UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, D.C. 20460

OFFICE OF
PREVENTION, PESTICIDES
AND TOXIC SUBSTANCES

MEMORANDUM

Date: July 2, 2003

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DP Barcode: D278386

Petition: 1F06313

Citation: 45405106 Grosshans, F. (2001) The Validation of BASF Method 471/0: The Determination of BAS 510F and the Metabolite M510F01 in Animal Matrices: Final Report: Lab Project Number: 42392: 471/0: 2000/1017223. Unpublished study prepared by BASF Aktiengesellschaft. 74 pages.

Sponsor: BASF Corporation

Background

The information contained herein was compiled by Dynamac Corporation (20440 Century Boulevard, Suite 100, Germantown MD 20874), contractor, under the supervision of RAB2/HED. This DER has undergone secondary review by RAB2, and reflects current HED and Office of Pesticide Programs (OPP) policies. This DER has also been peer-reviewed by PMRA/Canada.

Executive Summary

BASF Corporation has proposed **LC/MS/MS method 471/0** for the purposes of data collection for residues of BAS 510 F and its metabolite M510F01 in animal commodities. Briefly, animal

(1)

matrix samples are extracted with methanol. The extract is evaporated to dryness and subjected to enzyme hydrolysis using a mixture of β-glucuronidase and arylsulfatase solution. The hydrolysate is partitioned twice with ethyl acetate and the organic phase is applied to a C18 solid phase extraction (SPE) cartridge for cleanup. Further cleanup through a silica gel SPE cartridge may be used if necessary. The final eluate is evaporated to dryness and residues redissolved in acetonitrile (ACN) for LC/MS/MS analysis. MS/MS detection using the positive ionization mode monitors ion transitions from *m/z* 359 to 140 and 323 for BAS 510 F and *m/z* 343 to 140 and 307 for M510F01. Quantitation is conducted using an external calibration curve of BAS 510 F and M510F01 standards. The validated limit of quantitation (LOQ) was 0.010 ppm each for the residues of BAS 510 F and M510F01 in eggs, milk, and cream, and 0.025 ppm each for the residues of BAS 500 F and M510F01 in cow tissues (kidney, liver, and muscle) and fat.

Method validation was conducted for LC/MS/MS method 471/0 in egg, milk, cream, and cow kidney, liver, muscle, and fat. Method validation recoveries for residues of BAS 510 F ranged 78-97% in egg, 77-93% in milk, and 71-94% in cream fortified with BAS 510 F at 0.010 and 0.10 ppm, and 82-94% in cow kidney, 80-109% in cow liver, 82-97% in cow muscle, and 70-87% in cow fat fortified with BAS 510 F at 0.025 and 0.25 ppm. Method validation recoveries for residues of M510F01 ranged 75-100% in egg, 72-94% in milk, and 88-96% in cream fortified with BAS 510 F at 0.010 and 0.10 ppm, and 78-86% in cow kidney, 77-102% in cow liver, 85-91% in cow muscle, and 73-88% in cow fat fortified with M510F01 at 0.025 and 0.25 ppm.

A study to demonstrate the stability of standard solutions of BAS 510 F and various of its metabolites in ACN has been submitted separately (see DER of MRID 45405104). The recoveries of BAS 510 F, M510F01, M510F49, M510F51, and M510F53 following 62 days of storage, either refrigerated in the dark or at room temperature with daylight exposure, indicated no loss of concentration during storage. Based on these data, the petitioner recommended that standard solutions be stored no longer than 60 days. **As a condition of registration,** the petitioner should revise this LC/MS/MS method to recommend a 60-day maximum storage interval for standard solutions of reference standards, and submit a copy of the revised method to the Agency.

The petitioner conducted a study to investigate the efficiency of the enzymatic cleavage step of the LC/MS/MS data collection method using radiolabeled M510F02 (glucuronidase conjugate of M510F01). The recoveries demonstrate that the enzyme hydrolysis step of the LC/MS/MS method adequately converts M510F02 to M510F01 for analysis.

Radiovalidation of the extraction procedures of this LC/MS/MS method was not conducted. However, the extraction procedures of this data collection method are similar to those of the proposed enforcement method for animal commodities (MRID 45405103). Radiovalidation data to support that proposed enforcement method have been requested as a **condition of registration**. When those data have been submitted, they will be considered sufficient to also satisfy radiovalidation data requirements for method 471/0.

Provided this method is revised to impose a 60-day limitation on the storage of standard solutions of reference standards and **contingent upon** receipt of radiovalidation data, Method 471/0 is adequate for collection of residue data for BAS 510 F and M510F01 in animal commodities. A separate GC/ECD method has been proposed as the enforcement method for animal matrices (refer to the DER for MRIDs 45405102 and 45405103).

GLP Compliance

Signed and dated GLP, quality assurance, and data confidentiality statements were provided. The petitioner stated that the study was conducted in accordance with the GLP regulations established in Germany (Appendix 1 to §19a Section 1, Chemikaliengesetz of 25-July-1994; Official Bulletin/Federal Republic of Germany I 1994, page 1703) instead of U.S. EPA GLP regulations.

1. Materials and Methods

1.1. Test Substances

Table 1.1.1. List of Analytes Tested with the LC/MS/MS Method.

Common Name:	Nicobifen, ISO proposed (parent compound)	None assigned (hydroxy metabolite)	None assigned (glucuronide acid conjugate of M510F01)
IUPAC Name:	2-Chloro-N-(4'-chlorobiphenyl-2-yl)-nicotinamide	2-Chloro-N-(4'-chloro-5-hydroxy-biphenyl-2-yl)-nicotinamide	Not available; compound isolated from rat urine in the rat metabolism study
CAS Name:	3-Pyridinecarboxamide, 2-chloro-N-(4'chloro[1,1'-biphenyl]-2-yl)-	Not available	Not available
CAS Number:	188425-85-6	Not available	Not available
Company Name:	BAS 510 F	M510F01	M510F02
Other Synonyms:	BASF Registry No. 300355	BASF Registry No. 398794	

Table 1.1.2. Matrices Tested with the LC/MS/MS Method.	
Matrix	Matrix Form
Eggs	Obtained commercially; egg whites and yolk were homogenized in a blender and refrigerated.
Milk	Obtained commercially and refrigerated.
Cream	
Cow kidney	Obtained commercially; tissue and fat samples were homogenized in a beef grinder and frozen.
Cow liver	
Cow muscle	
Cow fat	

Table 1.1.3. Fortification Levels Tested with the LC/MS/MS Method.		
Matrices	BAS 510 F (ppm)	M510F01 (ppm)
Egg, milk, and cream	0.010 (LOQ)	0.010 (LOQ)
	0.10	0.10
Cow kidney, liver, muscle, and fat	0.025 (LOQ)	0.025 (LOQ)
	0.25	0.25

1.2. Methods

A description of method 471/0 follows. For method validation, processed (homogenized) samples of eggs, milk, and cow tissues and fat were fortified with a solution containing BAS 510 F and its M510F01 in acetonitrile (ACN). Samples were fortified prior to method extraction; however, the time interval from fortification to extraction was not reported.

Briefly, homogenized samples of eggs, milk, cream, and cow kidney, liver, muscle, and fat are extracted with methanol in a homogenizer and vacuum filtered through Celite. The filter cake is washed twice with additional methanol, and the filtrates and extracts combined. An aliquot of the combined extract is evaporated to dryness and residues redissolved in 0.1 M pH 5 sodium acetate buffer. The residues are then subjected to enzyme hydrolysis with a β-glucuronidase and arylsulfatase solution (for 1 hour at 37 C) to deconjugate the metabolite. Water, 1 M HCl, and sodium chloride are added, and residues partitioned twice with ethyl acetate. The combined ethyl acetate phase is filtered through sodium sulfate, evaporated to dryness, and residues redissolved in methanol:water (3:7, v:v) for cleanup through a C18 solid phase extraction (SPE) cartridge. Residues were eluted with water:methanol (3:7, v:v) under vacuum. If additional cleanup is required, the eluate is evaporated to dryness and residues redissolved in isohexane: dichloromethane (1:9, v:v) for further cleanup through a silica gel SPE cartridge; residues are eluted with dichloromethane:acetone (8:2, v:v) under vacuum. The eluate is evaporated to dryness and residues redissolved in ACN for LC/MS/MS analysis. The HPLC system utilizes a

High Purity Elite C18 column and a gradient mobile phase of water, methanol, and formic acid. MS/MS detection using the positive ionization mode monitors ion transitions from *m/z* 359 to 140 and 323 for BAS 510 F and *m/z* 343 to 140 and 307 for M510F01. Quantitation is conducted using an external calibration curve of BAS 510 F and M510F01 standards.

All samples for the method validation did not require the additional silica SPE cleanup step.

2. Results

2.1. Stability of Reference Materials

Stock solutions of the BAS 510 F and M510F01 standards in ACN were used for the fortification and calibration solutions. The storage conditions of the standard solutions were not addressed.

A study to demonstrate the stability of standard solutions of BAS 510 F and various of its metabolites in ACN has been submitted separately (see DER of MRID 45405104). The recoveries of BAS 510 F, M510F01, M510F49, M510F51, and M510F53 following 62 days of storage, either refrigerated in the dark or at room temperature with daylight exposure, indicated no loss of concentration during storage. Based on these data, the petitioner recommended that standard solutions be stored no longer than 60 days. **As a condition of registration,** the petitioner should revise this LC/MS/MS method to recommend a 60-day maximum storage interval for standard solutions of reference standards, and submit a copy of the revised method to the Agency.

2.2. Method Characteristics

2.2.1. Chromatography

The representative LC/MS/MS total ion chromatograms for egg, milk, cream, and cow tissue and fat samples indicated that the peak shape was generally good for BAS 510 F and M510F01. No interferences were observed in the control samples.

2.2.2. Linearity

The method linearity was good. The coefficients of determination (r^2) from representative calibration standard curves were ≥0.995 for BAS 510 F and M510F01 standard concentrations ranging 0.010-0.25 ppm each.

2.2.3. Specificity

An interference study is not required as the method employs an MS detector. No interferences were observed in the unfortified samples.

2.2.4. Method Limits

The limit of detection (LOD) was not determined. The validated limit of quantitation (LOQ) was 0.010 ppm each for the residues of BAS 510 F and M510F01 in eggs, milk, and cream, and 0.025 ppm each for the residues of BAS 500 F and M510F01 in cow tissues (kidney, liver, and muscle) and fat.

2.2.5. Analyte Recoveries

Table 2.2.5.1. Recovery of BAS 510 F and Its Metabolite M510F01 From Egg, Milk, and Cow Tissues and Fat Using the LC/MS/MS Data Collection Method 471/0.

Matrix	Fortification Level of Each Analyte (ppm)	Recoveries (%)			
		BAS 510 F	Mean ± SD	M510F01	Mean ± SD
Whole egg	0.010	78, 82, 84, 84, 86	88 ± 6	75, 80, 86, 87, 86	86 ± 7
	0.10	90, 92, 93, 95, 97		87, 81, 84, 100, 93	
Milk	0.010	82, 84, 85, 89, 90	87 ± 5	81, 92, 86, 94, 88	87 ± 6
	0.10	77, 89, 92, 93, 93		72, 89, 87, 85, 90	
Cream	0.010	71, 71, 72, 73, 74	81 ± 10	88, 88, 90, 91, 90	92 ± 3
	0.10	83, 90, 90, 92, 94		92, 95, 96, 96, 92	
Cow kidney	0.025	82, 82, 83, 83, 86	87 ± 5	82, 82, 79, 80, 85	82 ± 3
	0.25	85, 90, 92, 93, 94		78, 79, 83, 85, 86	
Cow liver	0.025	80, 81, 89, 91, 92	91 ± 8	95, 77, 101, 86, 95	91 ± 7
	0.25	87, 91, 94, 100, 109		88, 88, 89, 90, 102	
Cow muscle	0.025	82, 85, 86, 89, 91	90 ± 5	87, 91, 88, 91, 90	88 ± 2
	0.25	94, 94, 94, 94, 97		85, 86, 85, 87, 88	
Cow fat	0.025	76, 77, 78, 83, 86	80 ± 6	80, 78, 85, 78, 84	82 ± 5
	0.25	70, 81, 82, 86, 87		83, 73, 82, 87, 88	

[1] Recoveries for M510F01 are listed respective to the recoveries for BAS 510 F from the same sample.

2.2.6. Independent Laboratory Validation

ILV is not required for LC/MS/MS method 471/0 because it is used only for data collection. A separate **GC/ECD** method (German multi-residue method DFG S19) is proposed as the **enforcement method**; method validation and ILV have been submitted in support of the enforcement method (refer to the DER for MRIDs 45405102 and 45405103).

3. Discussion

3.1. Recovery and Repeatability

Method validation was conducted for the LC/MS/MS method 471/0 for analysis of residues of BAS 510 F and M510F01 in animal matrices (egg, milk, cream, and cow kidney, liver, muscle, and fat). Method validation recoveries for residues of BAS 510 F ranged 78-97% in egg, 77-93% in milk, and 71-94% in cream fortified with BAS 510 F at 0.010 and 0.10 ppm, and 82-94% in cow kidney, 80-109% in cow liver, 82-97% in cow muscle, and 70-87% in cow fat fortified with BAS 510 F at 0.025 and 0.25 ppm. Method validation recoveries for residues of M510F01 ranged 75-100% in egg, 72-94% in milk, and 88-96% in cream fortified with BAS 510 F at 0.010 and 0.10 ppm, and 78-86% in cow kidney, 77-102% in cow liver, 85-91% in cow muscle, and 73-88% in cow fat fortified with M510F01 at 0.025 and 0.25 ppm.

Overall recoveries were acceptable for residues of BAS 510 F and its metabolite M510F01 in animal matrices.

3.2. Method Efficiency

Radiovalidation of this method was not conducted. The extraction procedures of this data collection method are similar to those of the proposed enforcement method for animal commodities (MRID 45405103). Radiovalidation data to support the proposed enforcement method remain outstanding. When those data have been submitted, they will be considered sufficient to satisfy radiovalidation data requirements for method 471/0.

The petitioner has submitted a study to investigate the efficiency of the enzymatic cleavage step of the LC/MS/MS data collection method. Methanol extracts of egg, milk, and cow tissues and fat control samples were fortified with radiolabeled M510F02, the glucuronide acid conjugate of M510F01 isolated from rat urine. The glucuronide M510F02 was identified by MS and NMR in the rat metabolism study (MRID 45404918). Fortified samples were then processed through the enzyme hydrolysis, liquid/liquid partitioning, and C18 SPE cleanup steps as described for the LC/MS/MS method 471/0. Radioactive residues were analyzed by LSC and radio HPLC. Adequate recoveries demonstrate that the enzyme hydrolysis step of the LC/MS/MS method adequately converts M510F02 to M510F01 for analysis; recoveries are reported below.

Table 3.2.1. Recovery of M510F02 From Samples of Egg, Milk, and Cow Tissues and Fat Fortified with [^{14}C]M510F02 and Processed through the Enzyme Cleavage Step of the LC/MS/MS Method 471/0.

Matrices	% Recovery by LSC (radioactivity)	% Recovery by Radio HPLC (radioactivity quantitated as M510F01)
Egg	93	81
Milk	99	100
Cow kidney	99	95
Cow liver	99	92
Cow muscle	99	95
Cow fat	95	96

4. Deficiencies

As a **condition of registration**, this LC/MS/MS analytical method should be revised to recommend a 60-day maximum storage interval for standard solutions of reference standards. A copy of the revised method should be submitted to the Agency.

No radiovalidation data for the extraction efficiency of this method were provided. The radiovalidation data required as a **condition of registration** to support the proposed enforcement method on animal commodities (see DER for MRIDs 45405102/45405103) will be considered sufficient to also satisfy the radiovalidation data requirements for method 471/0.

5. References

None.