5 Information about the Study

5.1 Test System

The test system was soil (type 2.2 (loamy-sand). The specimen was obtained from LUFA Speyer; it was given the following number:

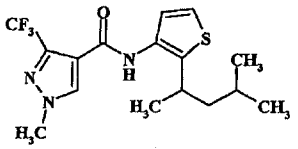
soil: 010/8225004

See also Table 1.

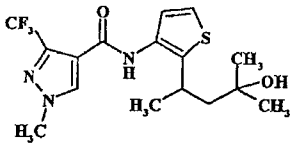
5.2 Test/Reference Items

These test / reference items were used for fortification and calibration purposes.

a) Penthiopyrad

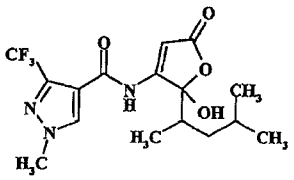
Product name	penthiopyrad
Product code	MTF-753
IUPAC-Name	(RS)-*N*-[2-(1,3-dimethylbutyl)-3-thienyl]-1-methyl-3-(trifluoromethyl)1*H*-pyrazole-4-carboximide
CAS No.	183675-82-3
Molecular formula	$C_{16}H_{20}F_3N_3OS$
Structure	
Molecular weight	359.42 g/mol
Batch No.	2100111
Date of analysis	SEP-06
Purity	99.8 %
Appearance	white crystal
Storage advice	cool and dark place
Expiry date	DEC-10

b) 753-A-OH

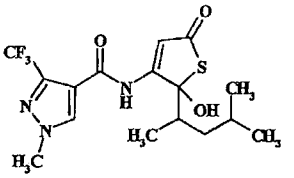
Product code	753-A-OH
Chemical name	*N*-[2-(3-hydroxy-1,3-dimethylbutyl) thiophen-3-yl]-1-methyl-3-trifluoromethyl-1*H*-pyrazole-4-carboxamide
IUPAC-Name	not given
CAS No.	not given
Molecular formula	$C_{16}H_{20}F_3N_3O_2S$
Structure	
Molecular weight	375.41 g/mol
Batch No.	092-050824-1
Date of analysis	26-JAN-07
Purity	100 %

Appearance	white powder
Storage advice	4 °C protected from light
Expiry date	31-DEC-10

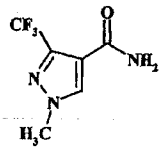
c) 753-F-DO

Product code	753-F-DO
Chemical name	*N*-[5-hydroxy-5-(1,3-dimethylbutyl)-2-oxo-2,5-dihydrofuran-4-yl]-1-methyl-3-trifluoromethyl-1*H*-pyrazole-4-carboxamide
IUPAC-Name	not given
CAS No.	not given
Molecular formula	$C_{16}H_{20}F_3N_3O_4$
Structure	
Molecular weight	375.35 g/mol
Batch No.	185-003-010-01
Date of analysis	20-MAR-07
Purity	91.0 %
Appearance	white powder
Storage advice	4 °C protected from light
Expiry date	31-DEC-08

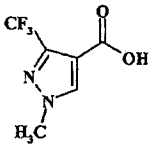
d) 753-T-DO

Product code	753-T-DO
Chemical name	*N*-[5-hydroxy-5-(1,3-dimethylbutyl)-2-oxo-2,5-dihydrothiophen-4-yl]-1-methyl-3-trifluoromethyl-1*H*-pyrazole-4-carboxamide
IUPAC-Name	not given
CAS No.	not given
Molecular formula	$C_{16}H_{20}F_3N_3O_3S$
Structure	
Molecular weight	391.42 g/mol
Batch No.	185-003-011-10
Date of analysis	20-MAR-07
Purity	98.4 %
Appearance	white powder
Storage advice	4 °C protected from light
Expiry date	31-DEC-09

e) PAM

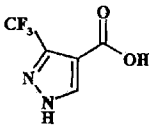
Product code	PAM
Chemical name	1-methyl-3-trifluoromethyl-1*H*-pyrazole-4-carboxamide
IUPAC-Name	not given
CAS No.	not given
Molecular formula	$C_6H_6F_3N_3O$
Structure	
Molecular weight	193.13 g/mol
Batch No.	152-050805-1
Date of analysis	26-JAN-07
Purity	100 %
Appearance	white powder
Storage advice	4 °C protected from light
Expiry date	31-DEC-10

f) PCA

Product code	PCA
Chemical name	1-methyl-3-trifluoromethyl-1*H*-pyrazole-4-carboxylic acid
IUPAC-Name	not given
CAS No.	not given
Molecular formula	$C_6H_5F_3N_2O_2$
Structure	
Molecular weight	194.11 g/mol
Batch No.	053-001207-1
Date of analysis	26-JAN-07
Purity	100 %
Appearance	white powder
Storage advice	4 °C protected from light
Expiry date	31-DEC-10

g) DM-PCA

Product code	DM-PCA
Chemical name	3-trifluoromethyl-1*H*-pyrazole-4-carboxylic acid
IUPAC-Name	not given
CAS No.	not given
Molecular formula	$C_5H_3F_3N_2O_2$

Structure	
Molecular weight	180.09 g/mol
Batch No.	133-050713-1
Date of analysis	26-JAN-07
Purity	98.28 %
Appearance	white powder
Storage advice	4 °C protected from light
Expiry date	31-DEC-10

The test / reference items and the data were supplied by the Sponsor.

Stock solutions and working solutions of the test / reference items were stored between + 4 °C and + 8 °C in capped vials protected from light. Usual safety precautions with chemicals were obeyed.

6 Purpose of the Study

The scope of this study was to perform an independent laboratory validation of the analytical method validated by ABC Laboratories, Inc.; 7200 E. ABC Lane; Columbia, Missouri 65202; USA under the ABC Study No. 63209 for the determination of penthiopyrad and its metabolites 753-A-OH, 753-F-DO, 753-T-DO, PAM, PCA and DM-PCA in soil.
The Test Facility did not have any previous experience in working with the method to be independently validated in this study.

7 Analytical Phase

7.1 Principle of the Method

The method validated under the ABC Study No. 63209 by A.G. Gant, ABC Laboratories Inc., dated 18 March 2008, was independently validated in this study.

Principle of the method:

Acetonitrile/water (4/1, v/v) was added to the soil specimen, followed by shaking at high speed and centrifugation. The extracts were combined and evaporated at approximately 40 °C to remove the acetonitrile. The concentrated extracts were transferred to a centrifuge tube and combined with ethyl acetate rinsates and 0.1 % acetic acid. The extracts were shaken and centrifuged before transferring the upper ethyl acetate layer into a new tube. Ethyl acetate was added to the aqueous phase, shaken and centrifuged to combine the ethyl acetate extracts. The extract was adjusted to volume with ethyl acetate, capped and mixed thoroughly. The ethyl acetate layers were transferred to centrifuge tubes and evaporated to dryness under a flow of nitrogen in a water bath at 40 °C or lower. The extracts were reconstituted in methanol and evaporated to < 0.5 mL under a flow of nitrogen in a water bath at 40 °C or lower. Methanol was added, the tubes were capped and mixed by hand before sonicating. Ultra pure water was added, the tubes were capped and mixed by hand before sonicating. Volume was adjusted with 50 % methanol/water and specimens filtered into HPLC vials and diluted for LC-MS/MS analysis.
The final determination of the residues of penthiopyrad and its metabolites 753-A-OH, 753-F-DO, 753-T-DO, PAM, PCA, DM-PCA was performed by LC-MS/MS, monitoring two parent-daughter ion transitions.

The LOQ of the method for penthiopyrad and its metabolites 753-A-OH, 753-F-DO, 753-T-DO, PAM, PCA, DM-PCA was 0.005 mg/kg for each analyte.

7.2 Deviations from the Method

Minor modifications were made in order to adapt the method to the laboratory situation at SGS INSTITUT FRESENIUS. Available chemicals and laboratory glassware were used, a few items of which deviated from those specified in the method but fulfilled the same purpose.

Chapter 4.4 Calculation

In deviation to the described calculation method, the results for the confirmatory ion transition of 753-F-DO, 753-A-OH and DM-PCA were calculated using the peak height instead of the peak area.
In case of 753-F-DO and 753-A-OH the same ion transition (376 → 177) is used for confirmatory purposes. It was impossible during this study to achieve fully to the baseline separated peaks, therefore the peak height was used for evaluation.
In case of DM-PCA a matrix peak eluted nearly at the same time as the substance peak and a reliable evaluation using the peak area was impossible. Using the peak height the evaluation showed satisfying results.

Calibration

In deviation to the used method the calibration curves for penthiopyrad, 753-T-DO, 753-F-DO, 753-A-OH and PAM were calculated and plotted by 2nd order (quadratic) regression.

7.3 Equipment and Materials

7.3.1 General Laboratory Equipment

- centrifuge	Jouan, model GR 422
- shaking machine	Bühler, model SM 25
- analytical balance	Sartorius, model CPA225D-OCE
- top loading balance	Sartorius, model 3100S
- top loading balance	Kern, model GS 3200-2
- ultrasonic bath	Bandelin, model DK 102
- magnetic stirrer	Heidolph, model 11 MR 2002
- rotating evaporator	various
- closed circuit refrigerator	Lauda, model WK 500
- diaphragm vacuum pump	Vacuubrand, model CVC 2
- ultra pure water unit	Millipore, model Synergy UV
- vortex	Heidolph, model Reax 2000
- nitrogen evaporator	Organomation, model 112
- bottle dispenser	Graf, model Fortuna Optifix
- micro pipettes	Gilson GmbH, model Microman
- centrifuge bottles	Nalgene, 125 mL
- centrifuge tube	various
- volumetric pipettes	various
- Pasteur pipettes	various
- volumetric flasks	various
- beakers	various
- round bottom flasks	various
- glass bottles	various
- funnels	various
- graduated cylinders	various
- sample vial, 1 mL, with	

PTFE sealed crimp-on caps	Burdich GmbH
- micro vial, 500 µL	fitting into the 1 mL sample vial
- sample vial, 20 mL	Burdich or equivalent

7.3.2 Reagents

- acetonitrile	Promochem, No. 2856
- ethyl acetate	Roth T164.1
- methanol	Roth T169.1
- ultra pure water	Millipore, Synergy UV
- formic acid	Merck, No. 1.00264
- acetic acid	Roth 3738.2

7.3.3 Preparation of the Mixtures

0.01 M formic acid:	0.38 mL conc. formic acid added to 1 L of ultra pure water and mixed
0.1 % acetic acid:	0.1 mL conc. acetic acid were dissolved in 100 mL of ultra pure water
acetonitrile/water (4/1, v/v):	800 mL of acetonitrile and 200 mL of ultra pure water, mixed
methanol/water (1/1, v/v):	500 mL of methanol and 500 mL of ultra pure water, mixed

7.4 Final Determination by LC-MS/MS

HPLC System:

System:	Agilent, No. 1200
Degasser:	Agilent G1322A
Pump:	Agilent G1311A
Autosampler:	CTC Analytics, HTC Pal
Column oven with 6 port valve:	Agilent G1316A
Control module:	Agilent G1323B

LC-MS/MS System:

System:	Applied Biosystems, API 4000
Vacuum pump:	Varian HS 602
Data system:	Applied Biosystems, Analyst, version 1.4.2

HPLC-Conditions:

Method 1 (determination of Penthiopyrad, 753-A-OH, 753-F-DO, 753-T-DO and PAM)

Column:	Phenyl Hexyl
Manufacturer/supplier:	Phenomenex
length:	150 mm
interior diameter:	4.6 mm
particle size:	3.0 µm
Split (detector/waste):	-
Autosampler temperature:	20 °C
Mobile phase C:	10 mmol formic acid
Mobile phase D:	methanol
Flow rate:	see table below
Stop time:	13.5 minutes
Oven temperature:	40 °C
Injection volume:	40.0 µL

Gradient time table:	Time [min]	% C	% D	Flow [mL/min]
	0.0	80	20	0.9
	0.1	80	20	0.9
	3.0	25	75	0.9
	4.0	20	80	0.9
	6.0	10	90	0.9
	6.1	1	99	0.9
	9.0	1	99	0.9
	9.1	80	20	0.9
	9.6	80	20	0.9
	13.5	80	20	0.9

Switching times for valve in column oven: 0 – 3.5 min (waste)
3.5 – 9 min (LC-MS/MS)
9.0 – 13.5 min (waste)

Method 2 (determination of PCA and DM-PCA)

Column: C18
Manufacturer/supplier: Phenomenex
length: 150 mm
interior diameter: 4.6 mm
particle size: 3.0 µm
Split (detector/waste): -
Autosampler temperature: 20 °C
Mobile phase C: 10 mmol formic acid
Mobile phase D: methanol
Flow rate: see table below
Stop time: 12.0 minutes
Oven temperature: 40 °C
Injection volume: 50.0 µL

Gradient time table:	Time [min]	% C	% D	Flow [mL/min]
	0.0	80	20	0.9
	0.5	80	20	0.9
	3.0	20	80	0.9
	3.8	1	99	0.9
	7.25	1	99	0.9
	7.5	80	20	0.9
	12.0	80	20	0.9

Switching times for valve in column oven: 0 – 3.5 min (waste)
3.5 – 6.5 min (LC-MS/MS)
6.5 – 12 min (waste)

LC-MS/MS Conditions:

		Penthiopyrad		753-A-OH	
Scan type:		MRM	MRM	MRM	MRM
Ionisation mode/polarity:		ESI positive	ESI positive	ESI positive	ESI positive
Curtain gas:	[psig]	15	15	15	15
Collision gas:	[psig]	5	5	5	5
Ion Spray voltage:	[V]	5000	5000	5000	5000
Temperature:	[°C]	450	450	450	450
Declustering potential:	[V]	61	61	36	36
Collision energy:	[V]	21	47	33	49
Collision cell exit potential:	[V]	16	10	10	12
Entrance potential:	[V]	10	10	10	10
Dwell:	[msec.]	50	50	150	150
Transition used for evaluation:	[m/z]	360 → 276	360 → 177	376 → 152	376 → 177

		753-F-DO		753-T-DO	
Scan type:		MRM	MRM	MRM	MRM
Ionisation mode/polarity:		ESI positive	ESI positive	ESI positive	ESI positive
Curtain gas:	[psig]	15	15	15	15
Collision gas:	[psig]	5	5	5	5
Ion Spray voltage:	[V]	5000	5000	5000	5000
Temperature:	[°C]	450	450	450	450
Declustering potential:	[V]	66	66	66	66
Collision energy:	[V]	17	35	35	19
Collision cell exit potential:	[V]	10	12	10	10
Entrance potential:	[V]	10	10	10	10
Dwell:	[msec.]	150	150	200	200
Transition used for evaluation:	[m/z]	376 → 182	376 → 177	392 → 177	392 → 182

		PAM		PCA	
Scan type:		MRM	MRM	MRM	MRM
Ionisation mode/polarity:		ESI positive	ESI positive	ESI negative	ESI negative
Curtain gas:	[psig]	15	15	15	15
Collision gas:	[psig]	5	5	5	5
Ion Spray voltage:	[V]	5000	5000	-4500	-4500
Temperature:	[°C]	450	450	450	450
Declustering potential:	[V]	51	51	-35	-35
Collision energy:	[V]	15	29	-32	-16
Collision cell exit potential:	[V]	10	12	-7	-7
Entrance potential:	[V]	10	10	-10	-10
Dwell:	[msec.]	500	500	350	150
Transition used for evaluation:	[m/z]	194 → 174	194 → 134	193 → 109	193 → 149

		DM-PCA	
Scan type:		MRM	MRM
Ionisation mode/polarity:		ESI negative	ESI negative
Curtain gas:	[psig]	15	15
Collision gas:	[psig]	5	5
Ion Spray voltage:	[V]	-4500	-4500
Temperature:	[°C]	450	450
Declustering potential:	[V]	-35	-35
Collision energy:	[V]	-16	-26
Collision cell exit potential:	[V]	-9	-7
Entrance potential:	[V]	-10	-10
Dwell:	[msec.]	150	350
Transition used for evaluation:	[m/z]	179 → 159	179 → 111

7.5 Preparation of Solutions for Fortification and Calibration

The stock, fortification and calibration solutions were stored in a refrigerator at +4 °C to +8 °C immediately after preparation in capped vials protected from light. The stability of the calibration solutions was proved by comparing the concentrations of one old solution and one freshly prepared solution by means of a single injection.

7.5.1 Stock and Fortification Solutions

Two stock solutions of each analyte (penthiopyrad and its metabolites 753-A-OH, 753-F-DO, 753-T-DO, PAM, PCA and DM-PCA) (including the one for the stability test) were prepared.

Example penthiopyrad:
On 31 July 2008, a stock solution of penthiopyrad was prepared in a volumetric flask by dissolving 11.62 mg in 10 mL of methanol, which resulted in a concentration of 1.160 mg/mL, taking into account a purity of 99.8 %.

Example 753-T-DO:
On 31 July 2008, a stock solution of 753-T-DO was prepared in a volumetric flask by dissolving 10.59 mg in 10 mL of methanol, which resulted in a concentration of 1.042 mg/mL, taking into account a purity of 98.4 %.

Example 753-F-DO:
On 31 July 2008, a stock solution of 753-F-DO was prepared in a volumetric flask by dissolving 10.84 mg in 10 mL of methanol, which resulted in a concentration of 0.986 mg/mL, taking into account a purity of 91.0 %.

Example 753-A-OH:
On 31 July 2008, a stock solution of 753-A-OH was prepared in a volumetric flask by dissolving 10.07 mg in 10 mL of methanol, which resulted in a concentration of 1.007 mg/mL, taking into account a purity of 100.0 %.

Example PAM:
On 31 July 2008, a stock solution of PAM was prepared in a volumetric flask by dissolving 12.42 mg in 10 mL of methanol, which resulted in a concentration of 1.242 mg/mL, taking into account a purity of 100.0 %.

Example PCA:
On 31 July 2008, a stock solution of PCA was prepared in a volumetric flask by dissolving 12.51 mg in 10 mL of methanol, which resulted in a concentration of 1.251 mg/mL, taking into account a purity of 100.0 %.

Example DM-PCA:
On 31 July 2008, a stock solution of DM-PCA was prepared in a volumetric flask by dissolving 11.65 mg in 10 mL of methanol, which resulted in a concentration of 1.145 mg/mL, taking into account a purity of 98.3 %.

On 13 August 2008 aliquots of the stock solutions were mixed up in a 10 mL volumetric flask and diluted with methanol, to obtain a standard mixture (SM).

This standard mixture was further diluted with methanol to following concentrations, which were used for fortification:

Substance	concentration [µg/mL]		
	SM	Solution II	Solution III
penthiopyrad	100.2	1.002	0.1002
753-T-DO	100.0	1.000	0.1000
753-F-DO	100.0	1.000	0.1000
753-A-OH	100.1	1.001	0.1001
PAM	100.1	1.001	0.1001
PCA	100.1	1.001	0.1001
DM-PCA	100.1	1.001	0.1001

7.5.2 Calibration Solutions

On 13 August 2008, the standard mixture (SM) (see chapter 7.5.1) was dissolved in methanol/ultra pure water (50/50; v/v) to concentrations of the following range:

Substance	concentration [µg/mL]		
	SM	highest	lowest
penthiopyrad	100.2	0.02004	0.0001002
753-T-DO	100.0	0.02	0.0001
753-F-DO	100.0	0.02	0.0001
753-A-OH	100.1	0.02002	0.0001001
PAM	100.1	0.02002	0.0001001
PCA	100.1	0.02002	0.0001001
DM-PCA	100.1	0.01001	0.0001001

7.6 Calculations

The detector signals were registered and integrated using the data system outlined in chapter 7.4. The peak area was taken into account to determine the amounts of penthiopyrad, 753-A-OH, 753-F-DO, 753-T-DO, PAM, PCA and DM-PCA in the specimens, apart from the determination of the confirmatory ion transition of 753-A-OH and 753-F-DO, where the peak height was used. The calibration curves were calculated from the peak area respectively the peak height of the calibration solutions with their corresponding concentrations of penthiopyrad, 753-A-OH, 753-F-DO, 753-T-DO and PAM, using equation (1), respectively equation (3) for PCA and DM-PCA.

(1) $y = a + bx + cx^2$

where y: peak area or height [integration units iu]

x: weight of analyte [ng]
a: constant [iu]
b: constant [iu/ng]
c: constant [iu/ng^2]

The concentration of penthiopyrad, 753-A-OH, 753-F-DO, 753-T-DO and PAM in the specimen was calculated using the transformed equation (2):

(2) $$x = -\frac{b}{2c} \pm \sqrt{\frac{y-a}{c} + \left(\frac{b}{2c}\right)^2}$$

(3) $$y = a + bx$$

where
y: peak area [integration units iu]
x: amount of analyte [ng]
a: ordinate intercept [iu]
b: slope [iu/ng]

The amount of PCA and DM-PCA in the specimen was calculated using the transformed equation (3):

(4) $$x = \frac{y-a}{b}$$

The concentration C of penthiopyrad, 753-A-OH, 753-F-DO, 753-T-DO, PAM, PCA and DM-PCA in the specimen was calculated from x using equation (5):

(5) $$C = \frac{x \cdot V_E \cdot A_1}{V_i \cdot A_2 \cdot W}$$

where
x : amount of analyte [ng]
C : analysed concentration of analyte in the specimen [mg/kg]
V_E : final volume (2.5 mL)
A_1 : total extract (50 mL)
V_i : injection volume (20 µL)
A_2 : aliquot (5 mL)
W : specimen weight (5 g)

The recovery data was calculated according to equation (6):

(6) $$R = \frac{C \cdot 100}{C_{for}}$$

where
R : recovery [%]
C : analysed concentration of analyte in the fortified specimen [mg/kg]
C_{for}: nominal concentration of analyte in the fortified specimen [mg/kg]

The recovery values reported in Tables 3 - 5 represent rounded results which were obtained from calculations based on the exact raw data. The detector signals of the control specimens (if any) were subtracted from the fortified specimens.

Example

The fortified soil specimen 010/8225004-A was extracted and analysed on 13 August 2008. The parameters of the calibration curve for penthiopyrad on the basis of the detected mass pair m/z 360 → m/z 276 were a = 3854 (ordinate intercept),

b = 7054254.06 and c= 1625186.7 derived from equation (1). The peak area of the analyte penthiopyrad corresponded to 140307 counts.

(2) $$x = -\frac{7054254.06}{2 \cdot 1625186.7} \pm \sqrt{\frac{140307 - 3854}{1625186.7} + \left(\frac{7054254.06}{2 \cdot 1625186.7}\right)^2}$$

$x = 0.0193$ ng

(5) $$C = \frac{0.0193 \cdot 2.5 \cdot 50}{20 \cdot 5 \cdot 5.04}$$

$C = 0.0048$ mg/kg

(6) $$R = \frac{0.0048 \cdot 100}{0.0050}$$

$R = 96$ %

Appendix 1 – Contacts

During the validation work the sponsor's monitor was contacted once to discuss the possibility to evaluate the results for the confirmatory transitions of 753-A-OH and 753-F-DO on the basis of peak height instead of peak area.