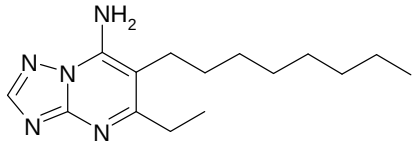
1. INTRODUCTION

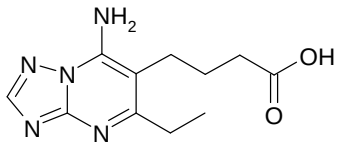
Objective:

To validate an analytical method for the determination of BAS 650 F and its metabolites M650F01, M650F02, M650F03, and M650F04 in soil at a limit of quantification (LOQ) of 0.01 mg/kg (per analyte), using LC/MS/MS for quantification and confirmation.

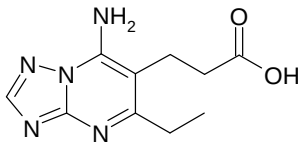
BAS 650 F



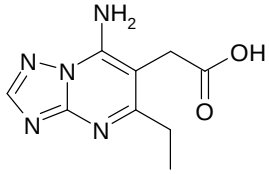
M650F01



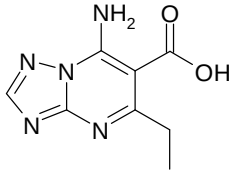
M650F02



M650F03



M650F04



Principles of the Method and Validation:

BASF method L0091, version 01, TP (technical procedure) 02, as obtained by the sponsor, using LC/MS/MS was employed.

The analytes are extracted from soil by shaking with acetonitrile followed by acetonitrile/water (50/50 v/v). An aliquot of the extract is diluted with acetonitrile/water (10/90 v/v). The final determination of BAS 650 F and its metabolites M650F01, M650F02, M650F03 and M650F04 is performed by LC/MS/MS.

For each analyte two parent-daughter ion MRMs for quantification and quantitative confirmation were monitored by LC/MS/MS. The validated method achieves a limit of quantification (LOQ) of 0.01 mg/kg and a limit of detection (LOD) of 0.001 mg/kg.

Method validation was accomplished by analyzing for two different soil types as sandy loam and clay and for each of the five analytes 2 blank control specimens, 5 replicate specimens fortified at LOQ, and 5 replicate specimens fortified at 10xLOQ.

2. EXPERIMENTAL

2.1 Test System

Two European standard soils (one sandy loam and one clay loam) were obtained from LUFA Speyer sieved (2 mm). Prior to use in this study, water was added as to establish approximately 40 % of the maximum water holding capacity. Soil characterization information is given in Appendix 2.

2.2 Test and Reference Items

Analytical standards used were obtained by the Sponsor. See Appendix 1 for information provided.

2.3 Solvents, Chemicals, Equipment, and LC/MS/MS Instrumentation

Solvent, Chemicals, and Miscellaneous:

Acetonitrile, methanol, all Promochem, HPLC grade. Millipore Water (ptrl Europe).
Acetic acid 100 %, Merck.
Folded filter 110 mm i.d., Machery-Nagel.

Equipment:

Analytical balance: Sartorius RC 210 D. Top loading balance Sartorius LP 620 S.

Ultrasonic bath: Elma Transsonic 700. Centrifuge: Hettich Rotixa 50 S.

IKA Horizontal shaker HS 260 B.

Typical glass and plastic ware and laboratory equipment.

LC/MS/MS Instrumentation:

Agilent 1200 Series HPLC system (vacuum solvent degasser, binary HPLC pump, column oven), and CTC Analytics HTC-Pal autosampler.

HPLC column: Waters XTerra, 50 mm length, 4.6 mm i.d., 3.5 µm particle size.
Pre-column: Phenomenex C_{18} RP, 4 x 3 mm.

Applied Biosystems MDS Sciex API 4000 triple quadrupole LC/MS/MS system with TurboIonspray ESI source. Analyst 1.4.2 Instrument control and data acquisition software.

2.4 Standard Solutions and Stability

2.4.1 Stock Solutions

Separate stock solutions of each analytical reference item were prepared in methanol by accurately weighing 10 mg (purity considered) in 10 mL volumetric flasks to obtain concentrations of 1.0 mg/mL.

2.4.2 Fortification and Calibration Solutions (exemplified)

A mixed intermediate solution was prepared in 10 mL of methanol by volumetric dilution of the stock solutions (100 µL each) to obtain concentrations of 10 µg/mL per analyte. The 10 µg/mL intermediate solution was further diluted in methanol (1.0 mL into 10 mL of methanol) to obtain a solution with concentrations of 1.0 µg/mL per analyte. The 10 µg/mL intermediate solution was used to fortify soil specimens at the 10xLOQ level of 0.1 mg/kg, dosing e.g. 0.05 mL to 5 g of soil dry mass. The 1 µg/mL mixed solution was used to fortify soil specimens at the LOQ level of 0.01 mg/kg, dosing e.g. 0.05 mL to 5 g of soil dry mass.

Examples for preparation of calibration solutions are given in the following table:

Take intermediate solution (µg/mL)	**Volume (mL)**	**Dilute with ACN/H_2O (1/9, v/v) to a final volume of (mL)**	**Concentration (ng/mL)**
10	0.10	10	100 (per analyte)
Take solution (ng/mL)	**Volume (mL)**	**Dilute with ACN/H_2O (1/9, v/v) to a final volume of (mL)**	**Concentration (ng/mL)**
100 (per analyte)	1.0	10	10 (per analyte)
10 (per analyte)	1.0	10	1.0 (per analyte)
10 (per analyte)	0.50	10	0.50 (per analyte)
10 (per analyte)	0.250	10	0.250 (per analyte)
10 (per analyte)	0.10	10	0.10 (per analyte)
1.0 (per analyte)	0.50	10	0.050 (per analyte)
1.0 (per analyte)	0.10	10	0.010 (per analyte)

All standard solutions were stored refrigerated when not in use.

2.4.3 Storage and Stability of Analytes

Stability for the analytes in extracts was demonstrated by consistent LC/MS/MS results throughout the duration of the experimental phase of the study and acceptable recoveries in the fortified samples within the range of 70-120 %.

Specifically, mixed solutions with nominal concentrations of 1.0 ng/mL per analyte were prepared in acetonitrile/water (1/9 v/v) from freshly prepared stock solutions at different times (always with same dilutions steps) to make conclusions about the stability of analytes in working solutions (see Table 3):

Separate stock solutions were prepared in methanol with concentrations of 1.0 mg/mL and were diluted to an intermediate mixed solution in 10 mL of methanol by volumetric dilution (100 µL each) to obtain concentrations of 10 µg/mL per analyte. This intermediate solution was further diluted in acetonitrile/water (1/9 v/v) (performing three dilution steps: 100 ng/mL > 10 ng/mL > 1 ng/mL) to obtain a 1 ng/mL solution:

K1481-60 prepared on the 8-May-2008 resulting in 48 days of refrigerated storage
K1481-86 prepared on the 02-Jun-2008 resulting in 23 days of refrigerated storage
K1481-111 prepared on the 24-Jun-2008 (freshly prepared solution)

Each solution was injected for three times and the average peak areas were compared:
As peak areas for the solution stored for 23 days were comparable with peak areas obtained

from the freshly prepared solution, it is concluded that stability of all five analytes for at least 23 days of refrigerated storage is given.
It is additionally demonstrated, that M650F03 and M650F04 are stable for at least 48 days after refrigerated storage.

2.5 Soil Extraction Method

Residue analysis of BAS 650 F and its metabolites M650F01, M650F02, M650F03 and M650F04 was conducted as described in method L0091 version 01, technical procedure TP02:

5 g soil dry mass (W) was weighed into a centrifuge bottle. Fortification is performed by dosing of fortification solutions: 50 µL of the 1 µg/mL (LOQ, 0.01 mg/kg) and 50 µL of the 10 µg/mL solution (10xLOQ, 0.1 mg/kg). 10 mL of acetonitrile were added, and the bottle shaken mechanically at about 225 rpm for 30 min. Thereafter the specimens were centrifuged at 4000 rpm for 5 min and the supernatant decanted and filtered through a folded filter into a 50 mL volumetric flask.

Subsequently the extraction and centrifugation is repeated twice with 20 mL acetonitrile/water (50/50, v/v). The supernatants are again decanted, filtered, and combined with the first extract in the 50-mL flask. The volume is adjusted with acetonitrile/water (10/90, v/v) to the mark (V_{Ex} = 50 mL) and the extract is mixed well.
A 0.1 mL aliquot (V_1) is diluted with acetonitrile/water (10/90, v/v) to a V_{End} of 1 mL for final LC-MS/MS determination.

2.6 LC/MS/MS Analysis

2.6.1 RP-HPLC Method

HPLC System	Agilent 1200 HPLC system (vacuum solvent degasser, binary HPLC pump, column oven), and CTC Analytics HTC-Pal Autosampler.			
HPLC Column	Waters XTerra C_{18}, 50 mm length, 4.6 mm i.d., 3.5 µm particle size. Pre-column: Phenomenex C_{18} RP, 4 x 3 mm. Column oven 30 °C.			
Injection Volume	50 µL.			
HPLC Method Pre-equilibration time of 2 min with 500 µL/min at 90% A : 10% B	Solvent A: 0.1 % acetic acid in water Solvent B: 0.1 % acetic acid in acetonitrile Mobile Phase Composition: Time (min) 0.00 7.00 9.00 9.10 11.0	 Flow rate (µL/min) 500 500 500 500 500	 % A 90 0 0 90 90	 % B 10 100 100 10 10

2.6.2 MS/MS Method

MS System	Applied Biosystems MDS Sciex API 4000 triple quadrupole LC/MS/MS system with TurboIonspray (ESI) source
Electrospray Ion Source Conditions	Source temperature: 550°C Curtain gas (CUR): 20 Nebulizer gas (GS1): 40 Turbo gas (GS2): 70 Ion spray voltage (IS): 4500 V Collision gas (CAD): 5 Entrance potential (EP): 10 V Resolution Q1 and Q3: Unit Dwell times: 100 msec (for M650F03: 200 msec)
MS/MS Conditions	MRMs proposed for quantification (Q) respectively confirmation (C):

Analyte	Q1 Mass (amu)	Q3 Mass (amu)	DP (V)	CE (eV)	CXP (V)
BAS 650 F					
Q	276.3	149.1	105	50	13
C	276.3	176.2	105	44	15
M650F01					
Q	250.2	232.0	46	31	22
C	250.2	149.1	46	49	12
M650F02					
Q	236.0	176.1	60	36	16
C	236.0	218.2	60	28	15
M650F03					
Q	222.2	176.1	41	31	16
C	222.2	204.1	61	23	16
M650F04					
Q	208.2	190.2	37	24	15
C	208.2	123.0	37	35	11

External calibration was used for quantification and confirmation of the analytes by LC/MS/MS. Calibrations were established with standard solutions prepared in acetonitrile / water (1/9, v/v) injected interspersed with soil extracts. The calibrations ranged from 0.01 ng/mL to 10 ng/mL with ≥ 5 concentration levels.

Linear or quadratic regression equations were generated with 1/x weighting, resulting in calibration functions with excellent correlation ($r \geq 0.998$), as exemplified in Figure 1 to Figure 3 calibration functions and diagrams.

LC/MS/MS chromatograms of standard solutions are shown in Figure 4 to Figure 8.

Figure 9 to Figure 13 (soil type: sandy loam) and Figure 14 to Figure 18 (soil type: clay) give examples of LC/MS/MS chromatograms of fortified (10xLOQ: 0.10 mg/kg, LOQ: 0.01 mg/kg) and blank control soil specimens.

2.7 Calculation of Results

2.7.1 Calculation of Concentrations

Concentrations in the final extracts (ng/mL) were determined by substituting the peak area responses into the regression equation, using the LC/MS/MS Analyst 1.4.2 Instrument control and data acquisition software.

2.7.2 Calculation of Residues

Calculations were performed by Excel with full precision; discrepancies may arise when recalculated with pocket calculator.

For the calculation of residues the following formula was used:

$R = c_{End} \times (V_{Ex} \times V_{End} / V_1 \times W) / 1000$ ng/µg

$= c_{End} \times$ Multiplier M

Where:

R: Analyte residue in mg/kg.

c_{End}: Final concentration of analyte in extract in ng/mL.
(where multiple injections were evaluated: mean).

W: Soil dry mass: 5 g.

V_{Ex}: Volume of extraction solvent: 50 mL.

V_1: Aliquot of V_{Ex}: 0.10 mL.

V_{End}: Volume of final extract used for LC/MS/MS: 1.0 mL.

M: Multiplier: 0.10.

Recoveries (Rec.) were calculated for the fortified specimens as follows:

$Rec. = (R / R_{fortified}) \times 100$ %

2.7.3 Example

The calculation is exemplified with a clay soil specimen fortified at 0.01 mg/kg or LOQ (P1481-46, see Table 2) with BAS 650 F (and its 4 metabolites).

The 5 g (W) soil specimen was extracted by shaking using acetonitrile and acetonitrile/water (V_{Ex} = 50 mL). An aliquot of 0.1 mL (V_1) was diluted with acetonitrile/water to obtain a final volume (V_{End}) of 1.0 mL for LC/MS/MS determination.

The final extract was examined for BAS 650 F by LC/MS/MS in run file P1481API#034 (Figure 14, middle). The Analyst software used a calibration function which was established by injecting calibration solutions interspersed with final extracts to calculate a final BAS 650 F concentration c_{End} of 0.078 ng/mL (276 m/z → 149 m/z), respectively, 0.080 ng/mL (276 m/z → 176 m/z).

Thus:

$R = c_{End} \times (V_{Ex} \times V_{End} / V_1 \times W) / 1000$ ng/µg

$= c_{End} \times$ Multiplier M

= 0.078 ng/mL x (50 mL x 1.0 mL) / (0.10 mL x 5 g) / 1000 ng/µg

= 0.078 ng/mL x 0.10 (mLxµg / ngxg)

= 0.0078 mg/kg

The result gave a recovery of 78 % for the ion transition 276 m/z → 149 m/z used for quantification.