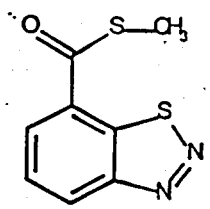
CGA 245704	RESIDUE METHOD VALIDATED	REM 172.08
DETERMINATION OF THE METABOLITE CGA 210007 BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)	SOIL	Januar 15, 1995 PP 2.533/Fo

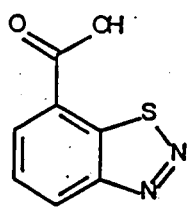
TABLE OF CONTENTS

CHEMICAL STRUCTURES

CGA 245704



CGA 210007



1. INTRODUCTION

1.1 Scope of the Method

The method allows the quantitative determination of the unchanged metabolite CGA 210007 in soil (See section 6.1, Figure 1 for structure and chemical name).
The lower practical level of quantitation by this method is 0.004 mg/kg of CGA 210007.

1.2 Principle of the Method

CGA 210007 is extracted by shaking a homogenized subsample of soil with a mixture of buffer solution pH 8 and methanol. An aliquot of the extract is diluted with brine and water and cleaned up by partition with a solvent mixture of hexane + tert-buthyl methyl ether 7 vol. + 3 vol. The organic phase which could contain CGA 245704 is discarded. The remaining solution is acidified and CGA 210007 is partitioned into the same solvent mixture as used in the first cleanup step. The final determination is performed by HPLC using a two-column switching system and UV-detection.

2. MATERIALS AND METHODS

Standard laboratory equipment is not listed. All equipment and chemicals mentioned herein can be substituted by suitable products of any origin. Prove suitability of reagents by analyzing reagent blanks.

2.1 Equipment

2.1.1 Lab-shaker, A. Kühner AG, 4000 Basel, CH.
2.1.2 Centrifuge, Megafuge 3.0 R, Heraeus Instruments GmbH Labortechnik, D-63405 Hanau.
2.1.3 Rotating evaporator, Büchi, Rotavapor RE, Büchi AG, Flawil, CH.
2.1.4 Vacuum system, Temo Duo 500 S, Temo AG, Küblis, CH or commercial water aspirator.
2.1.5 Circulation cooler, mgw Lauda WK 450, Messgeräte-Werke-Lauda, Dr. R. Wobser KG, Lauda-Königshofen, FRG, Cat. No. 7329-06.
2.1.6 Ultrasonic bath, Bransonic 220, Branson Cleaning Equipment Co., Parrot Drive, Shelton, CT 06484-0768, USA, represented in Switzerland by Dr. Bender & Hobein, Zürich.
2.1.7 High Performance Liquid Chromatograph: refer to section 2.4.

2.2 Reagents and Standards

Main suppliers' addresses: E. Merck AG, 6100 Darmstadt, FRG.
Fluka Chemie AG, 9470 Buchs, CH.
J. T. Baker Inc., Phillipsburg, N.J. 08864, USA.

2.2.1 Water, HPLC-grade, J. T. Baker Inc., Cat. No. 4218 or prepared in house, Ciba.
2.2.2 Phosphate buffer solution pH 8, Ciba: 0.063 mole of Na_2HPO_4 + 0.004 mole of KH_2PO_4 per liter water.
2.2.3 Methanol for extraction, Merck, Cat. No.6018 or distilled in house, Ciba.
2.2.4 Sodium chloride, analytical grade, Merck, Cat. 6404. Prepare a saturated solution of sodium chloride in water for HPLC (brine).
2.2.5 n-Hexane for residue analysis, Merck, Cat. No. 4371.
2.2.6 tert-Butyl methyl ether (TBME), puriss., analytical grade, Fluka, Cat. No. 20249.
2.2.7 Acetonitrile, LiChrosolv, chromatography grade, Merck, Cat. No. 14291.
2.2.8 ortho-Phosphoric acid, 85%, analytical grade, E. Merck, Cat. No. 573. Prepare solutions containing about 0.02 mole and 0.05 mole of phosphoric acid per liter HPLC water by diluting 2.3 g and 5.75 g concentrated acid, respectively, to 1 liter with water for HPLC.
2.2.9 Ammonium sulfate, analytical grade, Merck, Cat. No. 1217. Prepare a 0.05 M solution of ammonium sulfate by dissolving 6.6 g salt in one liter of 0.05 M phosphoric acid

2.2.10CGA 210007 reference substance for standardization. Prepare a stock solution containing 200 µg CGA 210007/mL acetonitrile LiChrosolv.

2.3 Analytical Procedure

2.3.1 Preparation of Sample

Remove big stones from soil samples. Homogenize laboratory samples (1 kg or more) by following suitable procedures. Determine the content of dry matter (e.g. dry a subsample of about 2g to constant weight at about 160 °C). Analyze the samples immediately after preparation or store them at about -20 °C until analysis .

For analysis, weigh a subsample corresponding to 15 g dry soil into a 100 mL centrifuge glass tube (e.g. weigh 18.1 g for a sample having a content of 82.9% dry matter.

2.3.2 Fortification

To regularly check the performance of the method, analyze also at least two fortified control samples with each series of analyses. To prepare these samples, add known amounts of CGA 210007 to control samples prior to extraction.

Select fortification levels to be one and ten times the lower practical level (limit of determination) or in the range of the expected residue levels. Make sure that control samples neither are contaminated nor show interfering signals.

To fortify samples with 0.004 and 0.04 mg/kg, prepare solutions of CGA 210007 containing 0.06 and 0.6 µg/mL in 0.02 M phosphoric acid, respectively, by appropriate dilution of the stock solution (cf. section 2.2.10).

To prepare fortified samples, add 1 mL of one of the solutions to an uncontaminated control sample. Proceed as described in section 2.3.3.

2.3.3 Extraction

Add 5 mL of phosphate buffer pH 8 to the sample. Add as much distilled methanol as to achieve a total volume of 60 mL taking into account the soil moisture content (e.g. for a sample having a content of 83.3% dry matter, weigh 18 g wet soil, add 5 mL of phosphat buffer pH 8 and 52 mL of distilled methanol, or 51 mL for fortified samples taking into account the solution of CGA 210007 added). Shake the tightly sealed glass tube for about 30 minutes (total volume of the extract: 60 mL) . Centrifuge the extract for about 3 min. by 2890 g (using the previously mentioned type of centrifuge, this value is reached at about 3500 rotations / min.)

2.3.4 Cleanup by Partition

Transfer 20 mL of the clear extract, corresponding to 5 g dry soil, to a 100 mL graduated cylinder with stopper. Add 4 mL of saturated sodium chloride solution and 50 mL of dest. water. Subsequently, add 15 ml of a mixture of n-hexane + TBME 7 vol. + 3 vol. (solvent mixture A) to the aqueous solution. Shake well for about 30 sec. Allow phases to separate and discard the organic phase by suction using a pipette and vacuum. Repeat the partition two more times with 15 mL of mixture A, each.

2.3.5 Cleanup by Acidic Partition

Add a 1 mL of 85% phosphoric acid and 25 mL of the solvent mixture A to the remaining aqueous phase (see section 2.3.4). Shake well for about 30 sec. and allow phases to separate. Transfer the organic phase into a 100 mL round bottom flask by means of a pipette. Repeat the partition two more times, with 25 mL of solvent mixture A, each. Each time transfer the organic phase into the same round bottom flask. Discard the aqueous phase.

2.3.6 Preparation of the Final Solution

Evaporate the combined organic phases (see section 2.3.5) just to dryness under reduced pressure using a rotating evaporator (water bath temp.: about 45°C). Dissolve the residue in 5 mL of 0.02 M phosphoric acid in HPLC water (final solution), using an ultrasonic bath.For quantitation, inject 50 µL of this solution into the chromatographic system.

2.4 Instrumentation

2.4.1 High Performance Liquid Chromatographic Systems (HPLC)

2.4.1.1 Determination with a HPLC Two-column Switching System

For the determination of CGA 210007, use a HPLC two-column switching system with UV-detector, pumps, autosampler-injector and columns as follows (all specified modules can be substituded by suitable equivalent ones):

Detector:	Spectroflow 783 (ABI Analytical Kratos Division, Ramsey, NJ07446, USA.
Pumps:	Two Shimadzu Solvent Delivery Modules LC-9A (Shimadzu Corporation, Analytical Instruments Division, Kyoto, Japan).
Valves:	Multiport Streamswitch "MUST" equipped with two low dead volume valves for column switching (Spark Holland, 7800 AJ Emmen, Holland) in connection with an automatic injection unit PROMIS II (Spark Holland).
Recorder:	Strip chart SE 120, dual channel (ABB, Goerz Metrawatt, 1101 Vienna, A).
Column oven:	Modell STH 585. Temperature range: 5 - 80 °C, Gynkotek GmbH
Remark:	A column oven is absolutely necessary in non thermostated laboratories to ensure constant retention times and a reproducible cut and transfer of the fraction containing the analyte (see below "Determination of Switching Interval").
Column 1:	Stainless steel tube, 10 cm length, 2 mm i.d., packed with Nucleosil SB (strongly basic anion exchanger), particle size 5 µm (Dr. H. Knauer KG, 6370 Oberursel, FRG, Cat. No.: B45 - Y91).
Remark:	A new column must be conditioned with the mobile phase 1 (see below) for at least 8 hours at a flow rate of 0.2 mL/min in order to obtain reproducible column performance
Column 2:	Stainless steel tube, 10 cm length, 2 mm i.d., packed with Nucleosil 100 C18 particle size 5 µm (Dr. H. Knauer KG, Cat. No.: B45 - Y76).
	For the HPLC system, connect pumps, injector, columns, valves and detector according to Figure 3 (see section 6.1).
Mobile phase 1:	0.05 M ammonium sulfate in 0.05 M phosphoric acid + acetonitrile LiChrosolv 85 vol. + 15 vol.
Mobile phase 2:	0.05 M phosphoric acid in water for HPLC + acetonitrile LiChrosolv 76 vol. + 24 vol.
Flow rate 1:	0.2 mL/min.
Retention time:	about 8.5 min. (column 1)

Flow rate 2:	0.2 mL/min.
Retention time:	about 17 min. (columns 1 and 2)
Injection vol.:	50 µL
Recorder:	10 mV full scale
Column oven:	23 °C
Chart speed:	0.5 - 1.0 cm/min.
Detector:	Wave length: 235 nm sensitivity: 0.005 aufs
Operation:	Operate the system manually or, preferably, automate it with a control unit (e.g. HP 3350 Laboratory Automation System, Hewlett-Packard)

Determination of Switching Interval

Determine actual switching times each time a series of samples is to be quantitated. Connect the outlet of column 1 directly to the UV-detector. Inject 50 µL of a 0.08 µg CGA 210007/mL standard solution (see section 2.4.2) and measure the begin and the end times of the peak of CGA 210007. Thereafter, connect column 2 to the detector and transfer that portion of eluate 1 which contains CGA 210007, e.g. portion between 7 min and 9 min from column 1 to column 2 (see section 6.2, page 12 for an example of measurement of switching times).

2.4.1.2 Alternative Chromatographic System

Residues of CGA 210007 may be confirmed by an alternative HPLC two-column switching system using a different column 1 and different mobile phases. For the determination of CGA 210007 by this system, use a HPLC two-column switching system with UV-detector, pumps and autosampler/injector as described in section 2.4.1.1. Columns and condition are as follows:

Column 1:	Stainless steel tube, 10 cm length, 2 mm i.d., packed with Nucleosil NH_2 particle size 5 µm (Dr. H. Knauer KG, 6370 Oberursel, FRG, Cat. No.: B45-Y82) .
Column 2:	Stainless steel tube, 10 cm length, 2 mm i.d., packed with Nucleosil 100 C18, particle size 5 µm (Dr. H. Knauer KG, Cat. No.: B45 - Y76).
Mobile phase 1:	0.02 M phosphoric acid in water for HPLC+ acetonitrile LiChrosolv 94 vol. + 6 vol.
Mobile phase 2:	0.02 M phosphoric acid in water for HPLC + acetonitrile LiChrosolv 76 vol. + 24 vol.
Flow rate 1:	0.2 mL/min.
Retention time:	about 6 min. (column 1)
Flow rate 2:	0.2 mL/min.
Retention time:	about 14 min. (columns 1 and 2)
Injection vol.:	50 µL

Recorder:	10 mV full scale
Column oven:	23°C
Chart speed:	0.5 - 1.0 cm/min.
Detector:	Wave length: 235 nm sensitivity: 0.005 aufs

Determination of Switching Interval

Determine actual switching times each time a series of samples is to be quantitated. Connect the outlet of column 1 directly to the UV-detector. Inject 50 µL of a 0.08 µg CGA 210007/mL standard solution (see section 2.4.2) and measure the begin and the end times of the peak of CGA 210007. Thereafter, connect column 2 to the detector and transfer that portion of eluate 1 which contains CGA 210007, e.g. portion between 5 min and 7 min from column 1 to column 2 (see section 6.2., page 14 for an example of measurement of switching times).

2.4.2 Preparation of Standard Solution and Calibration of the Chromatographic System

Standardize the chromatographic system each time a series of samples is to be quantitated. The range of the concentrations is depending on the range of residues to be determined. The lowest standard concentration is depending on the lower practical level. Calculate the lowest standard concentration (C) as follows:

$$C = \frac{L \times A}{Vf} \quad [\mu g/mL]$$

L: lower practical level [µg/g] or [mg/kg]
A: weight of soil cleaned up [g]
Vf: volume of the final solution [mL]

With the values proposed in this method, the lowest standard concentration is 0.004 µg CGA 210007/mL as calculated below:

$$C = \frac{0.004 \times 5}{5} = 0.004\ \mu g/mL$$

Prepare at least four standard solutions of different concentrations by appropriately diluting the stock solution of CGA 210007 (see section 2.2.10) with 0.02 M phosphoric acid in water for HPLC.

Select the concentrations as required; typical values are 0.08,0.04, 0.01 and 0.004 µg/mL. Inject 50 µL of each standard solution (corresponding to 4.0; 2.0; 0.5 and 0.2 ng CGA 210007, respectively). As the detector responce may change on injection of coextractives, it is recommended to inject standard and final solutions alternatively.

2.5 Interferences

No interferences which disturbed the determination of the CGA 210007 have been encountered so far.

2.6 Confirmatory Techniques

Alternative chromatographic system , as described in section 2.4.1.2.

2.7 Time Required for Analysis

A total of about 12 hours is required to work up a set of 12 samples to the point of HPLC injection. Automated HPLC analysis can be performed overnight.

2.8 Modifications and Potential Problems

None so far

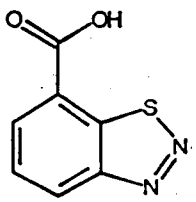
2.9 Calculation Procedure

Refer to REM 119.04 [5.1] for the detailed description of the recommended calculation procedures for the calibration of the chromatographic system and for the calculation of residues. Modification of REM 119.04: Quantitation of the fortified specimen at the lower practical level (limit of determination) may be performed by extrapolation of the calibration curve.

6.1 Figures

Figure 1: Structure and Chemical Name

CGA 210007



Benzo[1,2,3]Thiadiazole-7-carboxylic acid

$C_7H_4N_2O_2S$ Molecular mass: 180.18

Figure 2: Procedure Flow Diagram

15 g homogenized dry soil + 5 mL buffer pH 8 + 55* mL methanol in a 100 mL centrifuge glass tube
↓
shake 30 minutes
↓
centrifuge
↓
take an aliquot of 20 mL of the extract
(5 g dry soil equivalent)
↓
add 50 mL H_2O +4 mL brine and reextract with three portions
of 15 mL n-hexane + TBME 7 vol. + 3 vol., each
discard the organic phase
↓
add 1 mL of 85% phosphoric acid to the aqueous phase and extract CGA 210007 three times with
portions of 25 mL of n-hexane + TBME 7 vol. + 3 vol., each
collect the organic phase in a 100 mL round bottom flask
↓
evaporate to dryness
↓
dissolve the residue in 5 mL 0.02 M ortho-phosphoric acid.
↓
Quantitate by HPLC

* The moisture content of each soil sample has to be taken into account (see section 2.3.3)

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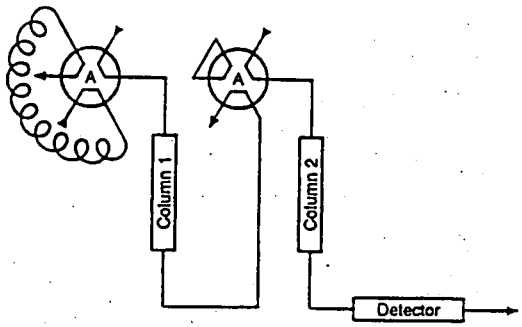
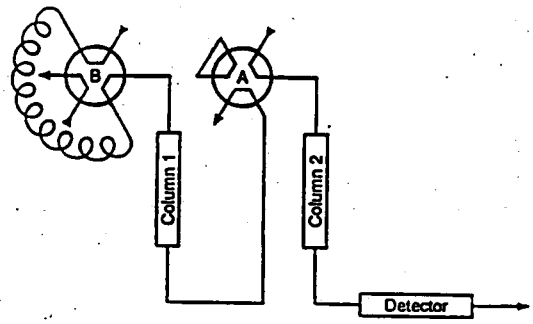
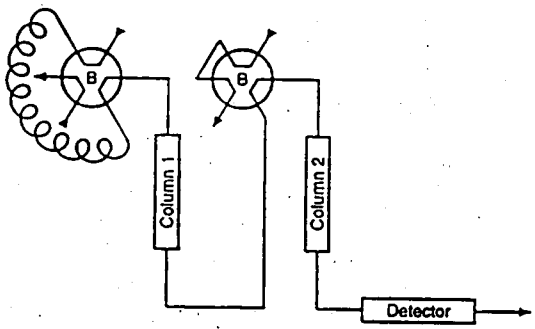
Figure 3: Setup of the Column Switching System



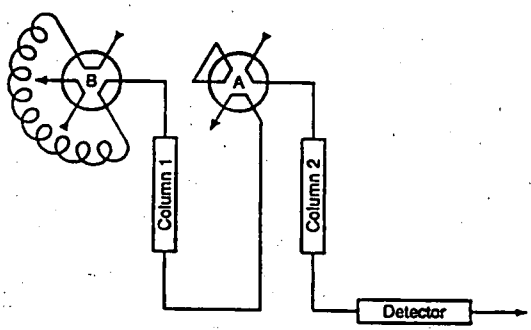
1) Loading sample into injection loop



2) Separation on first column



3) Transfer of "cut" to the second column



4) Analysis of "cut" on second column

EPL-BAS Modifications to Method REM 172.08

An unsuccessful recovery of CGA 210007 was achieved following the first attempt. The study director was contacted on March 31, 1997 with the unsuccessful results. The study director was also told at this time that plastic centrifuge bottles were substituted for glass due to breakage at high speeds. The study director requested a call to Pat Fleming of MVT. Ms. Fleming was called on April 1, 1997 with the following suggestions:

1. May need to purchase a commercial pH buffer 8 due to problems with the recipe in method.

2. MVT uses a hand rotation shaker.

3. Suggested using 125 mL separatory funnels for partitioning and 125 mL round bottoms for evaporation.

4. The samples were not evaporated to dryness. Low pressure was used to evaporate the samples.

5. Use only 95% Hexane , not 85%.

6. MVT uses plastic centrifuge bottles.

The study director said on April 4, 1997, to change the glassware and to not let the samples go dry. The following modifications were made to the method for the second attempt.

1. Plastic centrifuge bottles were used due to the glass centrifuge bottles breaking at such a high rotation. Step 2.3.3

2. A gyro shaker was used instead of a platform shaker which should have the same motion as a hand rotation shaker. Step 2.3.3

3. Glassware was changed from 100 mL cylinders to 125 mL separatory funnels. Step 2.3.4

4. The samples were partitioned using separatory funnels with the bottom layer of sample transferred to a beaker and the top layer transferred to a 150 mL round bottom flask. Step 2.3.6

5. The samples were not evaporated to dryness. Low pressure was used to evaporate the samples. Step 2.3.6

The prepared buffer was at pH 8 therefore a commercial pH 8 buffer was not needed. One hundred and fifty milliliter round bottoms were used instead of 125 mL due to

availability. EPL-BAS used 95% hexane. The study director was contacted on April 4, 1997 after the results for the second trial were low for the 10XLOQ. He suggested on April 9, 1997 to try a third attempt for the 10XLOQ since it was just under the 70% requirement.

On the third attempt the beakers used during the partitioning of the organic phase were rinsed after the aqueous phase was returned to the separatory funnel. The study director was notified on April 11, 1997 that the third trial was successful.

Analytical methodology, no matter what the source, generally requires the analyst to rely on his experience to make some interpretations and deal with problems that may occur with a method. The only problem with this method occurred when the glass centrifuge bottles broke during the centrifugation step. This problem was solved when plastic centrifuge bottles were substituted. A critical step with the method would be in step 2.3.6 in which the sample is rotovapped to dryness. The sample must not go to dryness but just to dryness. A small amount of water will remain on the round bottom flask. Low pressure must be maintained to achieve good results.