KIH-485/M-3, M-1 and M-25 Analytical Method in Cotton Processed Commodities as Described in
"Magnitude of the Residue of KIH-485 85 WG Herbicide in Cotton Processed Commodities,"
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These methods are used for the determination of Pyroxasulfone (KIH-485) and its metabolites M-3, M-1 and M-25 in cotton meal, hulls and refined oil. This method has demonstrated a limit of quantitation (LOQ) of 0.01 ppm of KIH-485, M-3, M-1 and M-25 in cotton raw agricultural and processed commodities (RACs) and processed commodities. The method for KIH-485 and M-3 has a limit of detection of 0.00037 ppm (0.0005 µg/mL in solution). The method for M-1 and M-25 has a limit of detection of 0.00030 ppm (0.0005 µg/mL in solution).

MATERIALS AND METHODS

Equipment

Glassware and Miscellaneous Equipment

Balance
Bottle, amber, with Teflon®-lined cap, 250 mL, 500 mL
Bottle, centrifuge
Centrifuge tube, glass, 50 mL
Centrifuge
Filter flask, 0.5 L
Filter paper, Whatman #4
Flask, round bottom, various sizes
Funnel, Büchner
Graduated glass centrifuge tubes, 15 mL
Graduated Cylinder, various sizes
SPE Cartridges, 12 mL, 1g ENVI-CARB (Supelco)
Separatory funnels, various sizes
Sonicator, Branson

Syringes, microliter, various sizes
Vacuum evaporator, Büchi Model RE111 with temperature-controlled bath, Brinkmann Instruments, Burlingame, CA
Vials, glass with Teflon®-lined cap
Volumetric flask, various sizes
Volumetric pipette, various sizes
Wrist-action shaker

Reagents and Reference Substance

Solvents/Reagents

All solvents were HPLC grade unless noted:
Acetonitrile
Citric Acid
Ethyl Acetate
Hexane
Methanol
Sodium Bicarbonate
Sodium Chloride
Sodium Sulfate, anhydrous
Water

Reference Substances

The reference standard of Pyroxasulfone (KIH-485, lot number LP001, PTRL Sample No. 1950W-001) was received from Kumiai Chemical Industry on July 29, 2009 with a stated purity of 99.84%. The expiration date on the certificate of analysis is April 25, 2013. The reference standard of M-1 (lot number 3, PTRL Sample No. 2123W-003) was received from Kumiai Chemical Industry on November 12, 2010 with a stated purity of 98.8%. The expiration date on the certificate of analysis is November, 2013. The reference standard of M-3 (lot number 6, PTRL Sample No. 2123W-004) was received from Kumiai Chemical Industry on November 12, 2010 with a stated purity of 99.3%. The expiration date on the certificate of analysis is November 2013. The reference standard of M-25 (lot number 2, PTRL Sample No. 1950W-004) was received from Kumiai Chemical Industry on July 29, 2009 with a stated purity of 98.74% and an

expiration date of November 21, 2013. The reference substances were stored frozen, when not in use.

Preparation of Sample

All cotton processed commodity samples were received frozen at PTRL West and remained frozen (<0ºC) until they were used for fortification and analysis.

Preparation of Standards

Stock solutions (1.0 mg/mL) of KIH-485, M-1 and M-3 reference standard were prepared in acetonitrile. M-25 stock solution (1.0 mg/mL) was prepared in water, as described under the "Method of Calculations" section. Mixed fortification solutions and calibrants were made by diluting the working solutions as described below. Microliter syringes, volumetric pipettes and volumetric flasks were used throughout. All stock and fortification solutions were stored at freezer temperature.

Fortification Procedure

Fortification of untreated matrices was conducted to determine the percent recovery within each sample set for KIH-485/M-3 and for M-1/M-25. A mixed 10 µg/mL fortification solution of KIH-485/M-3 was prepared by dilution, where 0.250 mL of 1.0 mg/mL KIH-485 and 0.250 mL of 1.0 mg/mL M-3 were combined and diluted to 25 mL with acetonitrile:water (1:1, v/v). A mixed 10 µg/mL fortification solution of M-1/M-25 was prepared by dilution, where 0.250 mL of 1.0 mg/mL M-1 and 0.250 mL of 1.0 mg/mL M-25 were combined and diluted to 25 mL with acetonitrile:water (1:1, v/v). Mixed 1.0 µg/mL fortification stocks were prepared by aliquoting 2.5 mL aliquots of the respective 10 µg/mL mixed working solutions and diluting to 25 mL with acetonitrile:water (1:1, v/v).

Fortification of KIH-485/M-3 and M-1/M-25 was conducted in duplicate within the sample sets. The following fortifications were conducted:

Fortification Level (mg/kg)	KIH-485/M-3	M-1/M-25
0.01	150 µL of 1.0 µg/mL	150 µL of 1.0 µg/mL
0.10	150 µL of 10 µg/mL	150 µL of 10 µg/mL

Preparation of Calibrant Standards

KIH-485/M-3 dilutions were made with acetonitrile:water (1:1, v/v).

Calibration Standard (ng/mL)	Concentration of Mixed KIH-485/M-3 Solution (µg/mL)	Volume of Stock Solution (mL)	Final Volume (mL)
100	10	0.1	10
50	10	0.05	10
25	1.0	0.25	10
10	1.0	0.1	10
5	1.0	0.05	10
2.5	0.1	0.25	10
1.0	0.1	0.1	10
0.5	0.1	0.05	10

M-1 and M-25 dilutions were made with acetonitrile:water (1:1, v/v)

Calibration Standard (ng/mL)	Concentration of Mixed M-1/M-25 Solution (µg/mL)	Volume of Stock Solution (mL)	Final Volume (mL)
100	10	0.100	10
50	10	0.05	10
25	1.0	0.25	10
10	1.0	0.10	10
5	1.0	0.05	10
2.5	0.1	0.25	10
1.0	0.1	0.10	10
0.5	0.1	0.05	10

Matrix-based Calibrants – prepared in control matrix final extract. The following are representative preparations followed throughout the study.

Calibration Standard (ng/mL)	Concentration of Stock Solution	Volume of Stock Solution	Volume Control Extract Added

	(ng/mL)	(µL)	(mL)
0.5	50	5	495
1	100	5	495
2.5	100	12.5	487.5
5.0	100	25	475
10	1000	5	495
25	1000	12.5	487.5

Matrix-based calibrants were prepared for any analyte as needed.

A calibration curve was generated with each sample set to determine linearity and to quantitate KIH-485, M-3, M-1 and M-25. See "Methods of Calculation" for example.

A. Cotton Meal Extraction Method - KIH-485, M-3, M-1 and M-25

1. Weigh 15 grams of processed sample into a 250 mL centrifuge bottle.
2. Spike as necessary.
3. Add 40 mL of DI water, stand at room temperature for 15 minutes. Add 120 mL of acetonitrile (ACN) and. Stand at room temperature for 1 hour.
4. Place samples on wrist action shaker for 30 minutes and then sonicate for 15 minutes.
5. Vacuum filter the mixture through filter paper (Whatman #4) in a Büchner funnel into 0.5 L side-arm flask. Rinse centrifuge bottle with 70 mL of ACN:water (3:1, v/v) and rinse the filter cake with this solvent and combine all filtrates in 0.5 L side-arm flask. Transfer quantitatively into clean 500 mL separatory funnel.
6. Partition sample with 120 mL hexane, allow layers to separate, discard upper hexane layer.
7. Collect ACN:water layer into a 250 mL graduated cylinder and adjust final volume to 240 mL with ACN:water (3:1, v/v). Transfer initial extracts to amber bottles.
8. Divide 240 mL initial extract into 160 mL and 80 mL aliquots. Store 80 mL aliquots in amber bottle for extraction of M-1/M-25.

KIH-485 and M-3 in Meal

9. Transfer 160 mL initial extract into a 500 mL concentration flask.

10. Concentrate by roto-evaporation to approximately 40 mL at 35°C. Ensure that all of the ACN has been removed.
11. Transfer the concentrate quantitatively into a 250 mL separatory funnel. Rinse the 500 mL concentration flask with 50 mL of hexane; add to separatory funnel. Add 5 mL of a saturated NaCl solution and 20 mL of 0.5M $NaHCO_3$ to the separatory funnel. Partition for 1 min. Allow the layers separate.
12. Transfer lower aqueous phase into an appropriate vessel.
13. Filter the upper hexane phase containing KIH-485 through ~30 g of anhydrous sodium sulfate supported by a plug of glass wool into a clean 500 mL concentrate flask.
14. Return the aqueous solution to the separatory funnel. Repeat hexane partition one more time, combining the organic extracts into the same 500 mL concentration flask from Step 13. Rinse the sodium sulfate with 20 mL of hexane.

M-3 Extraction

15. Return the aqueous solution to the separatory funnel for M-3 extraction.
16. Add 20 mL of 0.5M citric acid to the aqueous solution in the separatory funnel. Check the pH to ensure the aqueous solution is acidic. Partition for 1 min with 60 mL hexane:ethyl acetate (7:3,v/v).
17. Transfer the lower aqueous solution into an appropriate size vessel.
18. Filter the upper hexane:EtOAc layer through ~30 g of (fresh) anhydrous sodium sulfate supported by a plug of glass wool combining into the same concentration flask in Step 14.
19. Return aqueous solution to the separatory funnel. Repeat hexane:EtOAc partition, combining the organic extracts into the same 500 mL concentration flask in Step 18. Rinse the sodium sulfate with 20 mL of hexane: EtOAc (7:3, v/v).
20. Roto-evaporate to dryness at 35°C. Reconstitute with 10 mL of ACN. Sonicate to dissolve.
21. **SPE clean-up**
    - Condition SPE cartridge (12 mL 1g ENVI-CARB-, SUPELCO) with 12 mL of acetonitrile.
    - Load concentrate from Step 20 onto the conditioned cartridge. Collect the eluate into a 125 mL concentration flask.

- Elute the cartridge with 10 mL of acetonitrile into the same 125 mL concentration flask.
- Roto-evaporate to dryness at 35 °C.

22. Reconstitute with 5.0 mL of ACN:water (1:1 v/v). Sonicate to dissolve.
23. Microfilterfuge if needed. Dilute filtered sample as necessary. Aliquot in GC vial.

M-1/M-25 in Cotton Meal

24. Transfer 80 mL of initial extract into 250mL concentration flask. Roto - evaporate to a small volume (less than 5 mL) at ~40° C. Ensure that the ACN has been removed.
25. Complete sample workup as per Steps 40-48 for Refined Oil.

B. Cotton Hulls Extraction Method - KIH-485/M-3/M-1/M-25

1. Weigh 15.0 grams of processed hulls into 250 mL plastic bottles.
2. Spike samples, as needed.
3. Add 60 mL of water, let sample stand for 15 minutes. Add 180 mL of ACN, mix well and let stand at room temperature for ~ 1 hour.
4. Place samples on wrist action shaker for 30 minutes and then sonicate for 15 minutes.
5. Vacuum filter the mixture through filter paper (Whatman GF/A) in a Büchner funnel fitted into 0.5L side-arm flask. Rinse the 250 mL bottle with 100 mL of ACN:water (3:1, v/v), rinse the filtercake with this solvent and combine all filtrates in 0.5L side-arm flask. Transfer quantitatively into clean 1000 mL separatory funnel.
6. Partition sample with 200 mL hexane for ~1 minute, allow layers to separate, discard hexane layer.
7. Collect ACN:water layer into a 500 mL graduated cylinder and adjust final volume to 360 mL with ACN:water (3:1, v/v).
8. Transfer extract to an amber bottle and mix well.
9. Divide 360 mL sample into 240 mL and 120 mL aliquots.

KIH-485 and M-3 in Hulls

10. Transfer 240 mL aliquot to a 500 mL concentration flask.
11. Concentrate by roto-evaporation to approximately ~60 mL at ~35° C. Ensure that all acetonitrile has been removed.

12. Transfer the concentrate quantitatively into a 250 mL separatory funnel, rinse 250 mL concentration flask with 80 mL of hexane and add to separatory funnel. Add 10 mL saturated NaCl and 20 mL of 0.5M $NaHCO_3$, and shake for 1 min, allow the layers separate.
13. Transfer lower water layer into an appropriate vessel.
14. Filter the upper hexane containing KIH-485 layer through ~30 g of anhydrous sodium sulfate supported by a plug of glass wool into a clean 250 mL concentration flask.
15. Return water layer to separatory funnel. Repeat hexane extraction one more time, combining organic extracts into the clean 250 mL concentration flask.
16. Return water layer to separatory funnel for M-3 extraction.
17. Concentrate the KIH-485 containing organic extracts (Step 15) using roto-evaporation at ~35°C to dryness.
18. Reconstitute KIH-485 residue in 10 mL of acetonitrile.
19. Add 20 mL of 0.5M citric acid to the aqueous layer (Step 16). Check pH to insure the aqueous solution is acidic (~pH 4). Partition with 100mL hexane:ethyl acetate (EtOAc) (3:7, v/v) for ~1 minute.
20. Transfer lower water layer into an appropriate vessel**.**
21. Filter the upper hexane:EtOAc layer through ~30 g of anhydrous sodium sulfate supported by a plug of glass wool into a clean 500 mL concentration flask.
22. Return water layer to separatory funnel. Repeat hexane:EtOAc extraction combining organic extracts into the same 500 mL concentration flask
23. Concentrate using roto-evaporation to dryness. Reconstitute M-3 residue in 10 ml of acetonitrile.
24. SPE clean-up
    - Condition SPE cartridge (12mL 1g ENVI-CARB-, SUPELCO) with 6 mL of acetonitrile.
    - Load concentrate from steps 18 onto the conditioned cartridge. Collect the eluate into a 125 mL round bottom flask.
    - Elute the cartridge with 10 mL of acetonitrile into the same 125 mL concentration flask.
    - Load concentrate from steps 23 onto another conditioned cartridge. Collect the eluate into a same 125 mL round bottom flask from above.

- Elute the cartridge with 10 mL of acetonitrile into the same 125 mL concentration flask.
- Roto-evaporate the combined extract to dryness at ~35°C

25. Reconstitute the residue in 5.0 mL of ACN:water (2:1, v/v).
26. Sonicate well and aliquot to GC vials for analysis.

M-1/M-25 in Hulls

27. Transfer 25 mL of initial extract into a 250 mL concentration flask. Roto-evaporate to a small volume (less than 5 mL) at ~40°C. Ensure that the ACN has been removed.
28. Transfer the concentrated aqueous extract quantitatively to 15 mL graduated centrifuge tubes. Rinse the 250 mL concentration flask with 5 mL ethyl acetate and transfer to the 15 mL graduated tube contacting the concentrated extract and partition gently for 1 minute. Centrifuge for 5 minutes at 1500 rpm
29. Discard the upper ethyl acetate phase.
30. Adjust to a final volume of 5 mL with HPLC grade water. Transfer to amber bottle.
31. Microfilterfuge as needed. Diluted filtered sample if necessary. Aliquot to TC vial for analysis.

C. Refined Oil Extraction Method - KIH-485/M-3/M-1/M-25

1. Weigh out 10.0g aliquots of oil into 125mL flat bottom flasks.
2. Spike if necessary.
3. Transfer oil aliquots to 250mL separatory funnels
4. Add 2 x 80 mL each ACN:H_2O (3:1;v/v) to 125mL flat bottom flasks, rinse well each time and transfer to the 250mL separatory funnels containing oil samples.
5. Gently shake funnels for 1 minute, let phases to separate, oil phase at bottom, ACN:H_2O phase on top (cloudy yellow).
6. Drain lower oil phase layer to the same 125 mL flat bottom flask, add 50 mL of ACN:H_2O (3:1, v/v), sonicate for 2 minutes.
7. Drain upper ACN:H_2O phase layer from separatory funnel to 250 mL flat bottom flask.
8. Transfer oil and ACN:H_2O mixture from step #6 back to the same 250 separatory funnels. Gently shake for 1 minute, let phases to separate,

centrifuge mixture in 250 mL plastic bottles at 2500 rpm for 5 minutes if emulsion had formed.

9. Drain bottom oil phase layer to 125 mL flat bottom flask, discard oil.
10. Drain upper ACN:H_2O phase layer to same 250 mL flat bottom flask from step #7.
11. Transfer combined ACN:H_2O phase to 500mL separatory funnels. Add 150mL of hexane to the 250 mL flat bottom flask, rinse well and add to the 500mL separatory funnels.
12. Gently shake for 1 minute, let phases to separate.
13. Drain lower ACN:H_2O layer to 250 mL graduated cylinders, discard upper hexane layer.
14. Adjust volume to 210 mL with ACN:H_2O (3:1;v/v), transfer to 250 mL amber bottles and mix well. Use 140mL of the extract for KIH-485/M-3 extraction and 70mL for M-1/M-25 extraction.

KIH-485 and M-3 in Oil

15. Transfer 140 mL of extract from step #14 to 500 mL concentration flask.
16. Roto-evaporate around 40ºC to ~50 mL and ACN is gone.
17. Transfer the extract to 250 mL separatory funnels, add 20 mL 0.5M $NaHCO_3$ and 10 mL saturated NaCl, combine in the separatory funnel.
18. Rinse concentration flask with 80 mL of hexane, combine in the separatory funnel.
19. Gently shake the separatory funnels for 1 minute, allow phases to separate.
20. Collect the lower aqueous phase back into the 500 mL concentration flask. Collect the upper hexane phase layer through a bed of Na_2SO_4 into a 250 mL concentration flask
21. Repeat steps #18-20, collecting the filtrate into the same 250 mL concentration flask. Retain aqueous phase for M3 extraction.
22. Roto-evaporate hexane extract from step #21 at 35ºC to dryness.
23. Reconstitute with 10 mL ACN. Sonicate until solid fully dissolved.
24. Pre-Condition SPE column. Envi-Carb, 1gm/12 mL with 10 mL of ACN.
25. Pass the entire 10 mL ACN sample solution through the pre-condition cartridge collecting into a concentration flask, typically 125 mL.
26. Elute with 10 mL of ACN into the same flask.

27. Add 20 mL 0.5M citric acid to the aqueous phase (to become ~pH4) from step #21.
28. Transfer aqueous phase to 250 mL separatory funnel.
29. Partition with 100 mL hexane:EtOAc (3:7, v/v) gently shake for 1 minute.
30. Collect the lower aqueous phase back into the container. Collect the upper hexane:EtOAc (3:7, v/v) though a bed of Na_2SO_4 into a 500 mL concentration flask.
31. Repeat steps #29 and #30, collect the organic phase to the same 500 mL concentration flask. Discard aqueous phase.
32. Roto-evaporate hexane:EtOAc extract from step #31 at 35ºC to dryness.
33. Reconstitute with 10 mL ACN. Sonicate until solid fully dissolved.
34. Pre-Condition SPE column. Envi-Carb, 1gm/12mL with 10 mL of ACN.
35. Pass the entire 10 mL ACN sample solution through the pre-condition cartridge collecting into a concentration flask from step #25 and 26.
36. Elute with 10 mL of ACN into the same flask.
37. Roto-evaporate KIH-485 and M-3 (now combined) cleaned-up extract at ~35ºC to dryness.
38. Re-constitute with 5 mL ACN:H_2O (2:1, v/v). Sonicate well and aliquot to GC vials for analysis. Transfer the rest of extracts into amber glass bottles.

M-1 and M-25 in Oil

39. Transfer 70 mL of initial extract from step #14 to 500mL concentration flask. Roto-evaporate to ~20mL at 40 °C.
40. Transfer to 250 mL sep. funnel. Add 10mL of pH 5 acetate buffer.
41. Partition with 40 mL of EtOAc (rinse concentration flask prior adding to sep. funnel). Drain lower aqueous layer to the same 500mL concentration flask. Discard the EtOAc layer.
42. Add 10 mL saturated NaCl to each flask.
43. Partition with 50 mL ACN, drain aqueous layer to the same flask. Drain ACN layer through ~40 g of sodium sulfate in glass funnel, supported with glass wool plug into clean 250mL concentration flask.
44. Repeat partition one more time.
45. Roto-evaporate ACN extract to dryness at ~40°C.
46. Reconstitute in 2 mL of ACN:water (1:9, v/v). Sonicate to dissolve any solid in the flask.

47. Microfilterfuge final extracts. Transfer to GC vial for analysis.
48. Transfer the rest of the extract to another set of GC-vial. Mark as unfiltered.

LC/MS/MS Analysis of KIH-485, M-3, M-1 and M-25

SCIEX API3200 Components (HPLC/Turbo Ion Spray Mode) or equivalent:

LC Pump	Agilent 1100 Series Binary Pump, Model G1312A
Autosampler	Agilent 1100 Series Autosampler, Model G1313A or G1329A
Vacuum Degasser	Agilent 1100 Series Vacuum Degasser, Model G1379A

Analysis of KIH-485, M-3, M-1 and M-25

Column: Magic C18, 150 mm x 2.0 mm, 5µ 100A
with Upchurch ODS pre-column (1 cm x 3.2mm)

Injection Volume: 10 µL

Solvent System and Gradient Program:

Solvent A = Water (0.05% formic acid)
Solvent B = Methanol (0.05% formic acid)

Flow Rate: 0.23 mL/minute

Solvent Program:

Minutes	Solvent A	Solvent B
0.00	90.0	10.0
5.00	90.0	10.0
20.00	0.0	100.0
24.00	0.0	100.0
25.00	90.0	10.0
31.00	90.0	10.0

Period 1 settings: Experiment 1:

Q1 Mass (amu)	Q3 Mass (amu)	Dwell (msec)	CE	CXP
295	163	200	-27.0	-15.0
295	181	200	-33.0	-16.5
309	195	200	-26.0	-22.0
309	259	200	-17.0	-22.0

259	165	200	-25.0	-15.0
259	215	200	-14.0	-18.0

Period 2 settings: Experiment 1:

Q1 Mass (amu)	Q3 Mass (amu)	Dwell (msec)	CE	CXP
392	229	200	27.0	6.0
392	179	200	45.0	5.0

Representative Mass Spectrometer Settings

	Period 1	Period 2
	API 3200	API 3200
Scan Type:	MRM	MRM
Polarity:	Negative	Positive
Ion Source:	Turbo Spray	Turbo Spray
CUR:	40.0	40.0
CAD:	7.00	7.0
GS1:	70.0	70.0
GS2:	70.0	70.0
IS:	-4500.0	5500.0
TEMP:	700.0	700.0
EP:	-6.0	5.0/6.0

Retention Times: KIH-485 at ~20 minutes for m/z 229 + m/z 179, M-3 at ~19 minutes for m/z 215+m/z 165, M-1 at ~17 minutes for m/z 195 + m/z 259 and M-25 at ~15 minutes for m/z 163 + m/z 181

Separation of the analyte was achieved by high performance liquid chromatography. The analytes were identified by the coincidence of their retention times with the reference standards, and quantitated by integration of the peak areas.

A typical injection sequence for samples within a sample set analyzed by LC-MS/MS was: check calibrant, check calibrant, 0.5 ng/mL calibrant, 1.0 ng/mL calibrant, 2.5 ng/mL calibrant, reagent blank, control sample, 5 ng/mL calibrant, fortified control, 10 ng/mL calibrant, fortified control, 25 ng/mL calibrant, treated sample, treated sample, 50 ng/mL calibrant, QC calibrant.

Statistical Methods

The residue data included the following statistical calculations: averages, standard deviations, relative standard deviations and linear regression analysis.

Limit of Quantitation

The limit of quantitation was assigned as the lowest fortification level of analyte validated by the residue method. The KIH-485/M-3, M-1 and M-25 residue analysis methods have limits of quantitation (LOQ) of 0.01 mg/kg (ppm) in cotton processed commodities. The method for KIH-485 and M-3 has a limit of detection of 0.00037 ppm (0.0005 µg/mL in solution). The method for M-1 and M-25 has a limit of detection of 0.00030 ppm (0.0005 µg/mL in solution).

METHODS OF CALCULATION

Preparation of Stock Standards

$$\text{Volume of solvent (mL)} = \frac{(W) \times (P)}{(FC)}$$

where W = Milligrams of neat standard
P = Chemical purity of neat standard
FC = Final Concentration (mg/mL)

Residue on Cotton Matrices

Linear regression formula for KIH-485 peak area, calibration curve y = mx + b
where y = peak area
x = ng/mL KIH-485 injected
m = Slope
b = Calibration intercept

The residue on treated cotton matrix was calculated as follows:

$$\text{ppm KIH-485 (mg/kg)} = \frac{\text{ng/mL KIH-485} \times \text{Final vol. (mL)} \times 0.001\ \mu\text{g / ng}}{\text{Representative Sample Wt. (g)}}$$

where

Representative Wt. (g) = initial wt. (g) x (portion of extract (mL)/ total extract volume (mL))

Recoveries – KIH-485

$$\% \text{ Recovery} = \frac{\text{KIH - 485 Residue Detected (ppm) - ppm Control}}{\text{KIH - 485 Fortification Level (ppm)}} \times 100$$

Validity of the KIH-485, M-3, M-1 and M-25 residue analytical methods was established by acceptable recovery (70 - 120%) from fortified untreated control samples. Residues of KIH-485, M-3, M-1 and M-25 (ppm) in treated samples were calculated as for the fortified samples, without control subtraction.

An example calculation for the KIH-485 residue in the cotton refined oil (0.01 ppm) is shown below:

Linear regression analysis of the KIH-485 standards gave a curve with the equation x = (y – 423.96452189) ÷ 13205.884149 (r^2 = 0.9997). The ng/mL KIH-485 injected determined by this curve was:

ng/mL KIH-485 injected = [(145,213.4868 – 423.96452189) ÷ 13205.884149] = 11.0 ng/mL

$$\text{KIH-485 ppm (mg/kg)} = \frac{11.0 \text{ ng/mL} \times 5 \text{ mL} \times 0.001 \mu\text{g/ng}}{6.67 \text{ g}} = 0.008 \text{ ppm}$$

Where 6.67 g represents 140 mL of a 210 mL total extract of 10 grams of oil.

$$\text{Percent KIH-485 Recovery} = \frac{0.008 - 0 \text{ ppm}}{0.01 \text{ppm}} \times 100 = 80\%$$

Similar calculations were carried out for residue determination of M-1, M-3 and M-25 in cotton matrices.