phase, the contents were vortex mixed for 30 s, and 100  $\mu$ L was injected onto the HPLC column.

### HPLC analysis

Analyses were performed with an Isco LC-5000 syringe pump (Isco; Lincoln, NE) and a Rheodyne Model 7125 injector (Rheodyne; Berkeley, CA) connected to a Supelcosil LC-18 column (15 cm  $\times$  4.6-mm i.d., 5- $\mu$ m particle size) (Supelco; Bellefonte, PA). MGA was detected at 291 nm with an Applied Biosystems Model 1000S diode array detector (Foster City, CA). The mobile phase was CH<sub>3</sub>CN-H<sub>2</sub>O (5.5:4.5, v/v) at a flow rate of 1.0 mL/min. Chromatograms were recorded on a Hewlett-Packard (HP) Model 3396A integrator (Avondale, PA). Quantitation of MGA was accomplished by comparison of peak heights or areas (or both) with external standards.

### GC-MS analysis

The HFBA derivative of MGA was prepared according to the procedure of Stolker and co-workers (A.A.M. Stolker, et al. Submitted for publication.). The 2-mL MeOH eluant of the C<sub>18</sub> SPE column (see Procedure after SFE) was evaporated to dryness under a stream of nitrogen in a 2-mL Teflon-lined screw-capped vial at  $50^{\circ}\text{C}$ . Eighty microliters of acetone and 20  $\mu$ L HFBA were added to the residue, and the mixture was vortex mixed for 1 min and heated at  $60^{\circ}\text{C}$  for 1 h. After cooling, the contents of the vial, along with 100  $\mu$ L acetone to rinse the vial,

**Table II. A Comparison of Organic Solvent Consumption and Recoveries of Melengestrol Acetate from Fortified Fat Tissue (20–25 ppb) by Several Procedures**

Method*	Recoveries (% $\pm$ SD)	Solvent amount (L)	Solvent
FSIS	96.7	> 1.9	Hexane, acetone, acetonitrile
FDA	74.4 $\pm$ 8.0	> 2.2	Hexane, methanol, ethyl alcohol, methylene chloride, chloroform, ether, benzene
AOAC	93.0 $\pm$ 7.5	> 1.7	Hexane, acetone, benzene, acetonitrile
SFE	98.4 $\pm$ 4.5	0.012	Methanol

Abbreviations: FSIS, Food Safety and Inspection Service, United States Department of Agriculture; FDA, Food and Drug Administration; AOAC, Association of Official Analytical Chemists.

were transferred to a 0.3-mL micro Supelco reaction vessel (catalog no. 3-3291) and evaporated to dryness with a stream of nitrogen at room temperature. The residue was taken up in 10  $\mu$ L EtOAc–isooctane (5:95, v/v), and the vessel was capped with a hole cap fitted with a septum. The vessel contents were vortex mixed thoroughly and centrifuged, and 3  $\mu$ L was injected into the GC–MS system.

The GC–MS analyses were performed according to the procedure of Chichila and co-workers (2) using an HP Model 5890 GC equipped with an HP Model 7673 GC–SFC injector and an HP GC autosampler interfaced to the HP Model 5970 mass selective detector. The capillary column was a cross-linked methylsilicone gum HP Ultra-1 (12 m  $\times$  0.22-mm i.d., 0.3- $\mu$ m film thickness) (HP no. 109091A-101). The injector temperature was maintained at 260°C, and the interface temperature was 300°C. The oven temperature was set at 40°C, programmed at 30°C/min to 150°C, and then at 6°C/min to 300°C. The final temperature was held for 10 min. The presence of the 3-heptafluorobutyl enol ether of MGA (MGA–HFB) was confirmed by selected ion current monitoring for the molecular ion ( $m/z$  592) and five characteristic fragments ( $m/z$  533, 517, 489, 381, and 367) and their total absence in control fat tissue extracts.

#### Incurred tissues

Samples of bovine perirenal and visceral fat tissues containing varying levels of MGA were obtained from the FSIS. The samples had been extruded through a meat grinder for uniformity and analyzed for MGA concentrations by FSIS personnel using the solvent extraction procedure used by the regulatory agency (3).

#### Results and Discussion

The positive effect of in-line collection of analytes, versus off-line collection, on the background levels of HPLC chromatograms of supercritical fluid extracts from urine and liver has been demonstrated (8–10). In this study, in-line collection of analytes was necessitated by the nature of the sample matrix. SFE conditions determined to be ideal for the extraction of MGA also resulted in the extraction of 850–900 mg of fat per 1.0 g of tissue. Hence, without adsorptive extraction of MGA from the coextracted fat in the supercritical CO<sub>2</sub>, little would be gained by the SFE process.

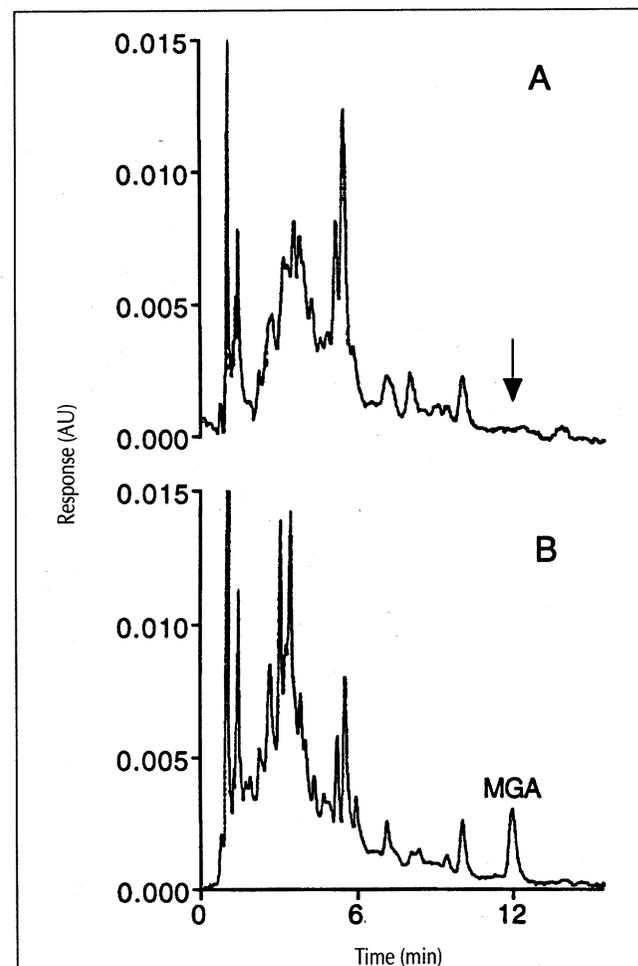
**Table III. Concentration of Incurred Residues of Melengestrol Acetate in Bovine Fat Tissue as Determined by Organic Solvent Extraction and SFE Procedures\***

Fat sample	Animal no.	Concentration (ppb $\pm$ SD)	
		Solvent (n = 3)	SFE (n = 5)
Visceral	6004	20	24.8 $\pm$ 1.1
Perirenal	6028	57	53.9 $\pm$ 1.1
Visceral	6036	85	89.4 $\pm$ 4.2
Perirenal	6036	108	97.7 $\pm$ 4.6

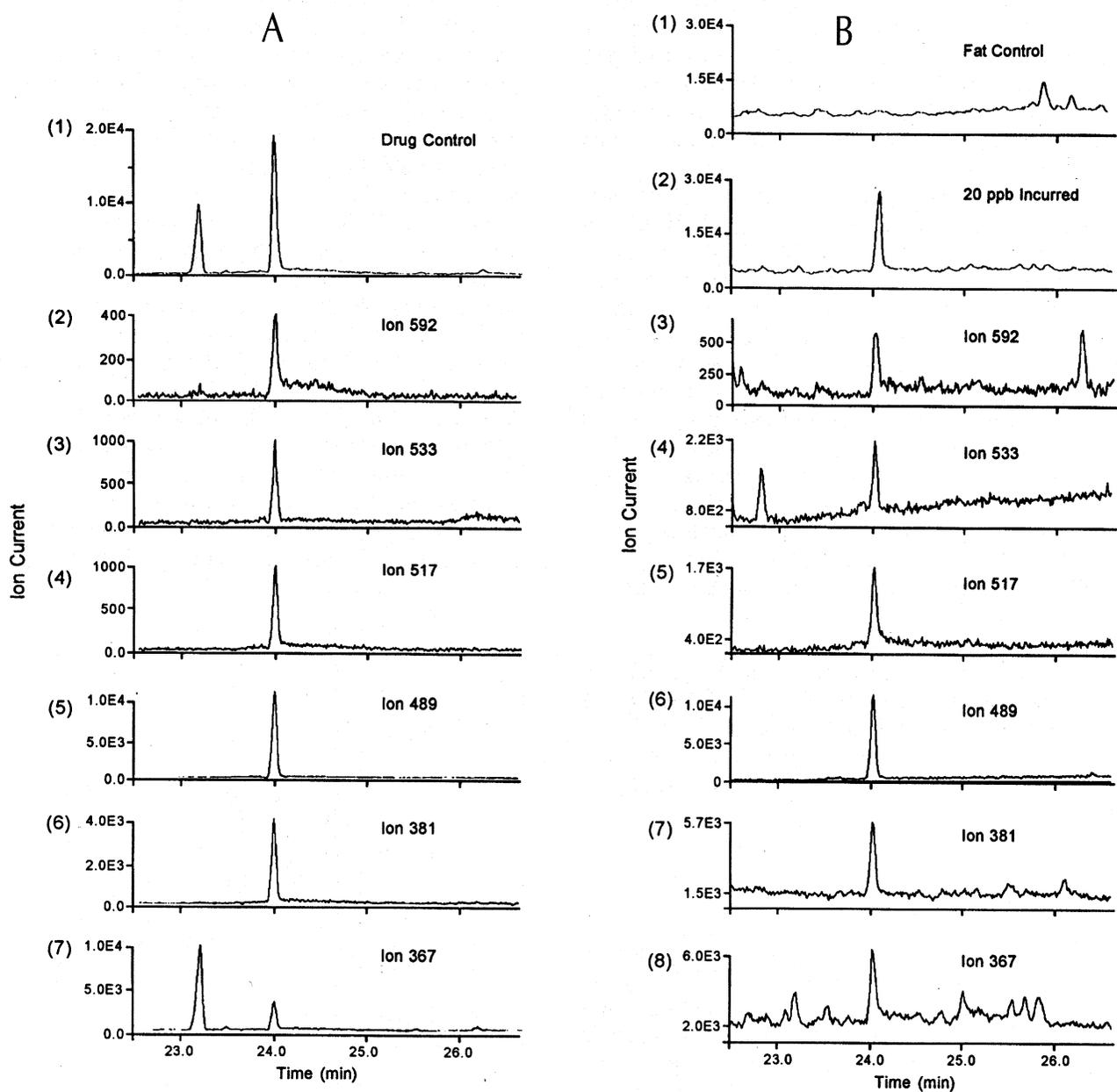
\* Food Safety Inspection Service method; see Reference 3.

The addition of H<sub>2</sub>O to the fatty tissue–hydromatrix mixture, although seemingly contradictory, improved the procedure in several ways. First, the added H<sub>2</sub>O facilitated the grinding of the fat tissue into the hydromatrix. Secondly, under the same SFE conditions, the added H<sub>2</sub>O increased the recovery of MGA by 10% and the amount of fat recovered off-line by 15%. Finally, the HPLC background levels improved in the presence of added H<sub>2</sub>O.

At present, a limitation of SFE as an analytical tool in drug residue analyses is the size of the sample feasibly extracted. Although 25-g tissue samples are not uncommon in solvent extraction procedures (see, for example, the FSIS and Association of Official Analytical Chemists [AOAC] methods of analyses for MGA [3,5]), SFE of tissues is performed on relatively small samples (1.0 g or less). To attain our goal for detecting MGA in fat tissue at or less than the tolerance level by HPLC–UV analysis, we found that it was necessary to concentrate the in-line Al<sub>2</sub>O<sub>3</sub> eluant eightfold, in contrast to previous studies in which the eluant was injected directly into the HPLC system (9,10 (A.M. Stolker, et al. Submitted for publication.)). This was accomplished by using a C<sub>18</sub> SPE column. Preliminary HPLC



**Figure 1.** High-performance liquid chromatographic chromatograms of supercritical carbon dioxide extracts of (A) a control sample of perirenal fat tissue (A) and visceral fat tissue (animal no. 6004) (B) containing incurred residues of melengestrol acetate (MGA) (reported concentration, 20 ppb; determined concentration, 24.8 ppb).



**Figure 2.** (A) GC-MS profiles of total (No. 1) and individual (No. 2–7) selected ion currents of a melengestrol acetate–heptafluorobutyric acid (MGA–HFB) standard (equivalent to 25 ppb) ( $t_R$ , 24 min). (B) Total selected ion current GC-MS profiles of control fat (No. 1) and MGA incurred fat tissue (No. 2) extracts and the individual selected ion current profiles (No. 3–8) of the incurred tissue extract (visceral fat; animal no. 6004).

studies on the concentrated extract revealed, however, unusually high background levels. The polypropylene wool used initially to layer the extraction vessel was found to be a major source of the contamination. The untreated wool was replaced with polyethylene discs or polypropylene wool that had been previously subjected to SFE. Although these measures improved the background, a contaminant chromatographing with MGA persisted. The contaminant was traced to either the  $\text{CO}_2$  or the air pump (or both) used in the instrument and was eliminated from the extract by placing 3.0 g of neutral  $\text{Al}_2\text{O}_3$  before the sample in the extraction vessel.

The fortification of fat tissue presents a problem not encountered with liver or muscle tissues in that solutions of an-

alytes readily permeate these tissues. The fortifying solutions in this study appeared to persist on the surface of the fat tissue until evaporation had occurred. The extent of actual penetration into the interior of the sample to produce a more uniform fortified sample is unknown. Table I summarizes the recovery data from perirenal fat fortified at 25, 50, and 100 ppb; the overall recovery of MGA at all fortification levels was 99.4% with a coefficient of variation of 4.14%. These recoveries compare favorably (Table II) with those attained from fortified tissues at the 20–25-ppb level by the FSIS (3), FDA (4), and AOAC (5) solvent extraction techniques. Table II further compares the organic solvent consumption and lists the solvents used in the four procedures. The benefits derived from the SFE pro-

cedure with regard to organic solvent consumption, as well as deposition of waste solvents, are readily apparent.

Table III compares the concentration of incurred residues of MGA in bovine visceral and perirenal fat samples as determined by the FSIS solvent extraction method and the SFE procedure reported here. In general, the results are comparable, and the differences that do exist, in these limited studies, appear to be dependent on the tissue (i.e., the SFE concentrations are slightly higher in visceral fat and lower in perirenal fat). Figure 1 shows typical HPLC chromatograms of SCCO<sub>2</sub> extracts of a control sample of perirenal fat (Figure 1A) and an incurred sample of visceral fat (Figure 1B).

The presence of MGA in the incurred samples was further determined by GC-MS of the HFB enol ester derivative. Figure 2A shows the total selected ion current chromatogram and selected ion current profiles of an MGA-HFB standard. Figure 2B shows typical total selected ion current chromatograms of a control and incurred fat extracts and selected ion current chromatograms of the later extract. The selected ion current profiles of the control extract were void of peaks for the molecular ion and the five characteristic fragments at the GC retention time of MGA-HFB.

## Conclusions

The results of this study demonstrate that SFE in conjunction with proper sample preparation and in-line trapping on adsorptive Al<sub>2</sub>O<sub>3</sub> is an alternative to solvent extraction of MGA residues from bovine fat tissue. Postextraction concentration of the extract with the use of a reversed-phase C<sub>18</sub> SPE column allows for HPLC-UV and GC-MS detection of residues that are at a level less than 25 ppb, which is the tolerance level for this analyte in edible tissues.

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