VALIDATION OF THE ANALYTICAL METHOD FOR THE DETERMINATION OF SAN 835 H AND ITS PHTHALAZINONE RESIDUES (M1 AND M5 METABOLITES) IN SOIL, 1995

1. SUMMARY

An analytical method has been developed by Sandoz Agro LTD, Market Area 1, ACES , Huningue for the determination of residues of SAN 835 H in soil. The method allows determination of the parent molecule and of the metabolites containing the common moiety phthalazinone (M1 and M5 metabolites), those metabolites being quantitated as phthalazinone.
The method consists of an extraction of the parent molecule and its M1 and M5 metabolites with acetone/ sodium bicarbonate solution. Then the extract is split in 2 aliquots and the acetone is evaporated. The aliquot to be analyzed for SAN 835 H is acidified then partitioned on an Extrelut column, followed by a SPE clean-up step on C18 column and HPLC analysis. The aliquot to be analyzed for phthalazinone is partitioned on an Extrelut column, cleaned on a Envi-Carb SPE column before analysis on GC/MS.
This method successfully passed a in-house validation .
The validation was performed by fortifying untreated soil samples with SAN 835 H and phthalazinone (M1 metabolite) at levels equivalent to the limit of determination of the described method (0.01 µg/g) and to ten times that level (0.1 µg/g). Additionally, 5 untreated soil samples were fortified with carbamoyl phthalazinone (M5 metabolite) at 10 times the limit of determination of the method. 5 untreated soil samples were fortified with SAN 835 H only at ten times the limit of determination of the method.
For each compound and each fortification level, 5 replicated samples were analyzed under repeatability conditions.

2. INTRODUCTION AND STUDY OBJECTIVES

SAN 835 H is an experimental herbicide being developed by Sandoz Agro Ltd for postemergence use in corn. SAN 835 H is a growth inhibitor that acts by inhibiting the auxin transport. From the results of a soil route and rate of degradation study, phthalazinone residues as the sum of phthalazinone (or metabolite M1) and carbamoyl phthalazinone (or metabolite M5) account for more than 10 % of the initial concentration of SAN 835 H applied. As a consequence, they have to be included in the residue definition.

An analytical method, described in this report, was developed to quantify the parent compound of SAN 835 H and the metabolites containing the phthalazinone common moiety in field soil samples.

The objective of the study reported here was to validate this analytical method developed in the testing facility. The accuracy, precision and repeatability of the method was to be determined.

This objective included to demonstrate that carbamoyl phthalazinone (M5 metabolite) can be quantitatively quantified as phthalazinone (M1 metabolite) in soil and that no significant amount of phthalazinone coming from degradation of SAN 835 H during the sample analysis procedure was observed.

3. DATES/ DEADLINES

Protocol signature :	26-Sep-95
First amendment:	31-Oct-95
Start of analytical work:	02-Oct-95
End of analytical work:	07-Nov-95
Final report:	07-May-96

4. PROJECT STAFF

Test facility site:	Name: Sandoz Agro LTD, Market Area 1, ACES Address: Sandoz Agro Europe, c/o Clariant, F-68330 Huningue FRANCE
Technician:	G. Golling
Study director:	Dr. MN. Carrier
Sponsor:	Dr. P. Hertl

5. ARCHIVING

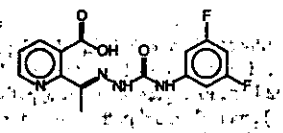
All analytical raw data including chromatograms, additional information, the study plans, the amendments and the final report are archived under the project number R 95-034 for a period of at least ten years in the archive of the test facility site.

6. TEST SUBSTANCE

SAN 835 H

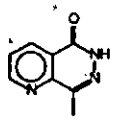
Systematic name: 2-[methyl((3,4-difluorophenylamino(carbonyl)hydrazono)methyl-a-pyridine carboxylic acid

Empirical formula: $C_{14}H_{12}F_2N_4O_3$



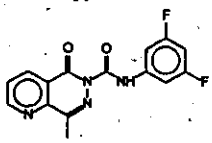
Molecular weight:	322 g/mol
Batch number :	5904-4-3
Purity:	98.1%
Expiry date:	11/97

phthalazinone (M1 metabolite of SAN 835H)
Systematic name: 8-methylpyrido(2,3-D)pyridazin-5(6H)-one
Empirical formula: $C_8H_7N_3O$



Molecular weight: 161 g/mol
Identification number: RS-M1-012095
Purity: 98.1%
Expiry date: 01/02/2000

Carbamoyl phthalazinone (M5 metabolite of SAN 835 H)
Systematic name: 6-((3,5-Difluorophenyl-carbamoyl-8-methyl-pyrido(2,3-d)-5-pyridazinone



Empirical formula: $C_{14}H_{10}F_2N_4O_2$
Molecular weight: 304 g/mol
Identification number: RS-M5-041395-1
Purity: 94.5 %
Expiry date: 09/05/2000

7. TEST SYSTEM

This study was performed using untreated field soil from a plot located at Leuggern , Switzerland. The sample was taken on June 6, 1995 on the control plot of site A of study R95-006 at day 0 just before the application to the treated sub-plot. The soil sample was received deep-frozen in good conditions on June 14, 1995, cut, sub-sampled, homogenized on June 21 and 22, 1995 and stored at a temperature below -20°C. The layer 0-10cm was used in this study. The soil parameters determined by Agrolab AG, Root, Switzerland are reported in Table 1 below.

Table 1: ***physico-chemical parameters of the test system***

% clay	19.6
% silt	55.2
% sand	25.2
pH (H_2O)	5.55
pH ($CaCl_2$)	5.05
CaCO3 content (%)	0.0
Organic Carbone content (%)	1.2
Cation exchange capacity (meq/100g)	8.0

8. EQUIPMENT

8.1 REAGENTS

Acetonitril gradient grade for chromatography,Lichrosolv , Merck art. 1.0030
Acetone GR, pro analysi , Merck art. 1.00014
Methanol for organic trace analysis, Suprasolv , Merck art. 1.06011
Ethyl acetate for organic residue analysis "Ultra-Resi analyzed" , JT Baker art. 9260-03
Dichloromethane for pesticides analysis, Pesti-pur , SDS art. 02922E21
Toluene for organic residue analysis "Baker Resi-analyzed" , JT Baker art. 9336-03
Sodium hydrogenocarbonate GR , Merck art. 1.06329
Filtration agent Clarcel , CECA
Diatomaceus earth Extrelut® 20 , Merck art. 11378
Supelclean™ Envi™-Carb SPE cartridges 0.25g, 3ml , Supelco, L.P.C.R. art. 5-7088
Bakerbond SPE C18 cartridges, 1 g, 6 ml, Baker art.7020-07

8.2 APPARATUS

Shaker , Edmund Bühler SM 25 and SM 25A
Rotary evaporator, Rotavapor R-124V, Büchi AG , CH
1 L bottle with screw cap, Verlabo 2000 , Strasbourg
13 cm diameter Büchner funnels , Store Sandoz Basel
11cm diam. round filters MN 640 M , L.P.C.R , Macherey-Nagel art. 203.011
various volumes graduated cylinders , Store Sandoz Basel
50 ml dropping funnels , Store Sandoz Basel
various volumes round bottom flask , Store Sandoz Basel
5 ml graduated tubes , Glasmechanic , CH
various volumes graduated pipettes , Store Sandoz Basel
Vacuum station for SPE columns, Vac Elut SPS 24 , Varian AG, Basel
Columns for Diatomaceous earth Extrelut® 20 , Merck art. 11737
Gas chromatography instrument with MS detector , Hewlett-Packard, HP5890/MSD 5972
HPLC instrument , Hewlett Packard, HP 1090 with DAD detector

9. STANDARD SOLUTIONS

All the stock solutions were stored at temperature equivalent to or below -20°C.
All the working solutions were stored at temperature below 5°C when not in use.

9.1 *For SAN 835H*

A stock solution of 10 mg/ml of SAN 835 H was prepared in acetone and working solutions of concentrations ranging from 0.05 µg/ml to 50 µg/ml in DMSO/ acetonitrile (5:95) were prepared by successive dilutions of the stock solution. The maximum dilution ratio used was 1 to 100. These working solutions were used for fortifications of untreated samples. They were stored at below 5°C for not more than 2 months.

These solutions were further diluted 5 times with water to give the standard solutions (in water/acetonitrile (8:2)) which were used as external standard calibration for HPLC analysis. These HPLC solutions were prepared just before use and were not stored.

9.2 *For phthalazinone (M1 metabolite)*

A stock solution of 1 mg/ml of phthalazinone in acetone and working solutions (concentration range: 10 µg/ml to 0.01 µg/ml) in toluene prepared by successive dilutions of the stock solution were used. The maximum dilution ratio used was 1 to 100.
These working solutions were used as standard solutions for external standard calibration for GC/MS analysis and also for fortification of untreated samples.

9.3 For carbamoyl phthalazinone (M5 metabolite)

A stock solution of 10 mg/ml in DMSO/ acetone (2:8) of carbamoyl phthalazinone was prepared. A working solution in acetone at a concentration of 5 µg/ml was prepared by successive dilutions of the stock solution and was used for fortification of untreated samples within 10 days of preparation. The maximum dilution ratio used was 1 to 100.

10. ANALYTICAL PROCEDURE

10.1 SAMPLE PREPARATION

The homogenized laboratory sample was prepared and used. Stones larger than 1 cm diameter were removed. 100 g of soil were let to dry overnight and weighed again to determine the weight of dry soil. This weight divided by five (to correspond to the amount analyzed in each aliquot) was used as soil weight (see point 13.2.3) for the calculation of the amount of residues.

10.2 FORTIFICATION

The untreated soil sample (50g) was fortified by direct addition of 1 ml of the appropriate solution of SAN 835 H (in DMSO/ acetonitrile= 5:95) , and /or phthalazinone (in toluene) or carbamoyl phthalazinone (in acetone) to the undried soil before extraction.

10.3 EXTRACTION

50 g of undried soil and about 10 g of Clarcel were weighed in a 1L screw-cap bottle. 50 ml of a 0.5% sodium hydrogenocarbonate solution and then 150 ml of acetone were added. The mixture was shaken for 15 minutes and filtered into a 1L round bottom flask under vacuum on a Büchner funnel containing a round filter moistened with acetone. The filter was rinsed with about 50 ml of acetone. The filtration cake was extracted again for 15 minutes with 200 ml acetone and then filtered in the same round bottom flask using another moistened filter. The cake was rinsed with about 50 ml of acetone.

The extract was adjusted to a definite volume with acetone and two aliquots each corresponding to 20 g of initial sample were withdrawn. The remaining solution was discarded. One aliquot was analyzed for residues of SAN 835H parent compound, the other one for phthalazinone residues.

10.4 ANALYSIS FOR SAN 835 H

The acetone was evaporated using the rotavapor under maximum vacuum and at a temperature of 20-30°C until about 20 ml of aqueous residue remained.

10.4.1 Clean-up on Extrelut column

An Extrelut column was filled with one dose of Extrelut® 20 material. 1 ml of 5N HCl was added to the aqueous residue , the mixture was swirled and transferred to a dropping funnel set over the Extrelut column. After checking that the volume was below 20 ml, the acidified solution was poured on the Extrelut silica, allowed to stand for 10 minutes and then eluted with 100 ml of ethyl acetate. The ethyl acetate was used to rinse the round bottom flask and the dropping funnel. The eluat was collected in a 250 ml round bottom flask, evaporated to dryness using the rotavapor under maximum vacuum and at a temperature of 20-30°C.

10.4.2 Clean-up on C18 column

10.4.2.1 Conditioning of the C18 column

A C18 column (1g /6 ml) was rinsed first with 2 volumes (=12 ml) of methanol and then with 2 volumes (=12 ml) of 0.5% sodium hydrogenocarbonate solution. The column was not allowed to dry.

10.4.2.2 Reversed Phase-Chromatography with C18

The residue of point 10.4.1 was reconstituted in 5 ml of the 0.5 % sodium hydrogenocarbonate solution. This solution was passed through the conditioned column using vacuum (the column was not allowed to dry). The round bottom flask was rinsed with 5 ml of the 0.5 % sodium hydrogenocarbonate solution , the rinse was added on the column. This rinse was repeated with 5 ml water. The column was then dried by applying vacuum and the eluate was discarded.
The column was eluted successively with 2 x 1 ml of the acetonitril/water mixture (2:8) bringing the column to dryness between each elution. The eluate was collected in a 5 ml graduated tube and the final volume was recorded. This solution was injected in HPLC.

10.5 ANALYSIS FOR PHTHALAZINONE

The acetone was evaporated using the rotavapor under maximum vacuum and at a temperature of 20-30°C until about 20 ml of aqueous residue remained.

10.5.1 Clean-up on Extrelut column

An Extrelut column was filled with one dose of Extrelut® 20 material. The aqueous residue was transferred to a dropping funnel set over the Extrelut column. After checking that the volume was below 20 ml, the solution was poured on the Extrelut silica, allowed to stand for 10 minutes and then eluted with 100 ml of ethyl acetate. The ethyl acetate was used to rinse the round bottom flask and the dropping funnel. The eluat was collected in a 250 ml round bottom flask, evaporate to dryness using the rotavapor under maximum vacuum and at a temperature of 40-50°C.

10.5.2 *Clean-up on Envi-Carb column*

10.5.2.1 Conditioning of the Envi-Carb column

An Envi-Carb column (0.25 g/3 ml) was rinsed first with 2 volumes (=6 ml) of methanol/ dichloromethane (2:8) and then with 2 volumes (= 6ml) of water. The column was not allowed to dry.

10.5.2.2 *Adsorption on Envi-Carb column*

The residue from point 10.5.1 was reconstituted in 3 ml water and passed through the conditioned column using vacuum (the column was not allowed to dry). The round bottom flask was rinsed with 2 x 3 ml water and the rinses were added on the column. The column was dried by applying vacuum and the eluate was discarded.
The column was eluted with 2 successive volumes (= 2 x 3 ml) of the mixture methanol/ dichloromethane (2:8) drying the column between each elution. The eluate was collected in a 50 ml round bottom flask. The solvent was evaporated to dryness using the rotavapor under maximum vacuum and at a temperature of 40-50°C. The residue was reconstituted in 2 ml of toluene.
The toluene solution was transferred to the GC vial using a Pasteur pipette with the tip covered with cotton in order to filter the carbon particles eluted from the SPE column. This solution was injected in GC/MS.

10.6 IMPORTANT REMARKS

The stability of SAN 835H is limited in solution and at high temperature.
As a consequence all the extracts containing SAN 835 H were stored at below 5°C overnight. It was found that a good timing to stop the analysis overnight was just after the clean-up step on Extrelut column when the sample is in solution in ethyl acetate. For stability reasons, final extracts ready for HPLC (in solution in acetonitrile/water (2:8)) need to be kept at a temperature below -18°C, if storage for more than 1 day is necessary.
Also when using the rotavapor with samples containing SAN 835 H, the temperature of the water bath was not set above 30°C and the temperature of the cooling fluid was set to -10°C. In order to reduce the time needed for the analysis of SAN 835 H, the analysis were performed in the following order:

a) Extrelut clean-up of the aliquot analyzed for SAN 835H (point 10.4) and of the aliquot analyzed for phthalazinone (point 10.5).
b) finalization of the analysis for SAN 835 H
c) Finalization of the analysis for phthalazinone

11. CHROMATOGRAPHIC DETERMINATION

11.1 Chromatographic determination using HPLC for SAN 835H

11.1.1 Clean-up of the required water

Only ultra-pure water was used (deionised water purified through Millipore purification system (Milli Q)). This water was used for the preparation of a 0.5 % trifluoroacetic acid solution and then this solution was filtered on a 10 g C18 SPE column before being used for HPLC.

11.1.2 Chromatographic determination

Column: Hypersil BDS-C18 , 5 µm, 250 x 4 mm
Hewlett-Packard , ref: 79926 OB 584
Temperature : 40°C

Mobile phase: Solvent A: Acetonitril
Solvent B: 0.5 % trifluoroacetic acid solution

Flow: 1 ml/minute

Gradient:

time	Acetonitril	0.5% TFA
Min. 0	10 %	90 %
Min. 2	10 %	90 %
Min. 22	50 %	50 %
Min. 25	50 %	50 %
Min. 27	80 %	20 %
Min. 29	80 %	20 %

Injection volume: 250 µl

Equilibration time: 10 minutes

Analysis time: 25 minutes

Retention time: about 17.8 minutes

Detector: Diode-Array detector or UV detector with variable wave length ,
wave length : 240 nm

11.2 Chromatographic determination using GC/MS for phthalazinone

Column: HP-5 MS , film thickness: 0.25 µm, lenght: 30 m, ID: 0.250 mm
Hewlett-Packard , ref: 19091S-433

Carrier gas: Helium

Temperature program: 2 min at 100° C
+ 15° C / min to 220° C,
220° C for 3 min.

Injector: 250° C

Detector: 280° C

Injected volume: 1 µl

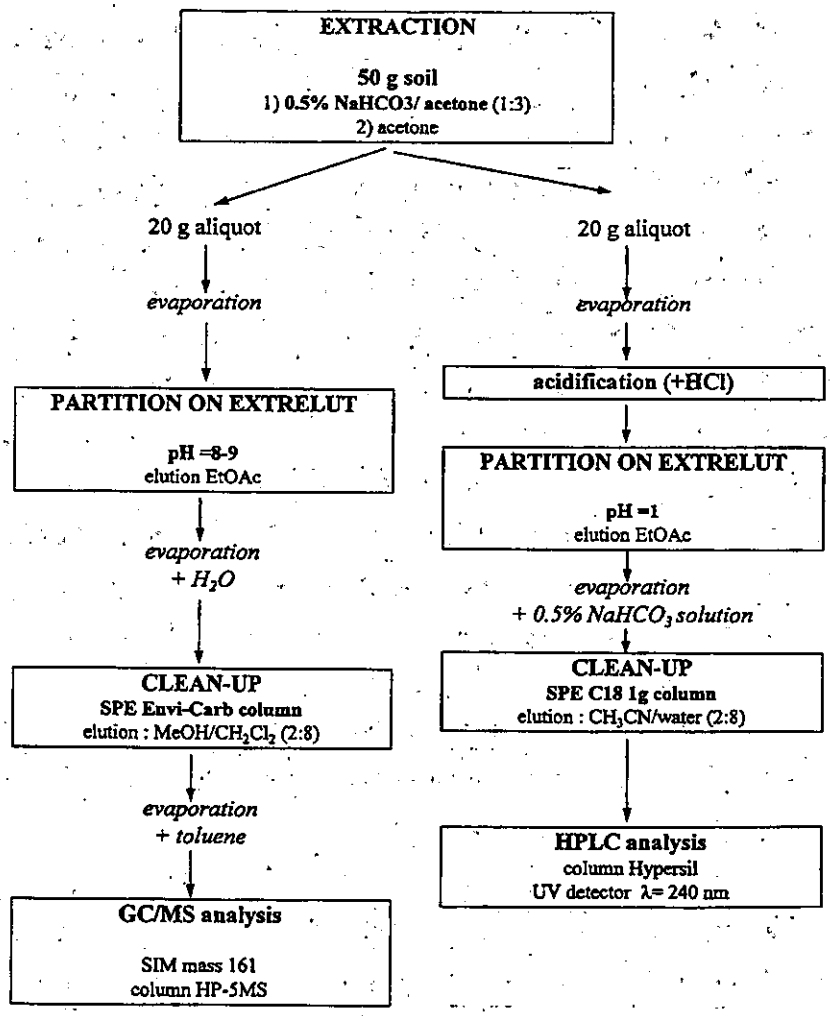
Analysis time: 13 minutes

Retention time: about 9 minutes

Detector: MSD

Single Ion Monitoring mass 161 (M^+)

12. FLOW CHART OF THE METHOD



13. EVALUATION OF RESULTS

13.1 Analysis sequence

Each analysis sequence injected was composed of the extracts from the samples spaced out with the external standards of the appropriate compound at selected concentrations. Each sequence started with one standard solution. The concentration of the standard solutions covered the expected working range.

13.2 Calculation of results

Concentrations in the sample extracts are quantified by their detector response with reference to an external standard calibration curve determined within the respective analytical sequence. The calibration curve is obtained by correlation of the detector responses of the external analytical standards with their corresponding concentrations (concentration range 0.005 to 10 µg/ml). A complete external standard calibration, consisting of at least ten different concentrations, was performed for each analytical sequence.

13.2.1 calculation of calibration curves

The data pairs are correlated by using the first or second order least square fitting functions (see equations 1 and 2).

$$y = a_0 + a_1 * x \qquad (1)$$

or

$$y = a_0 + a_1 * x + a_2 * x^2 \qquad (2)$$

where
x = concentration of analyte in the injected standard solution
y = detector response (in counts or mm)
a_0 = y-axis intercept
a_1 = first order correlation coefficient
a_2 = second order correlation coefficient.

Standard criteria for acceptance of the calibration functions was that the correlation coefficient R exceeded 0.99 and the coefficient of variation was smaller than 16 %.
The coefficient of variation was calculated by the following formula:

$$\text{Variation coefficient of calibration} = \frac{RSS}{S * X_{mean}}$$

where RSS: Residual Sum of Squares of the external standard calibration curve
S: Sensitivity a_1 (linear) or $a_1+2a_2*X_{mean}$ (quadratic) of the calibration function
X_{mean}: Mean of the external standard concentrations

13.2.2 Calculation of residue concentrations in sample extracts

The residue concentration x_u of the analyte was calculated using equations (3) or (4):

First order calibration function: $$x_u = \frac{y_u - a_0}{a_1} \quad (3)$$

Second order calibration function:

$$x_u = -\frac{a_1}{2a_2} \pm \sqrt{\left(\frac{a_1}{2a_2}\right)^2 - \left(\frac{a_0 - y_u}{a_2}\right)} \quad (4)$$

where
x_u = residue concentration in the sample extract [µg/ml]
y_u = detector response [counts or mm]

13.2.3 Calculation of residue concentrations in samples

The residue concentration C_u of the analyte in the sample was calculated using equation (5):

$$C_u = x_u * \frac{V_f}{W} \quad (5)$$

where
C_u = residual concentration of the analyte [in µg/g]
x_u = residue concentration in the sample extract [µg/ml]
V_f = final volume including all dilution steps [in ml]
W = dry weight of the sample analyzed [in g] (corresponding to a wet aliquot of 20g)

13.2.4 Calculation of recoveries

The recovery R is calculated using equation (6):

$$R = 100 * \frac{Cu}{Cs}\% \quad (6)$$

where
C_u = residual concentration of the analyte found in the fortified sample [in µg/g]
C_s = concentration of the analyte added to the fortified control sample [in µg/g]

13.3 Limit of determination/ detection

The upper limit of the working range of the external calibration function is defined by the maximum concentration used for calibration. The lower limit is the limit of determination of the actual analytical sequence calculated for each sequence as the lowest concentration being different from zero with a 95 % confidence level.
A limit of detection is also calculated as the lowest concentration being different from zero with a 50% confidence level.
The actual limit of detection (ng) and limit of determination (bg) in µg/g are calculated from these limits using equation (5).
Results are considered valid when they are within the working range of the calibration function.
All results from the fortified samples were within the acceptable range.