Determination of 4″-Deoxy-4″-(epimethylamino)avermectin B_1 Benzoate (MK-0244) and Its Delta 8,9-Isomer in Celery and Lettuce by HPLC with Fluorescence Detection

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An analytical method has been developed for the extraction, derivatization, and fluorescence HPLC determination of MK-0244 [4″-deoxy-4″-(epimethylamino)avermectin B_1 benzoate salt] and its delta 8,9-isomer from lettuce and celery at trace (parts per billion) levels. MK-0244 and its delta 8,9-isomer are extracted with methanol, and the extract is cleaned up on a reverse-phase C_8 cartridge followed by a liquid–liquid extraction with ethyl acetate before passing through a propyl sulfonyl cation-exchange cartridge. MK-0244 and its delta 8,9-isomer are then derivatized with trifluoroacetic anhydride/1-methylimidazole/acetonitrile reagent system to form a fluorescent derivative which is separated on a reverse-phase HPLC system and detected with a fluorescence detector. The average recoveries were 95 ± 10% for celery and 96 ± 8% for lettuce. For both lettuce and celery matrix, concentrations as low as 2 ppb (S/N = 10) can be detected in a 10-g sample. This method was used to determine the MK-0244 levels in samples from a lettuce field trial.

INTRODUCTION

The avermectin family of macrocyclic lactones are natural products prepared by fermentation (Burg et al., 1979). The major component of the fermentation product is avermectin B_1 (abamectin). Avermectin B_1 is a potent broad spectrum acaricide/insecticide (Campbell et al., 1983) but does not show high innate toxicity against some specific insect species. A novel second-generation avermectin, MK-0244, has shown potency against larvae, which are difficult to control effectively with abamectin or other currently available insecticides (Dybas and Babu, 1988).

MK-0244 [4″-deoxy-4″-(epimethylamino)avermectin B_1 benzoate salt] is a semisynthetic avermectin derivative and has been derived through chemical modification of abamectin. MK-0244 is the code designation of the benzoate salt. The designation MK-0243 is applied to the hydrochloride salt. Abamectin contains two secondary hydroxyl groups and one tertiary hydroxyl group. In the case of MK-0244, the secondary hydroxyl group at the 4″-position of abamectin is replaced by a 4″-(epimethylamino) group (Figure 1). Similar to abamectin, MK-0244 is amixture of two homologues designated B_{1a} and B_{1b}. These compounds differ by one methylene unit (CH_2) at the 25-position, wherein B_{1a} contains a *sec*-butyl group and B_{1b} contains an isopropyl group. The ratio of these two homologues in MK-0244 is minimum 80% B_{1a} and maximum 20% B_{1b} (Dybas and Babu, 1988).

It is known that liquid chromatographic methods employing UV detection lack the required sensitivity for the detection of avermectin-related compounds when present in complex matrices at the parts per billion level (Fox and Fink, 1985). Lower detection limits were achieved by Tolan et al. (1980), who derivatized 22,23-dihydroavermectin B_1 (ivermectin) with acetic anhydride to form a fluorescent compound. Tway et al. (1981) improved Tolan's method by using *N*-methylimidazole (NMIM) as a catalyst in dimethylformamide (DMF), thereby replacing pyridine which had been used as both catalyst and solvent. The NMIM catalyst allows the formation of the fluorophore in 1 h at 95 °C, which resulted in a much shorter and more reproducible derivatization procedure. The derivatization procedures (Tolan et al., 1980; Tway et al., 1981; Prabhu et al., 1991) were further modified; trifluoroacetic anhydride was used (Analytical Research, MSRDL, unpublished methods) in the presence of DMF and NMIM, effecting derivatization in 1 h at 30 °C. However, in all cases, the methods (Tolan et al., 1980; Tway et al., 1981; Prabhu et al., 1991; Analytical Research, unpublished methods) require sample preparation and cleanup both before and after the derivatization.

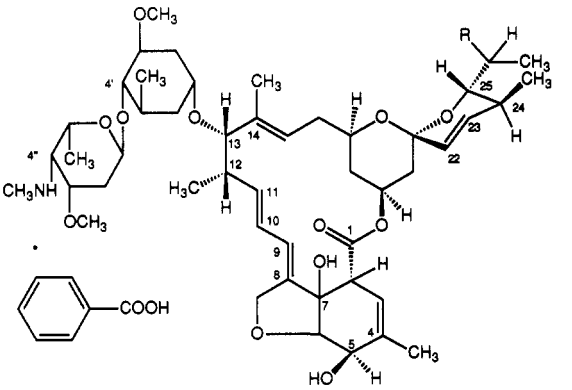

Figure 1. Structure of MK-0244 (benzoate salt). This modified fermentation product consists of two components: (a) component R = C_2H_5 ≥ 80%; (b) component R = CH_3 ≤ 20%.

The procedure was further shortened by de Montigny et al. (1990); they used acetonitrile (ACN) as the solvent with trifluoroacetic anhydride (TFAA) in the presence of the NMIM catalyst to derivatize ivermectin. The major advantage of this modification is that the derivatization is instantaneous at room temperature (RT) and no further sample cleanup is required following derivatization.

In the present work, a rapid method has been developed for the isolation, derivatization, and fluorescence HPLC quantitation of MK-0244 and its 8,9-isomer from lettuce and celery at the parts per billion level. The delta 8,9-isomer is one of the major photodegradation products of MK-0244 (L. Crouch, MSRDL, 1991, personal communication). The sample cleanup involves various solid-phase extraction procedures.

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The method has been validated for celery and lettuce samples fortified at various levels with MK-0244 and the 8,9-isomer. The method was used to determine incurred residues of MK-0244 and 8,9-isomer, where the head lettuce was treated with MK-0244. The derivatization step uses the TFAA/NMIM/acetonitrile reagent system with slight modifications (de Montigny et al., 1990).

EXPERIMENTAL PROCEDURES

Solvents and Reagents. All organic solvents were either Burdick and Jackson distilled in glass or nanograde quality. All other reagents were of analytical grade purity. Helium or nitrogen gas, the equivalent of Matheson extra dry compressed gas, was used to deaerate mobile phase or evaporate solvents. The following reagents were obtained from the source noted and were of the grade designated: 1-methylimidazole, Aldrich Chemical Co., 99% pure grade; phosphoric acid, E. M. Science, 85% solution, reagent grade; Sylon-CT, (dichlorodimethylsilane in toluene) Supelco, Inc.; trifluoroacetic anhydride (TFAA), Fisher, reagent grade; water, filtered distilled water was treated with a Milli-Q system including a Millipore Type HA 0.22-μm disk. The 1% ammonium acetate in methanol (w/v) solution can be stored at RT; however, the 1% ammonium acetate in water (w/v) solution has to be stored in the refrigerator. The derivatization reagent [1:2 (v/v) TFAA/ACN] has to be prepared fresh just before use. For each sample or standard tube, 0.3 mL of derivatization reagent was used. (*Note*: Extra care should be taken while handling TFAA reagent; avoid skin contact with this reagent.) The 1% phosphoric acid in methanol (w/v) solution can be stored at RT. The MK-0244 standard (L-656,748-038W003) with 92.1% B_{1a} (w/w) and 4.7% B_{1b} (w/w) was obtained from Merck Sharp and Dohme Research Laboratories (MSDRL) and stored at −20 °C.

Apparatus. The derivatization reaction was carried out in silylated 15-mL centrifuge tubes. The silylation procedure and the subsequent cleaning of the tubes are described elsewhere (Tway et al., 1980). All glassware used should be completely free of acidic and alkaline residues. The sources of specific items used were as follows: adapter, 1,3,6 mL, Analytichem International, P.N. 1213-1001; homogenizer, Polytron, Brinkmann Instruments Inc., Westbury, NY 11590; Bond-Elut C_8 (1000 mg) and propylsulfonyl Cartridges (500 mg), Analytichem International. A homemade device was made to continuously feed extract solution to the C_8 cartridges using the 1,3,6 mL adapters along with Teflon tubing (1/6 in. o.d., 2 ft long). The Teflon tubes were attached to the adapters by inserting them through the inner diameter of the adapter. These adapters with tubing attached were fitted into the C_8 cartridge (which was attached to the vacuum manifold) and the other end of the Teflon tube was inserted into the filter flask containing sample extract. When controlled vacuum was applied to the cartridge, the aqueous methanol extract was sucked into the cartridge continuously without overflowing. This device is not essential to the method but avoids the need to frequently refill the solid-phase extraction reservior.

Liquid Chromatography Instrumentation. A Spectra-Physics (SP) Model SP8700 XR liquid chromatographic pump complete with a SP Model SP8780XR autosampler and Kratos-Schoeffel Instruments Model FS950 fluorescence detector with a SP Model 4200 integrator or an equivalent system was used. A column heater such as the Fiatron CH-30 with a TC-55 controller was necessary. A 7μm, 15 mm length, 3.2 mm i.d., C_{18} standard Brownlee Labs guard column (RP-18 OD-GU obtained from Rainin Instrument Co., Inc.) or equivalent was used before the analytical column.

Chromatographic Conditions. The following settings were used: column, 15 cm length, 4.6 mm i.d. ES Industries, Chromegabond, C_{18} column; mobile phase, 7% H_2O in methanol (v/v), isocratic; column temperature, 30 °C; flow rate, 1.5 mL/min (usual pressure around 2200 psi).

Detector Settings. The following settings were used: excitation lamp, FSA110, standard 365 nm; standard Kratos flow cell, FSA210; excitation filter, FSA 403, 365-nm bandpass filters; emission filter, 418-nm cutoff filter; sensitivity range, 0.02 μA or higher; time constant, 6. The MK-0244 derivatives have retention times of about 8–11 min when the indicated conditions were used. The retention times of MK-0244 B_{1a} derivative and its 8,9-B_{1a} isomer derivative were identical and are believed to be the same compound.

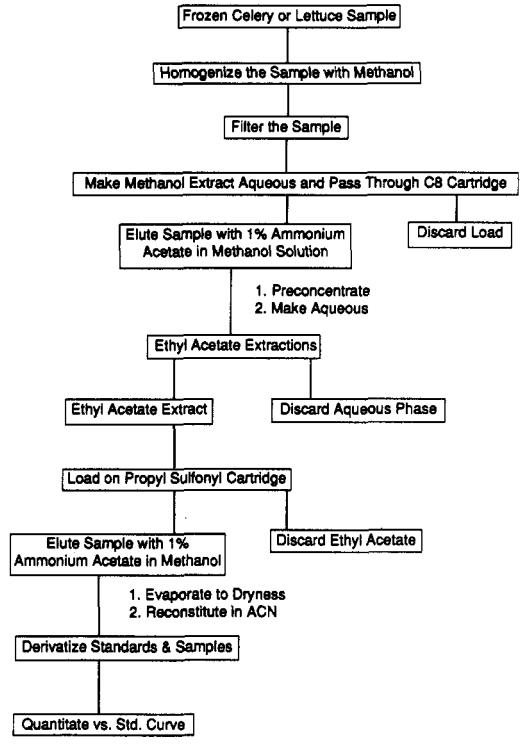

Figure 2. Flow diagram for the analysis of MK-0244 and its 8,9-isomer in lettuce and celery samples.

Stock Solutions and Standard Curve. The stock and standard solutions were prepared in acetonitrile. To prepare 2.0, 4.0, 6.0, 8.0, and 10.0 ng/mL calibration curve standards for the derivatization, 0.2, 0.4, 0.6, 0.8, and 1.0 mL of 50 ng/mL MK-0244 B_{1a} standard solution was transferred to separate silylated 15-mL tubes. The standards are stable for several months when stored in the freezer at −15 °C. The standards were derivatized with the samples and carried through all subsequent operations for injection on the HPLC. Derivatized standards and samples are stable for over 72 h when stored in the freezer at −15 °C.

EXTRACTION AND CLEANUP PROCEDURE

A flow diagram for the assay procedure is shown in Figure 2. Twelve samples can be completed up to HPLC injection in an 8-h day.

Homogenization. Samples should remain frozen throughout the sample homogenization steps. While the sample was processed, dry ice was added if the sample began to thaw. The entire sample was processed using a Cuisinart food processor or equivalent. The sample was ground until a homogeneous blend was attained. The sample homogenate was stored at −20 °C until used.

Extraction from Homogenate. Exactly 10 g of the homogenized sample was weighed into a 4-oz square bottle, and 25 mL of methanol was added. The sample was blended with the Polytron. The methanol and any accompanying macerate were poured into a 63-mm porcelain funnel fitted with a Whatman No. 50 filter paper which was pre-rinsed with water. By application of a vacuum, the filtrate was collected in a 500-mL filter flask. The probe was rinsed with 25 mL of methanol in the grinding bottle. The methanol rinsate was added to the porcelain funnel containing the filter cake, and the solvent was drawn through the cake. Another 25 mL of methanol was added to the bottle, and the rinsing steps were repeated. After these three passes of methanol (75 mL total volume), the filter cake was generally white in color, and the methanol filtrate was green. The empty sample bottle was rinsed with approximately 50 mL of deionized

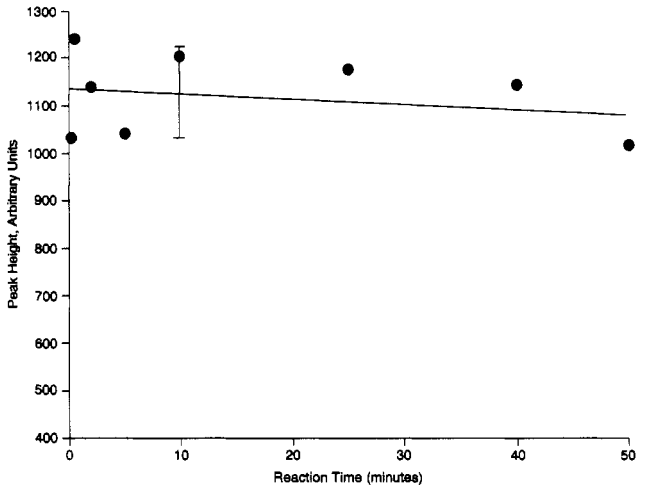

Figure 3. Effect of reaction time on the yield of the fluorescent derivative measured by fluorescence HPLC.

water and added to the flask containing the methanol filtrate and swirled to mix. The filtrate was diluted to a total volume of 500 mL by adding approximately 375 mL of deionized water and swirled gently to mix. After these steps, the methanol:water ratio is 15:85, which is critical to the performance of the SPE column.

Sample Cleanup and Preconcentration with C_8 Cartridge. One C_8 cartridge per sample was attached to a vacuum manifold. The cartridge was conditioned with 5 mL of methanol followed by 5 mL of distilled water. The aqueous methanol sample extract was loaded on the C_8 cartridge. This was done efficiently with the use of Teflon tubes and adapters described under Apparatus or similar large reserviors. This step takes about an hour. Flow through the cartridge should not exceed approximately 10 mL/min. The eluant was discarded. A graduated 15-mL centrifuge tube was placed in the sample collection rack. The sample from the cartridge was eluted with 10 mL of 1% ammonium acetate in methanol solution. The ammonium acetate in methanol eluate collected in the tubes was evaporated to approximately 1 mL; evaporation to less than 0.5 mL should be avoided.

Ethyl Acetate/Water–Methanol Partition. The 1 mL of evaporated eluate solution was diluted to 5 mL with the aqueous 1% ammonium acetate solution. To the diluted solution was added 5 mL of ethyl acetate. The tubes were stoppered and shaken for 1 min and subsequently centrifuged for 2 min at around 2000 rpm. The upper (ethyl acetate) layer was transferred to a clean centrifuge tube. The extraction of the aqueous ammonium acetate was repeated with a second 5 mL of ethyl acetate, and the ethyl acetate extracts were combined. The total volume of the ethyl acetate extracts was approximately 10 mL.

Sample Cleanup with Cation-Exchange Cartridge. One propylsulfonyl cation-exchange cartridge was attached per sample to a vacuum manifold. The cartridge was conditioned with 5 mL of each in the following order: 1% ammonium acetate in methanol, 1% phosphoric acid in methanol, deionized water, methanol, and ethyl acetate. The ethyl acetate extract was loaded onto the cartridge. The eluant was discarded. A 15-mL graduated centrifuge tube was placed in the sample rack under the column, and the sample was eluted from the cartridge with 5 mL of 1% ammonium acetate in methanol solution. The calibration on the test tube was used to make 5 mL final volume with 1% ammonium acetate in methanol solution. The tube was stoppered, vortexed for 10 s, and centrifuged for 1 min. The half-volume of the eluate was removed by a volumetric pipet to a 15-mL silylated centrifuge tube. The tube containing the remaining portion of the eluate was stoppered and stored in the freezer at −20 °C. The frozen fraction was used for repeat analyses, if necessary.

Derivatization of Samples or Standards. Samples removed in silylated tubes were evaporated to dryness under nitrogen purge at 70–85 °C. (Care must be taken to ensure that no moisture is left in the tubes.) The samples were reconstituted in acetonitrile. To the samples and the standards was added 0.1 mL of

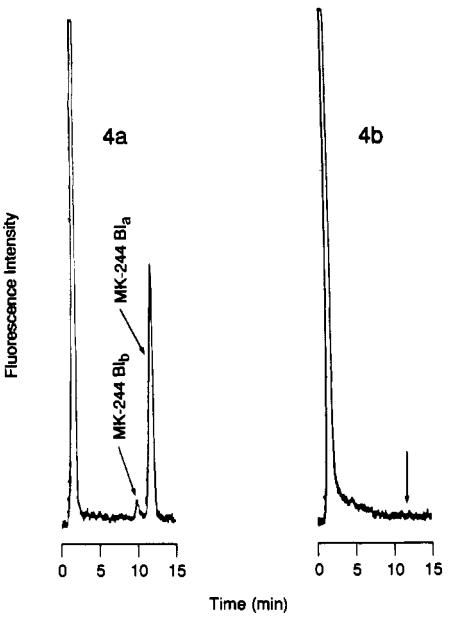

Figure 4. Typical chromatograms: (a) MK-0244 B_{1a} derivative standard 8 ng/mL; (b) control lettuce sample.

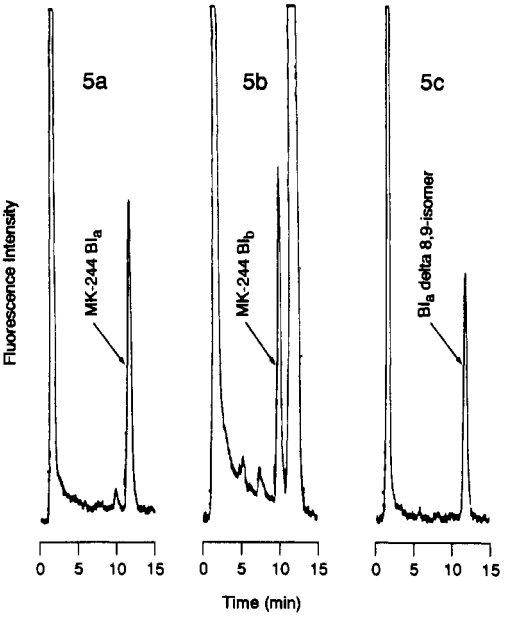

Figure 5. Typical chromatograms of control lettuce fortified with (a) 5.2 ng/g MK-0244 B_{1a}, (b) 4.5 ng/g MK-0244 B_{1b}, or (c) 5.5 ng/g MK-0244 delta 8,9-isomer.

1-methylimidazole reagent with a graduated pipet. The tubes were stoppered and then vortexed, sonicated for 5–10 s, and centrifuged for 2 min at 2000 rpm. All samples, standards, and the freshly prepared TFAA/ACN reagent tube (see Stock Solutions and Standard Curve) were placed in a Kryorack (ice temperature tube rack) for 10 min. After cooling, 0.3 mL of TFAA/ACN reagent was added to each sample and standard tube using a volumetric pipet. The tubes were stoppered, vortexed, and centrifuged for 1 min. (*Note*: Addition of the TFAA/ACN mixture caused a white fume or cloud above each solution. The fumes subside upon vortexing and standing.) The samples were allowed to sit at room temperature for about 10 min after addition of TFAA/ACN reagent. The samples generally turn light yellow, and standards turn very pale green. After 10 min, the standards were diluted to 5 mL with acetonitrile, yielding 2, 4, 6, 8, and 10 ng/mL MK-0244 B_{1a}, respectively. The samples were diluted to 5 mL or the appropriate volume on the basis of the expected residues (controls in 5 mL). Samples were vortexed to mix and centrifuged briefly prior to injection.

Quantitation. The standards were injected at the beginning

Table I. Recovery of MK-0244 B_{1a}, MK-0244 B_{1b}, and the Delta 8,9-Isomer B_{1a} from Lettuce

fortified with	fortification level, ng/g	calcd concn, ng/g	% recovery	precision, % RSD	% av recovery
MK-0244 B_{1a}	5.2	4.2, 4.4, 4.4, 4.3, 4.4	81, 85, 85, 83, 85	2.1	84
	23.5	21, 21, 23, 23, 23	89, 89, 98, 98, 98	5.2	94
	69.5	61, 63, 64, 64, 60	88, 91, 92, 92, 86	3.0	90
MK-0244 B_{1b}	4.5	4.1, 4.4, 4.3, 4.1, 4.6	91, 98, 96, 91, 102	4.9	96
delta-8,9 B_{1a}	5.5	5.6, 5.6, 6.4, 6.1, 5.4, 5.7	102, 102, 116, 111, 98, 104	6.3	106
	25.6	26, 24, 28, 25, 25	102, 94, 109, 98, 98	5.7	100
	73	48, 73, 81, 98, 80, 74	66, 100, 111, 134, 110, 101	21	104
control	0	0, 0, 0, 0, 0	NA[a]	NA	NA

[a] NA, not applicable.

Table II. Recovery of MK-0244 B_{1a}, MK-0244 B_{1b}, and the Delta 8,9-Isomer B_{1a} from Celery

fortified with	fortification level, ng/g	calcd concn, ng/g	% recovery	precision, % RSD	% av recovery
MK-0244 B_{1a}	5.2	5.3, 4.8, 4.5	102, 92, 87	8.1	94
	20.9	17, 17, 16, 15, 16	81, 81, 77, 72, 77	5.0	79
		17, 18, 16, 17	81, 86, 77, 81		
	87	75, 79, 81	86, 91, 93	4.0	90
MK-0244 B_{1b}	4.5	5.0, 5.0, 5.1	111, 111, 113	1.0	112
delta-8,9 B_{1a}	5.5	5.4, 5.3, 5.4	98, 96, 98	1.2	97
	14.6	14, 14, 11, 13 11	96, 96, 75, 89, 75	12.3	86
	25.6	27, 26, 24	105, 102, 94	5.7	100
	73	75, 74, 74	103, 101, 101	1.1	102
control	0	0, 0, 0	NA[a]	NA	NA

[a] NA, not applicable.

and at the end of the set to ensure stability of the HPLC system, the standards, and the samples. The residue was determined by reverse-phase liquid chromatography with fluorescence detection and quantitated by peak height measurements. A new set of standards were included with each set because the derivatized standards were not stable for long-term storage, and there were slight differences from day to day in the yield of the fluorescent product. The nanogram per milliliter for each unknown sample was read from the standard curve, and residue (in parts per billion) was calculated on the basis of the dilution and fraction of the sample derivatized.

The lower limit of detection for the method was 2 ng/g (S/N = 10), and the lower limit of quantitation was 5 ng/g (S/N = 25) for the MK-0244 B_{1a} component and/or the delta 8,9-isomer extracted from both celery and lettuce samples. At levels above 5 ng/g the MK-0244 B_{1b} component residues were quantitated in the same manner as MK-0244 B_{1a}/delta 8,9-isomer, using the MK-0244 B_{1a} standard curve.

RESULTS AND DISCUSSION

Liquid chromatography with fluorescence detection was chosen as the analytical method, combining a high degree of specificity due to the combination of chromogenic and chromatographic selectivities as well as sensitivity arising from the strong fluorescence of the derivative.

The derivatization step uses the TFAA/NMIM/acetonitrile reagent system (de Montigny et al., 1990). These conditions have been modified for the derivatization of MK-0244 and its 8,9-isomer. The reagent and sample (or standard) are first chilled to approximately 0 °C, and then the derivatization reaction is allowed to warm up to RT for 10 min. Use of the chilled reagent and samples gives better standard curves for MK-0244 B_{1a} as measured by the coefficient of determination.

A plot of derivatization yield (as detector response) vs reaction time is shown in Figure 3. Reaction times from 10 s to 50 min were evaluated. The reaction is complete instantaneously, and the same reaction yield is observed in each case. In the present work, a 10-min reaction time is used for convenience of operation. The reproducibility of the derivatization was confirmed at the 10-min reaction time for five different 10 ng/mL standard MK-0244 solutions. The relative standard deviation for these standard solutions measured as chromatographic peak height was less than 8%.

The analysis of the standards from 2 to 10 ng/mL showed a good correlation between the concentration (X) and peak height (Y) and with the coefficient of determination (r^2) averaging 0.995.

Samples of lettuce and celery matrices require purification steps prior to chromatographic determination. Several cleanup steps were tested. The combination of C_8 cartridge cleanup followed by liquid–liquid extraction and propylsulfonyl cation-exchange cartridge removes most of the polar and nonpolar matrix impurities that can interfere with the MK-0244 determination. The chromatographic behavior of MK-0244 is strongly influenced by water content in methanol. The RP-HPLC system used for the assay provides excellent separation. Representative chromatograms of MK-0244 standard and control lettuce sample after the cleanup steps are shown in Figure 4. Figure 5 shows representative chromatograms of control lettuce samples fortified with B_{1a} MK-0244, B_{1b} MK-0244, and delta 8,9-isomer after the cleanup steps were performed. The control lettuce and celery had no peaks that would interfere with the MK-0244 B_{1a}, B_{1b}, and 8,9-isomer peaks.

The method has been validated for both celery and lettuce samples. In each experiment, 10-g samples were fortified with MK-0244 or delta 8,9-isomer and homogenized with methanol before the cleanup and extraction steps were performed. Recoveries for MK-0244 and the B_{1a} 8,9-isomer were determined by spiking samples which were quantitated vs the B_{1a} component from the MK-0244 standards. The method showed good selectivity for the B_{1a} and B_{1b} components of MK-0244.

Table I gives the results of validation of the method for lettuce. Recoveries were good and ranged from 84 to 106% with precision that ranged from 2 to 21% RSD. Table II gives the results of method validation for celery. Recoveries were good and ranged between 79 and 112% with precision that ranged from 1 to 13% RSD. The method

Table III. Residue Levels (Nanograms per Gram) from the MK-0244 Head Lettuce Field Trial

days posttreatment	B_{1a} MK-0244/delta 8,9-isomer	
	1X rate	2X rate
0	80	151
1	13	23
3	5.9	6.9
7	ND[a]	NQ[b]

[a] ND, not detectable (<2 ng/g). [b] NQ, not quantitable (2 ng/g < NQ < 5 ng/g).

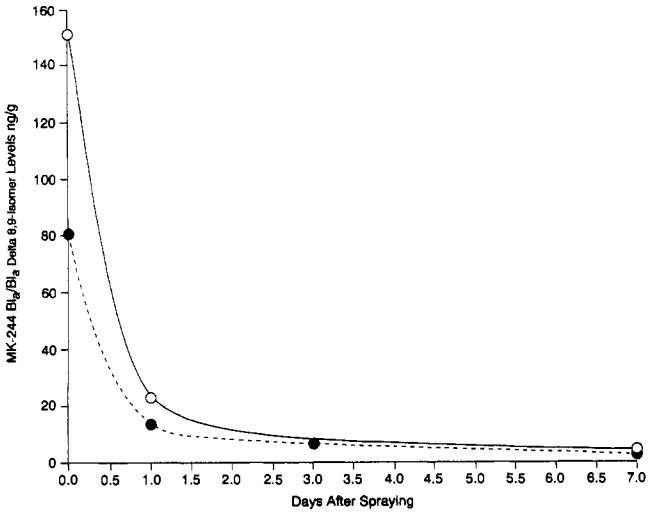

Figure 6. Dissipation curve for the depletion of MK-0244 B_{1a} and its delta 8,9-isomer in lettuce: (●) at the 1X level (0.015 lb/acre, dotted line); (○) at the 2X level (0.03 lb/acre, solid line).

could therefore be used for the determination of MK-0244 concentration ≥5 ppb in celery or lettuce.

The method was further used to determine MK-0244 levels on head lettuce in a field trial. The concentration levels observed are summarized in Table III. In this study head lettuce was subjected to seven weekly applications of MK-0244 at 0.015 and 0.03 lb of ai/acre with addition of 8 fluid oz/acre nonionic surfactant. Representative samples were from each treatment [untreated control, 0.015 lb of ai/acre (1X rate), 0.03 lb of ai/acre (2X rate)] beginning at 0 day (about 2 h following last application, after spray has dried) and at 1, 3, and 7 days after the last application. A sample consisted of one-fourth of a head from each of 12 heads in each treatment. After all of the samples were collected, they were stored at −20 °C until processed and analyzed.

The dissipation curves for MK-0244 on head lettuce shown in Figure 6 are given only to indicate the trend exhibited by the limited residue data. The solid line is shown for the 2X rate, and the dotted line is shown for the 1X rate. The MK-0244 level dissipated rapidly during the 0–1 day postapplication period and then dissipated thereafter at a slower rate. The mean half-life ($t_{1/2}$) values for the two curves were approximately 0.5 day for the initial rapid dissipation. At all times points higher concentration levels were observed for the higher (2X) treatment rate. No MK-0244 was detected in any of the control samples.

The results of these experiments show that detection and quantitation of parent MK-0244 and its major photodegration product delta 8,9-isomer at trace level are possible in celery and lettuce.

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