An LC/MS/MS Method for Improved Quantitation of the Bound Residues in the Tissues of Animals Orally Dosed with [^{14}C]Benomyl

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Livers of goats orally dosed with [phenyl(U)-^{14}C]benomyl contained radioactive residues which were not extractable using conventional, solvent-based extraction methods. We report a new residue method capable of enhanced extraction of benomyl-derived residues with selective and sensitive quantitation capability for methyl 4-hydroxybenzimidazol-2-ylcarbamate (4-HBC), methyl 5-hydroxybenzimidazol-2-ylcarbamate (5-HBC), and methyl benzimidazol-2-ylcarbamate (MBC). This method involves rigorous Raney-nickel reduction of hypothesized thioether bonds between benomyl residues and polar cellular components. Following acidic dehydration (desulfurization), the polar benomyl-derived residues are extracted into ethyl acetate and analyzed by LC/MS/MS. We have shown this method to be superior to alternative extraction approaches. When applied to goat liver tissue containing [phenyl(U)-^{14}C]benomyl-bound residues, the extraction efficiency of total radioactive residues was approximately 30%, and the major benomyl-derived residue was 5-HBC (91−95% of extractable residue) with minor levels of carbendazim (MBC) (5−9%). HPLC/LSC data were consistent with the LC/MS/MS data. The overall method satisfies U.S. regulatory requirements in extraction efficiency, selectivity in detection, and limits of quantitation for benomyl-bound residues.

Keywords: *Benomyl; benzimidazole; carbendazim; desulfurization; fungicide; mass spectrometry; metabolite; pesticide; Raney-nickel*

INTRODUCTION

Benomyl is the active ingredient in Benlate fungicide registered for the control of many important plant fungal diseases. A comprehensive review of benomyl metabolism was published recently (Anderson, 1999). Previous animal metabolism and residue work using benomyl had characterized carbendazim (MBC), methyl 5-hydroxybenzimidazol-2-ylcarbamate (5-HBC), and methyl 4-hydroxybenzimidazol-2-ylcarbamate (4-HBC) (Figure 1) as the major animal metabolites of benomyl (Kirkland, 1973; Monson, 1991). However, the existing residue method (Kirkland, 1973) for the above metabolites does not adequately quantitate liver residues, as less than 1% of these residues are extractable into organic solvents. Homogenization of the liver samples in water followed by centrifugation led to detection of a negligible amount of radioactivity in the supernatant, suggesting that the radioactivity due to liver bound residues was not due to carbendazim amino acid or small peptide conjugates (unpublished data). Hypothetically, a nucleophilic attack of an intracellular protein sulfhydryl group on the epoxide moiety of a benomyl intermediate residue generates a covalently linked bound benomyl residue (Figure 2). Literature on other compounds indicates that, in vivo, after hydrolysis of all amino acids in such complexes by soluble deacylases (i.e., peptidases), cysteine conjugate β-lyase can cleave the conjugate thioether bond in the β-position (Tateishi et al., 1978). This results in a thiol-containing metabolite, which can be excreted as such or conduct nucleophilic attacks on other biomolecules (Bakke, 1990).

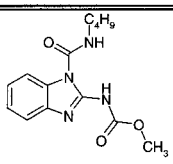
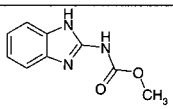
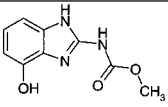
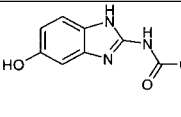
Common Name	Structure	IUPAC Name, Molecular Formula, and Molecular Weight
Benomyl		*IUPAC Name*: Methyl 1-(butylcarbamoyl)benzimidazol-2-ylcarbamate, *Molecular Composition*: $C_{14}H_{18}N_4O_3$, *Molecular Weight*: 290.32
Carbendazim (MBC)		*IUPAC Name*: Methyl benzimidazol-2-ylcarbamate, *Molecular Composition*: $C_9H_9N_3O_2$, *Molecular Weight*: 191.19
4-HBC		*IUPAC Name*: Methyl 4-hydroxybenzimidazol-2-ylcarbamate, *Molecular Composition*: $C_9H_9N_3O_3$, *Molecular Weight*: 207.19
5-HBC		*IUPAC Name*: Methyl 5-hydroxybenzimidazol-2-ylcarbamate, *Molecular Composition*: $C_9H_9N_3O_3$, *Molecular Weight*: 207.19

Figure 1. Chemical names and structures of analytes.

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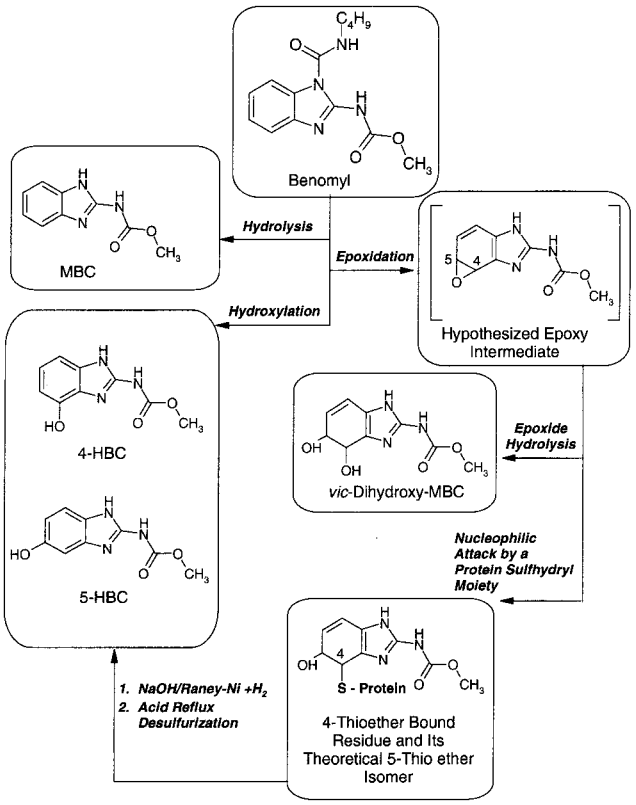

Figure 2. Proposed scheme for animal liver metabolism of benomyl into bound residues and their chemical liberation.

Alternatively, the thiol moieties of such metabolites may be methylated to thiomethyl groups by *S*-adenosylmethionine or form water-soluble glucuronide conjugates which can be excreted as deactivated sulfurs (Bakke, 1990).

Analogous reactions can be catalyzed using chemical reagents (Figure 2). In the presence of hydrogen, Raney-nickel can catalyze a hydrogenation reaction to yield thiols from thioethers (Jerina et al., 1970). These thiols may then be methylated using methyl iodide (Slaughter and Hanzlik, 1991) or dehydrated using acid desulfurization (Kirkland, 1973; Monson, 1991). Alternatively, a combination of base/methyl iodide can be used to yield the thiomethyl metabolites (Slaughter and Hanzlik, 1991). In both desulfurization methods described above, a basic solution should be used to hydrolyze the protein moiety down to a cysteine residue (Kirkland, 1973; Slaughter and Hanzlik, 1991) before the cleavage of thioether bonds. Another reason for using NaOH is that Raney-nickel is more stable in basic solutions.

Using radioactivity to trace benomyl metabolites, it was demonstrated that a Raney-nickel hydrogenation/acid-desulfurization step would render a considerable quantity of the previously bound residues organically soluble (Monson, 1991). This Raney-nickel method was modified and improved to provide us with a sensitive and reproducible residue method for routine quantitation of benomyl residues in animal tissues using LC/MS/MS. This method is an improvement on the existing method (Kirkland, 1973).

EXPERIMENTAL PROCEDURES

Reagents and Standards. The water used was obtained from the Milli-Q type I deionized, distilled water system (Millipore Corp., Bedford, MA). The HPLC grade acetonitrile, ethyl acetate, hexane, and sodium hydroxide (50% solution) were obtained from EM Science (Gibbstown, NJ). Sodium hydroxide pellet was purchased from VWR. Dimethylformamide, hydrochloric acid (HCl; 36−38% concentrated reagent), acetic acid (glacial, HPLC reagent grade), and ammonium acetate were obtained from J. T. Baker (Phillipsburg, NJ). Nickel−aluminum (Raney type), 50:50 w/w, was obtained from Alfa (Johnson Mathey, Wardhill, MA). Phosphoric acid (85%, HPLC grade) was purchased from Fisher. Hydrogen gas was obtained from MG Industries (Malvern, PA).

All analytical standards were internally synthesized at DuPont Agricultural Products and included MBC (98.8% pure), 4-HBC (99.9% pure), and 5-HBC (98.7% pure). For mass spectral use, stock standard solutions of 5-HBC and 4-HBC were prepared in acetonitrile/water/dimethylformamide (55:40:5, v/v/v), while that of MBC was prepared in distilled water. All stock solutions used for HPLC analysis were prepared in 6 N HCL. These stock solutions were stored under refrigeration (4 ± 2 °C) and replaced after 6 months or sooner if chromatography indicated significant degradation.

Instrumentation. *HPLC Analysis.* High-performance liquid chromatography was performed on a Hewlett-Packard series 1090 liquid chromatograph (Agilent Technologies, Little Falls, DE) equipped with a Raytest RAMONA-92 radiochemical detector (Raytest USA) and a Foxy fraction collector (ISCO, Lincoln, NE). The HPLC column used was a Hamilton semi-preparative PRP-1 HPLC column (30.5 cm × 7.0 mm) (Hamilton Co., Reno, NV) which was maintained at 40 °C while in use to minimize temperature variations and optimize peak separation. The flow rate was 3.0 mL/min of 0.25 M acetic acid for 20 min followed by a linear gradient to 50% 0.25 M acetic acid/acetonitrile over a period of 30 min. This was then followed by a linear gradient to 100% acetonitrile over a period of 10 min, which was maintained for a period of 5 min. A fraction collector was used to collect 1 min fractions which were then counted on a liquid scintillation counter to reconstruct a more precise radiochromatogram using RS1. For liquid scintillation, Atomlight Scintillation Cocktail solution (Packard Instrument Co., Meriden, CT), liquid scintillation counters (Packard Tricarb model 460, Packard Instrument Co.), and an RS1 integrated graphics and data analysis software package (Bolt, Beranek and Newman, Inc.) that operated on a VAX 11/785 computer (Digital Equipment Corp., Maynard, MA) were used.

LC/MS/MS. The LC/MS/MS instrumentation included a liquid chromatograph (Waters Corp., Milford, MA) Waters 616 HPLC system, consisting of model 616 and model 600S controllers, a Waters temperature control module, a Waters column oven heater module, and a Waters model 717 plus autosampler. The postcolumn addition included a Waters 590 programmable solvent delivery system, with an aqueous, in-line filter/degasser cartridge (VWR, Bridgeport, NJ) and an SSI Lo-Pulse pulse damper (Rainin Instrument Co., Inc., Emeryville, CA). The HPLC columns for LC/MS (Mac-Mod Analytical, Inc., Chadds Ford, PA) were a Zorbax SB-Phenyl guard cartridge, followed by a Zorbax SB-Phenyl analytical column (4.6 mm × 15 cm), followed by a Zorbax SB-Phenyl analytical column (4.6 mm × 25 cm). A Finnigan model TSQ7000 mass spectrometer (Finnigan MAT Corp., San Jose, CA), a Finnigan MAT thermospray LC interface module, model TSP-2, and digital DEC3000 model 300LX computer ICIS software (version 8.2.1) were used for mass spectrometry. For HPLC analysis, the column was maintained at 40 °C. The flow rate was 2 mL/min of 1:9 acetonitrile/0.1 M aqueous acetic acid for 7 min followed by a gradient leading to 9:1 acetonitrile/0.1 M aqueous acetic acid at the end of 35 min. There was a 2 min period of equilibrium back to the starting conditions.

Animal Treatment and Liver Preparation. Goat liver samples used in our studies were prepared by Battelle Memorial Institute (Columbus, OH). A single female goat was orally dosed with [phenyl(U)-^{14}C]benomyl once daily for five consecutive days. The actual dietary burden was 86.9 mg of benomyl/kg of feed based on an average daily food consumption of 1.45 kg of feed/day. After sacrifice, the liver was excised, homogenized, and divided into small subsamples which were shipped

frozen in 250 mL Nalgene centrifuge bottles. The concentration of total radioactive residue in the liver was 8.75 mg of benomyl/kg of feed, 23 h after the last dose (0.9% of the total oral dose). These liver samples were stored frozen below −15 °C until workup. During sampling, liver samples were kept partially or completely frozen depending on practical limitations of the equipment used at this point. Samples were not allowed to thaw completely, as the integrity of the residues might have been compromised. Beef (control) liver was purchased at a local supermarket.

Liver Digestion and Raney-Nickel Hydrogenation Workup. Approximately 5 g of homogenized liver was placed in a 500 mL round-bottom flask. After addition of boiling chips and a magnetic stir bar, 5 mL of deionized water was added and the solution was stirred to disperse the sample. Next, 30 mL of a 10% aqueous NaOH solution was added to the mixture slowly as it continued to stir. Approximately 1 g of nickel−aluminum powder (50:50, Raney type) was added to the stirring mixture in a slow and careful manner to prevent excessive foaming. (*Caution: Nickel−aluminum powder (50:50, Raney type) can undergo spontaneous combustion upon extensive exposure to air. Therefore, the weighing process for this material should be delayed as long as possible until the apparatus is ready to receive it.)* This was followed by an immediate capping of the apparatus with a rubber septum previously equipped with a balloon containing hydrogen gas to maintain a positive hydrogen pressure. (*Caution: The vessel should not be capped with the rubber septum unless the septum is equipped with the balloon via a syringe needle to relieve pressure buildup from evolution of hydrogen gas.)* The solution mixture was warmed to 60 ± 5 °C for 2 h in a water bath and then allowed to stir for 16 h. Next, the rubber septum was removed and the residual hydrogen gas in the balloon was emptied under a hood. Approximately 15 mL of a 85% phosphoric acid solution was added, and the pH was adjusted to 1 using phosphoric acid. This solution was refluxed for 1 h while being stirred vigorously. The pH was readjusted to 1 after 20 min, if there was a need to do so. At the end, the contents of the round-bottom flask were cooled and transferred to a centrifuge bottle.

Fortification. Because beef liver is considered to be the primary target for this method by the EPA, that tissue was used in our fortification studies. Four 15 g samples of the control beef liver were processed through the Raney-nickel step. Before the addition of phosphoric acid, the above solutions were combined and divided into 12 equal aliquots, each representing a 5 g liver sample. These 12 aliquots were then spiked to levels of 0.05, 0.60, 2.0, and 24 μg/mL (b.e.) of MBC and 5-HBC. The remaining aliquots were used as control beef liver samples.

Extraction and HPLC Analysis. The acidic digest was first defatted using 100 mL of hexane by shaking vigorously and centrifuging at 10000 rpm for 5 min. The hexane layer did not contain any radioactivity and was discarded. The pH of the aqueous phase was adjusted to 7.0−7.5 using 50% aqueous NaOH. This solution was extracted using 50 mL of ethyl acetate and centrifuged at 10000 rpm for 5 min. The ethyl acetate was separated from the aqueous phase in a separatory funnel, and the pellet was discarded as nickel-containing hazardous waste. This solvent partitioning was repeated for a total of three times. Ethyl acetate extracts were added to the separatory funnel each time. The aqueous layer was discarded as radioactive waste after LSC. The combined ethyl acetate extract was washed using 2 × 10 mL of deionized H_2O, reduced to dryness under vacuum, reconstituted in ethyl acetate to a final volume of 10 mL, and stored at −20 °C until further analysis.

HPLC analysis was performed after a 2 mL aliquot of the ethyl acetate extract was reduced to dryness under a stream of nitrogen. The dried residue was reconstituted in ≤0.4 mL of 1:1 acetonitrile/6 N HCl with the aid of a vortex mixer. After the solid particles were allowed to settle, the liquid was removed by pipet, filtered using Rainin microfilterfuge tubes (0.2 μm nylon filter; Rainin Instrument Co., Woburn, MA), and analyzed by HPLC.

For LC/MS analysis, 0.5 mL of the ethyl acetate solution was taken to dryness under a stream of nitrogen. The residue was redissolved in 2.5 mL of 1:9 acetonitrile/0.1 M acetic acid (sonication and vortex mixing). An approximately 1 mL aliquot of this sample was filtered into an autosampler vial using a 2.5 mL plastic syringe equipped with a 0.45 μm filter (Millex-HV13) prior to analysis. An aqueous 0.5 M ammonium acetate (NH_4Ac) solution was used as the postcolumn addition. All LC/MS solutions were filtered (≤0.45 μm).

Chromatographic Standard Preparation and Stability. Calibration solutions containing all analytes at concentrations over the range 0.002−0.1 μg/mL were used. A minimum of four standard concentrations were prepared within this range, selected to bracket expected residue levels. The typical linear range for calibration standards was 0.002−0.1 μg/mL (all analytes present at the same concentration). This is equivalent to residue levels in the original, 5 g sample of 0.028−1.4 ppm b.e. for 5-HBC (or 4-HBC) and 0.030−1.5 ppm b.e. for MBC. Standards typically yielded a linear response ($r^2 > 0.99$) over a range somewhat greater than 1 order of magnitude (but less than 2 orders of magnitude), such as the range 0.002−0.1 μg/mL.

Calibration solutions were stored in a refrigerator (4 ± 2 °C) and replaced weekly (≤7 days). Each set of samples analyzed included at least one control (an untreated and unfortified sample) which was similar to "investigation" samples. Each "official" sample analysis set began by injecting standards at three (or more) concentration levels. Only if (1) the relative standard deviation (RSD) for these standards was less than 20%, (2) the S/N was at least 10, and (3) the standard response (for 5-HBC) was a minimum of 25000 (area counts/ng)/mL for a 250 μL injection was the method considered to be operating correctly. The standards were injected after every four samples, and the last injection was always a standard. The range of standards always bracketed the residue levels determined in the samples. If a sample was too concentrated, it was diluted with 1:9 acetonitrile/0.1 M acetic acid (to be in the standard range) and reanalyzed. We considered this method valid if the RSD for the average response factor relevant to a particular sample was ≤20%.

RESULTS AND DISCUSSION

After the Raney-nickel treatment, the extraction efficiency of total radioactive residue (TRR) in the liver into the ethyl acetate fraction was about 31%. We were able to confirm the superiority of the Raney-nickel method (Monson, 1991) to the original Kirkland procedure (Kirkland, 1973) in which only about 1% of the total radioactive residue partitioned into the ethyl acetate phase.

In this procedure, after analysis of 11 liver samples, it was found that the approximate distribution of the radioactivity in the ethyl acetate, aqueous, and pellet fractions was 31.0 ± 4.3%, 39.0 ± 6.0%, and 25.4 ± 6.4%, respectively. Subsequent Raney-nickel workup of the pellet releases more radioactivity. The nature of the liberated radioactivity was consistent with the material released after the first Raney-nickel step (unpublished data). As can be observed, a total of 70% of the TRR in the goat liver was ultimately rendered soluble in water and/or ethyl acetate. A large volume, and hence a low analyte concentration, as well as a high salt content in the aqueous phase hindered further analysis of this fraction. HPLC analysis of the ethyl acetate phase using a radiochemical detector (Figure 3) indicated that 5-HBC was the primary benomyl-derived residue. The relative ratio of 5-HBC to MBC was 9:1 (Table 1). All ethyl acetate extracts were analyzed by HPLC/LSC within 57 days after extraction. HPLC/LSC analysis of the ethyl acetate extracts stored below −10 °C beyond 2 months indicated decomposition of 5-HBC and/or

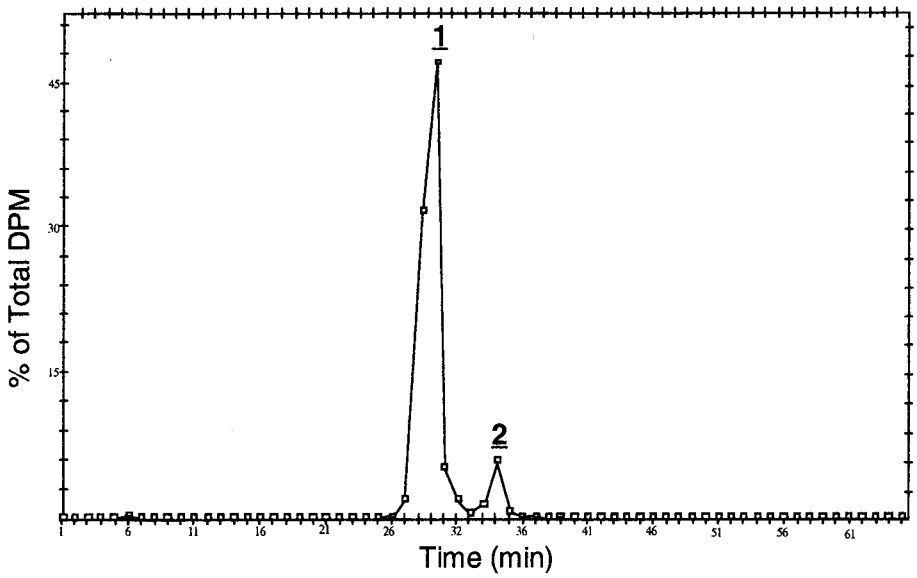

Figure 3. Representative reconstructed radiochromatogram following HPLC/LSC of an ethyl acetate extract of a goat liver homogenate. The peaks correspond to 5-HBC (1) and MBC (2).

Table 1. Liver Benomyl Equivalent Concentrations[a] (μg/g b.e.) Calculated on the Basis of the HPLC/LSC or LC/MS/MS Method

analytical method	MBC	5-HBC
HPLC/LSC	0.19 ± 0.11	1.68 ± 0.75
LC/MS/MS	0.17 ± 0.07	1.41 ± 0.37

[a] Values represent mean ± SD, $n = 5$.

MBC. Because of apparent instability of the analytes when stored in ethyl acetate for longer than 57 days, we recommend that the LC/MS/MS analysis be done within 30 days of extraction of the liver samples. Figure 4 represents an HPLC/MS/MS chromatogram of the same liver ethyl acetate extract shown in Figure 3. The LOQ for this method was estimated to be 0.05 ppm b.e. for each analyte. This LOQ is quite sufficient, relative to the current U.S. tolerance of 0.1 ppm (Code of Federal Regulations, 1995) for meat byproducts. The LOQ was primarily based on the S/N behavior of calibration standards, since the major source of noise was associated with the detection process (i.e., the MS instrument) and not the chemical background (matrix interference). The chemical background was essentially absent, for all practical purposes, in all mass channels. Consequently, sample extracts yielded chromatography that looked very similar to that of standards (Figure 4). As would be expected for the thermospray interface, the system response tended to drift over long periods of time (>6−8 h). Therefore, sample concentrations were determined using response factors from bracketed standards. The precursor and product ions corresponding to each analyte in our mass spectral analysis are listed in Table 2. The liver concentrations for MBC and 5-HBC calculated on the basis of the LC/MS/MS method are comparable to those reported from our HPLC/LSC (Table 1). The differences in concentrations of the two metabolites detected are likely due primarily to the different methods of reconstituting the samples prior to HPLC injec-

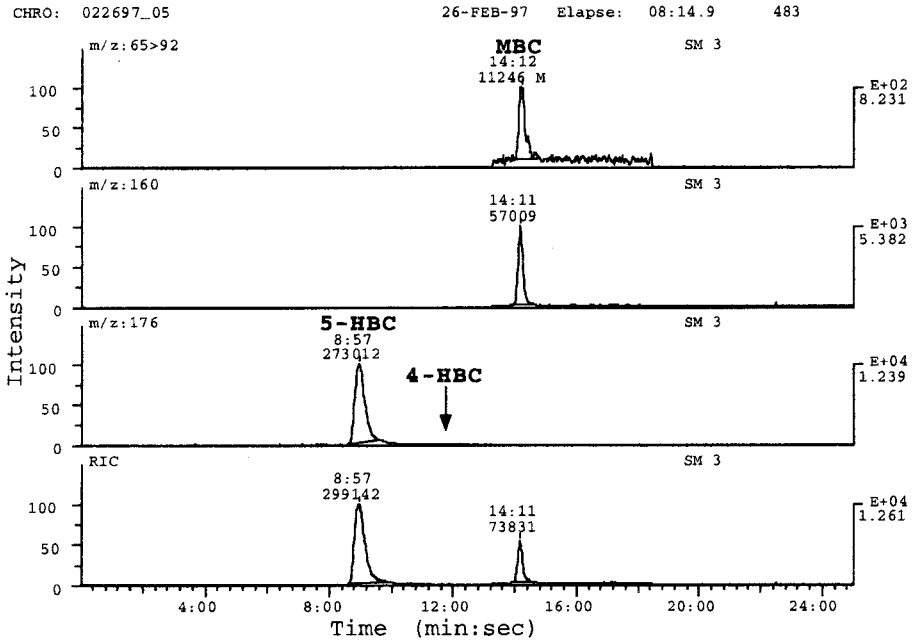

Figure 4. Representative LC/MS/MS of an ethyl acetate extract of a goat liver homogenate.

Table 2. Full-Scan Thermospray LC/MS and LC/MS/MS Spectral Data of 4-HBC, 5-HBC, and MBC Standards[a]

analyte	precursor ion (*m*/*z*) (LC/MS)	product ion (*m*/*z*) (LC/MS/MS)
5-HBC	207.8 $[M + H^+]^+$	176 [loss of methanol]
4-HBC	207.8 $[M + H^+]^+$	176 [loss of methanol]
MBC	192.1 $[M + H^+]^+$	160 [loss of methanol]
	134.1 $[M + H^+]^+$	65, 92

[a] A small amount of MBC thermally degrades to 2-aminobenzimidazole (2-AB), in the MS source. Hence, the appearance of *m*/*z* 134.1 ([M + H]+ for 2-AB) at the retention time of MBC. These additional ions were useful for confirmation purposes. Quantitation was based on the signal derived from the principal production associated with each analyte.

tion since the samples were redissolved in different solvents for compatibility with the two different HPLC methods.

We did not spike liver samples with standards to perform a fortification recovery study because the exact nature of the residue prior to Raney-nickel reduction and acid dehydration (desulfurization) is unknown. The native analyte is hypothesized as a sulfur conjugate between benomyl-derived residues and liver proteins. Because the nature of the native analyte is unknown, it is not meaningful to conduct fortification and recovery experiments to determine the precision and accuracy of the method. Additionally, replacement of the native analyte with surrogate analytes will yield erroneous results and interpretations. Indeed, spiking the control liver samples with nonradiolabeled MBC, 4-HBC, and 5-HBC resulted in poor recovery of these compounds probably due to nonspecific binding of these molecules to the active surface of our catalyst (data not shown). We, however, determined analyte recovery for all method steps following Raney-nickel reduction since no sample transfers occurred prior to this step. Hypothetically, MBC, 4-HBC, and 5-HBC are generated upon treatment of the mixture with Raney-nickel and phosphoric acid. No peaks were observed in the chromatograms of the control beef liver sample in our HPLC/LSC or LC/MS/MS studies. Recoveries for MBC and 5-HBC in fortified samples were found to be within a reasonable range for the four fortification levels. Average recoveries were 82% and 120% for MBC and 5-HBC, respectively. Finally, for recovery determinations, samples should be fortified after Raney-nickel reduction.

Special Precautions. Special care should be taken while handling hydrogen (either supplied from a cylinder or in situ generation from Raney-nickel). Raney-nickel itself may undergo spontaneous combustion upon exposure to air.

CONCLUSIONS

This residue method is well suited for the determination of benomyl residues (5-HBC, 4-HBC, and MBC) in ruminant liver. The data support the satisfactory performance of this method, with an expected LOQ of 0.05 ppm b.e. This LOQ is sufficient to meet the current U.S. tolerance of 0.1 ppm for meat byproducts. This method is considerably improved over previous methodologies. More specifically, the extraction of benomyl residues is greatly improved, selectivity of quantitation for the analytes (LC/MS/MS) is enhanced, and the three analytes 5-HBC, 4-HBC, and MBC are simultaneously determined.

ABBREVIATIONS USED

b.e., benomyl equivalent = [analyte] (MW of benomyl/MW of analyte); HPLC, high-performance liquid chromatography; LC/MS (/MS), HPLC with MS (/MS) detection; LOQ, limit of quantitation; LSC, liquid scintillation counting; MS, mass spectrometry; MS/MS, tandem mass spectrometry; MW, molecular weight; *m*/*z*, mass-to-charge ratio; ppm, parts per million; RSD, relative standard deviation; SD, standard deviation; S/N, signal-to-noise ratio; RS1, integrated graphics and data analysis software package that operates on a VAX 11/785 computer; TRR, total radioactive residue.

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