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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Petition: 1F06313

Citation: 45405102 Kampke-Thiel, K. (2001) Independent Laboratory Validation of the Adapted Multi-Residue Method DFG S19 for the Determination of BAS 510 F and its Metabolite, M510F01, in Animal Matrices: Final Report: Lab Project Number: 42397: 2000/1017226: P453G. Unpublished study prepared by BASF Aktiengesellschaft. 127 pages.

45405103 Class, T. (2001) Assessment and Validation of the Adapted Multi-Residue Method DFG S19 Determination of BAS 510 F and its Metabolite, M510F01, in Animal Matrices: Final Report: Lab Project Number: P/B 453 G: 2000/1017227: DFG S19. Unpublished study prepared by PTRL Europe. 88 pages.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

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and Office of Pesticide Programs (OPP) policies. This DER has also been peer-reviewed by PMRA/Canada.

Executive Summary

BASF Corporation has proposed a **GC/ECD** method for the enforcement of proposed tolerances for residues of BAS 510 F and its hydroxy metabolite, M510F01, in animal commodities. The method is an adaptation of German multiresidue **method DFG S19** (GC/ECD). Briefly, samples of homogenized animal matrices are extracted with methanol. An aliquot of the filtered extract is treated with enzymes (solution of β-glucuronidase and arylsulfatase) which induce the deconjugation of metabolite M510F02 (glucuronic acid conjugate of M510F01) to M510F01. Residues are partitioned into organic phase with the addition of water, acetone, sodium chloride, and ethyl acetate:cyclohexane (1:1, v:v). An aliquot of the organic phase is cleaned-up by gel permeation chromatography, followed by acetylation of the hydroxy metabolite M510F01 with acetic anhydride. Residues are then fractionated through a silica gel cartridge. Residues of BAS 510 F and the acetyl derivative of M510F01 are analyzed, either separately or combined, using GC/ECD; residues of the acetyl derivative of M510F01 are calculated as BAS 510 F equivalents. Quantitation is obtained using an external calibration curve of standards of BAS 510 F and the acetyl derivative of M510F01. The reported limit of detection (LOD) was ca 0.002 ppm in milk and 0.005 ppm for all other animal matrices. The limit of quantitation (LOQ) for BAS 510 F and the M510F01 acetyl derivative, expressed as BAS 510 F equivalents, was 0.010 ppm for each analyte in milk and 0.025 ppm for each analyte in eggs, and bovine tissues and fat.

Adequate method validation and independent laboratory validation (ILV) studies were conducted for this GC/ECD method for analysis of residues of BAS 510 F and its hydroxy metabolite M510F01 in animal matrices. Method validation recoveries of the residues of BAS 510 F and M510F01 (quantitated as the acetyl derivative and expressed as BAS 510 F equivalents) respectively ranged 85-126% and 70-97% in eggs, 68-96% and 75-110% in milk, 78-103% and 73-107% in bovine muscle, 77-115% and 67-109% in bovine liver, 78-105% and 79-103% in bovine kidney, and 76-131% and 67-106% in bovine fat fortified with a combination of BAS 510 F and M510F01 at the LOQ (0.010 ppm for milk and 0.025 ppm for egg and beef tissues) and 10x the LOQ (0.10 ppm for milk and 0.25 ppm for egg and beef tissues).

The recoveries obtained from the ILV studies are comparable to the recoveries obtained in method validation. ILV recoveries of residues of BAS 510 F and M510F01 (quantitated as the acetyl derivative and expressed as BAS 510 F equivalents) respectively ranged 91-101% and 105-136% (86-114% corrected) in milk and 71-77% and 86-99% in bovine liver fortified with a combination of BAS 510 F and M510F01 at the LOQ (0.010 ppm for milk and 0.025 ppm for liver) and 10x the LOQ (0.10 ppm for milk and 0.25 ppm for liver).

It was noted that quantifiable peaks, present at less than the method LOQ, eluted at the retention times of BAS 510 F and M510F01 in control kidney samples from the method validation study and control milk and liver samples from the GC/ECD ILV study, affecting validations at the LOQ level.

tissues and fat were fortified with a solution containing BAS 510 F and its metabolite M510F01 in acetonitrile (ACN). Samples were fortified prior to method extraction; however, the time interval from fortification to extraction and analysis was not reported.

Briefly, homogenized samples of eggs, milk, and bovine muscle, kidney, liver, and fat are extracted (2x) with methanol 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 aqueous and 0.1 M pH 5 sodium acetate buffer added. The residues are then subjected to enzyme hydrolysis with a β-glucuronidase and arylsulfatase solution (for 1 hour at 37° C) to deconjugate M510F02 (the glucuronic acid metabolite of M510F01) to M510F01. Following overnight refrigeration, water, acetone, sodium chloride, and ethylacetate:cyclohexane (1:1, v:v) are added. An aliquot of the organic phase is filtered through sodium sulfate and additional ethylacetate:cyclohexane added to the filtrate. The filtrate is then evaporated to aqueous, and ethylacetate, sodium sulfate:sodium chloride (1:1, w:w), and cyclohexane are added. The solution is filtered and applied to a gel permeation column for cleanup; residues are eluted with ethylacetate:cyclohexane (1:1, v:v). The eluate is evaporated to dryness and residues redissolved in dichloromethane. Acetic anhydride and pyridine are added and the mixture incubated at 45° C for 30 minutes to acetylate the hydroxy metabolite M510F01. The mixture is evaporated to dryness and residues redissolved in isooctane for silica gel column cleanup. Residue fractions eluted in toluene:acetone (95:5, v:v; primarily BAS 510 F) and toluene:acetone (8:2, v:v; BAS 510 F and acetylated M510F01) are collected. The collected fractions can be analyzed separately or combined. The fractions are concentrated to near dryness and volume adjusted with toluene for GC/ECD analysis. The GC system uses a Varian Chrompack CP Sil 8 column and electron capture detection. Quantitation is obtained using an external calibration curve of standards of BAS 510 F and the acetyl derivative of M510F01. The M510F01 acetyl derivative standards are calculated as BAS 510 F equivalents when determining the calibration curve; thus, residues of M510F01 are reported as BAS 510 F equivalents.

2. Results

2.1. Stability of Reference Materials

Stock solutions of the BAS 510 F, M510F01, and M510F01 acetyl derivative standards in ACN were stored refrigerated (4° C). The stability of the M510F01 acetyl derivative standard in ACN was demonstrated by comparison with fresh stock solutions prepared after one month; no degradation was observed. Fortification solutions containing BAS 510 F and M510F01 were prepared in ACN from stock solutions and the final calibration solutions containing BAS 510 F and M510F01 acetyl derivative were prepared in toluene from stock solutions. Both fortification and calibration solutions were stored at 4° C.

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 GC/ECD 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 GC/ECD chromatograms for egg, milk, and bovine tissues and fat samples indicate that the peak shape was generally good for BAS 510 F and the M510F01 acetyl derivative. No significant interferences were observed in the control samples; however, apparent residues of M510F01 were observed in kidney at 0.006-0.007 ppm. The retention times for BAS 510 F and the M510F01 acetyl derivative were 10.1-10.4 and 13.6-14.0 minutes, respectively.

2.2.2. Linearity

A quadratic equation was used for calculation of the calibration curve. The coefficients of determination (r^2) were >0.997 for BAS 510 F and >0.995 for the M510F01 acetyl derivative in representative calibration curves of BAS 510 F and M510F01 acetyl derivative standards at concentrations ranging 0.001-0.10 ppm of each analyte.

2.2.3. Specificity

The petitioner has submitted a **GC/MS** quantitation method using an ion trap mass spectrometer with selected ion storage (SIS) as a **confirmatory method**. Representative chromatograms were provided for standard solutions, calibration curves, and fortified egg, milk, and bovine muscle, liver, kidney, and fat extracts. Adequate recoveries for egg, milk, and bovine muscle, liver, kidney, and fat samples fortified with BAS 510 F and its metabolite M510F01 were achieved using the confirmatory method.

2.2.4. Method Limits

The limit of detection (LOD) was ca 0.002 ppm in milk and 0.005 ppm for all other animal matrices. The LOD for M510F01 in kidney may be considered higher (0.015 ppm) because of a consistent GC/ECD signal interfering with the metabolite at 0.007 ppm. The validated limit of quantitation (LOQ) for BAS 510 F and the M510F01 metabolite, expressed as BAS 510 F equivalents, was 0.010 ppm for each analyte in milk and 0.025 ppm for each analyte in eggs, and bovine tissues and fat.

A GC/MS quantitation method using an ion trap mass spectrometer with selected ion storage (SIS) was submitted as a **confirmatory method**. Representative chromatograms were provided for standard solutions, calibration curves, and fortified egg, milk, and bovine muscle, liver, kidney, and fat extracts. Adequate recoveries for egg, milk, and bovine muscle, liver, kidney, and fat samples fortified with BAS 510 F and its metabolite M510F01 were achieved using the confirmatory method.

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 GC/ECD 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.

Radiovalidation of this GC/ECD method was not conducted. It cannot be concluded that the extraction procedures of the metabolism study are similar to those of the proposed enforcement method. Therefore, a radiovalidation study will need to be conducted to support this proposed enforcement method (DFG S19); submission of the study may be made **a condition of registration.**

This GC/ECD method for the analysis of residues of BAS 510 F and its metabolite M510F01 in/on animal matrices has been forwarded to ACB/BEAD for a tolerance method validation (TMV) trial on milk, beef liver, and eggs. **Contingent upon** satisfactory results from the BEAD TMV and receipt of adequate radiovalidation data and a revised analytical method (to include a 60-day limit for the storage of standard solutions of reference standards), the method can be approved for tolerance enforcement purposes in animal matrices.

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 GC/ECD Method.		
Common Name:	Nicobifen, proposed (parent compound)	Hydroxy metabolite
IUPAC Name:	2-Chloro-N-(4'-chlorobiphenyl-2-yl)-nicotinamide	2-Chloro-N-(4'-chloro-5-hydroxybiphenyl-2-yl) nicotinamide
CAS Name:	3-Pyridinecarboxamide, 2-chloro-N-(4'chloro[1,1'-biphenyl]-2-yl)-	Not available
CAS Number:	188425-85-6	Not available
Company Name:	BAS 510 F	M510F01
Other Synonyms:	BASF Registry No. 300355	BASF Registry No. 398794

Table 1.1.2. Matrices Tested with the GC/ECD Method.	
Matrix	Matrix Form
Eggs	Obtained commercially; egg whites and yolk were mixed with a spatula.
Milk	Obtained commercially; milk was shaken for homogenization.
Bovine muscle	Obtained commercially; tissue and fat samples were minced.
Bovine liver	
Bovine kidney	
Bovine fat	

Table 1.1.3. Fortification Levels Tested with the GC/ECD Method.		
Matrices	Analytes (ppm) [1]	
	BAS 510 F	M510F01 (BAS 510 F equivalents)
Milk	0.010 (LOQ)	0.010 (LOQ)
	0.10	0.10
Egg, and bovine muscle, liver, kidney and fat	0.025 (LOQ)	0.025 (LOQ)
	0.25	0.25

[1] Samples were fortified with a solution containing equal amounts of BAS 510 F and M510F01.

1.2. Methods

A description of the proposed GC/ECD enforcement method follows. The method was adapted from the German multiresidue method (DFG S19) by PTRL Europe of Ulm, Germany. For method validation, processed (homogenized or minced) samples of eggs, milk, and bovine

2.2.5. Analyte Recoveries

Table 2.2.5.1. Recovery of BAS 510 F and Its Hydroxy Metabolite M510F01 From Egg, Milk, and Bovine Tissues and Fat Using the GC/ECD Method (DFG S19).

Matrix	Fortification Level of Each Analyte (ppm)	Recoveries as BAS 510 F equivalents (%)			
		BAS 510 F	Mean ± SD	M510F01[1]	Mean ± SD
Whole egg	0.025	85, 88, 92, 95, 126	93 ± 13	73, 70, 84, 76, 97	79 ± 12
	0.25	85, 86, 88, 89, 95		89, 88, 71, 72, 71	
Milk	0.010	68, 79, 83, 90, 92	85 ± 9	97, 75, 81, 104, 110	97 ± 12
	0.10	83, 86, 87, 90, 96		94, 93, 101, 108, 110	
Bovine muscle	0.025	89, 92, 96, 96, 103	90 ± 9	90, 75, 86, 107, 105	92 ± 14
	0.25	78, 80, 84, 85, 92		73, 83, 102, 100, 103	
Bovine liver	0.025	79, 84, 88, 88, 115	87 ± 13	88, 109, 84, 94, 72	88 ± 16
	0.25	77, 78, 83, 84, 92		99, 97, 97, 70, 67	
Bovine kidney[2]	0.025	78, 87, 92, 105, 105	91 ± 11	82, 86, 98, 79, 92	93 ± 6
	0.25	82, 82, 86, 96, 100		103, 100, 94, 103, 96	
Bovine fat	0.025	93, 93, 102, 107, 131	98 ± 15	85, 86, 75, 96, 84	86 ± 13
	0.25	76, 90, 93, 96, 101		67, 88, 90, 79, 106	

[1] Recoveries for M510F01 are listed respective to the recoveries for BAS 510 F from the same sample.
[2] Recoveries of M510F01 were corrected for background interference (0.007 ppm).

2.2.6. Independent Laboratory Validation

Independent laboratory validation (ILV) of the GC/ECD method was conducted on commercially obtained whole milk and bovine liver by BASF (Germany); these matrices were stated to be the most difficult to analyze. Samples of milk were fortified with equal amounts of BAS 510 F and its metabolite M510F01, each at 0.010 (LOQ) and 0.10 ppm, and samples of liver were fortified at 0.025 ppm (LOQ) and 0.25 ppm.

Control milk samples had quantifiable peaks below the method LOQ at the retention time of BAS 510 F (<0.003 ppm) and M510F01 (~0.005 ppm). For M510F01, this resulted in some recoveries >120%; correction of the recoveries for the interference yielded acceptable values. The first attempt at validation for liver failed because of low recoveries; in addition, control liver samples had quantifiable peaks below the method LOQ for both BAS 510 F (≤0.012 ppm) and M510F01 (≤0.001 ppm). The second validation attempt for liver also yielded control samples with detectable residues of BAS 510 F (≤0.005 ppm) and M510F01 (≤0.006 ppm). When the recoveries of liver samples fortified at the LOQ were corrected for these residues in the control samples, one recovery was below the acceptable 70-120% recovery range. Therefore, a third trial for liver was conducted. Adequate recoveries were achieved with the third attempt with liver

which incorporated the following changes to the method: (i) reduction of the amount of sodium sulfate used in the filtration of the organic phase and additional rinsing of the filter and flask; and (ii) a polymer column was used instead of a glass column for the silica gel column clean-up step. We note that the study was conducted in accordance with both U.S. (EPA) guidelines for ILV and European (EC) guidelines for residue analytical methods.

The laboratory did not report the time required to analyze samples using the GC/ECD method; however, it is reported in the method description that the extraction procedures and GC/ECD determination for 6-12 samples can be accomplished by one person over a 24-hour period.

Table 2.2.6. Recovery of BAS 510 F and Its Hydroxy Metabolite M510F01From Milk and Bovine Liver Using the GC/ECD Method (DFG S19) During Independent Laboratory Validation.

Matrix	Fortification Level of Each Analyte (ppm)	Uncorrected Recoveries as BAS 510 F equivalents (%)[1]			
		BAS 510 F	Mean ± SD	M510F01[2]	Mean ± SD
Whole milk	0.010	91, 95, 98, 98, 100	96 ± 3	136 (112), 121 (97), 112, 109, 128 (104)	115 ± 10
	0.10	91, 95, 98, 99, 100		108, 105, 116, 106, 113	
Bovine liver (Second attempt)	0.025	82 (63), 91, 95, 98, 106	97 ± 8	73 (50), 105, 96, 94, 95	95 ± 12
	0.25	95, 95, 97, 105, 108		86, 89, 88, 112, 111	
Bovine liver (Third attempt)	0.025	71, 72, 73, 77, 77	74 ± 2	94, 91, 86, 94, 93	92 ± 4
	0.25	73, 73, 74, 75, 76		89, 90, 96, 89, 99	

[1] Five samples were analyzed to fulfill EC guideline requirements. For certain samples, the corrected recovery (corrected for residues detected in the unfortified sample) is reported in parentheses.

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

3. Discussion

3.1. Recovery and Repeatability

Adequate method validation and independent laboratory validation studies were conducted for the GC/ECD method (DFG S19) for analysis of residues of BAS 510 F and its metabolite M510F01 in animal matrices. Method validation recoveries of the residues of BAS 510 F and M510F01 (quantitated as the acetyl derivative and expressed as BAS 510 F equivalents) respectively ranged 85-126% and 70-97% in eggs, 68-96% and 75-110% in milk, 78-103% and 73-107% in bovine muscle, 77-115% and 67-109% in bovine liver, 78-105% and 79-103% in bovine kidney, and 76-131% and 67-106% in bovine fat fortified with a combination of BAS 510 F and M510F01 at the LOQ (0.010 ppm for milk and 0.025 ppm for egg and beef tissues) and 10x the LOQ (0.10 ppm for milk and 0.25 ppm for egg and beef tissue).

The recoveries obtained from the ILV studies are comparable to the recoveries obtained in method validation. ILV recoveries of residues of BAS 510 F and M510F01 (quantitated as the acetyl derivative and expressed as BAS 510 F equivalents) respectively ranged 91-101% and 105-136% (86-114% corrected) in milk and 71-77% and 86-99% in bovine liver fortified with a combination of BAS 510 F and M510F01 at the LOQ (0.010 ppm for milk and 0.025 ppm for liver) and 10x the LOQ (0.10 ppm for milk and 0.25 ppm for liver).

It was noted that quantifiable peaks, present at less than the method LOQ, eluted at the retention times of BAS 510 F and M510F01 in control kidney samples from the method validation study and control milk and liver samples from the ILV study, affecting validations at the LOQ level.

3.2. Method Efficiency

Radiovalidation of the GC/ECD method was not conducted. The petitioner stated that the extractability of BAS 510 F and its metabolite M510F01 (present also as its glucuronic acid conjugate, M510F02) from animal matrices using methanol was demonstrated in the goat metabolism study (MRID 45405024) and the efficiency of enzymatic deconjugation of M510F02 (to M510F01) was demonstrated in the validation of the data collection method 471/0 (see DER for MRID 45405105).

In the goat metabolism study, the extractability of radioactive residues from goat matrices using methanol was adequate for milk (99.3% TRR), muscle (79.7% TRR), and kidney (81.3% TRR), but was low for fat (62.8% TRR) and liver (16.6% TRR). In addition, for milk, fat, and liver, the petitioner used alternate extraction procedures to characterize/identify residues. Therefore, it cannot be concluded that the extraction procedures of the metabolism study are similar to those of the proposed enforcement method (DFG S19). **A radiovalidation study needs to be conducted to support this proposed enforcement method (DFG S19); submission of the study may be made a condition of registration.**

4. Deficiencies

As a **condition of registration**, this GC/ECD 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.

A radiovalidation study of this method (DFG S19) needs to be conducted; submission of the study may be made a **condition of registration**.

5. References

None.