8. INTRODUCTION

RA department of Isagro GLP Test Facility conducted a study to validate a residue method for IR9792 (F9990) in drinking and surface water by LC/MS-MS with the quantification by using two ion transitions.
See Enclosure B for the Study Plan.

GUIDELINE REQUIREMENTS

The study was conducted according to the following guidelines:

- OECD guidance document on pesticide residue analytical methods; ENV/JM/MONO(2007)17;
- US EPA Ecological Effects Test Guideline OCSPP 850.6100: Environmental Chemistry Methods and Associated Independent Laboratory Validation;
- Regulation (EC) No 1107/2009;
- EU Council Directive 91/414/EEC amended by Commission Directive 96/68/EC;
- European Commission Guidance Document for Generating and Reporting Methods of Analysis in Support of Pre-Registration data Requirements for Annex II (part A, Section 4) and Annex III (part A, section 5) of directive 91/414, SANCO/3029/99;
- Guidance document on residue analytical methods; SANCO/825/00 rev. 8.1, European Commission, Directorate General Health and Consumer Protection, 16/11/2010;
- Regulation (EC) No 1107/2009 of the European Parliament and of the Council of 21 October 2009 concerning the placing of plant protection products on the market and repealing Council Directives 79/117/EEC and 91/414/EEC.

9. PROCEDURES

9.1 Identification

Drinking water was drawn directly from the municipal aqueduct. Surface water was drawn from the Ticino river at Cameri (NO), in the Northern Italy geographical area. The geographical coordinates and the map of drawing area are shown in Enclosure C.

Both waters were characterized by the external laboratory "THEOLAB S.p.A". The water characterization is reported in the Table 1.

All specimens were filtered on glass microfiber filters and stored in the dark, at t<+6 °C until the sample analysis.

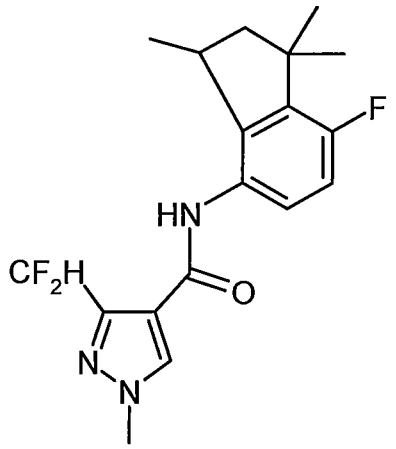
At the analysis time, each sample was assigned a lab code by RA, according to the SOP.

The sample history is reported in Table 7.

9.2 Test/Reference item

The test/reference item IR9792 (F9990) was provided by ISAGRO S.p.A. Chemical Discovery. Its certificate of analysis is included in Enclosure A.

Common name: **IR9792 (F9990)**



IUPAC name: 3-(difluoromethyl)-N-(7-fluoro-2,3-dihydro-1,1,3-trimethyl-1H-inden-4-yl)-1-methyl-1H-Pyrazole-4-carboxamide

Molecular formula and weight:	$C_{18}H_{20}F_3N_3O$	351.366 g/mole
Physical state:		powder, whitish
Batch No.:		30399/98
Expiry date:		31st October 2014
Purity (by HPLC):		99.72%[2]

An exactly weighed amount of IR9792 (F9990) (10.05 mg and 11.20 mg) was dissolved with methanol in a 100 mL volumetric flask for a 112.0 mg/L and 100.5 mg/L stock solutions (SS) of the reference item.

Starting from the SS above at concentration of 112.0 mg/L, several working standard solutions (WS) were prepared by proper dilution with the water.
These WS were injected in order to obtain the calibration curve.

SS and WS were stored in the dark in refrigerated conditions (T ≤ +6 °C) until use.

[2]According to internal laboratory procedures, no correction will be done for purity above 99%

9.3 Fortification

Starting from the SS, prepared as described in Section 9.2, the following *fortifying solutions* (FS) of IR9792 (F9990) were prepared:

- drinking water: two FS at concentrations of 0.1008 mg/L and 0.01008 mg/L were prepared by proper dilutions with water;
- surface water: two FS at concentrations of 0.1005 mg/L and 0.01005 mg/L) were prepared by proper dilutions with methanol.

Solutions were stored in the dark in refrigerated conditions (+1<t<+6 °C) until use.

The substrate volumes, the concentrations, the volumes added and the consequent spiking levels are described in Section 9.4.3.1 and in Table 2.

9.4 Analytical procedure

9.4.1 Reagents

- ✓ Analytical methanol, LC-MS Chromasolv®, for HPLC ≥ 99.9% (e.g.: Sigma Aldrich);
- ✓ Ammonium acetate ACS-for analysis (e.g.: Carlo Erba - Milan Italy);
- ✓ Formic acid 39% ACS-for analysis (e.g.: Carlo Erba - Milan Italy);
- ✓ Water, HPLC grade, purified with MILLI-Q MILLIPORE Italy;
- ✓ Nitrogen gas from Nitrogen generator;
- ✓ Nitrogen gas cylinder (99.95%).

9.4.2 Equipment

- ✓ Analytical balance (e.g. Mettler-Toledo XP105);
- ✓ Common analytical laboratory glassware and equipment for chemical laboratory;
- ✓ Glass microfiber filters (e.g. Whatman GF/C – cat. no. 1822-090);

- ✓ High performance liquid chromatograph equipped with degasser, binary pump, column thermostatic oven, automatic sampler and triple quadrupole mass detector (e.g.: HPLC 1200 + 6410 Mass detector Agilent Technologies);
- ✓ HPLC analytical column (e.g. Kinetex 2.6µ C18 100A 5µm, 50 x 4.6 mm + SecurityGuard Ultra Cartridge UHPLC C18 for 4.6 mm – Phenomenex);
- ✓ Technical balance (e.g. Sartorius LC 820 - Zeiss Milan Italy);
- ✓ Ultrasonic bath (SONICA 1200 M - SOLTEC DESE LAB - Piombino Dese Padova, Italy).

9.4.3 Method

9.4.3.1 Analytical method

For the validation, seven samples at the LOQ level and five samples at the 10xLOQ level were prepared as described below:

aliquots of 100 mL of untreated drinking and surface water were transferred into 100 mL volumetric flasks and added with 1 mL of the FS.

The substrate volume, the concentration, the volume added and the consequent spiking levels are summarized in the table below:

Level	Matrix	Volume (mL)	FS solution No.	FS solution conc. (µg/L)	FS volume added (mL)	Final conc (µg/L)
LOQ	Drinking water	100	3	10.08	1	0.00998
10xLOQ		100	2	100.8	1	0.09980
LOQ	Surface water	100	5	10.05	1	0.099505
10xLOQ		100	3	100.5	1	0.99505

Control and fortified samples were directly injected into the LC/MS-MS without filtration.

9.4.3.2 HPLC Method

The analysis was carried out by the HPLC/MS-MS technique.

The complete set of WS was injected in duplicate.

The regression equation generated from the WS was used to check the linearity of response.

The operative conditions used are summarized hereafter:

- *Column*: Kinetex 2.6 µ C18 100A 50x4.6 mm (Phenomenex)
- *Pre-column*: SecurityGuard Ultra Cartridge UHPLC C18 for 4.6 mm (Phenomenex)
- *Eluent*:
  - **A**: (aqueous 10 mM ammonium acetate + 0.2% formic acid) 40%
  - **B**: (methanol + 0.2% formic acid) 60%
- Elution conditions:

min	%B
0	60
3	80
3.5	60

- *Stop time*: 5 min
- *Post time:* 3 min
- *Flow rate*: 1.2 mL/min
- *Injection volume*: 10 µL
- *Column temp.*: 30 ± 0.8 °C
- *Retention time*: about 2.9 min (see Section 14.4 and *Panel 1*)

Mass spectrometric conditions

- *Instrument*: Agilent 1200 series + MSD 6410
- *Ionization mode*: ESI
- *Polarity*: positive

- *Molecular ion*: 352 m/z
- *Mass transitions*:
  - 352 → 256.1 m/z (1st transition used for quantitative/qualitative analysis)
  - 352 → 312.2 m/z (2nd transition used for quantitative/qualitative analysis)

Further details on instrument settings, operative conditions and mass spectra are reported in Section 14.

9.4.3.3 Calculations

Peaks were integrated and the calculation of the concentration of each injected solution was performed according to the calibration curve technique.

The regression equation generated by the calibration curve (type 1/x) was used to check both the linearity of response and to quantify the IR9792 (F9990).

The formula applied is:

$$R = \frac{A_{smp} - i}{s}$$

where:

R: residue (in µg/L);

A_{smp}: peak area of the sample (in arbitrary units ≡ a.u.);

s: slope of the calibration curve (in a.u./(µg/L);

i: intercept of the calibration curve (in a.u.);

The recovery % is calculated as follows:

$$\%\ recovery = \frac{R}{F} * 100\ \%$$

where:

R: residue found (in mg/kg);

F: quantity of IR9792 (F9990) added in the fortified samples (in µg/L).

For example, the residue levels measured in the fortified sample 1413/1R2 – ion 256.1 (as reported in Table 3) were calculated as follows:

$A_{mp:}$: *1st injection* 237 *2nd injection* 244
i: 34.1301
s: 2389.2046

1st injection

$$R = \frac{235 - 34.1301}{2389.2046} = 0.0841\ \mu g/L$$

2nd injection

$$R = \frac{249 - 34.1301}{2389.2046} = 0.0899\ \mu g/L$$

R mean= 0.0870 µg/L

$$\%\ recovery = \frac{0.0870}{0.0998} * 100\,\% = 87.2\,\%$$

The time required to supply and prepared 4 specimens and 24 procedural recoveries was approximately 38 person/hours.
The evaluation time required approximately 20 person/hours.
The time period from the preparation of the samples set until the completion of the data reporting was approximately 58 person/hours.

The LC/MS-MS run was 8 minutes, 28 samples and the corresponding samples for calibration required approximately 62 instrument/hours (considering at least two injections for each sample).

9.4.4 Important points

Solutions were sonicated whenever rinsing and washing operations were carried out.

Solvent injections were interspersed often in order to purge the column and the detector.

9.5 Method Validation

9.5.1 Specificity

The method was tested in several control samples in order to verify the capability to distinguish IR9792 (F9990) from interfering peaks present in the water substrates, if any.

9.5.2 Calibration

The calibration curve was checked in a concentration range to encompass the range of concentrations in the final samples.
For that purpose a concentration range was used to cover from 30% of the LOQ to 20% above the highest level.
The calibration curve was checked before the sample sequence.

9.5.3 Accuracy

The accuracy of the method was evaluated at two different concentrations, 0.10 µg/L and 1.0 µg/L. Overall accuracy was calculated for the entire data set.

According to the Office of Pesticide Programs – U.S. Environmental Protection Agency (2), to SANCO/3029/99 rev.4, 11/07/00, SANCO/825/00 rev. 8.1 (16/11/2010) and to OECD ENV/JM/MONO(2007)17, the set of samples was constituted by:

2 control samples	**(unfortified);**
7 samples	**at the limit of quantitative determination (LOQ);**
5 samples	**at 10 times LOQ.**

Although in the SANCO/825/00 rev 8.1[3], reports that in case of direct injection the recovery data cannot be calculated, in this study they were reported.

9.5.4 Precision – repeatability (r)

Precision was evaluated by calculating the %RSD at two fortification levels for all substrates. Overall precision of the method was calculated for the entire data set.

9.5.5 Limit of quantitative (LOQ) and qualitative (LOD) determination

The target quantitative limit of determination (LOQ) at 0.1 µg/L was tested for each substrate.

The limit of qualitative determination (named also "limit of detection" with the acronym LOD in "91/414/EEC Standard Terms and Abbreviation") was calculated as below and was used in turn to confirm the LOQ:

$$LOD = t_{0.99} \times S$$

$$LOQ = 3 \times LOD$$

[3] See paragraph 6 "Analytical methods for residues in water (Annex IIA, Point 4.2.3 of Directive 91/414/EEC; Annex Point IIA; Point 4.5 of OECD)".

where

t = one-tailed t-statistic at the 99% confidence level for n-1 replicates

S = Standard Deviation of n samples spikes at the estimated LOQ

9.6 Quantitative Sample Analysis

Quantitative analyses were performed according to the method described above.

The amount of each sample subject to the analytical procedure was 100 mL for drinking and surface water.

Final data were obtained by automatically averaging the concentrations of replicated injections.
Reference standard solutions were injected just before sample sequence (P suffixed samples) in order to check the chromatographic column.

The chronological complete sequences of injections and the primary data were archived with the raw data.

9.7 Graphical Software

All the graphical elaborations of data were carried out using the AGILENT MASS HUNTER Workstation Software (B.01.04) and/or the EXCEL software (Microsoft Office 2003 Professional Milan – Italy).

9.8 Measurement and data elaboration

Concentrations were automatically calculated by the integrator software AGILENT MASS HUNTER Workstation Software (B.01.04).

Data elaboration was carried out by EXCEL software (Microsoft Office 2003 Professional Milan – Italy).

The rounding of decimals, when not obtained by the above mentioned software, was executed following the F.W.Küster and A.Thiel guidelines[4].

The arithmetic means reported were automatically obtained by averaging the individual values with a greater number of decimals than usually shown in the tables.

[4] Küster and Thiel Tabelle Logaritmiche (Logarithmic Tables)–Ulrico Hoepli Editor Milan Italy.

Table 2. Spiking solutions for method validation recoveries

Substrate	FS conc. (µg/L)	FS volume (mL)	Substrate amount (mL)	Fortification level (µg/L)
drinking water	10.08	1.00	100	0.0998
	100.8	1.00	100	0.9980

Substrate	FS conc. (µg/L)	FS volume (mL)	Substrate amount (ml)	Fortification level (µg/L)
surface water	10.05	1.00	100	0.09950
	100.5	1.00	100	0.99505

14. Chromatographic section

14.1 Operative conditions of analysis[5]

Acquisition Method Info

Method Name	RA1413.m
Method Path	D:\MassHunter\methods\RA Methods\RA1407.m
Method Description	Default Method

Device List
ALS
Bin Pump
Column
MS QQQ
QQQ Mass Spectrometer

Ion Source	ESI
Tune File	atunes.tune.xml
Stop Mode	No Limit/As Pump
Stop Time	1
Time Filter	On
Time Filter Width	0.07

Time Segments

Time Seg #	Time	Scan Type	Ion Mode	Polarity	Div Valve	Delta EMV	Store
1	0	MRM	ESI	Positive	To MS	300	þ

Time Segment 1
Scan Segments

Compound Name	ISTD?	Prec Ion	MS1 Res	Prod Ion	MS2 Res	Dwell	Frag (V)	CE (V)
IR9792	"	352.4	Unit	312.2	Unit	200	146	17
IR9792	"	352.4	Unit	256.1	Unit	200	146	29

Source Parameters

Parameter	Value
Gas Temp (°C)	300
Gas Flow (l/min)	13
Nebulizer (psi)	20
Capillary (V)	4000

Chromatograms

Chrom Type	Label	Offset	Y-Range
TIC	TIC	0	10000000

Instrument Curves

Actual
Gas Flow

[5] In this section is reported the printout of instrumental data.

Capillary
Capillary Current
Chamber Current
Gas Temp
High Vac
MS1 Heater
MS2 Heater
Nebulizer
Pump1 Current
Pump2 Current
Rough Vac
Turbo1 Speed
Turbo2 Speed

Autosampler

Name	ALS	**Model**	G1329A
Ordinal #	1	**Options**	THM

Stop Time (min)	As Pump	**Post Time (min)**	Off
Injection Type	Standard Injection	**Injection Volume**	10
Overlap Time	Disable Overlapped Injection	**Draw Position**	0
Draw Speed	200	**Eject Speed**	200
Wash Vessel	N/A		
Ready Temp. Range		**Temp.**	
Contact 1	0		
Contact 2	0		
Contact 3	0		
Contact 4	0		

Binary Pump

Name	Bin Pump	**Model**	G1312A
Ordinal #	1	**Options**	

Stop Time (min)	5	**Post Time (min)**	3
Flow (µl/min)	1.2	**Pressure Min (bar)**	0
Pressure Max (bar)	400	**Max Flow Gradient (ml/min)**	100
Solvent A	Ammonium Acetate aq. 10mM + 0.2% FormicAcid		
Solvent Ratio A	40		
Compress. A (*10-6/bar)	46		
Stroke A	Auto		
Solvent B	MeOH + 0.2% FormicAcid		
Solvent Ratio B	60		
Compress. B (*10-6/bar)	115		
Stroke B	Auto		
Contact 1	0		

Contact 2 0
Contact 3 0
Contact 4 0
Pump Time Table

Time	Flow	Pressure	Solv Ratio B
0	No Change	No Change	60
3	No Change	No Change	80
3.5	No Change	No Change	60

Thermostated Column Compartment

Name Column **Model** G1316A
Ordinal # 1 **Options**

Stop Time (min) As Pump **Post Time (min)** Off
Left Temp. 30 **Right Temp.** Not Controlled
Left Ready With Any Temp **Right Ready** 0.8
Valve Position 1
Contact 1 0
Contact 2 0
Contact 3 0
Contact 4 0
Signals Selected

Description
Temperature of left heat exchanger