APPENDIX B

NORTH COAST LABORATORIES LTD

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	NAME	TITLE	DATE
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1.0 TITLE

Procedures for the extraction of VITAVAX, VITAVAX SULFOXIDE, and VITAVAX SULFONE (PLANTVAX) from soil; separation of components by HPLC; on-line, post-column photolysis and derivatization of the three analytes; and, subsequent determination of the analytes by fluorescence detection.

2.0 SCOPE

To set forth detailed procedures for the analysis of VITAVAX, VITAVAX SULFOXIDE, and VITAVAX SULFONE as individual analytes.

3.0 PURPOSE

To insure that all analyses of soils for VITAVAX, VITAVAX SULFOXIDE, and VITAVAX SULFONE are carried out in a consistant manner at NCL.

4.0 DEFINITIONS

4.1 On-Line, Post-Column Reactors: Chemical reactors which are located within a continuous system between the HPLC column and the detector.

4.2 Knitted Open Tubular Photochemical Reactor: Wrapped teflon tubing that carries the HPLC effluent around a pencil-lamp type UV source (mercury).

4.3 Packed-Bed, Single Bead String Reactor: Teflon tube which is packed with glass beads of slightly smaller diameter than the inside diameter of the tube. Packed-bed reactors delay the HPLC effluent and mix it with a reagent(s), when proper connections and pumping systems are supplied.

4.4 Fluorophor: Refers to the fluorescent derivatization product, which is detected by the system, in this case that of an amine and the reagent, Fluorescamine.

5.0 MATERIALS

5.1 Materials required for the extraction of VITAVAX, VITAVAX SULFOXIDE, and VITAVAX SULFONE from soils.

5.1.1 # 10 sieve.
5.1.2 Top loading balance capable of weighing to at least 0.1g.
5.1.3 Centrifuge tubes capable of holding 20g soil and 25 ml of acetone.
5.1.4 Wrist arm shaker.
5.1.5 Centrifuge capable of spinning tubes from section 5.1.3 to produce a clear soil extract.
5.1.6 25 ml graduated cylinders.
5.1.7 500 ml separatory funnels.
5.1.8 Separatory funnel rack.
5.1.9 Glass 60 degree funnels for 15 cm filter paper.
5.1.10 15 cm filter paper, grade #54 or equivalent.
5.1.11 250 ml boiling flasks.
5.1.12 50 ml & 25 ml Tilt-A-Pet repeating pipets.
5.1.13 Rotary evaporator capable of evaporating an acetone/methylene chloride solution.
5.1.14 25 ul, 100 ul and 2500 ul syringes.
5.1.15 Disposable, 3 cc, luer-lok syringes.
5.1.16 0.2 um syringe-tip filter.
5.1.17 1.5 ml or other suitable amber storage vials for final extract.
5.1.18 Pastuer pipets.
5.1.19 Several black plastic bags to use as light shields.

5.2 Materials required for on-line, post-column photolysis and derivatization of VITAVAX, VITAVAX SULFOXIDE, and VITAVAX SULFONE. (See Exhibit B for diagram of system.)

5.2.1 Dionex post-column dual reagent delivery system.
5.2.2 PTFE tubing, 1/16" O.D. x 0.03 or 0.02" I.D. for the wrap-around photolysis reactor.
5.2.3 Valco 0.03" connecting "T"s.
5.2.4 UV lamp. We use an Analamp 2 watt, Hg, low UV pencil lamp.
5.2.5 UV lamp power source.
5.2.6 Squirrel-cage fan, with variable speed control, to cool photolysis reactor.
5.2.7 Photolysis reactor cooling and UV protection chamber. We use 1 1/2" ABS pipe, with a thermometer to monitor the temperature.
5.2.8 Aluminum foil.

5.3 Materials required for HPLC analysis of VITAVAX, VITAVAX SULFOXIDE, and VITAVAX SULFONE.

5.3.1 HPLC,capable of ternary gradients, equipped with a C18 Econosphere, 3 um, 5 cm column in series with a C8 column of the same type and size, a Fluorichrom detector,an autosampler, and a data system capable of peak identification and quantification.

5.4 Chemicals required for the extraction of VITAVAX, VITAVAX SULFOXIDE, and VITAVAX SULFONE from soil.

5.4.1 Pesticide grade acetone.
5.4.2 Dithiothreitol. (10,000 ppm in methanol.)
5.4.3 Deionized water.
5.4.4 Pesticide grade methylene chloride.
5.4.5 Furnaced sodium sulfate.
5.4.6 HPLC grade water.
5.4.7 HPLC grade methanol.

5.5 Chemicals required for on-line, post-column reactions of VITAVAX, VITAVAX SULFOXIDE, and VITAVAX SULFONE.

5.5.1 HPLC grade water.
5.5.2 HPLC grade methanol.
5.5.3 HPLC grade dimethyl sulfoxide.
5.5.4 HPLC grade acetonitrile.
5.5.5 Fluorescamine.

5.6 Chemicals required for HPLC analysis.

5.6.1 HPLC grade water.
5.6.2 HPLC grade methanol.
5.6.3 HPLC grade acetonitrile.
5.6.4 Tetramethylammonium Acetate.

5.7 Chemicals required for the manufacture of standards.

5.7.1 VITAVAX neat standard, 99+ %. Available from Uniroyal Corp. Naugatuk, Conn.
5.7.2 VITAVAX SULFOXIDE neat standard, 94+ %. Available from Uniroyal Corp. Naugatuk, Conn.
5.7.3 VITAVAX SULFONE (PLANTVAX) neat standard, 99+%. Available from Uniroyal Corp. Naugatuk, Conn.
5.7.4 Chemicals required for the manufacture of stock standards.
5.7.4.1 Neat standard.
5.7.4.2 HPLC grade methanol.

5.7.5 Chemicals required for the manufacture of working standards.
5.7.5.1 Stock standard.
5.7.5.2 HPLC grade water.
5.7.5.3 HPLC grade methanol.
5.7.5.4 HPLC grade acetonitrile.

6.0 PROCEDURE

6.1 Flow chart of method. (See Exhibit A).

6.2 Procedure for the extraction of VITAVAX, VITAVAX SULFOXIDE, and VITAVAX SULFONE from soil.

6.2.1 Thaw soils in the dark. (VITAVAX and VITAVAX SULFOXIDE are susceptible to photooxidation.)

6.2.2 Sieve soil through #10 sieve.
6.2.3 Weigh out a 20g aliquot to the nearest 0.1g into a suitable centrifuge tube, see sec. 5.1.3 of this SOP.
6.2.4 Spike soil sample in centrifuge tube at this time if required. See sec. 6.
6.2.5 Add 25 ml pesticide grade acetone and 0.1 ml dithiothreitol (DTT).
6.2.6 Attach centrifuge tubes to wrist arm shaker clamps in a horizontal position. Cover shaker W/ black plastic.
6.2.7 Set shaker on medium ("5") shaking action.
6.2.8 Set shaker timer for 30 minutes.
6.2.9 After end of shaking period, place the centrifuge tubes in the centrifuge and spin at a suitable speed/time combination to yield a clear supernatent. For the Sorval centrifuge, set on "16" and take up to 12K RPM. Spin 5 minutes. This is sufficient to settle the soil suspension. Place 8 tubes at a time in the centrifuge and spin them while decanting the previous 4 tubes. CAUTION: DO NOT leave centrifuge unattended when set above "12". DO NOT allow RPM to exceed 12K at any time.
6.2.10 Pipet 20 ml of the supernatent into a 500 ml sep. funnel using 4 aliquots from a 5 ml macropipet.
6.2.11 NOTE: Drape black plastic over funnels to protect from UV.
6.2.12 Repeat steps 6.2.5 to 6.2.10, except add 20 ml acetone, no DTT, and shake for 20 min.
6.2.13 Repeat steps 6.2.5 to 6.2.10, except add 20 ml acetone, no DTT, and shake for 10 min.
6.2.13.1 Note:Total volume of supernatent in sep. funnel= 60 ml, not counting rinses. 60 ml out of 65 ml acetone added initially gives a dilution factor of 1:1.1 which must be taken into consideration on peak quantification.
6.2.14 Add 50 ml of deionized water to the supernatent.
6.2.15 Extract one time with 50 ml of methylene chloride.
6.2.15.1 Transfer acetone/methylene chloride phase (bottom layer) to a 250 ml boiling flask through about 2 teaspoons of furnaced sodium sulfate contained in a 60 degree funnel lined with the filter paper from sec. 5.1.10 in this SOP. NOTE: protect flasks from UV using black plastic.
6.2.16 Extract <u>two</u> more times with 25 ml of methylene chloride.
6.2.16.1 Transfer methylene chloride phase to 250 ml boiling flask through sodium sulfate as before.
6.2.16.2 After last 25 ml extraction rinse the sodium sulfate with about 10 ml methylene chloride.

6.2.17 Rotary evaporate the methylene chloride/acetone extract just to dryness @ 40° C and maximum vacuum. Note: Drape black plastic sheeting over rotery evaporator to protect from light.
6.2.17.1 Blow off any remaining vapors with N_2.
6.2.18 Add 1.50 ml 5:4:1, HPLC water: HPLC methanol: HPLC acetonitrile. Cap flask w/ $ stopper to prevent evaporation. swirl vigorously in 40 degree C waterbath 30 sec. Allow to sit at room temp. in dark a few (3 or 4) minutes to allow the contents of the flask to cool and settle.

6.2.19 Using a pastuer pipet, transfer the final extract to a 3 cc disposable syringe fitted with a 0.2 um syringe-tip filter.

6.2.19.1 Filter the final extract into an amber storage vial.

6.2.20 If final extract is unable to be analyzed immediately, store the vials in the freezer in the dark. **NOTE: Vitavax appears to easly photooxidize to the Sulfoxide upon exposure to light. Protect from light as much as possible.**

6.3 Manufacture of standards and spikes.

6.3.1 Manufacture of stock standards.

6.3.1.1 1,000 ppm primary stock standards for each component.

6.3.1.1.1 VITAVAX

6.3.1.1.1.1 Weigh out 0.0100g of the neat standard into a 10 ml volumetric flask.

6.3.1.1.1.2 Bring flask to volume with HPLC grade methanol. Cap flask and invert at least 5 times to mix.

6.3.1.1.2 VITAVAX SULFOXIDE

6.3.1.1.2.1 Follow procedures from 6.3.1.1.1, except weigh out 0.0106g because of the purity of the neat.

6.3.1.1.3 VITAVAX SULFONE (PLANTVAX)

6.3.1.1.3.1 Follow exactly the procedures from 6.3.1.1.1. **NOTE: Sections 6.3.1.2 and 6.3.1.3 describe manufacture of single component standards used for storage stability study. <u>STANDARDS USED FOR ACTUAL SAMPLE RUNS CONTAIN ALL THREE COMPONENTS IN A SINGLE MIXED STANDARD</u>. To manufacture the 100 PPM 3-component mix proceed exactly as per section 6.3.1.2 this SOP except add 1.0 ml. of each of the three primary standards to the 10 ml volumetric flask. To manufacture the 10PPM 3-component standard proceed exactly as per section 6.3.1.3 this SOP except use 1.0 ml of the 100 PPM 3-component standard rather than one of the single component standards. NOTE: Section 6.3.2 below describes the manufacture of single component working standards. To manufacture comparable values of 3 component working standards proceed exactly as per the directions in section 6.3.2 except use the 10 PPM mixed tertiary standard or the 100PPM mixed secondary standard in place of single component tertiary or secondary standards.**

6.3.1.2 100 ppm secondary stock standards for each component.
6.3.1.2.1 Using a 1.0 ml syringe, measure out 1.0 ml of the primary stock standard into a 10 ml volumetric flask and bring up to volume with methanol.
6.3.1.2.2 Cap flask and invert at least 5 times to mix.

6.3.1.3 10 ppm tertiary stock standards for each component.
6.3.1.3.1 Follow the procedures from sec. 6.3.1.2, except use 1.0 ml of the secondary stock standard rather than 1.0 ml of the primary.

6.3.2 Manufacture of working standards.

6.3.2.1 0.02 ppm working standards for each component.
6.3.2.1.1 Using a 100 ul syringe, measure out 40 ul of the tertiary stock standard into 1460 ul of 5:4:1, HPLC H_2O: HPLC CH_3OH: HPLC CH_3CN in a 1.5 ml amber vial.

6.3.2.2 0.1 ppm working standards for each component.
6.3.2.2.1 Using a 25 ul syringe, measure out 20 ul of the secondary stock standard into 1480 ul of 5:4:1, HPLC H_2O: HPLC CH_3OH: HPLC CH_3CN in a1.5 ml amber vial.

6.3.2.3 0.5 ppm working standards for each component.
6.3.2.3.1 Using a 10 ul syringe, measure out 10 ul of the primary stock standard into 1490 ul of 5:4:1, HPLC H_2O: HPLC CH_3OH: HPLC CH_3CN in a 1.5 ml amber vial.
NOTE: Section 6.3.3 below describes the manufacture of single component spikes for the storage stability study. SPIKES USED FOR ACTUAL SAMPLE RUNS CONTAIN ALL 3-COMPOMENTS. Manufacture spikes for sample runs exactly as directed in section 6.3.3.3 except use the appropriate 3-component 10 PPM tertiary or 100 PPM 3-component secondary standards in place of the single component tertiary and secondary standards.
NOTE:1. All spikes for actual sample runs will be at the 0.1 PPM level. 2. Two spikes per sample run will be prepared. 3. Three blanks per sample run will be prepared. 4. Blank values for the sulfoxide will be averaged and subtracted from any sulfoxide values found in a run.

6.3.3 Manufacture of spikes for 20g soil samples.
6.3.3.1 Spikes are to be of individual analytes onto separate 20 g soil samples.
6.3.3.2 0.02 ppm spike for each component.
6.3.3.2.1 Using a 100 ul syringe, measure out 40 ul of the tertiary (10 ppm) stock standard onto 20g soil sample in centrifuge tube.
6.3.3.3 0.1 ppm spike for each component.
6.3.3.3.1 Using a 20 ul syringe, measure out 20 ul of the secondary (100 ppm) stock standard onto 20g soil sample in centrifuge tube.

6.3.3.4 0.5 ppm spike for each component.
6.3.3.3.1 Using a 10 ul syringe, measure out 10 ul of the primary (1000 ppm) stock standard onto 20g soil sample in centrifuge tube.

6.4 On-line, Post-column Reactions Conditions.

6.4.1 Post-column Photolysis. See Exhibit B.
6.4.1.1 Pump: Pump number 2 of the Dionex dual reagent pump.
6.4.1.2 Reagent: 2:1:1, DMSO: H_2O: CH_3OH (photosensitizer).
6.4.1.3 Flow rate: 0.05 ml/min.
6.4.1.4 Reactor type: Open tube, knitted made of PTFE tubing from sec. 5.2.2 in this SOP.
6.4.1.5 Lamp:Analamp 2 watt, Hg, low wave UV pencil lamp, 1x18cm.
6.4.1.6 Lamp power source: Analamp power supply, model number 90-0001-01.
6.4.1.7 Reactor temperature range: 28-35° C. Note: Temperatures above 35° C will cause rapid breakdown of the PTFE reactor and bubble formation in the mobile phase.

6.4.2 Post-column Derivitization. See Exhibit B.
6.4.2.1 Pump: Pump number 1 of the Dionex dual reagent pump.
6.4.2.2 Reagent: 0.5g/L Fluorescamine in 4:1, CH_3CN: DMSO.
6.4.2.3 Flow rate: 0.20 ml/min.
6.4.2.4 Reactor type: Packed-bed, single bead string reactor.

6.4.3 Post-detection Hardware
6.4.3.1 Backpressure Regulator: 100 psi cartridge type.

6.5 Instrumental Analysis Conditions.

6.5.1 Instrument: OR-LC-1, a Varian 5020 Liquid Chromatograph, modified for ternary gradient capability. See SOP LE005 for general operational description.
6.5.2 Method Name: Vitavax
6.5.3 Method File: PJ20-003.MET
6.5.4 Run Time: 35 minutes.
6.5.5 Mobil Phase: A= H_2O, B= CH_3OH, C= CH_3CN + 0.1% TMA.OAc
6.5.6 Gradient: %ABC=85, 10, 5 - 45, 50, 5/30 min.
6.5.7 Flow Rate: 1.0 ml/min., LC program #1.
6.5.8 Loop Size: 75 ul.
6.5.9 Columns: C18 Econosphere cartridge, 3 um, 5 cm, Ordered from Alltech, stock number 71020, in series with, C8 Econsphere cartridge, 3 um, 5 cm, Ordered from Alltech,stock number 71023.
6.5.10 Col. Temp.: Ambient.
6.5.11 P max.: 350 atm.
6.5.12 P min.: 2 atm.
6.5.13 Detector: Varian Fluorichrom with filter wavelength selection and equipped with a Tungsten source lamp.
6.5.14 Det. Gain: Hi.
6.5.15 Det. Lamp: Hi.
6.5.16 Filters: CS 5-58 (360-460 nm) excitation, CS 3-70 (≥500 nm) emmision.

EXHIBIT A

SAMPLES AND EXTRACTS MUST BE PROTECTED FROM LIGHT TO PREVENT PHOTODEGRADATION OF VITAVAX.

Thaw soil

Sieve & weigh out 20g aliquots

spike

Add 25 ml acetone & 0.1 ml DTT

Shake for 30 min.

Shake 20 & 10 min. respectively

Centrifuge & decant 20 ml

Transfer to sep. fun. w/ 1x 2 ml acetone rinse of grad. cyl.

Total acetone extract= 60 ml

(NO) → Extract soil again, except add 20 ml acetone & no DTT

(YES)

Add 50 ml DI

Extract 1x w/ 50 ml CH_2Cl_2 and 2x w/ 25 ml. CH_2Cl_2 & acetone phase to boiling flask thru Na_2SO_4.

Rinse Na_2SO_4 w/about 10 ml CH_2Cl_2

Rotary evap. to dryness.

Add 1.5 ml 5:4:1, H_2O: CH_3OH: CH_3CN

Heat & swirl flask

Filter into amber vial

HPLC separation of analytes

DMSO Sensitization

Post-col. photolysis

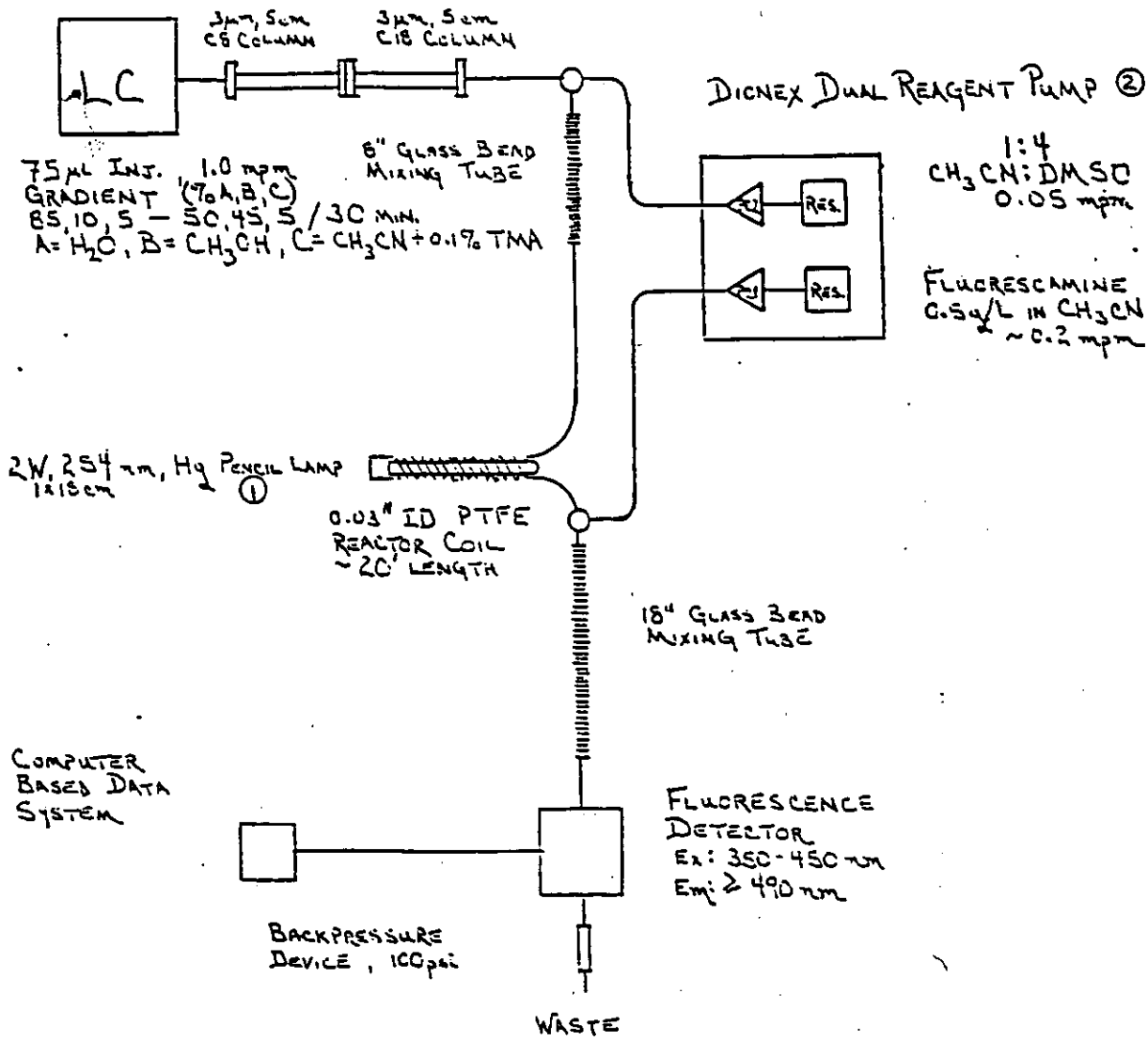
Post-col. derivitization

Fluorescence detection

END

SOP CODE VERSION
ME 025 02

EXHIBIT B



① See SOP
② See SOP

FOR GENERAL DESCRIPTION, PREP. AND OPERATIONAL INSTRUCTIONS FOR REACTOR.
FOR GEN. DESCRIPTION, PREP. & OPERATIONAL INSTRUCTIONS FOR REAGENT PUMP.

APPENDIX C

VITAVAX, VITAVAX SULFOXIDE AND PLANTVAX IN SOIL WORK-UP PROCEDURE

A 55 g sample is accurately weighted out and placed in a 800 ml beaker. 250 ml of methanol is added and shaken for 30 minutes using a Lab-Line shaker. The mixture is filtered through a 0.2 millipore teflon filter into a 500 ml round bottom flask. The solvent is removed using a rotary evaporator at 30°C. The residue is transferred into a 20 ml syringe by washing the flask with 3x 5 ml methanol. The combined washing is filtered through a C18 Sepak into a 50 ml pear shape flask. The solvent is removed using a rotary evaporator at 30°C. The residue is transferred into a 1 ml volumetric flask by washing the flask with small portions of methanol. The solution is analysed by HPLC.

Instrumentation:

Hatachi HPLC system equipped with two channel photodiode array detector and autosampler.

Column: Em Science Lichrosorb RP 18 (10 Micro) 25 cm x 4mm

Detector: uv at 0.25 aufs
Charnel one: 254 nm Attenuation 4
Channel two: 300 nm Attenuation 3

Solvent: 20% acetronitrile, 10% methnaol and 70% water
Flow rate: 1.5 ml per minute
Injection volume: 100 kmicroliter

After 30 minutes at the above solvent condition, the column is cleaned with methanol at the flow rate of 1.5 ml/minute for 25 minutes and reequilibrated for 10 minutes prior to the next sample injection.

Each sample is analysed twice, and the average value is reported.

A methanol blank is injected after the completion of each sample analysis for column clean out purposes.

APPENDIX D: EXAMPLE CALCULATIONS

Values on chromatograms are in PPM (ug/g) in the soil and are determined by comparison of areas under analyte peaks to areas under the standard peaks.

Values are calculated as follows:

1. Soil sample size: 20g
   Detection limit: 0.01 PPM (ug/g)
   Therefore the total amount of analyte to be detected in 20 g of sample is: (0.01 ug/g) (20 g) = 0.2 ug
2. Spikes - 20 ul of a 100 ng/ul mixed analyte standard is added to 20 g blank soil. and carried through the method. This is a ten times detection limit spike.
   (20 ul)(100 ng/ul)(1/20g) = 100 ng/g = 0.10 ug/g.
   Other values of spikes may be prepared by using the above described method, and adjusting the amount of standard used to an appropriate value.
3. Standards - See section 6.3 of method SOP (appendix B). for manufacture of standards.
4. Dilution - It should be noted that each sample, blank, and spike are diluted by a factor of 1.083. This dilution factor is automatically taken into account by the chromatography system, and may be seen on all sample, blank, and spike chromatograms. This factor arises because only 60 ml of the total extraction volume of 65 ml is removed from the soil samples. This leaves a dilution factor of 65/60 = 1.083 to account for. The reason for leaving 5 ml out of the 25 ml used for the first extraction in contact with the soil is to lessen the possibility of picking up some soil while pipeting off extractant after each extraction.
5. Sample Calculation - As an example of calculations utilized for this study NCL sample # 8901009-25A will be used. Vitavax sulfoxide is selected as the example analyte. All other analytes are calculated in a similar manner.
   The PPM in the soil (corrected for the dilution factor) is read off the chromatogram. and entered into the "uncorrected PPM" box on the raw data sheet. The reason it is entered into the "uncorrected PPM" box is that the result has yet to be corrected for any blank values which may occur. In this example, the uncorrected PPM value is 0.2066. Next, the blank value (in PPM),which is an average of three soil blanks which were run with each sample run, is entered in the raw data sheet column marked "blank PPM". For this run the average blank value is 0.0113 PPM. The blank value is subtracted from the "uncorrected PPM" and entered into the raw data sheet in the "corrected PPM" column. In this example the corrected PPM is: 0.2066 - 0.0113 = 0.1953.
   NOTE: the dilution factor on the raw data sheet is listed as "1" because it has automatically been taken into account by the chromatography data system, (or in the case of the method proof, the dilution factor was taken into account in the manufacture of the standards).

APPENDIX D: EXAMPLE CALCULATIONS

The % moisture is taken from the "Vitavax as aniline" workbook. The "correction factor, % moisture" is calculated as follows: (20 g) (0.112) = 2.24 g. This represents the weight of water in a 20 g soil sample. The weight of soil is then 20 g - 2.24 g = 17.76 g. The correction factor % moisture represents the number by which the corrected PPM must be multiplied to express the PPM analyte on a dry weight basis. In this case the correction factor,% moisture is: 20 g/17.76 g = 1.126. The PPM analyte on a dry weight basis is:(0.1953)(1.126) = 0.2199. This number is rounded to 3 significant figures and entered into the "reported PPM" column on the raw data sheet. This is the reported result for the analyte vitavax sulfoxide for NCL sample # 8901009-05a.