Document Title

Tier 2 Summary of the Metabolism and Residues Data for Flupyradifurone (BYI 02960)

- Part 1 of 3 -

Data Requirements

Regulation (EC) No 1107/2009

Regulatory Directive 2003-01/Canada/PMRA
OPPTS guidelines/US/EPA

Annex IIA
Section 4, Point 6.1 to Point 6.2.6
Document M

According to OECD format guidance for industry data submissions on plant protection products and their active substances

Date

2013-08-28
(updated EU version for Ctgb)

Author(s)

[redacted]

Bayer CropScience

OWNERSHIP STATEMENT

TABLE OF CONTENTS

IIA 6 Metabolism and Residues Data

IIA 6.1 Stability of residues

IIA 6.1.1 Stability of residues during storage of samples

As residue samples in trials with BYI 02960 on crops and animal were routinely stored frozen for longer periods of time prior to their analysis, the effects of frozen storage on the residue levels were investigated.

General remark:

In this summary section (KIIA 6.1.1), the name DFEAF will be used for the metabolite BYI 02960-difluoroethyl-amino-furanone, which is relevant to the tested residue definition:

Name	*Metab. No.*	*Standard "dossier name"*
DFEAF	M34	BYI 02960-difluoroethyl-amino-furanone

► **PLANT MATRICES**

The longest periods of frozen storage of samples from plant residue studies (field residue, processing, or rotational crop trials) are shown in Table 6.1.1-1 below. Table 6.1.1-1a summarizes the storage period of matrices obtained in European field trials, whereas table 6.1.1-1b refer to uses outside Europe (uses of the Global Joint Review Submission partner countries).

Table 6.1.1-1a: EU uses: Periods of frozen storage (approx. -18°C) of plant-based samples (between sampling and analysis) for matrices relevant to this dossier

Sample material		Longest storage duration (d)	Study	
Crop	Matrix		Report no.	Annex point KIIA…
lettuce	head	300	10-2503	6.6.3.1.1/01
	outer leaves inner head parts inner leaves washed inner leaves	224	10-3223	6.5.4.1/01
	washing water	225	10-3223	6.5.4.1/01
hops	green cone	219	10-3407	6.5.4.2/01
	dried cone	223	10-2225	6.3.1.2/01
	beer hops draff brewer's yeast	220	10-3407	6.5.4.2/01
apple	fruit	131	11-2077	6.3.1.3/02
	peeled fruit dried fruit peel	159	10-3171	6.5.4.4/01

Continued on next page…

Table 6.1.1-1a (cont'd): EU uses: Periods of frozen storage (5pprox.. -18°C) of plant-based samples (between sampling and analysis) for matrices relevant to this dossier

Sample material		Longest storage duration (d)	Study	
Crop	Matrix		Report no.	Annex point KIIA…
apple (con'd)	washed whole fruit raw juice juice raw sauce sauce washings wet pomace dried pomace retentate stain rest	264	10-3172	6.5.4.4/02
grape	bunch of grape	282	11-2090	6.3.1.4/04
	berry raisin must wine at bottling pomace raisin waste washings	273	10-3406	6.5.4.6/01
	jelly juice, pasteurised	260	10-3406	6.5.4.6/01
	wine at first taste test	338	10-3406	
blueberry	fruit	195	IR-4PR NO.10637	6.3.2.5/01
tomato	fruit	323	11-2087	6.3.1.5/02
	washed whole fruit peeled fruit raw juice juice raw puree puree preserve peel stain rest washings peeling water	320	10-3186	6.5.4.8/01
	dried fruit paste	259	10-3186	6.5.4.8/01
pepper	fruit	368	11-2081	6.3.1.6/03

Continued on next page…

Table 6.1.1-1a (cont'd): EU uses: Periods of frozen storage (6pprox.. -18°C) of plant-based samples (between sampling and analysis) for matrices relevant to this dossier

Sample material		Longest storage duration (d)	Study	
Crop	Matrix		Report no.	Annex point KIIA…
cucumber	fruit	226	11-2067	6.3.1.7/04
	washed whole fruit	231	10-3184	6.5.4.9/01
	fermented fruit			
	preserve			
	brine			
	washings			
watermelon	whole fruit	353	11-2075	6.3.1.8/04
	pulp peel	386	11-2075	6.3.1.8/04
barley	grain straw	480	10-2503	6.6.3.1.1/01
	green material	540	10-2503	6.6.3.1.1/01
carrot	root	257	10-2503	6.6.3.1.1/01
turnip	root (body)	180	10-2503	6.6.3.1.1/01
	leaf	177	10-2503	6.6.3.1.1/01
potato	tuber	200	11-2550	6.6.3.1.2/01
leek	Whole plant without roots	145	11-2551	6.6.3.1.3/01
cucumber	fruit	117	11-2552	6.6.3.1.4/01
onion	bulb	94	11-2553	6.6.3.1.5/01
bean	pod	114	11-2555	6.6.3.1.6/01
pea	dry seed	121	11-2556	6.6.3.1.7/01
winter rape	seed	117	11-2554	6.6.3.1.8/01

Table 6.1.1-1b: Uses of the GJR partner countries: Periods of frozen storage of plant-based samples (between sampling and analysis) for matrices relevant to this dossier

Sample material		Longest storage duration (d)	Study	
Crop	Matrix		Report no.	Annex point KIIA…
NAFTA countries				
orange	fruit	351	RARVY012	6.3.2.1/01
	peel			
	pulp	352		
	dried pomace	280	RARVY035	6.5.4.3/02
	washings	279		
	marmalade	276		
	washed peel	272		
	peel without oil	269		
	wet pomace	268		
	stain rest	267		
	raw juice	266		

Continued on next page…

Table 6.1.1-1b (cont'd): Uses of the GJR partner countries: Periods of frozen storage of plant-based samples (between sampling and analysis) for matrices relevant to this dossier

Sample material		Longest storage duration (d)	Study	
Crop	Matrix		Report no.	Annex point KIIA…
NAFTA countries				
orange (cont'd)	unwashed ripe peel	261	RARVY035	6.5.4.3/02
	oil			
	washed fruit			
	juice	259		
	dried pomace	246		
grapefruit	fruit	230	RARVY012	6.3.2.1/01
lemon	fruit	235		
mandarin	fruit	199	RARVP064	6.3.2.1/02
almond	nutmeat without shell	491	RARVY016	6.3.2.2/01
pecan	nutmeat without shell	364		
apple	fruit	170	RARVY013	6.3.2.3/01
pear	fruit	211		
grape	bunch of grapes	271	RARVY007	6.3.2.4/01
blueberry	fruit	179	IR-4PR No.10637	6.3.2.5/01
prickly pear cactus	fruit	99	IR-4PR No.10722	6.3.2.6/01
	pad	101		
potato	tuber	318	RARVY015	6.3.2.7/01
	crisps (chips)	216	RARVY038	6.5.4.16/01
	wet peel	214		
	cooked tuber with peel	212		
	cooking water			
	washings	211		
	steamed, mashed tuber	210		
	starch	203		
	flakes			
	peeled tuber	202		
	washed tuber	199		
tomato	fruit	266	RARVY022	6.3.2.8/01
bell pepper	fruit	247		
chili	fruit	189		
	dried fruit	197		
celery	stalk	238	RARVY005	6.3.2.9/01
	trimmed stalk			
bean	dry seed	243	RARVY028	6.3.2.11/01
pea	dry seed	259		

Continued on next page…

Table 6.1.1-1b (cont'd): Uses of the GJR partner countries: Periods of frozen storage of plant-based samples (between sampling and analysis) for matrices relevant to this dossier

Sample material		Longest storage duration (d)	Study	
Crop	Matrix		Report no.	Annex point KIIA…
NAFTA countries				
peanut	seed	217	RARVY010	6.3.2.12/01
	peanut butter	391	RARVY032	6.5.4.15/01
	meal	390		
	nut without shell			
	refined oil	389		
	roasted peanut	465		
soybean	meal	336	RARVY011	6.3.2.13/01
	defatted flour	266		
	dry seed			
	refined oil	262		
	milk			
cotton	undelinted seed	450	RARVY033	6.5.4.14/01
	gin byproduct	308		
	meal	454		
	hull	450		
	preclarified oil	445		
	neutralised crude oil	445		
	solv. extracted oil	441		
	refined oil	441		
barley	grain	401	RARVY001	6.3.2.15/01
corn	kernel and cob, husked	325	RARVY002	6.3.2.16/01
	kernel	269		
sorghum	grain	196	RARVY004	6.3.2.17/01
wheat	grain	390 (644)[1]	RARVY003	6.3.2.18/01
	dry pasta	426		
	fresh pasta			
	cooked dried pasta	411		
	cooked fresh pasta			
	whole meal	408		
	white flour			
	white bread	387		
	wholemeal bread			
	starch	412		
	cooking water	414		
	shorts	408		
	bran			
coffee	green bean	115	RARVP074	6.3.2.19/01
	roasted bean	121		
	instant coffee	119		
sugarcane	stalk	178	RARVX001	6.5.4.18/01
hops	kiln-dried cone	226	RARVY008	6.3.2.20/01

Continued on next page…

Table 6.1.1-1b (cont'd): Uses of the GJR partner countries: Periods of frozen storage of plant-based samples (between sampling and analysis) for matrices relevant to this dossier

Sample material		Longest storage duration (d)	Study	
Crop	Matrix		Report no.	Annex point KIIA…
Brazil				
coffee	bean	152	I11-008	6.3.2.19/02
orange	fruit	127	I11-006	6.3.2.1/02
Guatemala,				
coffee	green bean	113	RARVP075	6.3.2.19/01
Chile				
blueberry	fruit	242	IR-4PR NO.10637	6.3.2.5/01
Australia				
blueberry	fruit	271	IR-4PR NO.10637	6.3.2.5/01
potato	tuber	210	BCS-0358	6.3.2.7/03
sweet potato	tuber	104	BCS-0358	6.3.2.7/03
tomato	fruit	250	BCS-0348	6.3.2.8/02
New Zealand				
blueberry	fruit	262	IR-4PR NO.10637	6.3.2.5/01

1 one untreated grain sample was analysed after 644 days, all other samples were analysed within less than 500 days

Based on the fact that the use of BYI 02960 will be supported on a wide array of crops, storage stability data was required for the relevant residues of BYI 02960 in plant matrices. The freezer storage stability study covered a period of 18 months.

Report:	**KIIA 6.1.1/01, [redacted]; [redacted], B.C.; [redacted], A.M. 2012**
Title:	Storage stability of BYI 02960, difluoroacetic acid, and difluoroethyl-amino-furanone in plant matrices (18-month data)
Report No. & Document No.:	RARVP046-1, dated November 1, 2012 M-428412-02-1 (updated version of M-428412-01-1, dated April 3, 2012
Guidelines:	– EPA Ref. OPPTS 860.1380 Storage Stability Data – OECD Guideline for the Testing of Chemicals No. 506, Stability of Pesticide Residues in Stored Commodities – PMRA Residue Chemistry Guidelines, Reg. Dir. 98-02, Section 5, Storage Stability Data
GLP:	yes (certified laboratory)

I. Materials and Methods

To determine the freezer storage stability of the relevant residues of BYI 02960 in plant materials, individual 5-g control samples of orange fruit (high acid content), spinach leaves and tomato fruit (high water content), wheat grain (high starch content), bean seed (high protein content), coffee bean and soybean seed (high oil content), and sugar cane were separately spiked with 5.0 µg of either BYI 02960 parent compound, DFA, or DFEAF. Except for the day-0 analysis, samples were stored in glass containers in a freezer at an average temperature of -23°C (maximum -12°C with a three exceptions of very brief intervals at 6.5°C on April 14, at 4.2°C on August 24 and at 0.0°C on August 25, too short for the samples to thaw as documented by average temperatures no greater than-19°C on those days) for later use. For day-0 analysis, three treated samples of each material were chosen, as well as one control sample of each. Samples were then also analysed after nominal intervals of 1, 2½, 5-6, 12 and 18 months. (The intended final storage period to be covered in this study is 2 years.) At each of these intervals, two treated samples of each material were removed from storage and analyzed, as well as a control sample and two samples for concurrent recovery. Samples used for concurrent recoveries were prepared at the same time and stored in the same fashion as the control samples, and spiked on the day of analysis.

BYI 02960 and its metabolites were analytically determined using analytical method 01304 (cf. report RARVP013, [redacted] & [redacted], 2012; KIIA 4.3/03), which was validated prior to and parallel to the residue analysis of the samples. The LOQ was 0.01 mg/kg for parent and DFEAF and 0.05 mg/kg for DFA, expressed in BYI 02960 equivalents.

II. Findings

The mean values of the concurrent recovery rates per compound, sample material, and spiking level were in the range of 81-107%, with relative standard deviations in the range of 3-14%. Details of recovery data are shown in table 6.1.1-5.

At day 0, average residue recoveries of BYI 02960 ranged from 91-111% of nominal; of DFEAF ranged from 101-110% of nominal; and of DFA ranged from 79-101% of nominal. In samples analysed after approximately 12 months of frozen storage (362-372 days), storage stability recoveries, corrected to day 0, ranged from 81-114% for BYI 02960, 76-90% for DFEAF, and 74-104% for DFA.

In samples analysed after approximately 18 months of frozen storage (556-560 days), storage stability recoveries, corrected to day 0, ranged from 84-110% for BYI 02960, 81-102% for DFEAF, and 103-146% for DFA. At all sampling dates and in all sample materials, the relevant components of the residue of BYI 02960 were above 70%. Even in the case of the lower values in the given ranges, there was no evidence of any continued degradation of any of the analytes in any of the sample materials. Thus, all analytes can be considered stable in all relevant plant matrix types for a period of at least 18 months (556 to 560 days).

All storage stability results are summarised below in tables 6.1.1-2 to 6.1.1-4.

III. Conclusions

During a storage period of 18 months under deep-freezer conditions, the components of the relevant residues of BYI 02960 (including parent compound, DFEAF, and DFA) were stable in orange fruit, spinach leaves and tomato fruit, wheat grain, bean seed, coffee bean and soybean seed, and sugarcane, representing a wide array of plant-based sample materials. These results validate the residue values reported in all supervised field trials and processing studies with respect to storage stability of samples frozen prior to analysis.

(The storage stability study as reported and summarized in this section will be continued until a storage period of 2 years has been covered. The presented interim report submitted covers approx. 18 months of storage.)

Table 6.1.1-2: Summary of stability data for deep-frozen samples fortified with **BYI 02960** – samples fortified at 1 mg/kg

Matrix	Storage period (d)	Concurrent recovery (%)	Recovery in stored samples (%)		
			apparent	normalized to day 0	corrected for recovery
sugar cane	0	(100)	100	100	100
	29	101	92	92	91
	77	91	85	85	93
	149	98	94	94	96
	372	87	93	93	107
	559	89	92	92	104
coffee bean (green)	0	(100)	94	100	100
	33	97	93	99	96
	81	81	81	85	99
	152	85	104	110	122
	370	93	83	88	89
	560	91	94	100	104
orange fruit	0	(100)	96	100	100
	28	89	84	88	95
	77	86	95	98	111
	148	104	84	87	81
	365	102	80	83	79
	556	80	90	94	113
soybean seed	0	(100)	93	100	100
	28	93	93	101	100
	75	93	96	104	104
	148	91	97	105	106
	371	96	98	106	103
	558	95	89	96	93
navy bean	0	(100)	111	100	100
	26	91	107	96	117
	75	94	110	99	117
	148	100	123	111	123
	364	113	127	114	112
	558	100	94	84	94
tomato fruit	0	(100)	95	100	100
	28	90	92	97	103
	76	86	89	94	102
	148	107	105	111	98
	370	89	95	101	107
	558	102	94	99	92
spinach	0	(100)	91	100	100
	26	96	97	106	101
	75	90	85	93	94
	147	94	104	114	110
	364	119	97	106	81
	557	98	100	110	103

Continued on next page…

Table 6.1.1-2 (cont'd): Summary of stability data for deep-frozen samples fortified with **BYI 02960** – samples fortified at 1 mg/kg

Matrix	Storage period (d)	Concurrent recovery (%)	Recovery in stored samples (%)		
			apparent	normalized to day 0	corrected for recovery
wheat grain	0	(100)	94	100	100
	27	93	90	95	97
	76	89	82	87	93
	186	97	109	116	113
	362	92	77	81	83
	557	97	91	97	94

Table 6.1.1-3: Summary of stability data for deep-frozen samples fortified with **DFA** – samples fortified at 1 mg/kg

Matrix	Storage period (d)	Concurrent recovery (%)	Recovery in stored samples (%)		
			apparent	normalized to day 0	corrected for recovery
sugar cane	0	(100)	98	100	100
	29	97	96	99	100
	77	97	92	94	94
	149	86	100	102	116
	372	97	99	101	102
	559	93	137	140	147
coffee bean (green)	0	(100)	90	100	100
	33	90	89	98	99
	81	77	76	84	99
	152	80	94	104	118
	370	73	79	87	108
	560	85	108	120	128
orange fruit	0	(100)	95	100	100
	28	98	100	106	102
	77	89	99	105	112
	148	87	93	98	107
	365	94	98	103	104
	556	92	119	126	131
soybean seed	0	(100)	79	100	100
	28	89	71	89	79
	75	93	82	103	88
	148	75	98	124	131
	371	73	79	99	107
	558	79	87	110	111
navy bean	0	(100)	96	100	100
	26	96	99	104	104
	75	98	108	113	110
	148	79	100	104	127
	364	80	78	82	98
	558	99	128	134	130

Continued on next page…

Table 6.1.1-3 (cont'd): Summary of stability data for deep-frozen samples fortified with **DFA** – samples fortified at 1 mg/kg

Matrix	Storage period (d)	Concurrent recovery (%)	Recovery in stored samples (%)		
			apparent	normalized to day 0	corrected for recovery
tomato fruit	0	(100)	101	100	100
	28	99	96	95	97
	76	94	94	93	100
	148	84	98	96	117
	370	98	105	104	108
	558	102	134	133	132
spinach	0	(100)	100	100	100
	26	98	97	97	99
	75	90	87	87	97
	147	85	100	99	117
	364	70	74	74	106
	557	107	147	146	138
wheat grain	0	(100)	92	100	100
	27	97	98	107	102
	76	88	94	102	107
	186	78	98	107	126
	362	72	71	78	100
	557	88	116	126	132

Table 6.1.1-4: Summary of stability data for deep-frozen samples fortified with **BYI 02960-difluoroethylaminofuranone** – samples fortified at 1 mg/kg

Matrix	Storage period (d)	Concurrent recovery (%)	Recovery in stored samples (%)		
			apparent	normalized to day 0	corrected for recovery
sugar cane	0	(100)	110	100	100
	29	89	81	74	91
	77	93	88	80	95
	149	117	112	102	96
	372	87	92	85	108
	559	83	94	85	112
coffee bean (green)	0	(100)	101	100	100
	33	100	94	93	94
	81	89	88	87	99
	152	118	100	99	85
	370	90	86	85	95
	560	88	89	88	101

Continued on next page…

Table 6.1.1-4 (cont'd): Summary of stability data for deep-frozen samples fortified with **BYI 02960-difluoroethylaminofuranone** – samples fortified at 1 mg/kg

Matrix	Storage period (d)	Concurrent recovery (%)	Recovery in stored samples (%)		
			apparent	normalized to day 0	corrected for recovery
orange fruit	0	(100)	104	100	100
	28	100	109	105	109
	77	98	94	90	96
	148	119	110	105	92
	365	97	92	88	95
	556	97	85	81	87
soybean seed	0	(100)	107	100	100
	28	99	106	99	107
	75	103	94	87	91
	148	117	117	108	100
	371	84	85	79	101
	558	97	89	81	89
navy bean	0	(100)	102	100	100
	26	109	102	100	93
	75	107	93	91	87
	148	116	103	101	89
	364	93	88	86	94
	558	114	104	102	90
tomato fruit	0	(100)	110	100	100
	28	96	116	105	121
	76	103	100	91	98
	148	118	101	92	86
	370	97	84	76	87
	558	111	89	81	80
spinach	0	(100)	106	100	100
	26	107	109	103	102
	75	101	96	91	96
	147	114	109	102	95
	364	105	95	90	91
	557	102	95	89	93
wheat grain	0	(100)	103	100	100
	27	101	106	103	105
	76	96	91	88	94
	186	104	99	96	95
	362	92	90	88	98
	557	100	87	84	87

Table 6.1.1-5: Recovery data for the relevant residues of BYI 02960 in various plant matrices

Study No. GLP Year	Crop, Matrix	a.s./metabolite	n	Spike level (mg/kg)	Recovery (%)				
					Individual recoveries	Min	Max	Mean	RSD
RARVP046 GLP: yes 2011	spinach leaf	BYI 02960	13	1.0	84, 90, 99, 93, 99, 92, 89, 95,95, 115, 124, 99, 96	84	124	98	11
		difluoroacetic acid	13	1.0	101, 99, 101 97, 99, 90, 89, 87, 84, 69, 70, 106, 107	69	107	92	13
		BYI 02960-difluoroethyl-aminofuranone	13	1.0	102, 112, 104, 106, 108, 99, 102, 118, 110, 112, 98, 105, 99	98	112	106	6
	orange fruit	BYI 02960	13	1.0	101, 93, 95, 88, 89, 104, 67,111, 97, 107, 97, 75, 85	67	111	93	13
		difluoroacetic acid	13	1.0	94, 93, 96, 98, 99, 102, 76,87, 86, 94, 94, 90, 93	76	102	92	7
		BYI 02960-difluoroethyl-aminofuranone	13	1.0	104, 103, 105, 102, 98, 115, 82, 120, 119 101, 92, 99, 95	82	120	103	10
	soybean seed	BYI 02960	13	1.0	97, 90, 91, 95, 92, 92, 94, 88, 94, 96, 95, 98, 93	88	98	93	3
		difluoroacetic acid	13	1.0	66, 95, 76, 88, 90, 90, 96, 75, 75, 74, 73, 80, 77	66	96	81	12
		BYI 02960-difluoroethyl-aminofuranone	13	1.0	112, 109, 101, 92, 106, 104, 102, 116, 118, 84, 85, 104, 90	84	118	102	11
	navy bean	BYI 02960	13	1.0	105, 104, 125, 93, 90, 96, 93,104, 97, 106, 121, 100, 101	90	125	103	10
		difluoroacetic acid	13	1.0	96, 91, 100, 95, 96, 100, 96,79, 78, 82, 78, 99, 98	78	100	91	10
		BYI 02960-difluoroethyl-aminofuranone	13	1.0	109, 94, 103, 121, 98, 103, 111, 118, 115, 98, 88, 116, 113	94	121	107	10

Continued on next page…

Table 6.1.1-5 (cont'd): Recovery data for the relevant residues of BYI 02960 in various plant matrices

Study No. GLP Year	Crop, Matrix	a.s./metabolite	n	Spike level (mg/kg)	Recovery (%)				
					Individual recoveries	Min	Max	Mean	RSD
RARVP046 GLP: yes 2011	sugarcane	BYI 02960	13	1.0	97, 105, 99, 107, 96, 92, 89, 98, 99, 83, 90, 88, 89	83	107	95	7
		difluoroacetic acid	13	1.0	100, 97, 97, 95, 98, 98, 97, 85, 86, 96, 95, 92, 95	85	100	95	5
		BYI 02960-difluoroethyl-aminofuranone	13	1.0	116, 114, 100, 88, 90, 101, 84, 116, 117, 87, 86, 86, 81	84	117	97	14
	coffee bean, green	BYI 02960	13	1.0	92, 97, 95, 100, 94, 83, 80, 88, 83, 100, 86, 87, 95	80	100	91	7
		difluoroacetic acid	13	1.0	89, 89, 94, 92, 87, 77, 77, 80, 79, 72, 75, 79, 90	72	94	83	9
		BYI 02960-difluoroethyl-aminofuranone	13	1.0	103, 103, 97, 108, 92, 92, 86, 120, 117, 92, 89, 84, 94	84	120	98	12
	tomato fruit	BYI 02960	13	1.0	92, 108, 81, 88, 91, 86, 87, 105, 109, 97, 81, 103, 101	81	109	95	10
		difluoroacetic acid	13	1.0	99, 105, 100, 99, 99, 96, 91, 82, 85, 101, 94, 102, 102	82	105	97	7
		BYI 02960-difluoroethyl-aminofuranone	12	1.0	112, 108, 98, 94, 103, 102, 120, 116, 94, 99, 105, 117	94	120	106	8
	wheat grain	BYI 02960	13	1.0	97, 87, 99, 96, 90, 96, 82, 90, 104, 103, 82, 95, 98	82	104	94	8
		difluoroacetic acid	13	1.0	85, 98, 92, 95, 99, 97, 79, 81, 75, 70, 73, 85, 91	70	99	86	12
		BYI 02960-difluoroethyl-aminofuranone	13	1.0	97, 104, 107, 103, 99, 109, 84, 104, 104, 92, 92, 97, 102	84	109	100	7

► **ANIMAL MATRICES**

The longest storage periods for samples from animal residue studies (feeding studies) are shown in table 6.1.1-6 below:

Table 6.1.1-6: Periods of frozen storage (approx. -15 to -20°C) of animal-based samples (between sampling and analysis) for matrices relevant to this dossier

Sample material		Analyte group*	Longest storage duration (d)	Study	
Animal	Matrix			Report no.	Annex point IIIA…
ruminant	milk	A	16	RARVP050	6.4.3/01
		B	25		
	cream	A & B	11		
	whey	A	5		
		B	7		
	fat	A	14		
		B	40		
	kidney	A	22		
		B	41		
	liver	A	21		
		B	37		
	muscle	A	19		
		B	41		
	urine	A & B	33		
poultry	eggs	A & B	12	RARVP041	6.4.1/01
	fat	A & B	11		
	liver	A & B	11		
	muscle	A & B	11		
	excreta	A & B	18		

* analyte group A comprises BYI 02960, BYI 02960-acetyl-AMCP, and BYI 02960-OH
analyte group B comprises DFA

Based on the information presented here, no storage stability data was required for the relevant residues of BYI 02960 in most animal matrices. Only samples with DFA in bovine fat, kidney, liver, muscle, and urine; as well as with BYI 02960, BYI 02960-acetyl-AMCP, and BYI 02960-OH in bovine urine were stored for periods of > 1 month.

Report:	**KIIA 6.1.1/02, [redacted], S.M., & [redacted], A.M.; 2012**
Title:	BYI 02960 – Magnitude of the Residue in Dairy Cows
Report No. & Edition No.	RARVP050 M-428416-02-1
Guidelines:	– OPPTS 860.1480 – Meat/milk/poultry/eggs – OECD Guideline 505 – APVMA Residue Guideline No. 23 – DACO 7.5 – Meat/milk/poultry/eggs
GLP:	yes (certified laboratory)

I. Materials and Methods

To determine the freezer storage stability of the relevant residues of BYI 02960 in animal materials, individual control samples of bovine fat, kidney, liver, and muscle were separately spiked with DFA, at a nominal concentration of 0.20 mg/kg. Samples were stored in a freezer at a temperature of -15°C. For day-0 analysis, three treated samples of each material were chosen, as well as one control sample of each. Samples were then analysed after an interval of 43 days, in order to cover the longest period of storage for these matrices in the GLP feeding study. At this interval, two treated samples of each material were removed from storage and analyzed, as well as a control sample and two samples for concurrent recovery. Samples used for concurrent recoveries were prepared at the same time and stored in the same fashion as the control samples, and spiked on the day of analysis.

(In addition to the samples and matrices mentioned previously, urine samples were stored for 33 days between sampling and analysis. Although this is longer than one month, it is only very marginally so – an exceedance of only 10%. As the primary purpose of the urine residue values in the feeding study was to elucidate transfer factors for the two main components of the residue [BYI 02960 and DFA], the similarity of this very water-rich matrix with some of those included in the plant storage stability study [KIIA 6.1.1/01] indicates that the major residue components are, indeed, stable in frozen storage in urine.)

DFA was analytically determined using analytical method 01304 (cf. report RARVP013, [redacted] & [redacted], 2012; KIIA 4.3/03), which was validated prior to and parallel to the residue analysis of the samples. The LOQ of the method was 0.02 mg/kg for DFA, expressed in BYI 02960 equivalents.

II. Findings

The mean values of the concurrent recovery rates per compound, sample material, and spiking level were in the range of 60-88%, with standard deviations < 10%. Details of recovery data are shown in table 6.1.1-8.

At day 0, average residue recoveries of DFA ranged from 60-86% of nominal. In samples analysed after 43 days of frozen storage, storage stability recoveries, normalized to day 0, ranged from 100-109%. When corrected for concurrent recovery, they ranged from 97-100% There was no evidence of any continued degradation of DFA in any of the sample materials. Thus, it can be considered stable in all relevant animal matrix types over the tested period of 43 days.

All storage stability results are summarised below in table 6.1.1-7.

III. Conclusions

During a storage period of 43 days under deep-freezer conditions, DFA was stable in bovine fat, kidney, liver, and muscle. These results validate the residue values reported in the cattle feeding study with respect to storage stability of samples frozen prior to analysis. In addition, data from the plant storage stability study (KIIA 6.1.1/01) in watery matrices indicate that the crucial components of the residue in urine, BYI 02960 parent compound and DFA, are stable in storage, thus validating the residue values reported in the cattle feeding study.

Table 6.1.1-7: Summary of stability data for deep-frozen samples fortified with **DFA** – samples fortified at 0.20 mg/kg

Matrix	Storage period (d)	Concurrent recovery (%)	Recovery in stored samples (%)		
			apparent	normalized to day 0	corrected for recovery
bovine fat	0	-	85.8	100	100
	43	87.8	86.0	100.2	98.0
bovine kidney	0	-	68.7	100	100
	43	72.5	72.5	105.5	100
bovine muscle	0	-	65.3	100	100
	43	69.0	69.0	105.6	100
bovine liver	0	-	60.3	100	100
	43	68.0	65.8	109.0	96.7

Table 6.1.1-8: Concurrent recovery data for DFA in various animal matrices

Study No. GLP Year	Matrix	a.s./metabolite	n	Spike level (mg/kg)	Recovery (%) Individual recoveries	Min	Max	Mean	RSD
RARVP050 GLP: yes 2011	bovine fat	difluoroacetic acid	5	0.20	80, 87, 91, 88, 88	80	91	87	4.7
	bovine kidney	difluoroacetic acid	5	0.20	70, 72, 65, 73, 72	65	73	70	4.6
	bovine muscle	difluoroacetic acid	5	0.20	63, 66, 68, 68, 71	63	71	67	4.4
	bovine liver	difluoroacetic acid	5	0.20	58, 61, 63 71, 65	58	71	64	7.7

Additional recoveries for these matrices are presented with the feeding study, cf. KIIA 6.4.2/01

IIA 6.1.2 Stability of residues in sample extracts

The storage stability of residues of BYI 02960 in extracts was tested during development of the analytical methods.

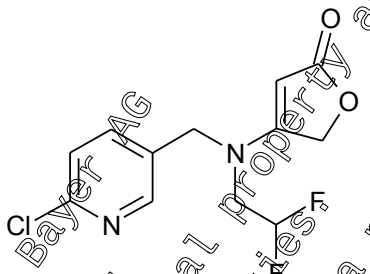
Since the validity of the methods depends on factors such as reproducibility and the possibility of interruption during the work process, it must be ensured that the stability during possible storage of samples in extracts is always guaranteed. Additionally, when conducting residue analysis on regular samples, the entire analytical procedure is routinely monitored by performing concurrent recoveries with each sample set.

During the course of the method validations, stability was shown in all tested matrix extracts for at least 4-8 days, when stored in the dark in a refrigerator at 4°C ± 3°C.

IIA 6.2 Metabolism, distribution and expression of residues

BYI 02960 (common name: flupyradifurone) is a new insecticide being developed by Bayer CropScience. The chemical structure and nomenclature for the active substance are provided below.

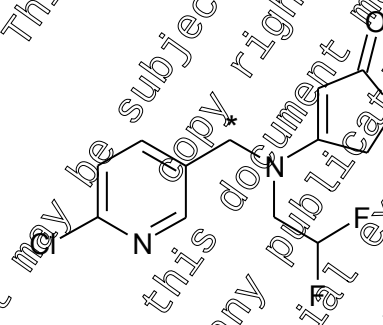
Chemical structure:



Common name:	Flupyradifurone
Company code:	BYI 02960
IUPAC name:	4-[[(6-chloropyridin-3-yl)methyl](2,2-difluoroethyl)amino]furan-2(5H)-one
CAS name:	2(5H)-furanone, 4-[[(6-chloro-3-pyridinyl)methyl](2,2-difluoroethyl)amino]-
CAS #:	951659-40-8
Empirical formula:	$C_{12}H_{11}ClF_2N_2O_2$
Molecular weight:	288.68 g/mol

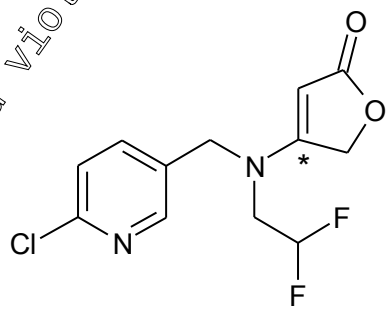
The metabolism of BYI 02960 has been investigated in target plants (apple, cotton, rice, tomato, potato) using different application techniques (foliar application, soil application, tuber treatment and granular application) and in confined rotational crops. The representative rotational crops were wheat, Swiss chard and turnips which were studied at three plant back intervals.
The metabolic fate of BYI02960 has also been investigated in livestock (lactating goats and laying hens) in addition to the rat.

As BYI 02960 contains separate ring systems, two different radiolabels were used in all plant and animal studies. These label positions are shown below:



* denotes position of radiolabel, labelled in the methyl group of the pyridinylmethyl moiety

[pyridinylmethyl-^{14}C]BYI 02960

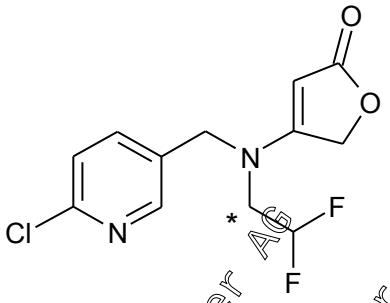


* denotes position of radiolabel, labelled in the 4-position of the furanone ring

[furanone-4-^{14}C]BYI 02960

A third radiolabel was used for one additional plant study after soil degradation studies indicated the formation of significant amounts of difluoroacetic acid (DFA) after application of BYI 02960 to soil. The fate of this radiolabel was investigated in a tomato study as a crop being representative for soil (drench) application. Two rat metabolism studies (one on absorption, distribution, excretion, and

metabolism in male rats and one on metabolism in organs and tissues of male and female rats) were also conducted with [ethyl-1-^{14}C]BYI 02960.



* denotes position of radiolabel, labelled in the ethyl group of the difluoroethyl aminomoiety

[ethyl-1-^{14}C]BYI 02960

Additionally, residue levels of difluoroacetic acid were estimated by LC-MS/MS according to the conditions of residue analytical method 01304 in selected samples collected in the plant and confined rotational crop metabolism studies conducted with either [pyridinylmethyl-^{14}C]BYI 02960 or [furanone-4-^{14}C]BYI 02960. Thus, DFA levels have been determined in all crops included in the BYI02960 metabolism program. For livestock tissues, difluoroacetic acid levels were estimated on basis of the rat data. Additionally, high resolution LC-MS analyses (non-GLP) were performed for selected samples and confirmed the estimations.

Numerous metabolites were identified in the metabolism studies. The chemical structures and report names used in the summaries are given at the very end of this summary and in the List of Metabolites presented in Document N.

All residue values given in mg/kg refer to parent compound equivalents if not indicated otherwise.

The overall summary of the metabolism in plants and livestock is given in KIIA 6.11.1.

IIA 6.2.1 In plants, at least three crops from three different crop categories

Metabolism studies were conducted in crops representing four different crop categories (fruits, root vegetables, oilseeds and cereals). The studies reflected the intended use patterns of the active substance for foliar and soil treatment (including tuber treatment, granular application and soil drench) as summarized in the table below.

Table 6.2.1-1: Crops and application techniques used in the metabolism studies

Crop group	Crop	Application technique
fruits	apple	foliar application
fruits	tomato	soil drench
root crops	potato	tuber treatment / in-furrow application
oilseeds	cotton	foliar application
cereals	rice	foliar application / granular application

In Europe, soil drench applications will not be developed, however, soil applications are important in other regions which will apply for import tolerances in future, e.g. NAFTA (USA, Canada) and South America (Brazil). Therefore, the metabolism studies covering soil drench applications will be presented in this dossier, as well. These studies also provide information on uptake of soil metabolites, which is also of interest for crops receiving early foliar applications where a portion of the spray solution will hit the ground.

Metabolism, distribution and expression of residues in tomato (soil drench)

Metabolism studies in tomatoes were conducted with [furanone-4-^{14}C]-, [pyridinylmethyl-^{14}C]- and [ethyl-1-^{14}C]BYI 02960:

Report:	**KIIA 6.2.1/01, [redacted]; 2011**
Title:	Metabolism of [furanone-4-^{14}C]BYI 02960 in tomatoes
Report No & Edition No	MEF-11/016 M-411352-01-3
Guidelines:	OECD 501 Metabolism in Crops US EPA Residue Chemistry Test Guideline OPPTS 860.1300: Nature of the Residue – Plants, Livestock PMRA Regulatory Directive Dir98-02: Residue Chemistry Guidelines Section 2: Nature of the Residue – Plants, Livestock Japanese MAFF, 12 Nousan 8147 European Parliament and Council Regulation (EC) No 1107/2009
GLP	yes

Executive Summary

The metabolism of [furanone-4-^{14}C]BYI 02960 in tomatoes was investigated according to the maximum envisaged use pattern globally. Four tomato plants were treated by soil drench application with [furanone-4-^{14}C]BYI 02960 formulated as an SL 200. The first application was performed at BBCH 15 (5th leaf on main shoot unfolded) and a second application at BBCH 51 (1st inflorescence visible and first bud erect). The single application rate corresponded to 300 g a.s./ha; the total application rate was 600 g a.s./ha.

At 6 to 36 days after the last application the flowers were sampled from one tomato plant, and at 69 to 92 days after the last application the fruits were harvested from the remaining three plants. The TRR values are shown in the following table:

Table 6.2.1-2: TRR values in tomato fruits and flowers after drench application of [furanone-4-^{14}C]BYI 02960

Matrix	Timing and Application	PHI (days)*	ppm (mg a.s. equiv./kg)
tomato fruits	two drench applications, at BBCH 15 and 14 days later; 2 x 300 g a.s./ha	69	0.096
tomato flowers		n.a.	0.721

* PHI: preharvest interval (corresponds to days after last treatment (DAT) at the start of harvest/sampling)
n.a. not applicable, flowers are not a RAC

The fruits and flowers were conventionally extracted four times with acetonitrile/water mixtures, and 84.8% and 93.6% of the TRR were released, respectively.

Parent compound and metabolites in the extracts of tomato flowers and fruits were analysed by HPLC. Identification was performed by HPLC and/or TLC co-chromatography with reference compounds as well as by comparison of HPLC profiles. Besides parent compound, all major metabolites (>10% of the TRR) and one minor metabolite were identified.

Parent compound was the main component in both matrices and represented 35.9% of the TRR in tomato fruits and 77.9% in tomato flowers. Two major metabolites were detected in tomato fruits: The natural compound glucose (or isomeric carbohydrates) represented 27.5% of the TRR and BYI 02960-difluoroethyl-amino-furanone represented 10.3% of the TRR. In tomato flowers, no radioactive glucose (or isomeric carbohydrates) was detected. BYI 02960-difluoroethyl-amino-furanone was detected as a minor metabolite and represented 9.2% of the TRR. The metabolite BYI 02960-OH-glyc was detected as minor metabolite in tomato fruits and flowers and represented 5.5% and 6.6% of the TRR, respectively.

[Furanone-4-^{14}C]BYI 02960 was moderately metabolised in tomatoes. The following metabolic routes were observed:

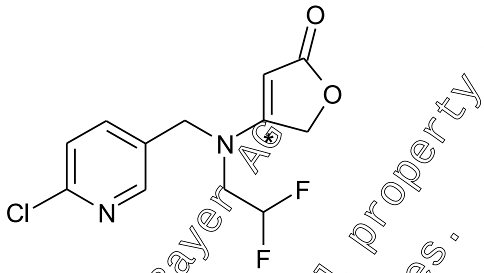
- complete degradation of the furanone moiety and incorporation of carbon atoms into the natural compound pool, i.e. into glucose (or isomeric carbohydrates),
- cleavage of the pyridinylmethylamine bond and formation of BYI 02960-difluoroethylamino-furanone and
- hydroxylation of the methylene group of the furanone moiety followed by conjugation with glucose

On the basis of these results, a metabolic pathway of [furanone-4-^{14}C]BYI 02960 in tomatoes can be proposed.

I. Materials and Methods

A. Materials

1. Test Material:

Chemical structure	 * position of the radiolabel
Radiolabelled test material	[furanone-4-^{14}C]BYI 02960
Specific radioactivity (before radiodilution) (after radiodilution)	 3.94 MBq/mg (106.46 µCi/mg) 1.31 MBq/mg (35.50 µCi/mg)
Chemical Purity	> 99% (HPLC)
Radiochemical purity	> 99% (HPLC and TLC)

The supplied radiolabelled test compound [furanone-4-^{14}C]BYI 02960 was dissolved in acetonitrile. Radiodilution and formulation of the test compound was performed prior to the first and the second application: Therefore, an adequate portion of the non-radiolabelled test compound was dissolved in an adequate volume of the stock solution of the radiolabelled test compound. The solution was evaporated to dryness. The remainder was dissolved in an adequate amount of SL 200 blank formulation using a magnetic stirrer. Preparation of each application solution was done by dissolving the formulation in water. The specific activity of each application solution was 1.31 MBq/mg (35.50 µCi/mg).

2. Soil: "Einheitserde T", pH ($CaCl_2$) = 5.8, 85% white moor peat and 15% clay, enriched with water soluble nutrient salt mixture (ENS)

3. Plant Tomato, variety "Philona", representative for fruiting crops

B. Study Design

Experimental conditions:

Four tomato plants (variety: Philona) were cultivated, each in a 30 L planting bucket with a surface diameter of 38 cm filled with "Einheitserde T". The plants were cultivated in the greenhouse of the test facility (controlled temperature, humidity and light conditions).

The tomato plants were treated with SL 200 formulated [furanone-4-^{14}C]BYI 02960 by drench application. Two applications were performed, each at an application rate of 300 g a.s./ha. The first application was conducted at a growth stage of BBCH 15 (5th leaf on main shoot unfolded) and the second at a growth stage of BBCH 51 (first inflorescence visible and first bud erect). The interval between the two applications was 14 days. The aqueous application solution was poured into a circular furrow prepared in the soil around each tomato plant. At each application, a volume of 200 mL was applied in total, corresponding to 132.64 MBq or to 100.0 mg a.s. (= 25.0 mg a.s./plant). Based on a

planting density of 12,000 tomato plants/ha in agricultural practice, the application rate corresponds to 300 g a.s./ha, which was anticipated to be the maximum single drench application rate in agricultural practice.

Sampling:
When the plants reached the growth stage BBCH 61 (first flower of the first inflorescence open), the open flowers were sampled from one of the four tomato plants in order to determine the nature of the residues in flowers. The flowers were collected with their receptacles and were stored in a freezer (≤ -18°C) on the same day. Sampling of newly opened flowers was continued 2 - 3 times a week over a total period of approx. four weeks until end of flowering (at BBCH 69: 9 or more inflorescences with open flowers). The tomato plant from which the flowers were cut was sampled, cut in pieces and stored in a freezer (≤ -18°C) for optional metabolite investigations. The frozen flowers were homogenized with a high speed blender in liquid nitrogen (Polytron). The homogenised flower sample was stored in a freezer (≤ -18°C) until extraction.

On the day when the three remaining tomato plants reached the growth stage BBCH 81 (10% of fruits show typical fully ripe colour), the ripe tomato fruits were harvested and were stored in a freezer (≤ -18°C). Harvesting of newly ripe fruits was continued 2 - 3 times a week over a total period of approx. three weeks until the plants produced no new fruits (at BBCH 89: fully ripe). The frozen fruits were cut in pieces and were homogenised with a high speed blender (Polytron). The homogenised tomato fruits samples was stored in portions in a freezer (≤ -18°C) until extraction.

C. Analytical Procedures
Extraction:
The homogenised tomato flowers were extracted three times with a mixture of acetonitrile/water (8:2, v/v) and one time with a mixture of acetonitrile/water (1:1, v/v), and a portion of the homogenised tomato fruits was extracted four times with a mixture of acetonitrile/water (8:2, v/v) using a high speed blender (Polytron). After each extraction step, extracts and solids were separated by centrifugation. The radioactivity in the extracts was determined by LSC and in the solids by combustion followed by LSC. The actual TRR value of the RAC was determined by summing up the radioactivity measured in the extracts and in the remaining solids.

All tomato fruit extracts were combined and the first two tomato flower extracts were combined. The combined extracts were subjected to a clean-up step using a pre-conditioned SPE RP 18 cartridge (Phenomenex, Strata C18-E). The flow-through fraction (percolate) was collected and the cartridge was rinsed with acetonitrile/water (8:2, v/v) and methanol/dichloromethane (1:1, v/v). The percolate and the acetonitrile/water fraction were combined and concentrated by rotary evaporation in vacuo. The concentrated extracts of fruits and flowers were analysed by HPLC.

Quantification:
Parent compound and metabolites in the extracts were analysed by reversed phase HPLC coupled to a radioactivity detector with a glass scintillator cell. The HPLC chromatograms (= metabolite profiles) were integrated for quantification of compounds.

Identification and characterisation:
In tomato fruits (RAC), the major components (parent compound and one major metabolite) were identified by co-chromatography with radiolabelled reference compounds using two independent chromatographic systems (reversed phase HPLC and normal phase TLC). Minor metabolites (residue levels ≤0.01 mg/kg) were identified by HPLC co-chromatography, only.

In tomato flowers (additional plant matrix), parent compound was also identified by HPLC and TLC co-chromatography (two independent chromatographic systems) with an authentic reference compound. The minor metabolites (<10% of the TRR) were identified by HPLC comparison (comparison of HPLC profiles of tomato fruits and tomato flowers) and by HPLC co-chromatography. The conjugate BYI 02960-OH-glyc was additionally cleaved by alkaline hydrolysis and its corresponding aglycon was identified by HPLC co-chromatography.

Storage stability:
Detailed evidence was provided in the report to show that the quantified pattern of parent compound and metabolites adequately reflected the residue components at harvest. The extraction experiments and the first HPLC analyses of tomato fruits and flowers were performed not later than two months after harvest. HPLC chromatograms recorded at different times during the study showed that the profiles of the extracts did not change significantly during the analytical work up (including identification and characterization of metabolites) which did not exceed a period of 6 months in total. In accordance with the OECD Guidance for the Testing of Chemicals 501 (2007), it was therefore concluded, that the residues in the extracts were sufficiently stable during the experimental period of the study and that the chromatograms represented the metabolic pattern in the samples at harvest.

II. Results and Discussion

The metabolism of [furanone-4-^{14}C]BYI 02960 was investigated in tomato fruits and flowers following two drench applications. The total radioactive residue (TRR) in fruits and flowers accounted for 0.096 mg/kg and 0.721 mg/kg, respectively. The major portion of radioactivity was extracted conventionally by acetonitrile/water mixtures (84.8% to 93.6% of the TRR) as shown in Table 6.2.1-3. Parent compound was the main residue in both extracts and was identified by HPLC and TLC co-chromatography (i.e. with two independent chromatographic systems) with an authentic reference compound.

In tomato fruits, two additional major metabolites (>10% of TRR and >0.01 mg/kg) were detected: a polar fraction which was assigned to glucose (or isomeric carbohydrates) and BYI 02960-difluoroethyl-amino-furanone. The identity of the carbohydrate was elucidated by HPLC and TLC co-chromatography before and after derivatisation with benzoyl chloride in pyridine. While correspondence with the reference compounds D-glucose and pentabenzoyl-D-glucose was clearly shown, the chromatographic methods were not considered selective enough to discriminate the configuration of the sugar moiety. Therefore, the isolated polar fraction was assigned more generally as glucose/carbohydrates. The minor metabolites BYI 02960-difluoroethyl-amino-furanone and BYI 02960-OH-glyc were identified by HPLC co-chromatography only, since their residue levels were rather low (≤0.01 mg/kg). The configuration of the hexose in BYI 02960-OH-glyc was identified

as D-glucose in the corresponding apple metabolism study due to the specific enzymatic treatment with ß-glucosidase.

In tomato flowers, the minor metabolites BYI 02960-difluoroethyl-amino-furanone and BYI 02960-OH-glyc were identified by HPLC comparison (comparison of HPLC profiles of tomato fruits and tomato flowers). For BYI 02960-difluoroethyl-amino-furanone, the assignment was additionally confirmed by HPLC co-chromatography. The conjugate BYI 02960-OH-glyc was cleaved by alkaline hydrolysis and the resulting aglycon BYI 02960-OH was additionally identified by HPLC co-chromatography.

The distribution of the parent compound and metabolites is shown in Table 6.2.1-4. In total, 79.2% and 93.6% of the TRR were identified in tomato fruits and flowers, respectively.

Table 6.2.1-3: Distribution of radioactivity in the extracts of the tomato matrices fruits and flowers

	tomato fruits		tomato flowers	
TRR [mg/kg] =	0.096		0.721	
	% of TRR	mg/kg	% of TRR	mg/kg
Conventionally extracted	84.8	0.081	93.6	0.675
Extract for analysis	83.5	0.080	93.6	0.675
Losses (not analysed)	1.3	0.001	---	---
Total extracted	**84.8**	**0.081**	**93.6**	**0.675**
Unextractable (PES*)	15.2	0.015	6.4	0.046
Accountability	**100.0**	**0.096**	**100.0**	**0.721**

* post extraction solids

Table 6.2.1-4: TRR values and distribution of parent compound and metabolites in tomatoes (fruit and flowers) after drench application of [furanone-4-^{14}C]BYI 02960

	tomato fruits		tomato flowers	
TRR [mg/kg] =	0.096		0.721	
Compound (BYI 02960-)	% TRR	mg/kg	% TRR	mg/kg
Conventional extraction				
BYI 02960 (parent compound)	35.9	0.034	77.9	0.561
glucose/carbohydrates	27.5	0.026	---	---
difluoroethyl-amino-furanone	10.3	0.010	9.2	0.066
OH-glyc	5.3	0.005	6.6	0.048
Total identified	**79.2**	**0.076**	**93.6**	**0.675**
unknown 1	4.3	0.004	---	---
Total characterised	**4.3**	**0.004**	**<0.1**	**<0.001**
Analysed extract(s)	83.5	0.080	93.6	0.675
Extract(s) not analysed	1.3	0.001	---	---
Total extracted	**84.8**	**0.081**	**93.6**	**0.675**
Unextractable (PES*)	15.2	0.015	6.4	0.046
Accountability	**100.0**	**0.096**	**100.0**	**0.721**

* post extraction solids

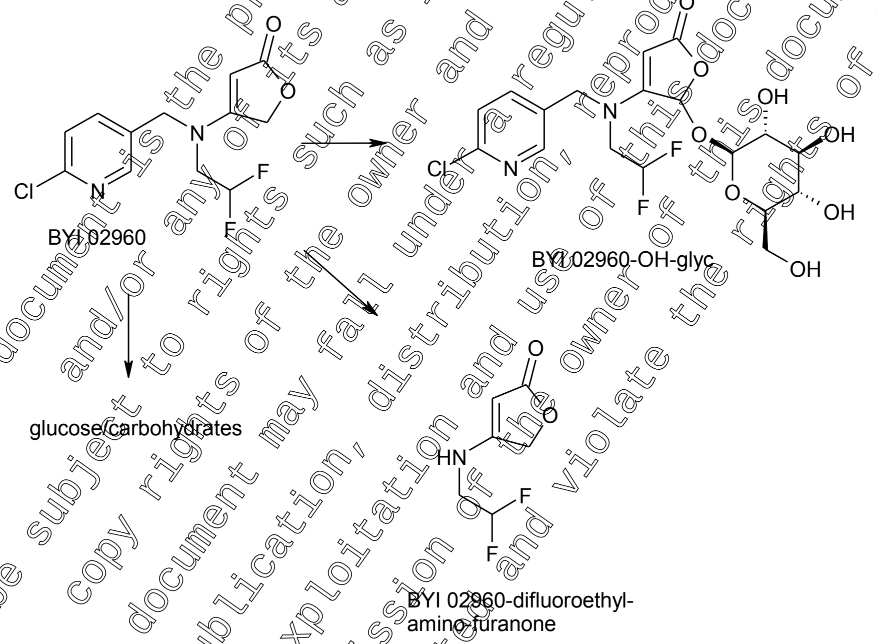
III. Conclusions

Based on the metabolites identified the following metabolic routes were deduced:

- complete degradation of the furanone moiety and incorporation of carbon atoms into the natural compound pool, e.g. into glucose/carbohydrates,
- cleavage of the pyridinylmethylamine bond and formation of BYI 02960-difluoroethylamino-furanone and
- hydroxylation of the methylene group of the furanone moiety followed by conjugation with glucose.

Thus, [furanone-4-^{14}C]BYI 02960 was metabolised moderately in tomatoes. On the basis of the results of this study it is concluded that the metabolism of [furanone-4-^{14}C]BYI 02960 in tomatoes is well understood and the following metabolic pathway is proposed.

Figure 6.2.1-1: Proposed metabolic pathway of [furanone-4-^{14}C]BYI 02960 in tomatoes



Report:	**KIIA 6.2.1/02, [redacted].; 2011**
Title:	Metabolism of [pyridinylmethyl-^{14}C]BYI 02960 in tomatoes
Report No & Edition No	MEF-11/182 M-411500-01-2
Guidelines:	OECD 501 Metabolism in Crops US EPA Residue Chemistry Test Guideline OPPTS 860.1300: Nature of the Residue – Plants, Livestock PMRA Regulatory Directive Dir98-02: Residue Chemistry Guidelines Section 2: Nature of the Residue – Plants, Livestock Japanese MAFF, 12 Nousan 8147 European Parliament and Council Regulation (EC) No 1107/2009
GLP	yes

Executive Summary

The metabolism of [pyridinylmethyl-^{14}C]BYI 02960 in tomatoes was investigated according to the maximum envisaged use pattern globally. Four tomato plants were treated by soil drench application with [pyridinylmethyl-^{14}C]BYI 02960 formulated as an SL 200. The first application was performed at BBCH 15 (5th leaf on main shoot unfolded) and a second application at BBCH 51 (first inflorescence visible and first bud erect). The single application rate corresponded to 300 g a.s./ha; the total application rate was 600 g a.s./ha.

At 3 to 36 days after the last application the flowers were sampled from one tomato plant and at 73 to 92 days after the last application the fruits were harvested from the remaining three tomato plants. The TRR values are shown in the following table:

Table 6.2.1-5: TRR values in tomato fruits and flowers after drench application of [pyridinylmethyl-^{14}C]BYI 02960

Matrix	Timing and Application	PHI (days)*	ppm (mg a.s. equiv./kg)
tomato fruits	two drench applications, at BBCH 15 and 14 days later: 2 x 300 g a.s./ha	73	0.130
tomato flowers		n.a.	1.254

* PHI: preharvest interval (corresponds to days after last treatment (DAT) at the start of harvest/sampling)
n.a. not applicable, flowers are not a RAC

The fruits and flowers were conventionally extracted four times with acetonitrile/water mixtures and 98.5% and 96.5% of the TRR were released, respectively.

Parent compound and metabolites in the extracts of tomato flowers and fruits were analysed by HPLC. Identification was performed by HPLC co-chromatography with reference compounds, by comparison of HPLC profiles or by HPLC-MS/MS analysis. Besides parent compound, two major metabolites and three minor metabolites were identified.

Parent compound was a major component in both matrices and represented 24.2% of the TRR in tomato fruits and 66.2% in tomato flowers. In tomato fruits, the label-specific metabolite BYI 02960-CHMP-di-glyc was the main constituent (37.1% of the TRR), followed by parent compound and the label-specific metabolite 6-CNA (13.2% of the TRR). Additionally, three minor metabolites

BYI 02960-CHMP-glyc (5.1% of the TRR), BYI 02960-OH-glyc (3.4% of the TRR) and BYI 02960-CHMP (3.3% of the TRR) were identified. In tomato flowers, parent compound represented the predominant portion besides four minor metabolites. The minor metabolites were identical with the metabolites identified in the tomato fruits, except for BYI 02960-CHMP, which was not present in flowers.

[Pyridinylmethyl-^{14}C]BYI 02960 was moderately metabolised in tomatoes. The following metabolic routes were observed:

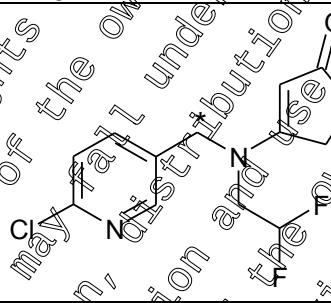
- cleavage of the pyridinylmethylamine bond followed by conjugation with carbohydrates or by oxidation of the methylene group to a carboxylic group, which were the major metabolic routes, and
- hydroxylation of the methylene group of the furanone moiety followed by conjugation with glucose, which was the minor metabolic route.

On the basis of these results, a metabolic pathway of [pyridinylmethyl-^{14}C]BYI 02960 in tomatoes can be proposed.

I. Materials and Methods

A. Materials

1. Test Material:

Chemical structure	 * position of the radiolabel
Radiolabelled test material	[pyridinylmethyl-^{14}C]BYI 02960
Specific radioactivity (before radiodilution) (after radiodilution)	4.37 MBq/mg (118.08 µCi/mg) 1.46 MBq/mg (39.37 µCi/mg)
Chemical Purity	> 99% (HPLC)
Radiochemical purity	> 98% (HPLC and TLC)

The supplied radiolabelled test compound [pyridinylmethyl-^{14}C]BYI 02960 was dissolved in acetonitrile. Radiodilution and formulation of the test compound was performed prior to the first and the second application. Therefore, an adequate portion of the non-radiolabelled test compound was dissolved in an adequate volume of the stock solution of the radiolabelled test compound. The solution was evaporated to dryness. The remainder was dissolved in an adequate amount of SL 200 blank formulation using a magnetic stirrer. Preparation of each application solution was done by dissolving the formulation in water. The specific activity of each application solution was 1.46 MBq/mg (39.37 µCi/mg).

2. Soil: "Einheitserde T", pH ($CaCl_2$) = 5.8, 85% white moor peat and 15% clay, enriched with water soluble nutrient salt mixture (ENS)

3. Plant Tomato, variety "Philona", representative for fruiting crops

B. Study Design

Experimental conditions:

Four tomato plants (variety: Philona) were cultivated, each in a 30 L planting bucket with a surface diameter of 38 cm filled with "Einheitserde T". The plants were cultivated in the greenhouse of the test facility (controlled temperature, humidity and light conditions). The tomato plants were treated with SL 200 formulated [pyridinylmethyl-^{14}C]BYI 02960 by drench application. Two applications were performed, each at an application rate of 300 g a.s./ha. The first application was conducted at a growth stage of BBCH 15 (5th leaf on main shoot unfolded) and the second at a growth stage of BBCH 51 (first inflorescence visible and first bud erect). The interval between the two applications was 14 days. The aqueous application solution was poured into a circular furrow prepared in the soil around each tomato plant. At each application, a total volume of 200 mL was applied, corresponding to 145.67 MBq or to 100.0 mg a.s. (= 25 mg a.s./plant) . Based on a planting density of 12,000 tomato plants/ha in agricultural practice, the application rate corresponds to 300 g a.s./ha, which was anticipated to be the maximum single drench application rate in agricultural practice.

Sampling:

When the plants reached the growth stage BBCH 61 (first flower of the first inflorescence open), the open flowers were sampled from one of the four tomato plants in order to determine the nature of the residues in flowers. The flowers were collected with their receptacles using scissors and were stored in a freezer (≤ -18°C) on the same day. Sampling of newly opened flowers was continued 2 - 3 times a week over a total period of approx. five weeks until the end of flowering (at BBCH 69: 9 or more inflorescences with open flowers). The tomato plant from which the flowers were cut was sampled, cut in pieces and stored in a freezer (≤ -18°C) for optional metabolite investigations. The frozen flowers were homogenized with a high speed blender in liquid nitrogen (Polytron). The homogenised flower sample was stored in a freezer (≤ -18°C) until extraction.

On the day when the three remaining tomato plants reached the growth stage BBCH 81 (10% of fruits show typical fully ripe colour), the ripe tomato fruits were harvested and were stored in a freezer (≤ -18°C). Harvesting of newly ripe fruits was continued 2 - 3 times a week over a total period of approx, three weeks until the plants produced no new fruits (at BBCH 89: fully ripe). The frozen fruits were cut in pieces and were homogenised with a high speed blender (Polytron). The homogenised tomato fruit samples were stored in portions in a freezer (≤ -18°C) until extraction.

C. Analytical Procedures

Extraction:

The homogenised tomato flowers were extracted three times with a mixture of acetonitrile/water (8:2, v/v) and one time with a mixture of acetonitrile/water (1:1, v/v) and a portion of the homogenised tomato fruits was extracted four times with a mixture of acetonitrile/water (8:2, v/v) using a high speed blender (Polytron). After each extraction step, extracts and solids were separated by centrifugation. The radioactivity in the extracts was determined by LSC, in the solids by combustion

followed by LSC. The actual TRR value of the RAC was determined by summing up the radioactivity measured in the extracts and in the remaining solids.

All tomato fruit extracts were combined, and the first two tomato flower extracts were combined. The combined extracts were subjected to a clean-up step using a pre-conditioned SPE RP 18 cartridge (Phenomenex, Strata C18-E). The flow-through fraction (percolate) was collected and the cartridge was rinsed with 100 mL of acetonitrile/water (8:2, v/v) and methanol/dichloromethane (1:1, v/v). The percolate and the acetonitrile/water fraction were combined and concentrated by rotary evaporation in vacuo. The concentrated extracts of fruits and flowers were analysed by HPLC.

Quantification:
Parent compound and metabolites in the extracts were analysed by reversed phase HPLC coupled to a radioactivity detector with a glass scintillator cell. The HPLC chromatograms (= metabolite profiles) were integrated for quantification of compounds.

Identification and characterisation:
Parent compound was identified in tomato fruits and flowers by reversed phase HPLC co-chromatography using a radiolabelled reference compound. Confirmation of the assignment by a second chromatographic method was shown in the tomato study performed with [furanone-4-^{14}C]BYI 02960. The main metabolite in tomato fruits was identified by LC-MS/MS analysis after isolation of the compound by semi-preparative HPLC. An additional major metabolite was identified by HPLC co-chromatography with an authentic reference compound. Confirmation of the assignment by a second chromatographic method was not needed due to its low residue level. All minor metabolites detected in fruits or flowers were identified either by reversed phase HPLC co-chromatography using authentic reference compounds or by HPLC comparison.

Storage stability:
The extraction experiments and the HPLC analyses of tomato fruits and flowers for quantitative evaluation were performed not later than 1.5 months after harvest. Extract stability was demonstrated by comparing the HPLC chromatograms recorded at different time points during the study. The profiles did not change significantly during the whole course of the study. It was therefore concluded, that the residues in the extracts were sufficiently stable during the experimental period of the study (which did not exceed a period of approx. 6 to 7 months in total) and that the chromatograms represented the metabolic pattern in the samples at harvest. Thus, no additional storage stability data have to be provided according to OECD Guidance for the Testing of Chemicals 501 (2007).

II. Results and Discussion

The metabolism of [pyridinylmethyl-^{14}C]BYI 02960 was investigated in tomato fruits and flowers following two drench applications. The total radioactive residue (TRR) in fruits, which represent the edible RAC, accounted for 0.130 mg/kg. The TRR was considerably higher in flowers and accounted for 1.254 mg/kg. A major portion of radioactivity was extracted conventionally by acetonitrile/water mixtures (96.5% to 98.5% of the TRR) as shown in Table 6.2.1-6. Parent compound was the main residue in flowers and the second major residue in fruits.

In tomato fruits, two major metabolites (>10% of TRR and >0.01 mg/kg) were detected: BYI 02960-CHMP-di-glyc (main compound in fruits) and 6-CNA. BYI 02960-CHMP-di-glyc was identified by LC-MS/MS analysis after isolation by semi-preparative HPLC. The metabolite 6-CNA was identified by HPLC co-chromatography using an authentic reference compound, as were the minor metabolite BYI 02960-CHMP and its conjugate BYI 02960-CHMP-glyc. The configuration of the carbohydrate in the conjugate was assigned to D-glucose in accordance to the reference compound used. The minor metabolite BYI 02960-OH-glyc was identified after isolation with semi-preparative HPLC and chromatographic comparison with radiolabelled reference compounds. For chromatographic comparison an acidic reversed phase HPLC method was used to ensure separation and differentiation from BYI 02960-acetic acid, a metabolite which was identified e.g. in the apple metabolism and confined rotational crops studies and which co-eluted with BYI 02960-OH-glyc using the neutral profiling method.

In tomato flowers, the same metabolites were detected as in the fruits, with the exception of BYI 02960-CHMP, which was not detected. All metabolites represented less than 10% of the TRR and were identified by HPLC co-chromatography with authentic reference compounds or by HPLC comparison.

The distribution of the parent compound and metabolites is shown in Table 6.2.1-7. In total, 86.3% and 96.5% of the TRR were identified in tomato fruits and flowers, respectively.

Table 6.2.1-6: Distribution of radioactivity in the extracts of the tomato matrices fruits and flowers after drench application of [pyridinylmethyl-^{14}C]BYI 02960

	tomato fruits		**tomato flowers**	
TRR [mg/kg] =	0.130		1.254	
	% of TRR	mg/kg	% of TRR	mg/kg
Conventionally extracted	98.5	0.128	96.5	1.209
Extract for analysis	98.5	0.128	96.5	1.209
Losses (not analysed)	---	---	---	---
Total extracted	**98.5**	**0.128**	**96.5**	**1.209**
Unextractable (PES*)	1.5	0.002	3.5	0.044
Accountability	**100.0**	**0.130**	**100.0**	**1.254**

* post extraction solids

Table 6.2.1-7: TRR values and distribution of parent compound and metabolites in tomatoes (fruit and flowers) after drench application of [pyridinylmethyl-^{14}C]BYI 02960

	tomato fruits		**tomato flowers**	
TRR [mg/kg] =	0.130		1.254	
Compound (BYI 02960-)	% TRR	mg/kg	% TRR	mg/kg
Conventional extraction				
BYI 02960 (parent compound)	24.2	0.031	66.2	0.829
6-CNA	13.2	0.017	7.0	0.087
CHMP-di-glyc	37.1	0.048	8.0	0.100
CHMP-glyc	5.1	0.007	9.5	0.119
CHMP	3.3	0.004	---	---
OH-glyc	3.4	0.004	5.9	0.073
Total identified	**86.3**	**0.112**	**96.5**	**1.209**
unknown 1	5.5	0.007	---	---
unknown 2	3.8	0.005	---	---
unknown 3	3.0	0.004	---	---
Total characterised	**12.3**	**0.016**	**<0.1**	**<0.001**
Analysed extract(s)	98.5	0.128	96.5	1.209
Extract(s) not analysed	---	---	---	---
Total extracted	**98.5**	**0.128**	**96.5**	**1.209**
Unextractable (PES*)	1.5	0.002	3.5	0.044
Accountability	**100.0**	**0.130**	**100.0**	**1.254**

* post extraction solids

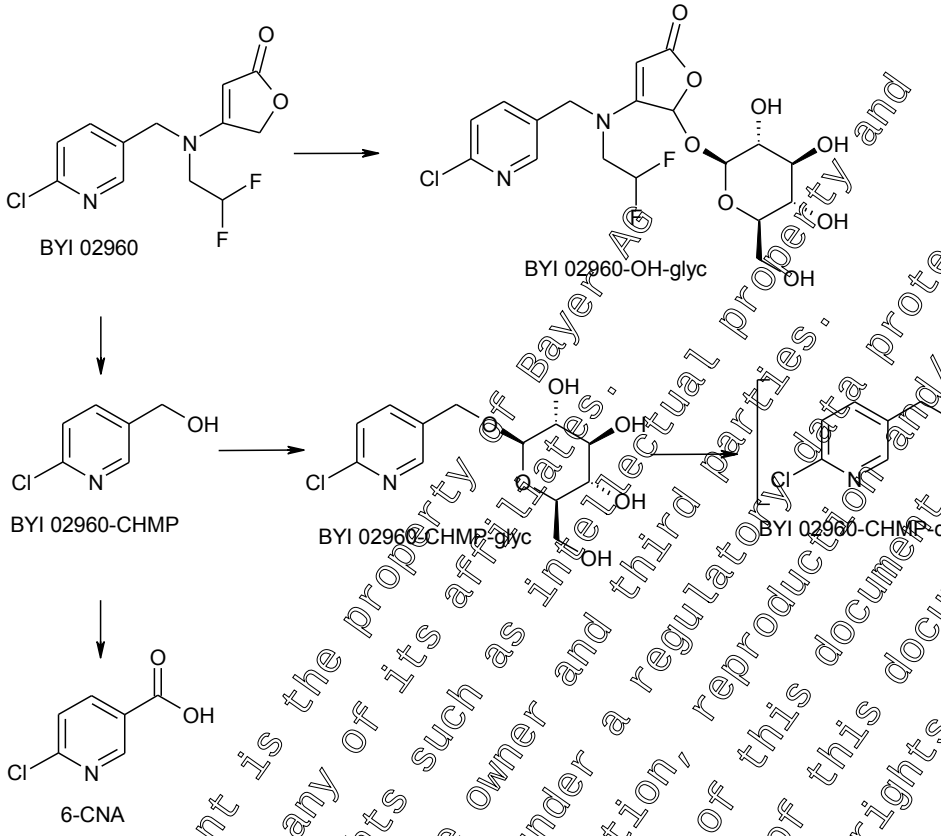
III. Conclusions

Based on the metabolites identified the following metabolic routes were deduced:

- cleavage of the pyridinylmethylamine bond followed by conjugation with carbohydrates or followed by oxidation of the methylene group to a carboxylic group, which were the major metabolic routes, and
- hydroxylation of the methylene group of the furanone moiety followed by conjugation with glucose, which was the minor metabolic route.

Thus, [pyridinylmethyl-^{14}C]BYI 02960 was metabolised moderately in tomatoes. On the basis of the results of this study it is concluded that the metabolism of [pyridinylmethyl-^{14}C]BYI 02960 in tomatoes is well understood and the following metabolic pathway is proposed.

Figure 6.2.1-2: Proposed metabolic pathway of [pyridinylmethyl-^{14}C]BYI 02960 in tomatoes



Report:	**KIIA 6.2.1/03, [redacted], [redacted].; 2011**
Title:	Metabolism of [ethyl-1-^{14}C]BYI 02960 in tomatoes
Report No & Edition No	MEF-11/498 M-413996-01-2
Guidelines:	OECD 501 Metabolism in Crops US EPA Residue Chemistry Test Guideline OPPTS 860.1300: Nature of the Residue – Plants, Livestock PMRA Regulatory Directive Dir98-02: Residue Chemistry Guidelines Section 2: Nature of the Residue – Plants, Livestock Japanese MAFF, 12 Nousan 8147 European Parliament and Council Regulation (EC) No 1107/2009
GLP	yes

Executive Summary

The metabolism of [ethyl-1-^{14}C]BYI 02960 in tomatoes was investigated according to the maximum envisaged use pattern. Three tomato plants were treated by soil drench application with [ethyl-1-^{14}C]BYI 02960 formulated as an SL 200. The first application was performed at BBCH 14-15 (4th to 5th leaf on main shoot unfolded) and a second application at BBCH 51-59 (first to 9th inflorescence visible and first bud erect). The single application rate corresponded to 300 g a.s./ha; the total application rate was 600 g a.s./ha.

The flowers were sampled from one tomato plant at 1 to 36 days after the last application and the fruits were harvested from the remaining two tomato plants at 56 to 86 days after the last application. The TRR values are shown in the following table:

Table 6.2.1-8: TRR values in tomato fruits and flowers after drench application of [ethyl-1-^{14}C]BYI 02960

Matrix	**Timing and Application**	**PHI (days)***	**ppm (mg a.s. equiv./kg)**
tomato fruits	two drench applications, at BBCH 14-15 and 14 days later; 2 x 300 g a.s./ha	56	0.201
tomato flowers		n.a.	2.230

* PHI: preharvest interval (corresponds to days after last treatment (DAT) at the start of harvest/sampling)
n.a. not applicable, flowers are not a RAC

The fruits and flowers were extracted conventionally. Three extraction steps with acetonitrile/water mixtures released 99.5% and 98.3% of the TRR from fruits and flowers, respectively.

Parent compound and metabolites in the extracts of tomato flowers and fruits were analysed by HPLC. Identification was performed by HPLC and/or TLC co-chromatography with reference compounds, as well as by comparison of HPLC profiles. Besides parent compound, one major metabolite and two minor metabolites were identified.

The label-specific metabolite difluoroacetic acid (DFA) was the main component detected in both matrices and represented 86.6% of the TRR in tomato fruits and 59.8% in tomato flowers. Since difluoroacetic acid was also detected as a major soil metabolite in the aerobic soil degradation studies, it can be expected that at least a part of the residues detected in tomato fruits and flowers originated

from the uptake of the soil metabolite. Parent compound was the second major component in tomato fruits and flowers and represented 10.0% and 33.0% of the TRR, respectively. BYI 02960-OH-glyc and BYI 02960-difluoroethyl-amino-furanone were minor metabolites (<5% of the TRR) detected in fruits and flowers.

[Ethyl-1-^{14}C]BYI 02960 was metabolised to a significant extent in tomatoes. The following metabolic routes were observed:

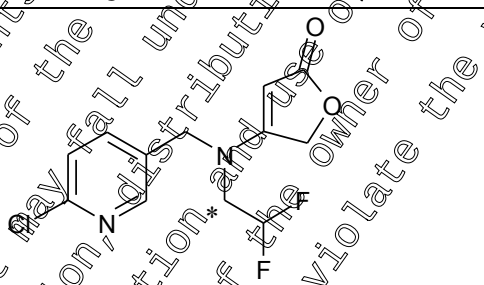
- oxidative cleavage of the difluoroethylamine bond and formation of difluoroacetic acid (this degradation process occurs also in the soil and thus uptake of difluoroacetic acid from the soil is supposed as well)
- cleavage of the pyridinylmethylamine bond and formation of BYI 02960-difluoroethylamino-furanone and
- hydroxylation of the methylene group of the furanone moiety followed by conjugation with glucose

On the basis of these results, a metabolic pathway of [ethyl-1-^{14}C]BYI 02960 in tomatoes can be proposed.

I. Materials and Methods

A. Materials

1. Test Material:

Chemical structure	 * position of the radiolabel
Radiolabelled test material	[ethyl-1-^{14}C]BYI 02960
Specific radioactivity (before radiodilution) (after radiodilution)	 3.93 MBq/mg (106.28 µCi/mg) 1.31 MBq/mg (35.46 µCi/mg)
Chemical Purity	> 99% (HPLC)
Radiochemical purity	> 99% (HPLC and TLC)

The supplied radiolabelled test compound [ethyl-1-^{14}C]BYI 02960 was dissolved in acetonitrile. Radiodilution and formulation of the test compound was performed prior to the first and the second application. Therefore, an adequate portion of the non-radiolabelled test compound was dissolved in an adequate volume of the stock solution of the radiolabelled test compound. The solution was evaporated to dryness. The remainder was dissolved in an adequate amount of SL 200 blank formulation using a magnetic stirrer. Preparation of each application solution was done by dissolving the formulation in water. The specific activity of each application solution was 1.31 MBq/mg (35.46 µCi/mg).

2. Soil: "Einheitserde T", pH ($CaCl_2$) = 5.8, 85% white moor peat and 15% clay, enriched with water soluble nutrient salt mixture (ENS)

3. Plant Tomato, variety "Philona", representative for fruiting crops

B. Study Design

Experimental conditions:

Three tomato plants (variety: Philona) were cultivated each in a 30 L planting bucket with a surface diameter of 38 cm. The planting buckets have been filled with "Einheitserde T". The plants were cultivated in the greenhouse of the test facility (controlled temperature, humidity and light conditions).

The tomato plants were treated with SL 200 formulated [ethyl-1-^{14}C]BYI 02960 by drench application. Two applications were performed, each at an application rate of 300 g a.s./ha. The first application was conducted at a growth stage of BBCH 14-15 (4th to 5th leaf on main shoot unfolded) and the second at a growth stage of BBCH 51-59 (1st to 9th inflorescence visible and first bud erect). The interval between the two applications was 14 days. The aqueous application solution was poured into a circular furrow prepared in the soil around the tomato plant. At each application, a total volume of 150 mL was applied, corresponding to 98.50 MBq or to 75.1 mg a.s. (= 25 g a.s./plant). Based on a planting density of 12,000 tomato plants/ha in agricultural practice, the application rate was 300 g a.s./ha. The total rate corresponds to the anticipated maximum application rate in agricultural practice.

Sampling:

When the plants reached the growth stage BBCH 61 (first flower of the first inflorescence open), the open flowers were sampled from one of the three tomato plants in order to determine the nature of the residues in flowers. The flowers were collected with their receptacles using scissors and were stored in a freezer (≤ -18°C) on the same day. Sampling of newly opened flowers was continued 2 - 3 times a week over a total period of approx. four weeks until end of flowering (at BBCH 69: 9 or more inflorescences with open flowers). The tomato plant from which the flowers were cut was sampled, cut in pieces and stored in a freezer (≤ -18°C) for optional metabolite investigations. The frozen flowers were homogenized with a high speed blender in liquid nitrogen (Polytron). The homogenised flower sample was stored in a freezer (≤ -18°C) until extraction.

On the day when the two remaining tomato plants reached the growth stage BBCH 81 (10% of fruits show typical fully ripe colour), the ripe tomato fruits were harvested and were stored in a freezer (≤ -18°C) on the same day. Harvesting of newly ripe fruits was continued 2 - 3 times a week over a total period of approx. four weeks until the plants produced no new fruits (at BBCH 89: fully ripe). The frozen fruits were crushed and homogenised with a high speed blender (Polytron). The homogenised tomato fruits samples was stored in portions in a freezer (≤ -18°C) until extraction.

C. Analytical Procedures

Extraction:

The homogenised tomato flowers and tomato fruits were extracted three times with a mixture of acetonitrile/water (8:2, v/v) using a high speed blender (Polytron). After each extraction step, extracts and solids were separated by centrifugation. The radioactivity in the extracts was determined by LSC, in the solids by combustion followed by LSC. The actual TRR value of the RAC was determined by summing up the radioactivity measured in the extracts and in the remaining solids.

The first two tomato fruit extracts were combined and as well the first two tomato flower extracts. The combined extracts were subjected to a clean-up step using a pre-conditioned SPE RP 18 cartridge (Phenomenex, Strata C18-E). The flow-through fraction (percolate) was collected and the cartridge was rinsed with 100 mL of acetonitrile/water (8:2, v/v) and methanol/dichloromethane (1:1, v/v). The percolate and the acetonitrile/water fraction were combined and concentrated by rotary evaporation in vacuo. The concentrated extract was analysed by HPLC.

Quantification:
Parent compound and metabolites in the extracts were analysed by reversed phase HPLC coupled to a radioactivity detector with a glass scintillator cell. The HPLC chromatograms (= metabolite profiles) were integrated for quantification of compounds.

Identification and characterisation:
Parent compound and metabolites were identified by co-chromatography with reference compounds or by comparison of chromatographic profiles. For identification of the label-specific main metabolite, three different stationary phases were used for TLC co-chromatography. Thus, dissimilar TLC systems confirmed the assignment un ambiguously.

Storage stability:
The extraction experiments and the first HPLC analysis of the tomato fruits were performed within approx. 1.5 months after harvest. The time period between harvest and analysis was approx. 3 months for tomato flowers. Thus, no additional storage stability data have to be provided according to OECD Guidance for the Testing of Chemicals 501 (2007).

All extracts were analysed on the next day after the start of extraction. Extract stability was demonstrated by comparing the HPLC chromatograms recorded at different times during the study. The profiles did not change significantly during the analytical work. In the corresponding tomato metabolism studies performed with [pyridinylmethyl-^{14}C]BYI 02960 and [furanone-4-^{14}C]BYI 02960, it was shown that the profiles of tomato fruits and flowers extracts did not significantly change during a period of at least six months. It was therefore concluded that the residues in the extracts were sufficiently stable during the experimental period of the study and that the chromatograms represented the metabolic pattern in the samples at harvest.

II. Results and Discussion

The metabolism of [ethyl-1-^{14}C]BYI 02960 was investigated in tomato fruits and flowers following two drench applications. The total radioactive residue (TRR) in fruits, which represent the edible RAC, accounted for 0.201 mg/kg. The TRR was considerably higher in flowers and accounted for 2.230 mg/kg. By far the main portion of radioactivity was extracted conventionally by acetonitrile/water mixtures (99.5% to 98.3% of the TRR) as shown in Table 6.2.1-9. The label-specific metabolite difluoroacetic acid (DFA) was the main residue in both extracts, followed by parent compound. Two additional minor metabolites (<5% of TRR) were identified in tomato fruits and flowers: BYI 02960-difluoroethyl-amino-furanone and BYI 02960-OH-glyc.

Parent compound was identified in tomato fruits and flowers by HPLC co-chromatography using a non-radiolabelled reference compound and by comparison of the metabolite profiles of all tomato studies conducted with BYI 02960 labelled in the different moieties of the molecule. In the tomato metabolism study with [furanone-4-^{14}C]BYI 02960, parent compound was additionally identified in tomato fruits and flowers by normal phase TLC co-chromatography as different chromatographic technique. The main metabolite difluoroacetic acid was identified after semi-preparative isolation by TLC co-chromatography with an authentic reference compound. Two dissimilar systems (normal phase and reversed phase TLC) and one modified normal phase system were applied. TLC co-chromatography with three different stationary phases showed chromatographic correspondence of the radioactivity in the isolated fraction with the radiolabelled reference compound difluoroacetic acid. In tomato flowers this metabolite was identified by comparison of the HPLC profiles of fruits and flowers.

The minor metabolite BYI 02960-difluoroethyl-amino-furanone was identified in tomato fruits by reversed phase HPLC co-chromatography with a non-radiolabelled reference compound. This metabolite was identified in tomato flowers by comparison of the two profiles. The minor metabolite BYI 02960-OH-glyc was identified in tomato fruits and flowers by comparison of the HPLC profile with a corresponding profile obtained in the tomato metabolism study performed with [furanone-4-^{14}C]BYI 02960. In the latter study, the metabolite BYI 02960-OH-glyc had been identified in tomato fruits by reversed phase HPLC co-chromatography with a radiolabelled reference compound and additionally in tomato flowers by co-chromatography of the aglycon after alkaline hydrolysis with a non-radiolabelled reference compound.

The TRR and the distribution of parent and metabolites in the extracts is shown in Table 6.2.1-10. In total, 99.5% and 98.3% of the TRR were identified in the tomato fruits and flowers, respectively.

Table 6.2.1-9: Distribution of radioactivity in the extracts of the tomato matrices fruits and flowers after drench application of [ethyl-1-^{14}C]BYI 02960

	tomato fruits		**tomato flowers**	
TRR [mg/kg] =	0.201		2.230	
	% of TRR	mg/kg	% of TRR	mg/kg
Conventionally extracted	99.5	0.200	98.3	2.192
Extract for analysis	99.5	0.200	98.3	2.192
Losses (not analysed)	n.q.	n.q.	n.q.	n.q.
Total extracted	**99.5**	**0.200**	**98.3**	**2.192**
Unextractable (PES*)	0.5	0.001	1.7	0.037
Accountability	**100.0**	**0.201**	**100.0**	**2.230**

* post extraction solids

n.q. not quantified

Table 6.2.1-10: TRR values and distribution of parent compound and metabolites in tomatoes (fruit and flowers) after drench application of [ethyl-1-^{14}C]BYI 02960

	tomato fruits		**tomato flowers**	
TRR [mg/kg] =	0.201		2.230	
Compound (BYI 02960-)	% TRR	mg/kg	% TRR	mg/kg
Conventional extraction				
BYI 02960 (parent compound)	10.0	0.020	33.0	0.736
difluoroacetic acid	86.6	0.174	59.8	1.334
difluoroethyl-amino-furanone	2.2	0.004	3.1	0.068
OH-glyc	0.6	0.001	2.4	0.054
Total identified	**99.5**	**0.200**	**98.3**	**2.192**
Total characterized	**---**	**---**	**---**	**---**
Analysed extract(s)	99.5	0.200	98.3	2.192
Extract(s) not analysed	**---**	**---**	**---**	**---**
Total extracted	**99.5**	**0.200**	**98.3**	**2.192**
Unextractable (PES*)	0.5	0.001	1.7	0.037
Accountability	**100.0**	**0.201**	**100.0**	**2.230**

* post extraction solids

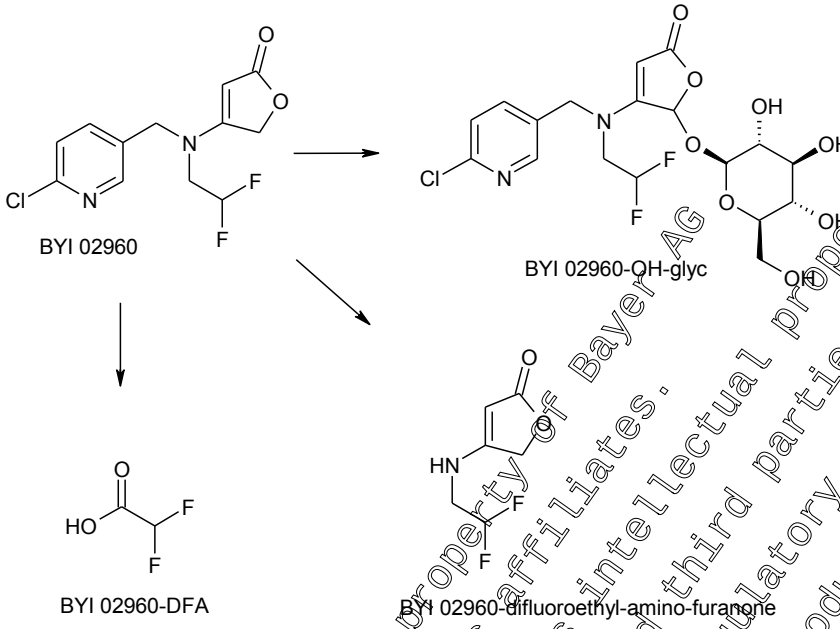
III. Conclusions

Based on the metabolites identified the following metabolic routes were deduced:

- oxidative cleavage of the difluoroethylamine bond and formation of difluoroacetic acid,
- cleavage of the pyridinylamine bond and formation of BYI 02960-difluoroethyl amino-furanone, and
- hydroxylation of the methylene group of the furanone moiety followed by conjugation with glucose.

[Ethyl-1-^{14}C]BYI 02960 was metabolised rather extensively in the present study. Since it is known that parent degrades to difluoroacetic acid in soil, the difluoroacetic acid residue is probably the result of both uptake of this metabolite from the soil and degradation of the parent compound BYI 02960 in the plant. On the basis of the results of this study it is concluded that the metabolism of [ethyl-1-^{14}C]BYI 02960 in tomatoes is well understood and the following metabolic pathway is proposed.

Figure 6.2.1-3: Proposed metabolic pathway of [ethyl-1-^{14}C]BYI 02960 in tomatoes



Overall Conclusions Tomato (soil drench)

The metabolism of the insecticide BYI 02960 was investigated in tomato fruits and tomato flowers in three studies following two soil drench applications of (1) [furanone-4-^{14}C]BYI 02960, (2) [pyridinylmetyl-^{14}C]BYI 02960 or (3) [ethyl-1-^{14}C]BYI 02960. Total radioactive residue was measured in fruits and flowers and metabolites identified or characterized dependent on levels found.

(1) Label 1: [furanone-4-^{14}C]BYI 02960
At harvest, the total radioactive residue (TRR) in the tomato fruits was low (0.096 mg/kg). Parent compound was the main component detected. Besides parent compound, two major label-specific metabolites were present. The natural compound glucose (or isomeric carbohydrates) and BYI 02960-difluoroethyl-amino-furanone. Furthermore, two minor metabolites were detected. One of them was identified as the non-label specific metabolite BYI 02960-OH-glyc. In tomato flowers, the same metabolites were identified as in the fruits, with the exception of glucose which was only present in fruits. Although the same metabolites were detected, the proportions differed significant. Most significant was that parent compound was by far the main constituent in flowers and represented nearly 80% of the TRR.

On basis of the metabolites identified, the metabolic pathway was deduced. One major metabolic route was the complete degradation of the furanone moiety and incorporation of the carbon atoms into the natural compound pool, i.e. most probably into glucose. Cleavage of the pyridinylmethylamine bond and formation of BYI 02960-difluoroethylamino-furanone was also an important route. The cleavage of the molecule was also detected in the tomato studies conducted with the other two radiolabels. In the study with [ethyl-1-^{14}C]BYI 02960, BYI 02960-difluoroethylamino-furanone was detected in nearly identical concentrations and in the study with [pyridinylmetyl-^{14}C]BYI 02960, BYI 02960-

CHMP and 6-CNA were identified as corresponding counterparts. A minor metabolic route was the hydroxylation of the methylene group of the furanone moiety followed by conjugation with glucose. This metabolic route resulted in the non-label specific metabolite BYI 02960-OH-glyc, which was identified in corresponding concentrations in the tomato study performed with [ethyl-1-^{14}C]- and [pyridinylmethyl-^{14}C]BYI 02960. Thus, the results of the present metabolism study in tomatoes are in very good conformity with the results of the corresponding studies performed with [ethyl-1-^{14}C]- and [pyridinylmethyl-^{14}C]BYI 02960.

(2) Label 2: [pyridinylmethyl-^{14}C]BYI 02960

At harvest, the total radioactive residue (TRR) in the tomato fruits, representing the edible raw agricultural commodity (RAC), was low (0.130 mg/kg). Three major components were detected in the fruits: Metabolite BYI 02960-CHMP-di-glyc, parent compound BYI 02960 and 6-CNA. Besides the major components, six minor metabolites were detected, three of them were identified: BYI 02960-CHMP and BYI 02960-CHMP-glyc, the label-specific precursor metabolites of BYI 02960-CHMP-di-glyc, and BYI 02960-OH-glyc, a metabolite common to all three radiolabels tested. In tomato flowers, the same metabolites were identified as in the fruits, with the exception of BYI 02960-CHMP. However, the proportions of the compounds differed significantly. Most significant was that parent compound was by far the main constituent and represented more than 60% of the TRR.

The major metabolic route was cleavage of the pyridinylmethylamine bond followed by conjugation with carbohydrates or by oxidation of the methylene group to a carboxylic group. Cleavage of the molecule was also confirmed in the tomato study performed with [furanone-4-^{14}C]- and [ethyl-1-^{14}C]BYI 02960. A minor metabolic route was hydroxylation of the methylene group of the furanone moiety followed by conjugation with glucose. This metabolic route resulted in the non-label specific metabolite BYI 02960-OH-glyc, which was identified in corresponding concentrations in the tomato studies performed with [furanone-4-^{14}C]- and [ethyl-1-^{14}C]BYI 02960. Thus, the results of the present metabolism study in tomatoes are in good conformity with the results of the corresponding studies performed with the other radiolabels.

(3) Label 3: [ethyl-1-^{14}C]BYI 02960

At harvest, the total radioactive residue (TRR) in the tomato fruits, representing the edible raw agricultural commodity (RAC), was 0.201 mg/kg. The TRR in flowers accounted for 2.230 mg/kg. These TRR values were significantly higher compared to those obtained in the tomato metabolism studies conducted with [pyridinylmethyl-^{14}C]BYI 02960 and [furanone-4-^{14}C]BYI 02960. Since it is known that parent degrades to difluoroacetic acid in soil, the difluoroacetic acid residue is probably the result of both uptake of this metabolite from the soil and degradation of the parent compound BYI 02960 in the plant. Difluoroacetic acid was by far the main compound detected in fruits and flowers, followed by parent compound. Additionally, two minor metabolites were detected. They were identified as BYI 02960-difluorethyl-amino-furanone and BYI 02960-OH-glyc.

Parent compound and all metabolites not specific to the [ethyl-1-^{14}C]-label were detected in comparable amounts as in the tomato metabolism studies conducted with the other radiolabels. The major metabolic route in the present study observed was oxidative cleavage of the difluoroethylamine bond and formation of difluoroacetic acid. Cleavage of the pyridinylamine bond was also observed and led to the formation of BYI 02960-difluoroethyl amino-furanone. The latter molecular cleavage

was also confirmed in the tomato study with [furanone-4-^{14}C]BYI 02960 and as well in the tomato study with [pyridinylmethyl-^{14}C]BYI 02960, in which BYI 02960-CHMP and 6-CNA were detected as corresponding counterparts. A minor metabolic route was hydroxylation of the methylene group of the furanone moiety followed by conjugation with glucose. This metabolic route resulted in the non-label specific metabolite BYI 02960-OH-glyc, which was identified in comparable concentrations in the other two tomato studies performed with [furanone-4-^{14}C]- and [pyridinylmethyl-^{14}C]BYI 02960. Thus, the results of the present metabolism study in tomatoes are in good conformity with the results of the corresponding studies performed with the other radiolabels.

When considering the results from all metabolism studies conducted on tomato, it can be concluded that BYI02960 is rather extensively metabolised in this crop. A total of 6 major and 6 minor metabolites were found, and all major and 3 minor have been identified. The distribution of parent compound and metabolites in the edible commodity tomato fruits is summarized in Table 6.2.1-11.

Table 6.2.1-11: TRR values and distribution of parent compound and metabolites in tomato fruits after drench application of radiolabelled BYI 02960

	tomato fruits					
Radiolabel	[furanone-4-^{14}C]		[pyridinylmethyl-^{14}C]		[ethyl-1-^{14}C]	
TRR [mg/kg] =	0.096		0.130		0.201	
Compound (BYI 02960-)	% TRR	mg/kg	% TRR	mg/kg	% TRR	mg/kg
BYI 02960	35.9	0.034	24.2	0.031	10.0	0.020
difluoroacetic acid					*86.6*	*0.174*
glucose/carbohydrates	*27.5*	*0.026*				
6-CNA			*13.2*	*0.017*		
CHMP-di-glyc			*37.1*	*0.048*		
CHMP-glyc			*5.1*	*0.007*		
CHMP			*3.3*	*0.004*		
difluoroethyl-amino-furanone	*10.3*	*0.010*			*2.2*	*0.004*
OH-glyc	5.5	0.005	3.4	0.004	0.6	0.001
Total identified	**79.2**	**0.076**	**86.3**	**0.112**	**99.5**	**0.200**
Total characterised	**4.3**	**0.004**	**12.2**	**0.016**	**--**	**---**
Analysed extract(s)	83.5	0.080	98.5	0.128	99.5	0.200
Extract(s) not analysed	1.3	0.001	--	--	--	---
Total extracted	**84.8**	**0.081**	**98.5**	**0.128**	**99.5**	**0.200**
Unextractable (PES*)	15.1	0.015	1.5	0.002	0.5	0.001
Accountability	**100.0**	**0.096**	**100.0**	**0.130**	**100.0**	**0.201**

* post extraction solids

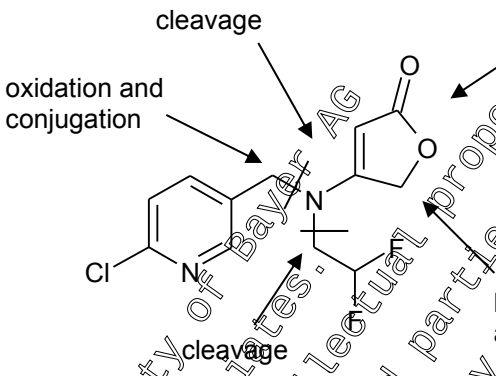
Label specific metabolites are printed in italics.

On basis of the metabolites identified, biotransformation of BYI02960 in tomato proceeds by the following pathways:

- oxidative cleavage of the difluoroethylamine bond and formation of difluoroacetic acid
- complete degradation of the furanone moiety and incorporation of carbon atoms into the natural compound pool, e.g. into glucose/carbohydrates
- cleavage of the pyridinylmethylamine bond and formation of BYI 02960-difluoroethylamino-furanone and its corresponding counterpart BYI 02960-CHMP, which was either conjugated with carbohydrates or oxidised to 6-chloronicotinic acid (6-CNA)
- hydroxylation of the furanone moiety followed by conjugation with glucose

The positions involved in the metabolic degradation are summarised in the following figure.

Figure 6.2.1-4: Positions involved in metabolic degradation of BYI 02960 in tomato fruits and flowers



Metabolism, distribution and expression of residues in potato (tuber treatment and in-furrow application)

Metabolism studies in potatoes were conducted with [furanone-4-^{14}C]- and [pyridinylmethyl-^{14}C]BYI 02960:

Report:	**KIIA 6.2.1/04, [redacted].; 2011**
Title:	Metabolism of [furanone-4-^{14}C]BYI 02960 in potatoes
Report No & Edition No	MEF-10/769 M-415234-01-2
Guidelines:	OECD 501 Metabolism in Crops US EPA Residue Chemistry Test Guideline OPPTS 860.1300: Nature of the Residue – Plants, Livestock PMRA Regulatory Directive Dir98-02 Residue Chemistry Guidelines Section 2: Nature of the Residue – Plants, Livestock Japanese MAFF, 12 Nousan 8147 European Parliament and Council Regulation (EC) No 1107/2009
GLP	yes

Executive Summary

The metabolism of [furanone-4-^{14}C]BYI 02960 in potatoes was investigated according to the maximum envisaged use pattern using two different methods of application. In one experiment, the seed potato tubers were treated with [furanone-4-^{14}C]BYI 02960 formulated as an FS 480 at an application rate of 100 g a.s./dt (= 254 g a.s./ha, seed density 25 dt/ha). In the other experiment, [furanone-4-^{14}C]BYI 02960, formulated as an SL 200, was sprayed in the furrow onto the soil at an application rate of 626 g a.s./ha (seed density 22 dt/ha) prior to planting of the seed potatoes. The potato tubers were harvested at maturity in both experiments. Concurrently, the leaves and roots as well as the remainders of the seed potatoes were sampled. The TRR values determined are shown in the following table:

Table 6.2.1-12: TRR values in potato tubers and the remaining part of the plant after tuber treatment or in-furrow application of [furanone-4-^{14}C]BYI 02960

Matrix	Timing and Application	PHI (days)*	ppm (mg a.s. equiv./kg)
potato tubers	tuber treatment at planting (BBCH 03), 100 g a.s./dt	97	0.078
potato leaves and roots		97	6.97
remainders of the seed potatoes		97	36.21
potato tubers	in-furrow application at planting (BBCH 03), 626 g a.s./ha	97	0.171
potato leaves and roots		97	7.01
remainders of the seed potatoes		97	3.43

* PHI: preharvest interval (corresponds to days after last treatment (DAT) at the start of harvest/sampling)

The potato tubers (edible raw agricultural commodity) were extracted conventionally. Four extraction steps with acetonitrile/water mixtures released 67.0% of the TRR in the tuber treatment experiment and 75.3% of the TRR in the in-furrow treatment experiment.

Parent compound and metabolites in the extracts of potato tubers were analysed by HPLC. Identification was performed by HPLC co-chromatography with reference compounds and by comparison of HPLC profiles. Parent compound represented 40.0% and 56.9% of the TRR in potato tubers. Besides parent compound, which was the predominant compound in the extracts, two minor metabolites representing less than 10% of the TRR and accounting for less than 0.01 mg/kg were identified: BYI 02960-OH-glyc and BYI 02960-difluoroethyl-aminofuranone.

[Furanone-4-^{14}C]BYI 02960 was moderately metabolised in potatoes. The following metabolic routes were observed:

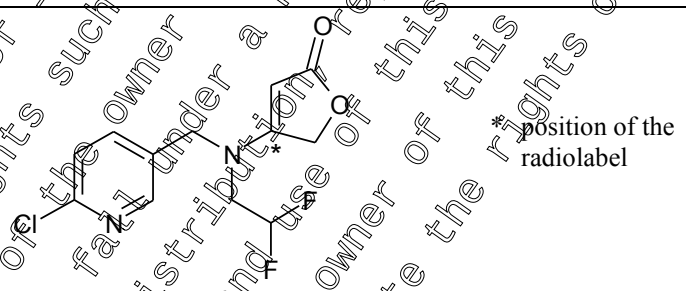
- hydroxylation of the methylene group of the furanone moiety followed by conjugation with glucose, and
- cleavage of the pyridinylmethylamine bond.

On the basis of these results, a metabolic pathway of [furanone-4-^{14}C]BYI 02960 in potatoes can be proposed.

I. Materials and Methods

A. Materials

1. Test Material:

Chemical structure	
Radiolabelled test material	[furanone-4-^{14}C]BYI 02960
Specific radioactivity	3.94 MBq/mg (106.46 µCi/mg)
Chemical Purity	> 99% (HPLC)
Radiochemical purity	> 99% (HPLC and TLC)

The supplied radiolabelled test compound [furanone-4-^{14}C]BYI 02960 was dissolved in acetonitrile. Formulation of the test compound was performed prior to the application. For both formulations (FS 480 and SL 200), adequate parts of this stock solution were transferred into special glass vials and evaporated to dryness. For the preparation of the FS 480 formulation, the blank formulation was added to the test item and was homogenized using a ball mill. Afterwards the mixture was suspended in water by stirring or swirling. For the preparation of the SL 200 formulation, the liquid blank formulation was added to the test item using a magnetic stirrer. The mixture was diluted with water to get the aqueous application solution.

2. Soil: "[redacted] 4" (sandy loam soil from Germany), pH ($CaCl_2$) = 6.8, 69% sand, 18% silt and 13% clay, 1.2% organic carbon, cation exchange capacity (CEC) of 8.3 meq/100 g

3. Plant Potato, variety "Cilena", representative for root crops

B. Study Design

Experimental conditions:

Two experiments were performed representing the intended application types (tuber treatment and in-furrow application). Each experiment was conducted with three seed potatoes (variety: Cilena) in a planting container with a surface area of 0.5 m^2. The plants were cultivated in the glass-roofed vegetation area of the test facility. The plants were grown similar to natural temperature and light conditions, but protected from rainfall. The plants were watered by pouring onto the soil in the planting containers.

<u>Tuber treatment experiment:</u>

The formulated test compound was applied to the seed potatoes at a target rate of 10 g a.s./dt. For this purpose, three seed potatoes were treated with a defined volume of the aqueous application suspension. The potatoes were placed in the furrows before application to prevent any loss of the application suspension. To ensure a regular treatment, the seed potatoes were treated on the upper side, allowed to dry, then turned and treated on the other side. The furrow was closed when the application suspension was dried on the seed potatoes. A total volume of 763.2 µL was applied, corresponding to 50.2 MBq or to 12.7 mg a.s.. The actual seed treatment rate was 10.0 g a.s./dt, corresponding to 254 g a.s./ha. The seed density was 25 dt/ha.

<u>In-furrow treatment experiment:</u>

For the in-furrow treatment experiment, a defined volume of the aqueous application solution was filled into the bottle of a hand pump sprayer. The application solution was sprayed onto the soil in the furrow. The empty pump sprayer was rinsed with 3 mL water, 0.5 mL of the rinse was sampled for radioactivity measurement and the rest of the rinse was sprayed onto the soil in the furrow. Three seed potatoes were placed into the furrow and the furrow was closed. The empty pump sprayer was again rinsed with 3 mL water. The remaining radioactivity in the rinsing solution was determined by LSC. The losses were subtracted from the initial amount of radioactivity. An amount of 123.3 MBq or 31.3 mg a.s. was applied to the three potatoes, corresponding to an actual application rate of 626 g a.s./ha. The seed density was 22 dt/ha.

Sampling:

Potato tubers were sampled as raw agricultural commodity (RAC) from the tuber treatment experiment and from the in-furrow treatment experiment at maturity of the potato plants (BBCH 97). The leaves and roots, as well as the remainders of the seed potatoes were sampled at the same time to determine the TRR by combustion analysis.

The potato tubers were allowed to dry at room temperature for one night. Adhering soil was removed. The potato tuber samples from the two experiments were each washed with 1 L of water and the radioactivity in the wash water was determined. The potato tubers were cut into small cubes. The cubes were randomized by shuffling and stored in a freezer (≤ -18°C) in aliquots.

The leaves and roots as well as the remainders of the seed potatoes were cut in pieces, liquid nitrogen was added and the mixtures were homogenised using a high speed blender (Polytron PT6000). The powdered samples were stored in a freezer (≤ -18°C).

C. Analytical Procedures

Extraction:

The cut potato tubers of the tuber treatment and the in-furrow treatment experiment were extracted three times with a mixture of acetonitrile/water (8:2, v/v) and one time with a mixture of acetonitrile/water (1:1, v/v) using a high speed blender (Polytron PT6000). The radioactivity in the extracts was determined by LSC and in the solids by combustion followed by LSC. The actual TRR value of the RAC was determined by summing up the radioactivity measured in the extracts and in the remaining solids. The first two extracts of each experiment were combined and subjected to a clean-up step using an SPE RP 18 cartridge (Phenomenex, Strata C18-E, 20 g), which was conditioned with acetonitrile beforehand. The flow-through fraction (percolate) was collected and the cartridge was rinsed with 100 mL of acetonitrile/water (8:2, v/v) and methanol/dichloromethane (1:1, v/v). The percolate and the acetonitrile/water fraction were combined and concentrated by rotary evaporation in vacuo for HPLC analysis.

The remaining solids after conventional extraction of the potato tubers in the in-furrow treatment experiment were subjected two times to exhaustive extraction with acetonitrile/water (1:1, v/v) under microwave assistance (120°C for 20 min.). After each extraction step, extracts and solids were separated by centrifugation. The radioactivity in the extracts was determined by LSC, in the solids by combustion followed by LSC. The two extracts were combined and concentrated by rotary evaporation in vacuo for HPLC analysis. In the tuber treatment experiment, the solids remaining after conventional extraction amounted to 0.026 mg/kg, only. Moreover, since the distribution of the radioactivity in the extracts and solids after conventional extraction was nearly identical within the in-furrow and the tuber treatment experiments and the conventional extracts showed a very similar metabolite pattern, exhaustive extraction of the solids of the tuber treatment experiment was not considered necessary.

Quantification:

Parent compound and metabolites in the extracts were analysed by reversed phase HPLC coupled to a radioactivity detector with a glass scintillator cell. The HPLC chromatograms (= metabolite profiles) were integrated for quantification of compounds.

Identification and characterisation:

Parent compound and metabolites were identified by co-chromatography with reference compounds and/or by comparison with the HPLC profiles obtained in the potato metabolism study with [pyridinylmethyl-^{14}C]BYI 02960.

Storage stability:

The conventional extractions and the first HPLC analyses of the potato tuber extracts were performed not later than three months after harvest. The extracts were analysed after two and four days following the start of extraction. The solids after conventional extraction were extracted with microwave assistance and were analysed within five and a half months after harvest.

According to OECD Guidance for the Testing of Chemicals 501 (2007), storage stability data are not necessary for samples analysed within 6 months of collection. Therefore it was concluded that the data

in hand provide adequate evidence to show the stability of the compounds and no additional investigations deemed necessary.

In addition, the extract stability was demonstrated by comparing the HPLC chromatograms recorded at different time points during the study. The profiles did not change significantly during the whole course of the study. The stability of extracts was demonstrated for parent and metabolites for at least two months which covers the time period of all analytical investigations including identification and characterisation of metabolites.

II. Results and Discussion

The metabolism of [furanone-4-^{14}C]BYI 02960 was investigated in potato tubers following two different soil application methods. In one experiment, tubers of seed potatoes were treated with [furanone-4-^{14}C]BYI 02960 formulated as an FS 480 at an application rate of 10.0 g a.s./dt (=254 g a.s./ha). In the other experiment [furanone-4-^{14}C]BYI 02960, formulated as an SL 200, was sprayed in the furrow onto the soil prior to planting of the seed potatoes at an application rate of 626 g a.s./ha..

At harvest, the total radioactive residues (TRR) in potato tubers were low after tuber treatment and after in-furrow application and accounted for 0.078 mg/kg and 0.171 mg/kg, respectively. The TRR values in the remaining plant parts (leaves, roots and remainders of the seed potatoes) were considerably higher. These plant parts were only sampled for optional analysis to support identification of metabolites, if needed.

A major portion of radioactivity in the potato tubers was extracted conventionally by acetonitrile/water mixtures (67.0% to 69.0% of the TRR) as shown in Table 6.2.1-13. Additionally, 6.3% of the TRR was released after exhaustive extraction of the remaining solids with microwave assistance as shown for the in-furrow experiment.

Parent compound was the main residue in both extracts. Two additional minor metabolites (<10% of TRR and <0.01 mg/kg) were detected: BYI 02960-difluoroethyl-amino-furanone and BYI 02960-OH-glyc. The assignment of parent compound and metabolite BYI 02960-OH-glyc was based on the identification achieved in the potato study with [pyridinylmethyl-^{14}C]BYI 02960. The HPLC profiles were compared and corresponding peaks were assigned. In the potato study with [pyridinylmethyl-^{14}C]BYI 02960, all major metabolites were identified by co-chromatography with authentic reference compounds using two different chromatographic systems (HPLC and TLC), and minor metabolites were identified by HPLC co-chromatography. In the present study, the identification of parent compound (main constituent in the profile) was confirmed additionally by HPLC co-chromatography with an authentic reference compound. Co-chromatographic investigations were performed with the extracts obtained after tuber treatment and after in-furrow application.

The minor, label-specific metabolite BYI 02960-difluoroethyl-amino-furanone was identified by HPLC co-chromatography with an authentic reference compound in the tuber extract obtained after in-furrow application. In the profile of the tuber treatment experiment, the metabolite was assigned by comparison of the two profiles.

The TRR values and the distribution of the parent and metabolites is shown in Table 6.2.1-14. In total, 50.8% and 64.2% of the TRR were identified in potato tubers, respectively.

Table 6.2.1-13: Distribution of radioactivity in the extracts of the potato tubers after tuber treatment or in-furrow application of [furanone-4-^{14}C]BYI 02960

	tuber treatment		**in-furrow application**	
	potato tubers		**potato tubers**	
TRR [mg/kg] =	0.078		0.171	
	% of TRR	mg/kg	% of TRR	mg/kg
Conventionally extracted	67.0	0.052	69.0	0.118
Extract for analysis	63.1	0.049	65.3	0.111
Losses (not analysed)	3.9	0.003	3.8	0.006
Microwave extraction	---	---	6.3	0.011
Extract for analysis	---	---	6.2	0.011
Losses (not analysed)	---	---	0.1	0.001
Total extracted	**67.0**	**0.052**	**75.3**	**0.129**
Unextractable (PES*)	33.0	0.026	24.7	0.042
Accountability	**100.0**	**0.078**	**100.0**	**0.171**

* post extraction solids

Table 6.2.1-14: TRR values and distribution of parent compound and metabolites in potato tubers after tuber treatment and in-furrow application of [furanone-4-^{14}C]BYI 02960

	tuber treatment		in-furrow application	
	potato tubers		potato tubers	
TRR [mg/kg] =	0.078		0.171	
Compound (BYI 02960-)	% TRR	mg/kg	% TRR	mg/kg
Conventional extraction				
BYI 02960 (parent compound)	40.0	0.031	50.6	0.086
difluoroethyl-amino-furanone	4.2	0.003	2.9	0.005
OH-glyc	6.6	0.005	4.4	0.007
Subtotal identified	**50.8**	**0.039**	**57.9**	**0.099**
unknown 1	7.2	0.006	5.3	0.009
unknown 2	1.7	0.001	---	---
unknown 3	2.4	0.002	1.9	0.003
unknown 4	1.1	0.001	---	---
Subtotal characterised	12.4	0.010	7.4	0.013
Conventional extracts not analysed	3.9	0.003	3.8	0.006
Total conventional extraction	**67.0**	**0.052**	**69.0**	**0.118**
Microwave extraction				
BYI 02960 (parent compound)	---	---	6.3	0.011
Subtotal identified	**---**	**---**	**6.2**	**0.011**
Microwave extracts not analysed	---	---	0.1	<0.001
Total microwave extraction	**---**	**---**	**6.3**	**0.011**
Total identified	**50.8**	**0.039**	**64.2**	**0.110**
Total characterised	**12.4**	**0.010**	**7.4**	**0.013**
Analysed extract(s)	63.1	0.049	71.5	0.122
Extracts not analysed	3.9	0.003	3.8	0.006
Total extracted	**67.0**	**0.052**	**75.3**	**0.129**
Unextractable (PES*)	33.0	0.026	24.7	0.042
Accountability	**100.0**	**0.078**	**100.0**	**0.171**

* post extraction solids

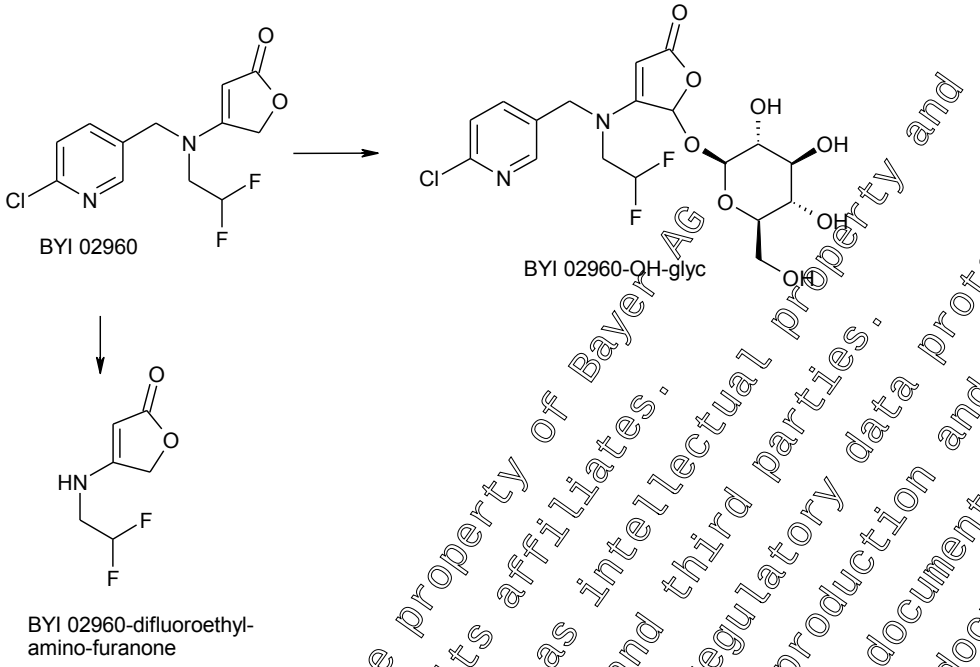
III. Conclusions

Based on the metabolites identified the following metabolic routes were deduced:

- hydroxylation of the methylene group of the furanone moiety followed by conjugation with glucose, and
- cleavage of the pyridinylmethylamine bond

Thus, [furanone-4-^{14}C]BYI 02960 was metabolised moderately in potatoes. On the basis of the results of this study it is concluded that the metabolism of [furanone-4-^{14}C]BYI 02960 in potatoes is well understood and the following metabolic pathway is proposed.

Figure 6.2.1-5: Proposed metabolic pathway of [furanone-4-^{14}C]BYI 02960 in potatoes



Report:	**KIIA 6.2.1/05, [redacted].; 2011**
Title:	Metabolism of [pyridinylmethyl-^{14}C]BYI 02960 in potatoes
Report No & Edition No	MEF-10/710 M-415078-01-2
Guidelines:	OECD 501 Metabolism in Crops US EPA Residue Chemistry Test Guideline OPPTS 860.1300: Nature of the Residue – Plants, Livestock PMRA Regulatory Directive Dir98-02: Residue Chemistry Guidelines Section 2: Nature of the Residue – Plants, Livestock Japanese MAFF, 12 Nousan 8147 European Parliament and Council Regulation (EC) No 1107/2009
GLP	yes

Executive Summary

The metabolism of [pyridinylmethyl-^{14}C]BYI 02960 in potatoes was investigated according to the maximum envisaged use pattern. Two different methods of application were covered in this study. In one experiment, the seed potato tubers were treated with [pyridinylmethyl-^{14}C]BYI 02960 formulated as an FS 480 at an application rate of 10.0 g a.s./dt (= 270 g a.s./ha, seed density 27 dt/ha). In the other experiment, [pyridinylmethyl-^{14}C]BYI 02960, formulated as an SL 200, was sprayed in the furrow onto the soil at an application rate of 626 g a.s./ha (seed density 28 dt/ha) prior to planting of the seed potatoes. The potato tubers were harvested at maturity in both experiments. Concurrently, the leaves and roots as well as the remainders of the seed potatoes were sampled. The TRR values determined are shown in the following table:

Table 6.2.1-15: TRR values in potato tubers and the remaining part of the plant after tuber treatment or in-furrow application of [pyridinylmethyl-^{14}C]BYI 02960

Matrix	**Timing and Application**	**PHI (days)***	**ppm (mg a.s. equiv./kg)**
potato tubers	tuber treatment at planting (BBCH 03), 10.0 g a.s./dt	97	0.076
potato leaves and roots		97	8.40
remainders of the seed potatoes		97	33.33
potato tubers	in-furrow application at planting (BBCH 03), 626 g a.s./ha	97	0.115
potato leaves and roots		97	12.44
remainders of the seed potatoes		97	6.91

* PHI: preharvest interval (corresponds to days after last treatment (DAT) at the start of harvest/sampling)

The potato tubers (edible raw agricultural commodity) were extracted conventionally. Four extraction steps with acetonitrile/water mixtures released 93.4% of the TRR in the tuber treatment experiment and 90.4% of the TRR in the in-furrow treatment experiment.

Parent compound and metabolites in the extracts of potato tubers were analysed by HPLC. Major compounds were identified by co-chromatography using two independent chromatographic systems (reversed phase HPLC and normal phase TLC). Minor metabolites were identified by HPLC co-chromatography or by HPLC comparison. Parent compound represented 40.2% and 44.1% of the TRR in potato tubers. Besides parent compound, only 6-CNA was detected as major compound. All other metabolites detected represented less than 10% of the TRR and accounted for less than 0.01 mg/kg.

Nevertheless, five metabolites were identified in the tuber extract after in-furrow application and four thereof in the extract after tuber treatment.

[Pyridinylmethyl-^{14}C]BYI 02960 was moderately metabolised in potatoes. The following metabolic routes were observed:

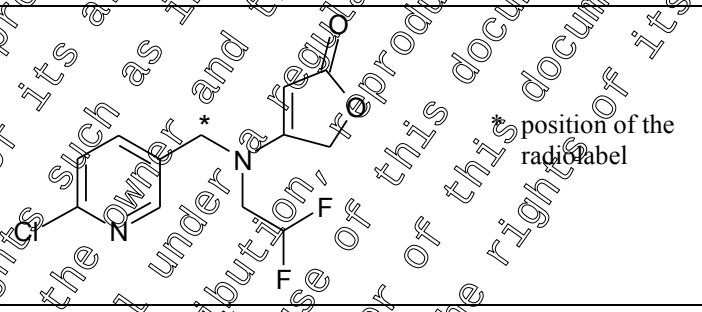
- hydroxylation of the methylene group of the furanone moiety followed by conjugation with glucose, and
- cleavage of the pyridinylmethylamine bond followed by conjugation with carbohydrates or by oxidation of the methylene group to a carboxylic group.

On the basis of these results, a metabolic pathway of [pyridinylmethyl-^{14}C]BYI 02960 in potatoes can be proposed.

I. Materials and Methods

A. Materials

1. Test Material:

Chemical structure	
Radiolabelled test material	[pyridinylmethyl-^{14}C]BYI 02960
Specific radioactivity	4.37 MBq/mg (118.08 µCi/mg)
Chemical Purity	> 99% (HPLC)
Radiochemical purity	> 99% (HPLC and TLC)

The supplied radiolabelled test compound [pyridinylmethyl-^{14}C]BYI 02960 was dissolved in acetonitrile. Formulation of the test compound was performed prior to the application. For both formulations (FS 480 and SL 200), adequate parts of this stock solution were transferred into special glass vials and evaporated to dryness. For the preparation of the FS 480 formulation, the blank formulation was added to the test item and was homogenized using a ball mill. Afterwards the mixture was suspended in water by stirring or swirling. For the preparation of the SL 200 formulation, the liquid blank formulation was added to the test item using a magnetic stirrer. The mixture was diluted with water to get the aqueous application solution.

2. Soil: [redacted] 4" (sandy loam soil from Germany), pH ($CaCl_2$) = 6.8, 69% sand, 18% silt and 13% clay, 1.2% organic carbon, cation exchange capacity (CEC) of 8.3 meq/100 g,

3. Plant Potato, variety "Cilena", representative for root crops

B. Study Design

Experimental conditions:

Two experiments were performed representing the intended application types (tuber treatment and in-furrow application). Each experiment was conducted with three seed potatoes (variety: Cilena) in a planting container with a surface area of 0.5 m^2. The plants were cultivated in the glass-roofed vegetation area of the test facility. The plants were grown similar to natural temperature and light conditions, but protected from rainfall. The plants were watered by pouring onto the soil in the planting containers.

<u>Tuber treatment experiment:</u>

The formulated test compound was applied to the seed potatoes at a target rate of 10 g a.s./dt. For this purpose, three seed potatoes were treated with a defined volume of the aqueous application suspension. The potatoes were placed in the furrows before application to prevent any loss of the application suspension. To ensure a regular treatment, the seed potatoes were treated on the upper side, allowed to dry, then turned and treated on the other side. The furrow was closed when the application suspension was dried on the seed potatoes. A total volume of 787.2 µL was applied, corresponding to 59.1 MBq or to 13.5 mg a.s.. The actual seed treatment rate was 10.0 g a.s./dt, corresponding to 270 g a.s./ha. The seed density was 27 dt/ha.

<u>In-furrow treatment experiment:</u>

For the in-furrow treatment experiment, a defined volume of the aqueous application solution was filled into the bottle of a hand pump sprayer. The application solution was sprayed onto the soil in the furrow. The empty pump sprayer was rinsed with 3 mL water, 0.5 mL of the rinse was sampled for radioactivity measurement and the rest of the rinse was sprayed onto the soil in the furrow. Three seed potatoes were placed into the furrow and the furrow was closed. The empty pump sprayer was again rinsed with 3 mL water. The remaining radioactivity in the rinsing solution was determined by LSC. The losses were subtracted from the initial amount of radioactivity. An amount of 136.6 MBq or 31.3 mg a.s. was applied to the three potatoes, corresponding to an actual application rate of 626 g a.s./ha. The seed density was 28 dt/ha.

Sampling:

Potato tubers were sampled as raw agricultural commodity (RAC) from the tuber treatment experiment and from the in-furrow treatment experiment at maturity of the potato plants (BBCH 97). The leaves and roots, as well as the remainders of the seed potatoes were sampled at the same time to determine the TRR by combustion analysis.

The potato tubers were allowed to dry at room temperature for one night. Adhering soil was removed. The potato tuber samples from the two experiments were each washed with 1 L of water and the radioactivity in the wash water was determined. The potato tubers were cut into small cubes. The cubes were randomized by shuffling and stored in a freezer (≤ -18°C) in aliquots.

The leaves and roots as well as the remainders of the seed potatoes were cut in pieces, liquid nitrogen was added and the mixtures were homogenised using a high speed blender (Polytron PT6000). The powdered samples were stored in a freezer (≤ -18°C).

C. Analytical Procedures

Extraction:

The cut potato tubers of the tuber treatment and the in-furrow treatment experiment were extracted three times with a mixture of acetonitrile/water (8:2, v/v) and one time with a mixture of acetonitrile/water (1:1, v/v) using a high speed blender (Polytron PT6000). The radioactivity in the extracts was determined by LSC, in the solids by combustion followed by LSC. The actual TRR value of the RAC was determined by summing up the radioactivity measured in the extracts and in the remaining solids. The first two extracts were combined and subjected to a clean-up step using an SPE RP 18 cartridge (Phenomenex, Strata C18-E, 20 g), which was conditioned with acetonitrile beforehand. The flow-through fraction (percolate) was collected and the cartridge was rinsed with 100 mL of acetonitrile/water (8:2, v/v) and methanol/dichloromethane (1:1, v/v). The percolate and the acetonitrile/water fraction were combined and concentrated by rotary evaporation in vacuo for HPLC analysis.

Quantification:

Parent compound and metabolites in the extracts were analysed by reversed phase HPLC coupled to a radioactivity detector with a glass scintillator cell. The HPLC chromatograms (= metabolite profiles) were integrated for quantification of compounds.

Identification and characterisation:

Major compounds (parent compound and one metabolite) were identified using two independent chromatographic systems (reversed phase HPLC and normal phase TLC). Minor metabolites (<10% of the TRR and representing <0.01 mg/kg) were identified by HPLC co-chromatography with authentic reference compounds or by HPLC comparison.

The metabolite profiles of the potato tubers of the tuber treatment and the in-furrow application experiments were very similar. Thus, identification of metabolites was performed either in the extract of the in-furrow application experiment or in the tuber treatment experiment. Assignment of the according metabolite in the complementary experiment was completed by comparing the profiles.

Storage stability:

All extraction experiments and the first HPLC analyses were performed within three months after harvest of the potato tubers. The extracts were analysed after two and five days following the start of extraction. According to OECD Guidance for the Testing of Chemicals 501 (2007), storage stability data are not necessary for samples analysed within 6 months of collection. Therefore it was concluded that the data in hand provide adequate evidence to show the stability of the compounds and no additional investigations deemed necessary.

In addition, the extract stability was demonstrated by comparing the HPLC chromatograms recorded at different times during the study. The profiles did not change significantly during the whole course of the study. The stability of extracts was demonstrated for parent and metabolites for at least three months which covers the time period of all analytical investigations including identification and characterisation of metabolites.

II. Results and Discussion

The metabolism of [pyridinylmethyl-^{14}C]BYI 02960 was investigated in potato tubers following two different soil application methods. In one experiment, tubers of seed potatoes were treated with [pyridinylmethyl-^{14}C]BYI 02960 formulated as an FS 480 at an application rate of 10.0 g a.s./dt (= 270 g a.s./ha). In the other experiment [pyridinylmethyl^{14}C]BYI 02960, formulated as an SL 200, was sprayed in the furrow onto the soil prior to planting of the seed potatoes at an application rate of 626 g a.s./ha.

At harvest, the total radioactive residues (TRR) in potato tubers were low after tuber treatment and after in-furrow application and accounted for 0.076 mg/kg and 0.115 mg/kg, respectively. The TRR values in the remaining plant parts (leaves, roots and remainders of the seed potatoes) were considerably higher. These plant parts were only sampled for optional analysis to support identification of metabolites, if needed.

A high portion of radioactivity in the potato tubers was extracted conventionally by acetonitrile/water mixtures (93.4% to 90.4% of the TRR) as shown in Table 6.2.1-16. HPLC analysis of the extracts revealed that the metabolite profiles of the potato tubers after tuber treatment and in-furrow application were nearly identical – the metabolite patterns showed no significant difference, and even the proportions of the compounds were nearly identical. Major components detected in the HPLC profiles (>10% of the TRR and >0.01 mg/kg) were parent compound and the metabolite BYI 02960-6-CNA. Both components were identified by co-chromatography with two different chromatographic systems. They were identified in the tuber extract of each experiment by reversed phase HPLC and in the tuber extracts of the in-furrow treatment experiment by normal phase TLC using radiolabelled reference compounds. The minor, label-specific metabolites BYI 02960-CHMP-glyc and BYI 02960-CHMP were identified by HPLC co-chromatography with non-radiolabelled reference compounds. The minor, label-specific metabolites BYI 02960-6-CNA-glycerol-glcA and BYI 02960-CHMP-di-glyc were identified by HPLC comparison. The HPLC profiles were compared to that of wheat straw of the 1st rotation of the confined rotational crops study and to that of tomato fruits of the tomato metabolism study. The metabolites had been identified with spectroscopic methods in these studies.

The minor metabolite BYI 02960-OH-glyc was identified by HPLC co-chromatography after semi-preparative isolation. Co-chromatography was performed using an acidic reversed phase HPLC method to ensure separation from BYI 02960-acetic acid, a metabolite co-eluting with BYI 02960-OH-glyc using the profiling method, as shown in the apple metabolism and the confined rotational crop studies. The configuration of the conjugated hexose of BYI 02960-OH-glyc was identified unambiguously as D-glucose in the apple metabolism study conducted with [pyridinylmethyl-^{14}C]BYI 02960.

The TRR and the distribution of parent compound and metabolites in the extracts is shown in Table 6.2.1-17. In total, 80.5% and 80.9% of the TRR were identified in the potato tubers after tuber treatment or in-furrow application, respectively.

Table 6.2.1-16: Distribution of radioactivity in the extracts of the potato tubers after tuber treatment or in-furrow application of [pyridinylmethyl-^{14}C]BYI 02960

	tuber treatment		**in-furrow application**	
	potato tubers		**potato tubers**	
TRR [mg/kg] =	0.076		0.115	
	% of TRR	mg/kg	% of TRR	mg/kg
Conventionally extracted	93.4	0.071	90.4	0.104
Extract for analysis	89.8	0.068	86.7	0.100
Losses (not analysed)	3.7	0.003	3.6	0.004
Total extracted	**93.4**	**0.071**	**90.4**	**0.104**
Unextractable (PES*)	6.6	0.005	9.6	0.011
Accountability	**100.0**	**0.076**	**100.0**	**0.115**

* post extraction solids

Table 6.2.1-17: TRR values and distribution of parent compound and metabolites in potato tubers after tuber treatment and in-furrow application of [pyridinylmethyl-^{14}C]BYI 02960

	tuber treatment		**in-furrow application**	
	potato tubers		**potato tubers**	
TRR [mg/kg] =	0.076		0.115	
Compound (BYI 02960-)	% TRR	mg/kg	% TRR	mg/kg
Conventional extraction				
BYI 02960 (parent compound)	40.7	0.031	44.1	0.051
6-CNA	21.5	0.016	18.4	0.021
6-CNA-glycerol-gluA	---		2.3	0.003
CHMP-di-glyc	4.4	0.003	5.3	0.006
CHMP-glyc	3.7	0.003	2.4	0.003
CHMP	3.9	0.003	3.9	0.004
OH-glyc	6.7	0.005	4.7	0.005
Total identified	**80.5**	**0.061**	**80.9**	**0.093**
unknown 1	1.9	0.001	---	---
unknown 2	2.3	0.002	2.8	0.003
unknown 3	5.1	0.004	3.0	0.003
Total characterised	**9.3**	**0.007**	**5.8**	**0.007**
Analysed extract(s)	89.8	0.068	86.7	0.100
Extracts not analysed	3.7	0.003	3.6	0.004
Total extracted	**93.4**	**0.071**	**90.4**	**0.104**
Unextractable (PES*)	6.6	0.005	9.6	0.011
Accountability	**100.0**	**0.076**	**100.0**	**0.115**

* post extraction solids

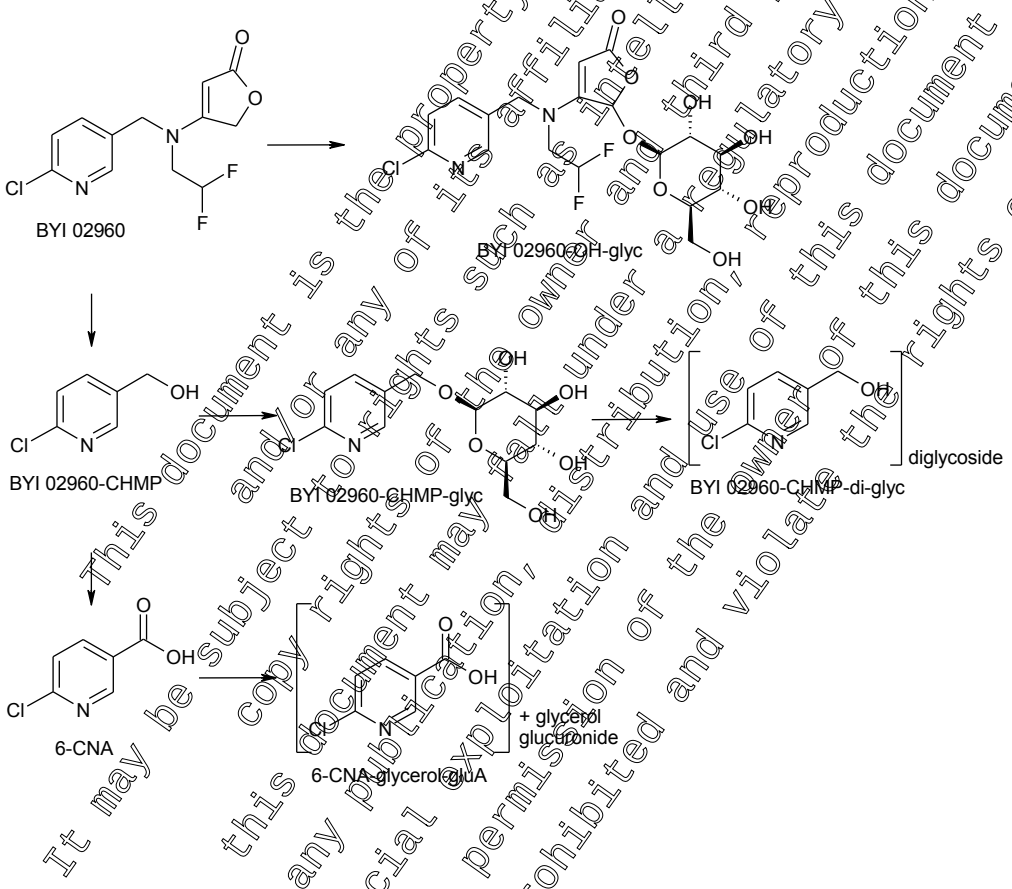
III. Conclusions

[Pyridinylmethyl-^{14}C]BYI 02960 was moderately metabolised in potatoes. Two metabolic routes were observed:

- cleavage of the pyridinylmethylamine bond followed by conjugation with carbohydrates or by oxidation of the methylene group to a carboxylic group and conjugation with carbohydrates or derivatives, which were the major metabolic routes, and
- hydroxylation of the methylene group of the furanone moiety followed by conjugation with glucose, which was the minor metabolic route.

On the basis of the results of this study it is concluded that the metabolism of [pyridinylmethyl-^{14}C]BYI 02960 in potatoes is well understood and the following metabolic pathway is proposed.

Figure 6.2.1-6: Proposed metabolic pathway of [pyridinylmethyl-^{14}C]BYI 02960 in potatoes



Overall Conclusions Potato (tuber treatment and in-furrow application)

The metabolism of the insecticide BYI 02960 was investigated in potatoes in two studies following tuber or soil application with (1) [furanone-4-^{14}C]BYI 02960 or (2) [pyridinylmethyl-^{14}C]BYI 02960. In both studies, the total radioactive residues (TRR) in the potato tubers, representing the edible raw agricultural commodity (RAC), were low after tuber treatment and after in-furrow application. The metabolite profiles of all tuber extracts were very similar, and at harvest, the predominant portion was always parent compound BYI 02960. However, subsequent analysis of the extracts on the non-

radiolabelled metabolite difluoroacetic acid - which cannot be detected with the radiolabels used - revealed even higher concentrations of this metabolite compared to the parent compound.

(1) Label 1: [furanone-4-^{14}C]BYI 02960
Besides parent compound, six minor metabolites were detected, two of them were identified: BYI 02960-OH-glyc, a metabolite common to both radiolabels tested and BYI 02960-difluoroethyl-aminofuranone, a metabolite specific to the furanone-label. The metabolites identified were also detected in tomato fruits after soil application and in the confined rotational crop studies.

BYI 02960 was metabolised moderately in potatoes: Hydroxylation of the methylene group of the furanone moiety was observed followed by conjugation with glucose and cleavage of the pyridinylmethylamine bond. Cleavage of the molecule was also confirmed in the potato study performed with [pyridinylmethyl-^{14}C]BYI 02960 illustrating that the results of the metabolism studies were in good conformity.

(2) Label 2: [pyridinylmethyl-^{14}C]BYI 02960
Only one major metabolite (6-CNA) was detected besides parent compound. Additionally, eight minor metabolites were detected, five thereof were identified: BYI 02960-OH-glyc, a metabolite common to both radiolabels tested, and the label-specific metabolites BYI 02960-CHMP, BYI 02960-CHMP-glyc, BYI 02960-CHMP-di-glyc and 6-CNA-glycerol-gluA. These metabolites or at least the aglycons were also detected in tomato fruits after soil application indicating the same metabolic degradation paths.

The major metabolic routes in this study were cleavage of the pyridinylmethylamine bond followed by conjugation with carbohydrates or by oxidation of the methylene group to a carboxylic group. Cleavage of the molecule was also confirmed in the potato study performed with [furanone-4-^{14}C]BYI 02960. A minor metabolic route was hydroxylation of the methylene group of the furanone moiety followed by conjugation with glucose. This metabolic route resulted in the non-label specific metabolite BYI 02960-OH-glyc, which was identified in nearly identical concentrations in the potato study performed with [furanone-4-^{14}C]BYI 02960. Thus, the results of the present metabolism study in potatoes are in good conformity with the results of the corresponding study performed with [furanone-4-^{14}C]BYI 02960 and as well with all metabolism studies being representative for soil application.

When considering the results from both metabolism studies conducted on potato, it can be concluded that BYI02960 is moderately metabolised in this crop. A total of 2 major and 10 minor metabolites were found, and all major and 6 minor have been identified. The distribution of parent compound and metabolites in the edible commodity potato tuber is summarized in Table 6.2.1-18.

Table 6.2.1-18: TRR values and distribution of parent compound and metabolites in potato tuber after tuber treatment and in-furrow application of BYI 02960

	Potato tuber							
Radiolabel	[furanone-4-^{14}C]				[pyridinylmethyl-^{14}C]			
	tuber treatment		in-furrow appl.		tuber treatment		in-furrow appl.	
TRR [mg/kg] =	0.078		0.171		0.076		0.115	
Compound (BYI 02960-)	% TRR	mg/kg	% TRR	mg/kg	% TRR	mg/kg	% TRR	mg/kg
BYI 02960	40.0	0.031	56.9	0.097	40.2	0.031	44.1	0.051
6-CNA					*21.5*	*0.016*	*18.4*	*0.021*
6-CNA-glycerol-gluA					---	---	*2.3*	*0.003*
CHMP-di-glyc					*4.4*	*0.003*	*5.3*	*0.006*
CHMP-glyc					*3.7*	*0.003*	*2.4*	*0.003*
CHMP					*3.9*	*0.003*	*3.9*	*0.004*
difluoroethyl-amino-furanone	*4.2*	*0.003*	*2.9*	*0.005*				
OH-glyc	6.6	0.005	4.4	0.007	6.7	0.005	4.7	0.005
Total identified	**50.8**	**0.039**	**64.2**	**0.11**	**80.5**	**0.061**	**80.9**	**0.093**
Total characterised	**12.4**	**0.01**	**7.4**	**0.013**	**9.3**	**0.007**	**5.8**	**0.007**
Analysed extract(s)	63.1	0.049	71.5	0.122	89.8	0.068	86.7	0.100
Extract(s) not analysed	3.9	0.003	3.8	0.006	3.7	0.003	3.6	0.004
Total extracted	67.0	0.052	75.3	0.129	93.4	0.071	90.4	0.104
Unextractable (PES*)	33.0	0.026	24.7	0.042	6.6	0.005	9.6	0.011
Accountability	**100.0**	**0.078**	**100.0**	**0.171**	**100.0**	**0.076**	**100.0**	**0.115**

* post extraction solids

Label specific metabolites are printed in italics.

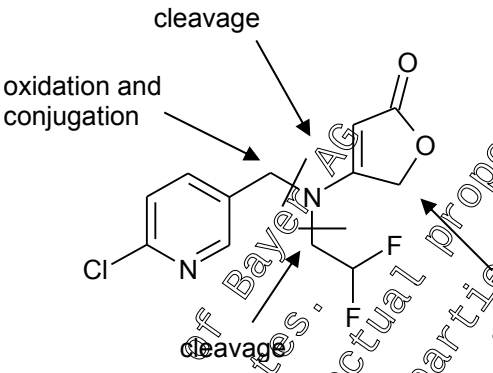
In order to gain information on the fate of the difluoroethane moiety of BYI 02960, the tuber extracts obtained in the potato metabolism study with [pyridinylmethyl-^{14}C]BYI 02960 were additionally analysed for non-radiolabelled difluoroacetic acid by LC-MS/MS according to residue method 01304 (see KIIA 6.2./12). High levels of this metabolite were found. After tuber treatment and in-furrow application, difluoroacetic acid accounted for 0.39 mg a.s. equiv/kg and 0.54 mg a.s. equiv/kg in potato tubers. These concentrations are by a factor of approx. 10 higher compared to those of the parent compound indicating that difluoroacetic acid is the main residue in potato tubers. Since it is known that BYI 02960 degrades to difluoroacetic acid in soil, the high concentration of difluoroacetic acid is probably the result of both uptake of this metabolite from the soil and degradation of the parent compound BYI 02960 in the plant.

On basis of the metabolites identified, biotransformation of BYI 02960 in potato proceeds by the following pathways:

- oxidative cleavage of the difluoroethylamine bond and formation of difluoroacetic acid
- cleavage of the pyridinylmethylamine bond and formation of BYI 02960-difluoroethylamino-furanone and its corresponding counterpart BYI 02960-CHMP, which was either conjugated with carbohydrates or oxidised to 6-chloronicotinic acid (6-CNA), which was conjugated as well
- hydroxylation of the furanone moiety followed by conjugation with glucose

The positions involved in the metabolic degradation are summarised in the following figure.

Figure 6.2.1-7: Positions involved in metabolic degradation of BYI 02960 in potato tubers



Metabolism, distribution and expression of residues in apple (foliar application)

Report:	**KIIA 6.2.1/06, [redacted].; 2011**
Title:	Metabolism of [furanone-4-^{14}C]BYI 02960 in apples
Report No & Edition No	MEF-11/499 M-422562-01-1
Guidelines:	OECD 501 Metabolism in Crops US EPA Residue Chemistry Test Guideline OPPTS 860.1300: Nature of the Residue – Plants, Livestock PMRA Regulatory Directive Dir98-02: Residue Chemistry Guidelines Section 2: Nature of the Residue – Plants, Livestock Japanese MAFF, 12 Nousan 8147 European Parliament and Council Regulation (EC) No 1107/2009
GLP	yes

Executive Summary

The metabolism of [furanone-^{14}-4-C]BYI 02960 formulated as an SL 200 was investigated in apples after foliar spray application. In one experiment (single application experiment), two apple trees were treated at an actual application rate of 86 g a.s. per hectare and per meter canopy height (86 g a.s./(ha x m CH) or also referred to 86 g a.s./ha/m CH) at the end of flowering (BBCH 69). In another experiment, one apple tree was treated twice with 86 g a.s./(ha x m CH), one time at the end of flowering (BBCH 69) and a second time at 14 days before harvest.

At maturity the apple fruits were harvested. Concurrently, the leaves were sampled to support metabolism investigation. The TRR values are shown in the following table:

Table 6.2.1-19: TRR values in apple fruits and leaves after foliar application of furanone-4-^{14}C]BYI 02960

Matrix	**Timing and Application**	**PHI (days)***	**ppm (mg a.s. equiv./kg)**
apple fruits	one foliar spray application at BBCH 69, 86 g a.s./(ha x m CH)	98	0.280
apple leaves		98	38.957
apple fruits [1]	two foliar spray applications, at BBCH 69 and 14 days PHI, 2 x 86 g a.s./(ha x m CH)	14	1.133
apple fruits [2]		14	1.286
apple leaves		14	102.919

* PHI: preharvest interval (corresponds to days after last treatment (DAT) at the start of harvest/sampling)
[1] determined from the extraction experiment with prior surface wash
[2] determined from the extraction experiment without prior surface wash

The apple fruits and leaves were extracted with acetonitrile/water mixtures. The remaining solids after conventional extraction of apple fruits were additionally submitted to exhaustive extraction with microwave assistance and/or to enzymatic treatment with cellulase. In total, 86.5% to 96.5% of the TRR was extracted from the apple matrices. The apple fruits of the double application experiment were additionally surface-washed with dichloromethane and then extracted with acetonitrile/water mixtures. A portion of 7.5% of the TRR was removed by the surface wash, another portion of 81.9% was extracted conventionally and 3.9% of the TRR was extracted after treatment with cellulase.

Parent compound and metabolites in the extracts of apple fruits and leaves were analysed by HPLC. Identification was performed by comparing the HPLC profiles with those obtained in the apple metabolism study with [pyridinylmethyl-^{14}C]BYI 02960, or by HPLC co-chromatography with authentic reference compounds. In total, 79.9% to 92.7% of the TRR was identified in apple fruits and leaves. As expected, the residues were dominated by parent compound in the double application experiment due to the late second application. However, irrespective of the amounts, the vast majority of metabolites was identical in the single and the double application experiments.

Glucose, or an isomeric carbohydrate, was the only major metabolite (> 10% of the TRR) in apple fruits, occurring at up to 71.7% of the TRR. The metabolite BYI 02960-OH-glyc was the only major metabolite in apple leaves, occurring at 36.1% and 17.3% of the TRR. All other metabolites were minor. Four basic metabolic routes were detected:

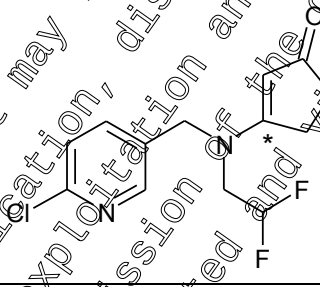
- hydroxylation of the furanone or the difluoroethyl moiety followed by conjugation,
- complete degradation of the furanone moiety and incorporation of carbon atoms into the natural compound pool, i.e. into glucose/carbohydrates,
- cleavage of the pyridinylmethylamine bond, and
- oxidative degradation of the furanone moiety to an acetic acid group followed by conjugation with a carbohydrate.

On the basis of these results, a metabolic pathway of [furanone-4-^{14}C]BYI 02960 in apples can be proposed.

I. Materials and Methods

A. Materials

1. Test Material:

Chemical structure	 * position of the radiolabel
Radiolabelled test material	[furanone-^{14}C]BYI 02960
Specific radioactivity (before radiodilution) (after radiodilution)	 3.94 MBq/mg (106.46 µCi/mg) 1.97 MBq/mg (53.24 µCi/mg)
Chemical Purity	> 99% (HPLC)
Radiochemical purity	> 99% (HPLC and TLC)

The test compound was formulated as an SL 200. In order to prepare an appropriate stock solution, the supplied radiolabelled test compound [furanone-4-^{14}C]BYI 02960 was dissolved in acetonitrile. Radiodilution and formulation of the test compound was performed prior to the first and the second application: Therefore, an adequate portion of the non-radiolabelled test compound was dissolved in an adequate volume of the stock solution of the radiolabelled test compound. The solution was evaporated to dryness. The remainder was dissolved in an adequate amount of SL 200 blank

formulation using a magnetic stirrer. Preparation of each application solution was done by dissolving the formulation in water. The specific activity of each application solution was 1.97 MBq/mg (53.24 µCi/mg).

2. Soil: "[redacted]4" (sandy loam soil from Germany), pH ($CaCl_2$) = 6.8, 69% sand, 18% silt and 13% clay, 1.2% organic carbon, cation exchange capacity (CEC) of 8.3 meq/100 g,

3. Plant Apple, variety "James Grieve", representative for fruiting crops

B. Study Design

Experimental conditions:

Two experiments were performed with a total of three apple trees of the variety "James Grieve". The experiments represented the intended foliar spray application scenarios of BYI 02960 in orchards. In one experiment (single foliar spray application), the apple trees were treated at a target application rate of 75 g a.s./(ha x m CH) (canopy height) at the end of flowering (BBCH 69). In the other experiment (double foliar spray application), one apple tree was treated two times. The target application rate was 75 g a.s./(ha x m CH) at each application. The first application was conducted at the end of flowering (BBCH 69) and the second at 14 days before harvest. The total target rate corresponds to the anticipated maximum application rate. The apple trees used in the present study had a canopy height (CH) of 0.5 m and were cultivated in the sandy loam soil "[redacted]4" in a planting container with a surface area of 0.091 m^2. The plants were grown in the glass-roofed vegetation area of the test facility which allows cultivation similar to natural temperature and light conditions, but protected from rainfall. The plants were watered according to their needs.

<u>Single foliar spray application (BBCH 69):</u>

Two apple trees with a canopy height of 0.5 m were treated each with 10 mL of the aqueous application solution using a hand pump sprayer. Prior to the application, each apple tree was covered with a protective plastic wrap to prevent radioactivity spreading into the vegetation area. After application, the hand pump sprayer was rinsed with water and acetonitrile and the plastic wrap was rinsed with methanol. The radioactivity in the rinse solutions was determined and subtracted from the amount in the application solution. A total amount of 28.2 MBq was applied per tree, corresponding to 14.3 mg a.s. From a planting density of 3000 trees/ha an actual application rate of 86 g a.s./(ha x m CH) was calculated.

<u>Double foliar spray application (BBCH 69 & 14 days before harvest):</u>

One apple tree with a canopy height of 0.5 m was treated for the first time with 10 mL of the aqueous application solution using a hand pump sprayer at the end of flowering (BBCH 69). A second application was performed at 14 days before harvest. Again, 10 mL of the aqueous application solution was applied. As described above, the hand pump sprayer and the protective plastic wrap were rinsed to determine the losses of radioactivity due to the application. A total amount of 28.2 MBq was applied at the first application and 28.1 MBq were applied at the second application. Thus 14.3 mg a.s. were applied at each treatment. From a planting density of 3000 trees/ha a total application rate (comprising both applications) of 172 g a.s./(ha x m CH) was calculated.

Sampling:
Apple fruits of both experiments were sampled at maturity (BBCH 87- 89). On the same day, the leaves were cut off the trees with scissors. The leaves were stored in a freezer (≤ -18°C) on the day of harvest until extraction.

All apple fruits of the single application experiment were cut in pieces. The pieces were mixed and divided into aliquots. The apple aliquots were stored in a freezer (≤ -18°C) until needed for extraction. The apple fruits of the double application experiment were separated into two subsamples. Approx. one third of the whole apple fruit sample was stored in a freezer (≤ -18°C) on the day of harvest. The remaining whole fruits were stored overnight at room temperature. On the next day the apples were subjected to a surface wash with dichloromethane.

C. Analytical Procedures
Extraction:
Single application experiment (apple fruits):
The apple pieces were extracted three times with a mixture of acetonitrile/water (8:2, v/v) and one time with a mixture of acetonitrile/water (1:1, v/v) using a high speed blender. The radioactivity in the extracts was determined by LSC, in the solids by combustion followed by LSC. The actual TRR value of the RAC was determined by summing up the radioactivity measured in the extracts and in the remaining solids. All extracts were combined and subjected to a clean-up step using an SPE RP 18 cartridge (Phenomenex, Strata C18-E, 20 g), which was conditioned with acetonitrile beforehand. The flow-through fraction (percolate) was collected and the cartridge was rinsed with acetonitrile/water (8:2, v/v) and methanol/dichloromethane (1:1, v/v). The percolate and the acetonitrile/water fraction were combined and concentrated by rotary evaporation in vacuo. The pH value of the concentrate was adjusted to pH 7 with ammonium carbonate before chromatographic analysis by HPLC. The solids remaining after the conventional extraction steps were submitted two times to a microwave-assisted extraction step with acetonitrile/water (1:1, v/v). After each extraction step, extracts and solids were separated by centrifugation. The extracts were combined and concentrated by rotary evaporation in vacuo. Prior to HPLC analysis, an aliquot of the extract was treated with cellulase to reduce viscosity. Additionally, the solids remaining after microwave-assisted extraction were suspended in water and the mixture was concentrated by rotary evaporation in vacuo to remove acetonitrile. Cellulase was added and the mixture was stirred at room temperature for 3 days. The aqueous phase was separated by centrifugation and adjusted to pH 7 with ammonium carbonate for HPLC analysis.

Double application experiment (apple fruits):
The subsample of the whole apple fruits which was stored at room temperature was subjected to a surface wash with dichloromethane. An aliquot of the dichloromethane surface wash was dispersed with 5 mL of water by ultrasonic treatment. The dichloromethane was removed by a nitrogen stream and the remaining concentrate was analysed by HPLC. The washed apples were cut into pieces. The apples pieces were mixed, divided into aliquots and stored in a freezer (≤ -18°C). Extraction, purification and concentration of the extract were performed exactly as described for the single application experiment. The resulting sample was adjusted to pH 7 with ammonium carbonate for HPLC analysis.

Additionally, three whole apples of the double application experiment were extracted without prior surface wash. The frozen apples were crushed with a wooden hammer and homogenised under liquid nitrogen using a high speed blender. An aliquot of the apple mush was extracted for final analysis. Extraction, purification and concentration of the extract were performed exactly as described for the single application experiment. The resulting sample was adjusted to pH 7 with ammonium carbonate for HPLC analysis.

Aliquots of the apple leaves of the single and double application experiment were conventionally extracted as described for the fruits of the single application experiment. The clean-up procedure and the concentration step were also identical with the one applied for the fruit samples. The resulting sample was adjusted to pH 7 with ammonium carbonate for HPLC analysis.

Quantification:
Parent compound and metabolites in the extracts were analysed by reversed phase HPLC coupled to a radioactivity detector with a glass scintillator cell. The HPLC chromatograms (= metabolite profiles) were integrated for quantification of compounds.

Identification and characterisation:
Identification of parent compound and metabolites common to both radiolabels was based on the metabolite profiles obtained in the apple metabolism study with [pyridinylmethyl-^{14}C]BYI 02960. In this study parent compound and all metabolites common to both radiolabels were identified by two independent chromatographic methods (reversed phase HPLC and normal phase TLC) or by LC-MS/MS after semi-preparative isolation of the compounds. Thus assignment of compounds in the present study was possible by comparing the metabolite profiles.

The only major label-specific metabolite present in the current study was identified by TLC co-chromatography before and after derivatisation, whereas a minor label-specific metabolite was identified by HPLC co-chromatography with an authentic reference compound.

Storage stability:
All extraction and surface wash experiments and the first HPLC analyses of the apple fruits were performed within 4 months after harvest. The extraction experiments and the first HPLC analyses of the apple leaves were also performed within 4 months after harvest. Thus, no additional tests are necessary to prove the stability of the relevant residues in frozen matrices. The extracts were analysed after 1 to 6 days following start of extraction and the surface wash were analysed 14 days after washing.

Comparison of the HPLC chromatograms recorded at different times during the study showed that the profiles of the extracts did not change significantly during the analytical work up, which did not exceed approx. nine months.

It was therefore concluded, that the residues in the matrices and in the extracts were sufficiently stable during the experimental period of the study and that the chromatograms represented the metabolic pattern in the samples at harvest.

II. Results and Discussion

The metabolism of [furanone-4-^{14}C]BYI 02960 was investigated in apple fruits and leaves following two different spray scenarios. In one experiment (single application experiment), two apple trees were treated at an actual application rate of 86 g a.s./(ha x m CH) (CH = meter canopy height) at the end of flowering (BBCH 69). In the other experiment, one apple tree was treated two times with an actual application rate of 86 g a.s./(ha x m CH), one time at the end of flowering (BBCH 69) and a second time at 14 days before harvest. The total application rate in the double application experiment was 172 g a.s./(ha x m CH). The actual single application rates were slightly above the anticipated maximum rate of 75 g a.s./(ha x m CH).

Apple fruits were harvested for analysis from the single application experiment at 98 days after the treatment and from the double application experiment at 14 days after the last treatment. Concurrently, the leaves were sampled to support the analysis of metabolites. The apple fruit samples of the double application experiment were investigated with and without a surface wash step.

The TRR level in the apple fruits, which represent the edible RAC, was 0.280 mg/kg in the single application experiment. In the double application experiment, the apple fruits had a TRR of 1.133 mg/kg (extraction including surface wash) and 1.286 mg/kg (extraction without surface wash). The TRR values of the apple leaves were high: 38.957 mg/kg were found in the leaves of the single application experiment and 102.919 mg/kg in the leaves of the double application experiment. Apple leaves were only sampled to support the identification of metabolites.

The radioactive residues were efficiently extracted with acetonitrile/water mixtures after conventional and exhaustive extraction procedures (86.5% to 96.5% of the TRR) as shown in Table 6.2.1-20 and Table 6.2.1-21. The apple fruits of the double application experiment were additionally surface-washed with dichloromethane and then extracted conventionally with acetonitrile/water mixtures. A portion of 7.5% of the TRR was removed by the surface wash and another portion of 81.9% was extracted conventionally indicating a good uptake of the product and its systemic behaviour.
HPLC analysis of the surface wash and the conventional extracts of apple fruits and leaves after both spray scenarios revealed that all metabolite profiles were very well comparable with those of the study conducted with [pyridinylmethyl-^{14}C]BYI 02960. Thus, identification was performed generally by comparison of profiles.

The main compound in apple fruits of the single application experiment was the natural compound glucose (or a corresponding isomeric carbohydrate). It was also a major component in the in apple fruits of the double application experiment. It was isolated by semi-preparative HPLC and identified by TLC co-chromatography before and after derivatisation. ^{14}C-glucose and ^{14}C-pentabenzoyl-D-glucose were used as authentic reference compounds. While correspondence with these reference compounds had clearly been shown, the methods were not considered selective enough to discriminate the configuration of the sugar moiety. Therefore, this label-specific fraction has been assigned more generally as glucose/carbohydrates.

As expected, parent compound was the main component in the extracts of apple fruits and leaves of the double application experiment. In the surface wash, parent was by far the predominating component, as well. The high amount of parent compound was due to the fact that the product was sprayed on the developed fruits at 14 days before harvest. Assignment of parent compound was confirmed in the extracts and in the surface wash of apple fruits by HPLC co-chromatography.

All other metabolites detected in the raw agricultural commodity fruit were minor metabolites. They did not exceed 10% of the TRR in the single and the double application experiment and were assigned by comparison of profiles, if possible. Metabolites common to both radiolabels tested as BYI 02960-acetic acid-glyc, BYI 02960-OH-glyc, BYI 02960-acetic acid, BYI 02960-difluoroethyl-OH-glyc and BYI 02960-OH had been assigned by this means. The two metabolites BYI 02960-OH-glyc and BYI 02960-acetic acid co-eluted in one peak if analysed with the profiling method. Chromatographic separation has been achieved with an acidic reversed phase HPLC method in the apple metabolism study conducted with [pyridinylmethyl-^{14}C]-BYI 02960. The ratios which had been determined for the two metabolites in the different matrices were transferred to the profiles of the current study.

The label-specific metabolite BYI 02960-difluoroethyl-amino-furanone was identified in the extract of apple fruits of the double application experiment by HPLC co-chromatography with a non-radiolabelled reference compound. BYI 02960-difluoroethyl-amino-furanone and glucose/carbohydrates were assigned in the other extracts of the apple matrices by comparison of the HPLC profiles.

The distribution of the radioactive residues in the extracts is shown in Table 6.2.1-20 and Table 6.2.1-21. In total, 83.4% to 92.7% of the TRR were identified in the apple fruits and 79.9% to 87.5% in apple leaves, respectively as summarised in Table 6.2.1-22 and Table 6.2.1-23.

Table 6.2.1-20: Distribution of radioactivity in the extracts of apple fruits and leaves after a single foliar application of [furanone-4-^{14}C]BYI 02960

	single application experiment			
	apple fruits		**apple leaves**	
TRR [mg/kg] =	0.280		38.957	
	% of TRR	mg/kg	% of TRR	mg/kg
Conventionally extracted	59.8	0.168	94.3	36.725
Extract for analysis	58.7	0.164	94.0	36.604
Losses (not analysed)	1.1	0.003	0.3	0.121
Microwave extraction	9.5	0.027	---	---
Extract for analysis	9.4	0.026	---	---
Losses (not analysed)	<0.1	<0.001	---	---
Cellulase extract	17.2	0.048	---	---
Total extracted	**86.5**	**0.242**	**94.3**	**36.725**
Unextractable (PES*)	13.5	0.038	5.7	2.232
Accountability	**100.0**	**0.280**	**100.0**	**38.957**

* post extraction solids

Table 6.2.1-21: Distribution of radioactivity in the surface wash and the extracts of apple fruits and leaves after a double foliar application of [furanone-4-^{14}C]BYI 02960

	double application experiment					
	apple fruits incl. surface wash		**apple fruits without surface wash**		**apple leaves**	
TRR [mg/kg] =	1.133		1.286		102.919	
	% of TRR	mg/kg	% of TRR	mg/kg	% of TRR	mg/kg
Surface wash with DCM	7.5	0.085	---	---	---	---
Conventionally extracted	81.9	0.928	91.9	1.182	96.5	99.280
Extract for analysis	81.6	0.925	91.2	1.173	96.0	98.843
Losses (not analysed)	0.3	0.003	0.7	0.009	0.4	0.436
Cellulase extract	3.9	0.044	---	---	---	---
Total extracted	**93.3**	**1.057**	**91.1**	**1.182**	**96.5**	**99.280**
Unextractable (PES*)	6.7	0.076	8.1	0.104	3.5	3.639
Accountability	**100.0**	**1.133**	**100.0**	**1.286**	**100.0**	**102.919**

* post extraction solids

Table 6.2.1-22: TRR values and distribution of parent compound and metabolites in apple fruits and leaves after a single foliar treatment of [furanone-4-^{14}C]BYI 02960

	single application			
	apple fruits		apple leaves	
TRR [mg/kg] =	0.280		56.715	
Compound (BYI 02960-)	% TRR	mg/kg	% TRR	mg/kg
Conventional extraction				
BYI 02960 (parent compound)	7.4	0.021	26.0	10.138
glucose/carbohydrates	50.3	0.141	2.5	0.991
~~difluoroethyl-amino-furanone~~	~~3.2~~	~~0.009~~	—	
acetic acid-glyc	0.3	0.001	6.4	2.486
OH-glyc	0.4	0.001	36.1	14.062
acetic acid	0.2	0.001	2.5	0.986
difluoroethyl-OH-glyc	---	---	5.8	2.264
OH	---	---	0.6	0.244
Subtotal identified	**58.7**	**0.164**	**79.9**	**31.141**
unknown 1	---	---	0.3	0.120
unknown 3	---	---	1.7	0.646
unknown 4	---	---	---	---
unknown 5	---	---	2.6	1.002
unknown 6	---	---	1.6	0.609
unknown 7	---	---	1.5	0.578
unknown 8	---	---	4.9	1.895
Subtotal characterised	---	---	14.0	5.463
Conventional extracts not analysed	1.1	0.003	0.3	0.121
Total conventional extraction	**59.8**	**0.168**	**94.3**	**36.725**
Microwave extraction				
glucose/carbohydrates	6.4	0.018		
difluoroethyl-amino-furanone	1.0	0.003		
Subtotal identified	**7.5**	**0.021**		
unknown 2	2.0	0.005		
Subtotal characterised	2.0	0.005		
Microwave extracts not analysed	<0.1	0.001		
Total microwave extraction	**9.5**	**0.027**	**---**	**---**
Cellulase digestion				
glucose/carbohydrates	15.0	0.042		
difluoroethyl-amino-furanone	2.2	0.006		
Subtotal identified	**17.2**	**0.048**		
Subtotal characterised	---	---		
Total cellulase extraction	**17.2**	**0.048**	**---**	**---**
Total identified	**83.4**	**0.234**	**79.9**	**31.141**
Total characterised	**2.0**	**0.005**	**14.0**	**5.463**
Analysed extract(s)	85.3	0.239	94.0	36.604
Extracts not analysed	1.1	0.003	0.3	0.121
Total extracted	**86.5**	**0.242**	**94.3**	**36.725**
Unextractable (PES*)	13.5	0.038	5.7	2.232
Accountability	**100.0**	**0.280**	**100.0**	**38.957**

* post extraction solids

Table 6.2.1-23: TRR values and distribution of parent compound and metabolites in apple fruits and leaves after two foliar treatments of [furanone-4-^{14}C]BYI 02960

	double application experiment					
	apple fruits incl. surface wash		apple fruits without surface wash		apple leaves	
TRR [mg/kg] =	1.133		1.286		102.919	
Compound (BYI 02960-)	% TRR	mg/kg	% TRR	mg/kg	% TRR	mg/kg
Surface wash						
parent compound	6.7	0.076				
glucose/carbohydrates	0.2	0.002				
OH-glyc	0.2	0.002				
acetic acid	0.1	0.001				
OH	0.1	0.001				
Subtotal identified	**7.2**	**0.082**				
unknown 8	<0.1	<0.001				
unknown 9	0.3	0.003				
Subtotal characterised	0.3	0.003				
Total surface wash	7.5	0.085				
Conventional extraction						
parent compound	64.7	0.733	73.6	0.946	57.9	59.547
glucose/carbohydrates	13.6	0.154	14.2	0.182	3.6	3.687
difluoroethyl-amino-furanone	---	---	0.2	0.003	0.7	0.736
acetic acid-glyc	0.8	0.009	0.5	0.007	4.2	4.274
OH-glyc	1.0	0.012	1.1	0.014	17.3	17.856
acetic acid	0.6	0.007	0.7	0.009	1.2	1.214
difluoroethyl-OH-glyc	---	---	---	---	2.1	2.118
OH	0.9	0.010	0.8	0.010	0.6	0.630
Subtotal identified	**81.6**	**0.925**	**91.1**	**1.171**	**87.5**	**90.063**
unknown 1	---	---	---	---	0.6	0.646
unknown 2	---	---	---	---	---	---
unknown 3	---	---	---	---	0.5	0.474
unknown 4	---	---	---	---	0.5	0.530
unknown 5	---	---	---	---	1.9	1.960
unknown 6	---	---	---	---	0.8	0.851
unknown 7	---	---	---	---	0.5	0.501
unknown 8	---	---	---	---	2.5	2.609
unknown 9	---	---	0.1	0.002	1.2	1.210
Subtotal characterised	0.3	0.003	0.1	0.002	8.5	8.781
Conv. extracts not analysed	0.3	0.003	0.7	0.009	0.4	0.436
Total conv. extraction	**81.9**	**0.928**	**91.9**	**1.182**	**96.5**	**99.280**
Cellulase digestion						
glucose/carbohydrates	3.3	0.037				
difluoroethyl-amino-furanone	0.6	0.007				
Subtotal identified	**3.9**	**0.044**				
Subtotal characterised	---	---				
Total cellulase extraction	**3.9**	**0.044**	**---**	**---**	**---**	**---**
Total identified	**92.7**	**1.051**	**91.1**	**1.171**	**87.5**	**90.063**
Total characterised	**0.3**	**0.003**	**0.1**	**0.002**	**8.5**	**8.781**

Table continued on next page…

	double application experiment					
	apple fruits incl. surface wash		apple fruits without surface wash		apple leaves	
Analysed extract(s)	93.0	1.054	91.2	1.173	96.0	98.843
Extracts not analysed	0.3	0.003	0.7	0.009	0.4	0.436
Total extracted	**93.3**	**1.057**	**91.9**	**1.182**	**96.5**	**99.280**
Unextractable (PES*)	6.7	0.076	8.1	0.104	3.5	3.639
Accountability	**100.0**	**1.133**	**100.0**	**1.286**	**100.0**	**102.919**

* post extraction solids

III. Conclusions

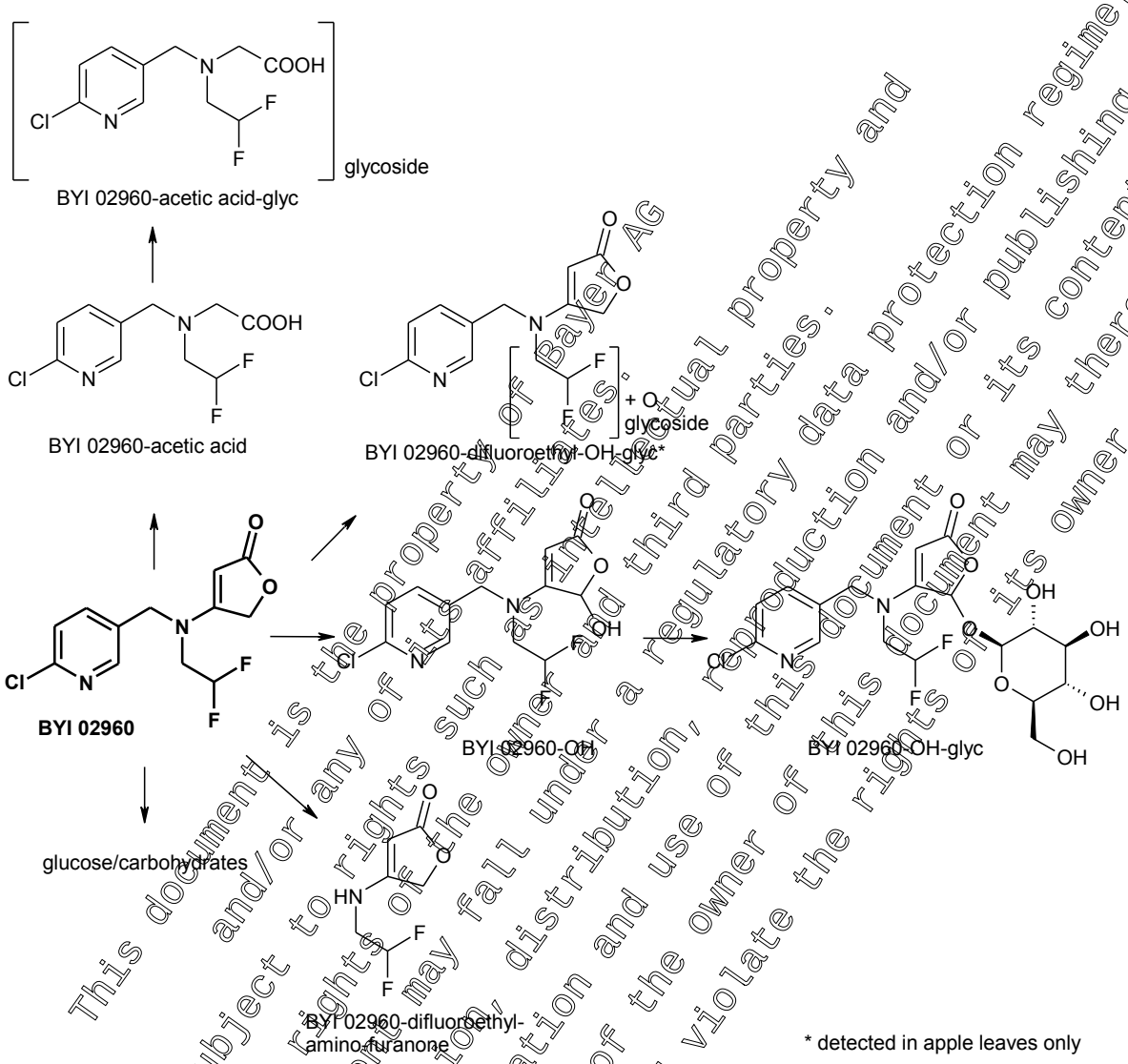
As expected, the residues were dominated by parent compound in the double application experiment, whereas the metabolite pattern was very similar in both experiments.

Four major metabolic routes of [furanone-4-^{14}C]BYI 02960 were observed in apples:

- hydroxylation of the furanone or the difluoroethyl moiety followed by conjugation with carbohydrates,
- complete degradation of the furanone moiety and incorporation of carbon atoms into the natural compound pool, i.e. into glucose/carbohydrates,
- oxidative degradation of the furanone moiety to an acetic acid group followed by conjugation with a carbohydrate, and
- cleavage of the pyridinylmethylamine bond.

On the basis of the results of this study it is concluded that the metabolism of [furanone-4-^{14}C]BYI 02960 in apples is well understood and the following metabolic pathway is proposed.

Figure 6.2.1-8: Proposed metabolic pathway of [furanone-4-^{14}C]BYI 02960 in apples



Report:	**KIIA 6.2.1/07, [redacted].; 2011**
Title:	Metabolism of [pyridinylmethyl-^{14}C]BYI 02960 in apples
Report No & Edition No	MEF-11/198 M-414678-01-2
Guidelines:	OECD 501 Metabolism in Crops US EPA Residue Chemistry Test Guideline OPPTS 860.1300: Nature of the Residue – Plants, Livestock PMRA Regulatory Directive Dir98-02: Residue Chemistry Guidelines Section 2: Nature of the Residue – Plants, Livestock Japanese MAFF, 12 Nousan 8147 European Parliament and Council Regulation (EC) No 1107/2009
GLP	yes

Executive Summary

The metabolism of [pyridinylmethyl-^{14}C]BYI 02960 formulated as an SL 200 was investigated in apples after foliar spray application. In one experiment (single application experiment), two apple trees were treated at an actual application rate of 87 g a.s./ha per meter canopy height (87 g a.s./(ha x m CH)) at the end of flowering (BBCH 69). In another experiment, one apple tree was treated two times, one time at an actual application rate of 87 g a.s./(ha x m CH) at the end of flowering (BBCH 69) and a second time at an application rate of 85 g a.s./(ha x m CH) at 14 days before harvest. The total application rate in the double application experiment was 172 g a.s./(ha x m CH).

At maturity the apple fruits were harvested. Concurrently, the leaves were sampled to support metabolism investigation. The TRR values are shown in the following table:

Table 6.2.1-24: TRR values in apple fruits and leaves after foliar application of [pyridinylmethyl-^{14}C]BYI 02960

Matrix	Timing and Application	PHI (days)*	ppm (mg a.s. equiv./kg)
apple fruits	one foliar spray application at BBCH 69, 87 g a.s./(ha x m CH)	98	0.079
apple leaves		98	56.715
apple fruits [1]	two foliar spray applications at BBCH 69 and 14 days PHI, 87 and 85 g a.s./(ha x m CH)	14	1.868
apple fruits [2]		14	0.545
apple leaves		14	134.841

* PHI: preharvest interval (corresponds to days after last treatment (DAT) at the start of harvest/sampling)
[1] determined from the extraction experiment with prior surface wash
[2] determined from the extraction experiment without prior surface wash

The apple fruits and leaves were extracted with acetonitrile/water mixtures releasing 94.2% to 99.2% of the TRR. A subsample of the apple fruits of the double application experiment was surface-washed with dichloromethane (11.0% of the TRR) and then extracted conventionally with acetonitrile/water mixtures (88.2% of the TRR). The high proportion of residues in the conventional extracts indicates a good uptake of the product and demonstrates its systemic properties.

Parent compound and metabolites in the extracts of apple fruits and leaves were analysed by HPLC. Identification of compounds was performed by LC-MS/MS, by HPLC and/or TLC co-chromatography with reference compounds, and as well as by comparison of HPLC profiles. Parent compound, the

major metabolites and several minor metabolites were identified. In total, 74.8% to 98.5% of the TRR was identified in apple fruits and leaves.

Parent compound was the main component in all chromatographic profiles and represented 43.1% to 88.4% of the TRR in the fruits and 24.5% and 48.2% of the TRR in the leaves. Nevertheless, a large number of metabolites was detected; 14 metabolites were detected in fruits (all <10% of the TRR) and 24 metabolites in leaves (two metabolites represented >10% of the TRR, all other metabolites were minor). Three basic metabolic routes were detected:

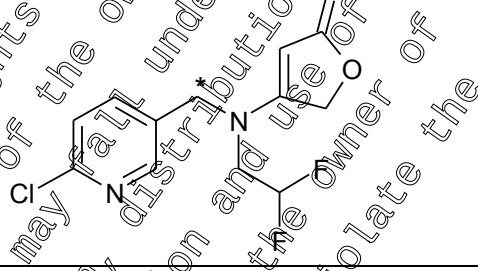
- hydroxylation of the furanone or the difluoroethyl moiety followed by conjugation with carbohydrates,
- cleavage of the pyridinylmethylamine bond followed by conjugation with carbohydrates or by oxidation of the methylene group to a carboxylic group, and
- oxidative degradation of the furanone moiety to an acetic acid group followed either by conjugation with a carbohydrate or by further degradation of the moiety

On the basis of these results, a metabolic pathway of [pyridinylmethyl-^{14}C]BYI 02960 in apples was proposed.

I. Materials and Methods

A. Materials

1. Test Material:

Chemical structure	 * position of the radiolabel
Radiolabelled test material	[pyridinylmethyl-^{14}C]BYI 02960
Specific radioactivity (before radiodilution) (after radiodilution)	4.37 MBq/mg (118.08 µCi/mg) 2.18 MBq/mg (58.92 µCi/mg)
Chemical Purity	> 99% (HPLC)
Radiochemical purity	> 98% (HPLC and TLC)

The test compound was formulated as an SL 200. Therefore the supplied radiolabelled test compound [pyridinylmethyl-^{14}C]BYI 02960 was dissolved in acetonitrile. Radiodilution and formulation of the test compound was performed prior to the first and the second application: Therefore, an adequate portion of the non-radiolabelled test compound was dissolved in an adequate volume of the stock solution of the radiolabelled test compound. The solution was evaporated to dryness. The remainder was dissolved in an adequate amount of SL 200 blank formulation using a magnetic stirrer. Preparation of each application solution was done by dissolving the formulation in water. The specific activity of each application solution was 2.18 MBq/mg (58.92 µCi/mg).

2. Soil: “[redacted]4” (sandy loam soil from Germany), pH ($CaCl_2$) = 6.8, 69% sand, 18% silt and 13% clay, 1.2% organic carbon, cation exchange capacity (CEC) of 8.3 meq/100 g,

3. Plant Apple, variety “James Grieve”, representative for fruiting crops

B. Study Design

Experimental conditions:

Two experiments were performed with a total of three apple trees of the variety "James Grieve". The experiments represented the intended foliar spray application scenarios of BYI 02960 for fruits. In one experiment (single foliar spray application), the apple trees were treated at a target application rate of 75 g a.s./(ha x m CH) (canopy height) at the end of flowering (BBCH 69). In the other experiment (double foliar spray application), one apple tree was treated two times. The target application rate was 75 g a.s./(ha x m CH) at each application. The first application was conducted at the end of flowering (BBCH 69) and the second at 14 days before harvest. The total target rate corresponds to the anticipated maximum application rate. The apple trees had a canopy height (CH) of 0.5 m and were cultivated in the sandy loam soil "[redacted]4" in a planting container with a surface area of 0.091 m^2. The plants were grown in the glass-roofed vegetation area of the test facility which allows cultivation similar to natural temperature and light conditions, but protected from rainfall. The plants were watered according to their needs.

<u>Single foliar spray application (BBCH 69):</u>

Two apple trees with a canopy height of 0.5 m were treated each with 10 mL of the aqueous application solution using a hand pump sprayer. Prior to the application, each apple tree was covered with a protective plastic wrap to prevent radioactivity spreading into the vegetation area. After application, the hand pump sprayer was rinsed with water and acetonitrile and the plastic wrap was rinsed with methanol. The radioactivity in the rinse solutions was determined and subtracted from the amount in the application solution. A total amount of 31.5 MBq was applied per tree, corresponding to 14.5 mg a.s. From a planting density of 3000 trees/ha an actual application rate of 87 g a.s./(ha x m CH) was calculated.

<u>Double foliar spray application (BBCH 69 & 14 days before harvest):</u>

One apple tree with a canopy height of 0.5 m was treated for the first time with 10 mL of the aqueous application solution using a hand pump sprayer at the end of flowering (BBCH 69). A second application was performed at 14 days before harvest. Again, 10 mL of the aqueous application solution was applied. As described above, the hand pump sprayer and the protective plastic wrap were rinsed to determine the losses of radioactivity due to the application. A total amount of 31.5 MBq was applied at the first application and 30.9 MBq were applied at the second application. Thus 14.5 mg a.s. and 14.2 mg a.s. were applied. From a planting density of 3000 trees/ha a total application rate (comprising both applications) of 172 g a.s./(ha x m CH) was calculated.

Sampling:

Apple fruits of both experiments were sampled at maturity (BBCH 87- 89). On the same day, the leaves were cut off the trees with scissors. The leaves were stored in a freezer (≤ -18°C) on the day of harvest until extraction.

All apple fruits of the single application experiment were cut in pieces. The pieces were mixed and divided into aliquots. The apple aliquots were stored in a freezer (≤ -18°C) until needed for extraction. The apple fruits of the double application experiment were separated into two subsamples. Approx. one third of the whole apple fruits was stored in a freezer (≤ -18°C) on the day of harvest. The remaining whole fruits were stored overnight at room temperature. On the next day the apples were subjected to a surface wash with dichloromethane.

C. Analytical Procedures

Extraction:

Single application experiment (apple fruits):

The apple pieces were extracted three times with a mixture of acetonitrile/water (8:2, v/v) and one time with a mixture of acetonitrile/water (1:1, v/v) using a high speed blender. The radioactivity in the extracts was determined by LSC, in the solids by combustion followed by LSC. The actual TRR value of the RAC was determined by summing up the radioactivity measured in the extracts and in the remaining solids. The first two extracts were combined and subjected to a clean-up step using an SPE RP 18 cartridge (Phenomenex, Strata C18-E, 20 g), which was conditioned with acetonitrile beforehand. The flow-through fraction (percolate) was collected and the cartridge was rinsed with acetonitrile/water (8:2, v/v) and methanol/dichloromethane (1:1, v/v). The percolate and the acetonitrile/water fraction were combined and concentrated by rotary evaporation in vacuo. The pH value of the concentrate was adjusted to pH 7 with ammonium carbonate before chromatographic analysis by HPLC.

Double application experiment (apple fruits):

The subsample of the whole apple fruits which was stored at room temperature was subjected to a surface wash with dichloromethane. An aliquot of the dichloromethane surface wash was dispersed with 1 mL of water by ultrasonic treatment. The dichloromethane was removed by a nitrogen stream and the remaining concentrate was analysed by HPLC. The washed apples were cut into pieces. The apples pieces were mixed, divided into aliquots and stored in a freezer (≤ -18°C). Extraction, purification and concentration of the extract were performed exactly as described for the single application experiment. The resulting sample was adjusted to pH 7 with ammonium carbonate for HPLC analysis.

Additionally, three whole apples of the double application experiment were extracted without prior surface wash. The frozen apples were crushed with a wooden hammer and homogenised under liquid nitrogen using a high speed blender. An aliquot of the apple mush was extracted for final analysis. Extraction, purification and concentration of the extract were performed exactly as described for the single application experiment. The resulting sample was adjusted to pH 7 with ammonium carbonate for HPLC analysis.

Aliquots of the apple leaves of the single and double application experiment were extracted as described for the fruits of the single application experiment. The clean-up procedure and the concentration step were also identical with the one applied for the fruit samples. The resulting sample was adjusted to pH 7 with ammonium carbonate for HPLC analysis.

Quantification:
Parent compound and metabolites in the extracts were analysed by reversed phase HPLC coupled to a radioactivity detector with a glass scintillator cell. The HPLC chromatograms (= metabolite profiles) were integrated for quantification of compounds.

Identification and characterisation:
Generally, major metabolites were identified by two independent chromatographic methods (reversed phase HPLC and normal phase TLC) or by LC-MS/MS after semi-preparative isolation of the compounds.

Since the profiles of the apple leaves showed higher levels of metabolites than the profiles of apple fruits (in terms of mg/kg), several metabolites were isolated with semi-preparative HPLC from the extract of apple leaves of the single treatment experiment. The isolated compounds were identified with spectroscopic methods, even if they were minor. The isolated and identified metabolites were furthermore used as reference compounds for co-chromatographic identification of metabolites in apple fruits. Other minor metabolites (<10% of the TRR and representing <0.01 mg/kg in edibles) were identified by HPLC co-chromatography with authentic reference compounds or by HPLC comparison.

Storage stability:
All extraction and surface wash experiments and the first HPLC analyses of the apple fruits were performed within 3 months after harvest. The extraction experiments and the first HPLC analyses of the apple leaves were performed within 4 months after harvest. Thus, no additional tests are necessary to prove the stability of the relevant residues in frozen matrices. The extracts were analysed after 1 to 3 days following start of extraction and the surface wash was analysed 13 days after washing.

Comparison of the HPLC chromatograms recorded at different times during the study showed that the profiles of the extracts did not change significantly during the analytical work up, which did not exceed approx. eight months.

It was therefore concluded, that the residues in the matrices and in the extracts were sufficiently stable during the experimental period of the study and that the chromatograms represented the metabolic pattern in the samples at harvest.

II. Results and Discussion

The metabolism of [pyridinylmethyl-^{14}C]BYI 02960 was investigated in apple fruits and leaves following two different spray scenarios. In one experiment (single application experiment), two apple trees were treated at an actual application rate of 87 g a.s./(ha x m CH) (CH = meter canopy height) at the end of flowering (BBCH 69). In the other experiment, one apple tree was treated two times, one time at an actual application rate of 87 g a.s./(ha x m CH) at the end of flowering (BBCH 69) and a second time at an application rate of 85 g a.s./(ha x m CH) at 14 days before harvest. The total application rate in the double application experiment was 172 g a.s./(ha x m CH). The actual single application rates were slightly above the anticipated maximum rate of 75 g a.s./(ha x m CH).

Apple fruits were harvested for analysis from the single application experiment at 98 days after the treatment and from the double application experiment at 14 days after the last treatment. Concurrently, the leaves were sampled to support the analysis of metabolites. The apple fruit samples of the double application experiment were investigated with and without a surface wash step.

The TRR level in the apple fruits, which represent the edible RAC, was low in the single application experiment: only 0.079 mg/kg was found. In the double application experiment, the apple fruits had a TRR of 1.868 mg/kg (extraction including surface wash) and 0.545 mg/kg (extraction without surface wash). The difference of the TRR values is presumably due to the low amount of apples harvested and extracted and a partly inhomogeneous spray distribution.

The TRR values of the apple leaves were high: 56.715 mg/kg were found in the leaves of the single application experiment and 134.841 mg/kg in the leaves of the double application experiment. Apple leaves were only sampled to support the identification of metabolites.

The main portion of radioactivity in the apple fruits and leaves was extracted conventionally by acetonitrile/water mixtures (94.2% to 98.7% of the TRR) as shown in Table 6.2.1-25 and Table 6.2.1-26. The apple fruits of the double application experiment were additionally surface-washed with dichloromethane and then extracted conventionally with acetonitrile/water mixtures. A portion of 11.0% of the TRR was removed by the surface wash and another portion of 88.2% was extracted conventionally (see also Table 6.2.1-26).

HPLC analysis of the surface wash and the conventional extracts of apple fruits and leaves after both spray scenarios revealed that all metabolite profiles were very well comparable. Thus, identification was performed generally in the fruit or leaf extract of the single application experiment and metabolites were assigned in the other extracts (including the surface wash) by comparison.

The main compound in apple fruits and leaves of the single and the double application experiment was parent compound. It was identified in the extracts of apple fruits of both experiments and in the extract of apple leaves of the single application experiment by HPLC co-chromatography with a radiolabelled reference compound. For confirmation, parent compound was additionally identified in the extract of apple fruits of the single application experiment by TLC co-chromatography on silica gel.

All other metabolites detected in the raw agricultural commodity fruit were minor metabolites. They did not exceed 10% of the TRR in the single and the double application experiment. However, since all metabolites were detected in higher levels in the extract of leaves, the leaf extract of the single application experiment was fractionated and the fractions were subjected to LC-MS/MS analysis. Thus, metabolites BYI 02960-CHMP-glyc, BYI 02960-difluoroethyl-OH-glyc, BYI 02960-difluoroethanamine, BYI 02960-acetic acid-glyc and BYI 02960-OH-glyc and the corresponding aglycons BYI 02960-acetic acid and BYI 02960-OH were identified. The isolated metabolites were used for HPLC co-chromatography to confirm their occurrence in apple fruits.

Mass spectroscopic investigations of the isolated metabolite BYI 02960-CHMP-glyc showed that the one HPLC peak was represented by two isomers, presumably with different configurations of the conjugated glycoside moiety. The major isomer was assigned to the glucose conjugate of BYI 02960-

CHMP. This configuration of the carbohydrate was confirmed in the tomato metabolism study and thus it was assumed that it is most probably the preferred configuration in all plants. Another conjugate of BYI 02960-CHMP, the minor metabolite BYI 02960-CHMP-di-glyc was identified by comparison of apple and tomato profiles, too.

LC-MS/MS analysis showed as well that the two metabolites BYI 02960-OH-glyc and BYI 02960-acetic acid co-eluted in one peak using the neutral HPLC profiling method. Chromatographic separation of the compounds was achieved with an acidic reversed phase HPLC method. For sub-quantification the peaks with the two metabolites were isolated from the apple fruits and leaves of the single application experiment by semi-preparative HPLC using the neutral profiling method. Re-analyses of the fraction with the acidic HPLC method separated the two components. The ratios obtained in the extracts of the single application experiment were then used for sub-quantification of the compounds in the double application experiment. The very similar pattern in the profiles of the two experiments (neglecting the amounts of parent compound) justified the transfer of the ratios. The identification of BYI 02960-OH-glyc and BYI 02960-acetic acid was additionally confirmed by comparison with reference compounds isolated and identified in the confined rotational crops study.

Following LC-MS/MS analysis, supportive experiments were performed to fully elucidate the chemical structures of the metabolites BYI 02960-OH-glyc and BYI 02960-OH. Enzymatic treatment with ß-glucosidase cleaved the conjugate BYI 02960-OH-glyc. HPLC co-chromatography of the cleavage product with the reference compound BYI 02960-OH located the conjugating hydroxy group at the 5-position of the furanone moiety. Concurrently, the successful enzymatic cleavage revealed D-glucose as the configuration for the conjugated hexose. For the metabolite BYI 02960-difluoroethyl-OH-glyc, the position of the hydroxy group being the link for the conjugation could not be exactly determined by LC-MS/MS. However, the fragmentation pattern indicated hydroxylation and conjugation in the difluoroethyl side chain.

The minor metabolites BYI 02960-6-CNA and BYI 02960-CHMP were identified in apple fruits of the single application experiment by HPLC co-chromatography with authentic reference compounds. Following identification of metabolites by LC-MS/MS analysis and co-chromatography or comparison with reference compounds, assignment to peaks in other profiles was completed by comparison of all profiles

The distribution of the radioactive residues in the extracts is shown in Table 6.2.1-25 and Table 6.2.1-26. In total, 78.9% to 98.5% of the TRR were identified in the apple fruit after foliar application as summarized in Table 6.2.1-27 and Table 6.2.1-28.

Table 6.2.1-25: Distribution of radioactivity in the extracts of apple fruits and leaves after a single foliar application of [pyridinylmethyl-^{14}C]BYI 02960

	single application experiment			
	apple fruits		**apple leaves**	
TRR [mg/kg] =	0.079		56.715	
	% of TRR	mg/kg	% of TRR	mg/kg
Conventionally extracted	94.2	0.074	96.7	54.859
Extract for analysis	94.2	0.074	96.5	54.727
Losses (not analysed)	---	---	0.2	0.132
Total extracted	**94.2**	**0.074**	**96.7**	**54.859**
Unextractable (PES*)	5.8	0.005	3.3	1.855
Accountability	**100.0**	**0.079**	**100.0**	**56.715**

* post extraction solids

Table 6.2.1-26: Distribution of radioactivity in the extracts of apple fruits and leaves after a double foliar application of [pyridinylmethyl-^{14}C]BYI 02960

	double application experiment					
	apple fruits incl. surface wash		**apple fruits without surface wash**		**apple leaves**	
TRR [mg/kg] =	1.868		0.545		134.841	
	% of TRR	mg/kg	% of TRR	mg/kg	% of TRR	mg/kg
Surface wash with DCM	11.0	0.205	---	---	---	---
Conventionally extracted	88.2	1.648	98.7	0.538	98.4	132.635
Extract for analysis	88.1	1.645	98.0	0.534	97.8	131.847
Losses (not analysed)	0.2	0.003	0.6	0.004	0.6	0.788
Total extracted	**99.2**	**1.853**	**98.7**	**0.538**	**98.4**	**132.635**
Unextractable (PES*)	0.8	0.015	1.3	0.007	1.6	2.206
Accountability	**100.0**	**1.868**	**100.0**	**0.545**	**100.0**	**134.841**

* post extraction solids

Table 6.2.1-27: TRR values and distribution of parent compound and metabolites in apple fruits and leaves after a single foliar treatment of [pyridinylmethyl-^{14}C]BYI 02960

	single application			
	apple fruits		apple leaves	
TRR [mg/kg] =	0.079		56.715	
Compound (BYI 02960-)	% TRR	mg/kg	% TRR	mg/kg
Conventional extraction				
BYI 02960 (parent compound)	43.1	0.034	24.5	13.882
6-CNA	5.0	0.004	---	---
CHMP-di-glyc	---	---	0.6	0.342
CHMP-glyc	4.7	0.004	14.4	8.141
CHMP	4.0	0.003	1.3	0.727
acetic acid-glyc	3.5	0.003	5.1	2.891
OH-glyc	4.9	0.004	19.9	11.278
acetic acid	3.0	0.002	1.4	0.767
difluoroethyl-OH-glyc	1.4	0.001	6.4	3.631
AMCP-difluoroethanamine	8.4	0.007	0.4	0.255
OH	0.8	0.001	0.9	0.484
Total identified	**78.9**	**0.062**	**74.8**	**42.399**
unknown 1	2.7	0.002	---	---
unknown 2	6.6	0.005	---	---
unknown 3	3.5	0.003	1.8	1.000
unknown 4	2.0	0.002	3.6	2.052
unknown 5	---	---	---	---
unknown 6	---	---	1.1	0.634
unknown 7	---	---	0.6	0.322
unknown 8	---	---	---	---
unknown 9	---	---	2.6	1.477
unknown 10	---	---	1.3	0.762
unknown 11	---	---	0.5	0.295
unknown 12	---	---	---	---
unknown 13	---	---	---	---
unknown 14	---	---	0.6	0.353
unknown 15	---	---	0.6	0.315
unknown 16	---	---	1.2	0.682
unknown 17	---	---	0.7	0.403
unknown 18	---	---	2.4	1.386
unknown 19	---	---	1.9	1.088
unknown 20	0.6	<0.001	2.5	1.445
unknown 21	---	---	0.2	0.114
Total characterised	**15.3**	**0.012**	**21.7**	**12.328**
Analysed extract(s)	94.2	0.074	96.5	54.727
Extracts not analysed	---	---	0.2	0.132
Total extracted	**94.2**	**0.074**	**96.7**	**54.859**
Unextractable (PES*)	5.8	0.005	3.3	1.855
Accountability	**100.0**	**0.079**	**100.0**	**56.715**

* post extraction solids

Table 6.2.1-28: TRR values and distribution of parent compound and metabolites in apple fruits and leaves after two foliar treatments of [pyridinylmethyl-^{14}C]BYI 02960

	double application experiment					
	apple fruits incl. surface wash		apple fruits without surface wash		apple leaves	
TRR [mg/kg] =	1.868		0.545		134.841	
Compound (BYI 02960-)	% TRR	mg/kg	% TRR	mg/kg	% TRR	mg/kg
Conventional extraction						
parent compound	88.4	1.652	85.6	0.467	48.2	64.981
6-CNA	0.5	0.009	1.5	0.008	0.3	0.436
CHMP-di-glyc	---	---	---	---	0.2	0.327
CHMP-glyc	0.5	0.010	0.9	0.005	7.3	9.842
CHMP	0.7	0.013	0.8	0.004	0.6	0.775
acetic acid-glyc	0.6	0.012	0.8	0.004	5.0	6.799
OH-glyc	1.3	0.024	1.7	0.009	15.4	20.729
acetic acid	0.8	0.015	1.1	0.006	1.0	1.480
difluoroethyl-OH-glyc	---	---	---	---	4.9	6.674
AMCP-difluoroethanamine	4.5	0.085	4.1	0.023	0.8	1.015
OH	1.0	0.020	1.0	0.005	0.7	0.944
Total identified	**98.5**	**1.839**	**97.6**	**0.532**	**84.5**	**113.933**
unknown 1	---	---	---	---	---	---
unknown 2	---	---	---	---	---	---
unknown 3	0.2	0.004	---	---	0.8	1.070
unknown 4	---	---	---	---	2.0	2.652
unknown 5	---	---	---	---	0.2	0.222
unknown 6	---	---	---	---	0.3	0.406
unknown 7	---	---	---	---	0.4	0.601
unknown 8	---	---	---	---	0.4	0.504
unknown 9	---	---	---	---	1.6	2.150
unknown 10	---	---	---	---	0.5	0.721
unknown 11	---	---	---	---	0.5	0.654
unknown 12	---	---	---	---	0.2	0.323
unknown 13	0.3	0.005	---	---	0.4	0.518
unknown 14	---	---	---	---	---	---
unknown 15	---	---	---	---	---	---
unknown 16	---	---	---	---	1.0	1.380
unknown 17	---	---	---	---	0.5	0.669
unknown 18	---	---	---	---	1.7	2.291
unknown 19	---	---	---	---	0.8	1.091
unknown 20	0.1	0.002	0.5	0.002	1.7	2.248
unknown 21	---	---	---	---	0.3	0.413
Total characterised	**0.6**	**0.011**	**0.5**	**0.002**	**13.3**	**17.914**
Analysed extract(s)	99.0	1.850	98.0	0.534	97.8	131.847
Extracts not analysed	0.2	0.003	0.6	0.004	0.6	0.788
Total extracted	**99.2**	**1.853**	**98.7**	**0.538**	**98.4**	**132.635**
Unextractable (PES*)	0.8	0.015	1.3	0.007	1.6	2.206
Accountability	**100.0**	**1.868**	**100.0**	**0.545**	**100.0**	**134.841**

* post extraction solids

III. Conclusions

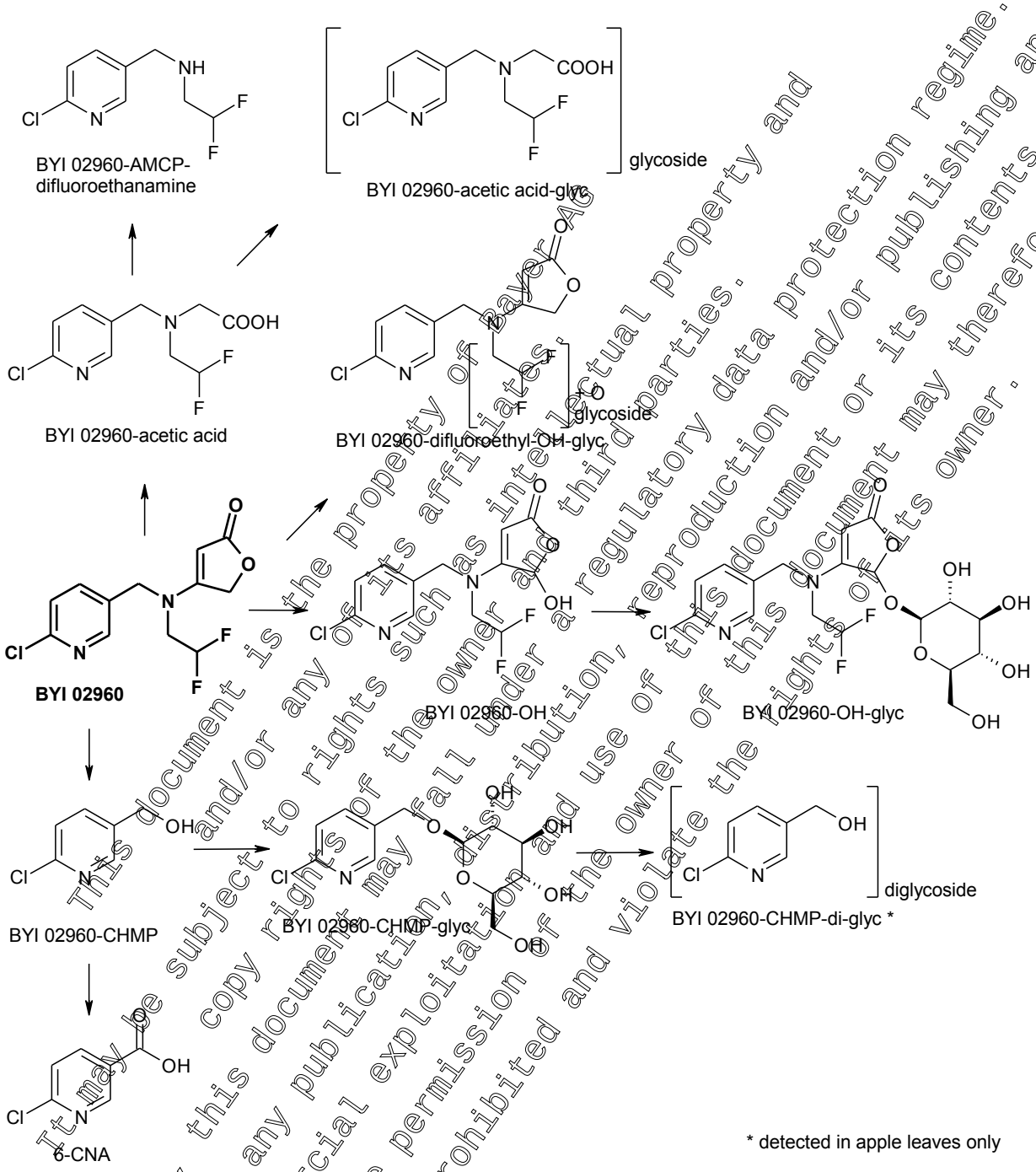
As expected, the residues were dominated by parent compound in the double application experiment, whereas the metabolite pattern was the same in both experiments.

Three major metabolic routes of [pyridinylmethyl-^{14}C]BYI 02960 were observed in apples:

- hydroxylation followed by conjugation with carbohydrates,
- cleavage of the pyridinylmethylamine bond followed by conjugation with carbohydrates or by oxidation of the methylene group to a carboxylic group, and
- oxidative degradation of the furanone moiety to an acetic acid group followed either by conjugation with a carbohydrate or by further degradation of the moiety.

On the basis of the results of this study it is concluded that the metabolism of [pyridinylmethyl-^{14}C]BYI 02960 in apples is well understood and the following metabolic pathway is proposed.

Figure 6.2.1-9: Proposed metabolic pathway of [pyridinylmethyl-^{14}C]BYI 02960 in apples



Overall Conclusions Apple (foliar application)

The metabolism of the insecticide BYI 02960 was investigated in apple fruits in two studies following foliar application with (1) [furanone-4-^{14}C]BYI 02960 or (2) [pyridinylmethyl-^{14}C]BYI 02960. In order to gain information on the fate of the difluoroethane moiety of BYI 02960, the extracts obtained in the apple metabolism study with [furanone-4-^{14}C]BYI 02960 were additionally analysed for non-radiolabelled difluoroacetic acid by LC-MS/MS according to residue method 01304 (see KIIA

6.2./12). In both metabolism studies, single and double application experiments were done. In the single application experiment, apple trees were treated once at the end of flowering (BBCH 69), whereas in the double application experiment, the trees were additionally treated with the same rate 14 days before harvest. As expected, in both studies the residues in the double application experiment were dominated by parent compound.

Label (1): [furanone-4-^{14}C]BYI 02960

In the single application experiment a natural compound (glucose/carbohydrates) was by far the main compound in apple fruits, whereas parent compound was predominant in leaves. Nevertheless, the metabolite patterns were very similar in fruits and leaves in both experiments. Besides parent compound and glucose/carbohydrates, each of the other metabolites in apple fruits represented less than 4% of the TRR. In total, 5 and 6 metabolites were identified in the single and the double application experiment, respectively. In apple leaves, BYI 02960-OH-glyc was identified as major metabolite, each of the other metabolites (6 to 7) represented less than 7% of the TRR. Overall, four major metabolic routes were detected: (1) hydroxylation of the parent compound (either the furanone moiety or the difluoroethyl moiety) followed by conjugation with carbohydrates, (2) oxidative degradation of the furanone moiety to an acetic acid group followed either by conjugation with a carbohydrate or by further degradation of the moiety, (3) complete degradation of the furanone moiety and incorporation of carbon atoms into the natural compound pool, i.e. into glucose/carbohydrates, and (4) cleavage of the pyridinylmethylamine bond. The first two routes led to the non label-specific metabolites BYI 02960-acetic acid and BYI 02960-OH, their corresponding glycosides and to BYI 02960-difluoroethyl-OH-glyc, which was detected in apple leaves only. These metabolites were also detected in the apple metabolism study with [pyridinylmethyl-^{14}C]BYI 02960. Molecule cleavage led to the label-specific metabolite BYI 02960-difluoroethyl-amino-furanone and degradation of the furanone moiety finally to glucose/carbohydrates. The corresponding counterparts BYI 02960-CHMP and BYI 02960-AMCP-difluoroethanamine were detected in the apple metabolism study with [pyridinylmethyl-^{14}C]BYI 02960. Thus, the results of the present metabolism study in apples are in very good conformity with the results of the corresponding study performed with [pyridinylmethyl-^{14}C]BYI 02960.

(2) Label 2: [pyridinylmethyl-^{14}C]BYI 02960

Parent compound was the main compound detected in both experiments in apple fruits at harvest. All metabolites represented less than 10% of the TRR. Nine metabolites were identified in fruits from which BYI 02960-AMCP-difluoroethanamine was the most prominent one, followed by 6-CNA (single application experiment) or BYI 02960-OH-glyc (double application experiment). Overall, three major metabolic routes were detected: (1) Oxidative degradation of the furanone moiety to an acetic acid group followed either by conjugation with a carbohydrate or by further degradation of the moiety; (2) hydroxylation of the parent compound (either the furanone moiety or the difluoroethyl moiety) followed by conjugation with carbohydrates and (3) cleavage of the pyridinylmethylamine bond followed by conjugation with carbohydrates or by oxidation of the methylene group to a carboxylic group. The first two routes led to the non label-specific metabolites BYI 02960-acetic acid and BYI 02960-OH, their corresponding glycosides and to BYI 02960-difluoroethyl-OH-glyc. These metabolites were also detected in the apple metabolism study with [furanone-4-^{14}C]BYI 02960. Molecule cleavage led to the label-specific metabolites BYI 02960-AMCP-difluoroethaneamine, 6-CNA, BYI 02960-CHMP and its corresponding glycoside. The corresponding counterpart BYI 02960-

difluoroethyl-amino-furanone was detected in the apple metabolism study with [furanone-4-^{14}C]BYI 02960. Thus, the results of the present metabolism study in apples are in good conformity with the results of the corresponding study performed with [furanone-4-^{14}C]BYI 02960.

When considering the results from both metabolism studies conducted on apple, it can be concluded that BYI02960 is rather extensively metabolised in this crop. A total of 3 major (one in apple fruits and 2 in leaves) and more than 20 minor metabolites (5 thereof in fruits) were found. All major and 10 minor metabolites have been identified. The distribution of parent compound and metabolites in the edible commodity apple fruit is summarized in Table 6.2.1-29.

Table 6.2.1-29 TRR values and distribution of parent compound and metabolites in apple fruit after foliar application of BYI 02960

	apple fruits							
Radiolabel	[furanone-4-^{14}C]				[pyridinylmethyl-^{14}C]			
	single appl.		double appl. w/o surface wash		single appl.		double appl. w/o surface wash	
TRR [mg/kg] =	0.280		1.286		0.079		0.545	
Compound (BYI 02960-)	% TRR	mg/kg	% TRR	mg/kg	% TRR	mg/kg	% TRR	mg/kg
BYI 02960	7.4	0.021	73.6	0.946	43.1	0.034	85.6	0.467
glucose/carbohydrates	*71.7*	*0.201*	*14.2*	*0.182*				
6-CNA					*5.0*	*0.004*	*1.5*	*0.008*
CHMP-glyc					*4.1*	*0.004*	*0.9*	*0.005*
CHMP					*4.0*	*0.003*	*0.8*	*0.004*
difluoroethyl-amino-furanone	*3.1*	*0.009*	*0.3*	*0.003*				
acetic acid-glyc	0.3	0.001	0.5	0.007	3.5	0.003	0.8	0.004
OH-glyc	0.4	0.001	1.1	0.014	4.9	0.004	1.7	0.009
acetic acid	0.2	0.001	0.7	0.009	3.0	0.002	1.1	0.006
AMCP-difluoroethanamine					*8.4*	*0.007*	*4.1*	*0.023*
difluoroethyl-OH-glyc					1.4	0.001	---	---
OH	---	---	0.8	0.01	0.8	0.001	1.0	0.005
Total identified	**83.4**	**0.234**	**91.1**	**1.171**	**78.9**	**0.062**	**97.6**	**0.532**
Total characterised	**2.0**	**0.005**	**0.1**	**0.002**	**15.3**	**0.012**	**0.5**	**0.002**
Analysed extract(s)	85.3	0.239	91.2	1.173	94.2	0.074	98.0	0.534
Extract(s) not analysed	1.2	0.003	0.7	0.009	---	---	0.6	0.004
Total extracted	86.5	0.242	91.9	1.182	94.2	0.074	98.7	0.538
Unextractable (PES*)	13.5	0.038	8.1	0.104	5.8	0.005	1.3	0.007
Accountability	**100.0**	**0.280**	**100.0**	**1.286**	**100.0**	**0.079**	**100.0**	**0.545**

* post extraction solids

Label specific metabolites are printed in italics.

Analysis of apple fruit and leave extracts on the non-radiolabelled metabolite difluoroacetic acid revealed that this metabolite represents a significant proportion of the residue. In apple fruits, difluoroacetic acid accounted for 0.69 mg a.s. equiv./kg in the single application experiment and represented by far the highest residue. In the double experiment difluoroacetic acid accounted for 0.12 mg a.s. equiv./kg and was thus a main metabolite. Only parent compound and the natural compound glucose were detected in higher concentrations. In leaves quite high concentrations of

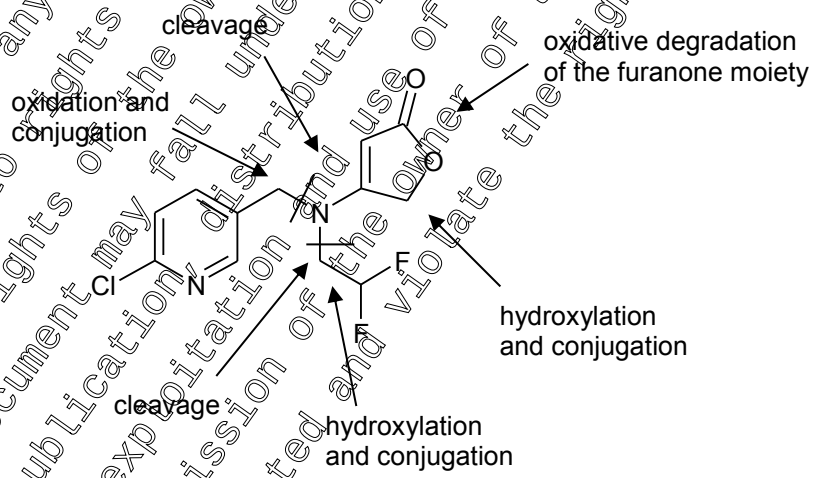
difluoroacetic acid were found (1.86 mg a.s. equiv/kg in the single application experiment and 1.35 mg a.s. equiv./kg in the double application experiment), however the proportions were low compared to the total radioactive residue detected.

On basis of the metabolites identified, biotransformation of BYI 02960 in apple proceeds by the following pathways:

- oxidative cleavage of the difluoroethylamine bond and formation of difluoroacetic acid
- oxidative degradation of the furanone moiety to an acetic acid group followed either by conjugation with a carbohydrate or by further degradation of the moiety
- complete degradation of the furanone moiety and incorporation of carbon atoms into the natural compound pool, e.g. into glucose/carbohydrates
- cleavage of the pyridinylmethylamine bond and formation of BYI 02960-difluoroethylamino-furanone and its corresponding counterpart BYI 02960-CHMP, which was either conjugated with carbohydrates or oxidised to 6-chloronicotinic acid (6-CNA)
- hydroxylation of the furanone or difluoroethane moiety followed by conjugation with glucose or another hexose

The positions involved in the metabolic degradation are summarised in the following figure.

Figure 6.2.1-10: Positions involved in metabolic degradation of BYI 02960 in apple fruits and leaves



Metabolism, distribution and expression of residues in cotton (foliar application)

Metabolism studies in cotton were conducted with [furanone-4-^{14}C]-, and [pyridinylmethyl-^{14}C]BYI 02960:

Report:	**KIIA 6.2.1/08, [redacted], [redacted]; 2011**
Title:	Metabolism of [furanone-4-^{14}C]BYI 02960 in cotton after spray application
Report No & Edition No	MEF-11/392 M-421625-01-2
Guidelines:	OECD 501 Metabolism in Crops US EPA Residue Chemistry Test Guideline OPPTS 860.1300: Nature of the Residue – Plants, Livestock PMRA Regulatory Directive Dir98-02: Residue Chemistry Guidelines Section 2: Nature of the Residue – Plants, Livestock Japanese MAFF, 12 Nousan 8147 European Parliament and Council Regulation (EC) No 1107/2009
GLP	yes

Executive Summary

The metabolism of [furanone-4-^{14}C]BYI 02960 formulated as an SL 200 was investigated in cotton after foliar spray application. In one experiment (single application experiment), five cotton plants were treated at an actual application rate of 209 g a.s./ha when the sixth true leaf was unfolded (BBCH 16), i.e. at an early growth stage. In another experiment, one cotton plant was treated two times, one time at an actual application rate of 209 g a.s./ha at BBCH 15 and a second time at an application rate of 176 g a.s./ha at 14 days before harvest of the cotton bolls (when more than 90% of the bolls were open and approx. 50% to 70% of the leaves were discoloured or fallen; BBCH 95 - 97). The total application rate in the double application experiment was 385 g a.s./ha.

Samples were taken from both experiments. Cotton seeds and gin trash were the raw agricultural commodities (RACs) harvested at maturity of the seeds in this study. Lint was collected as additional sample material at the same time. Additionally, one plant of the single application experiment was sampled at an intermediate growth stage (two to four vegetative side shoots were visible; BBCH 22 – 24) to support the identification of metabolites and to possibly obtain additional information on the metabolic degradation behaviour of BYI 02960. The TRR values of all plant matrices determined are shown in the following table:

Table 6.2.1-30: TRR values in cotton (intermediate, gin trash, lint, seeds) after foliar application of [furanone-4-^{14}C]BYI 02960

Matrix	**Timing and Application**	**PHI (days)***	**ppm (mg a.s. equiv./kg)**
intermediate	one spray application, 209 g a.s./ha (at BBCH 16)	28	12.391
gin trash		169	0.191
lint		169	0.009
seeds		169	0.013
gin trash	two spray applications, 209 g a.s./ha (at BBCH 15) 176 g a.s./ha (at BBCH 95 – 97)	14	2.767
lint		14	4.993
seeds		14	0.016

* PHI: preharvest interval (corresponds to days after last treatment (DAT) at the start of harvest/sampling)

The radioactive residues were extracted with acetonitrile/water mixtures. In the case of seeds, the samples were extracted with heptane beforehand. When necessary, additional exhaustive extraction steps were applied to the solids of the first extraction. Extraction efficiencies ranged from 23.4% of the TRR for seeds (single application experiment) to 96.6% for lint (double application experiment). The profiles of the extracts comprising a reasonable amount of radioactivity were recorded by HPLC and all major and several minor components were identified. Identification of parent compound and metabolites common to both radiolabels was based on the metabolite profiles obtained in the cotton metabolism study with [pyridinylmethyl-^{14}C]BYI 02960. Additionally, all assignments made in the present study were confirmed by HPLC co-chromatography using authentic reference compounds. The identification rates ranged from 70.3% of the TRR for gin trash (single application experiment) to 86.0% for lint (double application experiment). [Furanone-4-^{14}C]BYI 02960 was metabolised moderately in cotton. Parent compound was the most prominent component and represented approx. 40 – 70% of the TRR in all matrices analysed. The sum of metabolites BYI 02960-OH-glyc and BYI 02960-acetic acid represented another major fraction in all matrices and accounted for approx. 14% – 25% of the TRR. In gin trash of the single application experiment, BYI 02960-OH was detected as additional major metabolite representing approx. 13% of the TRR. All other metabolites detected were minor or trace components.

The following metabolic routes of [furanone-4-^{14}C]BYI 02960 were observed in cotton:

- hydroxylation of the methylene group of the furanone moiety followed by conjugation,
- oxidative opening of the furanone moiety to an acetic acid group followed by further oxidation or conjugation with a carbohydrate (e.g. glycosylation), and
- halogenation (mostly bromination, to a minor extent chlorination) of the furanone moiety

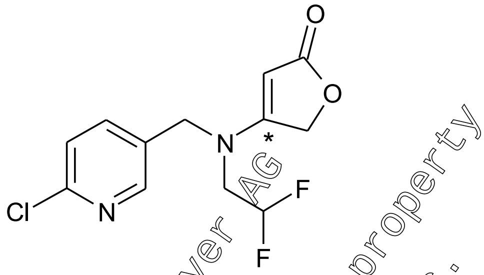
Halogenation of the furanone moiety of the active substance probably occurred in the soil which was supported by the fact that small amounts of halogenated parent compound were identified in the aerobic soil degradation studies. Due to the early timing of the first application, a significant portion of the parent compound could have reached the soil and been subjected to soil metabolism processes.

On the basis of these results, a metabolic pathway of [furanone-4-^{14}C]BYI 02960 in cotton can be proposed.

I. Materials and Methods

A. Materials

1. Test Material:

Chemical structure	 * position of the radiolabel
Radiolabelled test material	[furanone-4-^{14}C]BYI 02960
Specific radioactivity	3.94 MBq/mg (106.46 µCi/mg)
Chemical Purity	> 99% (HPLC)
Radiochemical purity	> 98% (HPLC and TLC)

The supplied radiolabelled test compound [furanone-4-^{14}C]BYI 02960 was dissolved in acetonitrile. Formulation of the test compound was performed prior to each application. For the preparation of the SL 200 formulation, an adequate portion of the stock solution was transferred into a special glass vial and evaporated to dryness. The liquid blank formulation was added to the test item using a magnetic stirrer. The mixture was diluted with water to get the aqueous application solution.

2. Soil: "Einheitserde T" (standard soil containing 2 kg/m^3 of a water soluble salt mixture), pH ($CaCl_2$) = 5.8, 85% white moor peat, 15% clay, containing NO_3^-, NH_4^+, P_2O_5, K_2O, Fe, Mn, B, Cu, Mo, Zn; distributer: [redacted], Germany

3. Plant Cotton, variety "Carmen", Gossypium hirsutum, representative for oil seeds

B. Study Design

Experimental conditions:

Two experiments were performed with a total of six cotton plants of the variety "Carmen". The experiments represented the intended foliar spray application scenarios of BYI 02960 in cotton. In one experiment (single foliar spray application), the plants were treated at a target application rate of 200 g a.s./ha when the sixth true leaf was unfolded (BBCH 16). In the other experiment (double foliar spray application), one cotton plant was treated two times. The target application rate was 200 g a.s./ha at each application. The first application was conducted at BBCH 15 and the second at 14 days before harvest. The total target rate of 400 g a.s./ha corresponds to the anticipated maximum application rate. The cotton plants were cultivated in the standard soil "Einheitserde T" in planting pots with a surface area of approx. 0.076 m^2 and a volume of 15 L. The plants were grown in the greenhouse of the test facility which allows cultivation similar to natural temperature and light conditions. The plants were irrigated according to their needs.

Sampling:
In both experiments cotton seeds (= undelinted seeds), lint and gin trash were harvested at maturity of the seeds (BBCH 99). The cotton bolls (capsules) were cut from the plants and the seeds were separated manually from the lint. No chemical process followed to delint the seeds. The remaining plants (= gin trash) were cut off just above the soil surface and were shredded into small pieces. The gin trash chaff was combined with the empty capsules which had contained the lint and the seeds. Gin trash and seed samples were further processed using a Polytron homogenizer and liquid nitrogen. The homogenized samples were stored in a freezer (≤ -18°C) until analysis. Lint samples of the double application experiment were not submitted to any sample preparation steps and were stored in a freezer (≤ -18°C) until analysis.

In the single application experiment, additionally an intermediate was sampled at an early growth stage (two to four vegetative side shoots were visible; BBCH 22 – 24) to support the identification of metabolites. The plant was cut off just above the soil surface and was cut in small pieces prior to extraction and analysis.

C. Analytical Procedures
Extraction:
Aliquots of all sample matrices were extracted three times with a mixture of acetonitrile/water (8:2, v/v) using a high speed blender. The cotton seed samples were additionally extracted with heptane prior to the first acetonitrile/water extraction step. The radioactivity in the extracts was determined by LSC and in the solids by combustion followed by LSC. The actual TRR value of the RAC was determined by summing up the radioactivity measured in the extracts and in the remaining solids. All extracts were combined. The intermediate and the gin trash extracts were subjected to an SPE clean-up step, whereas the seed and the lint extracts were directly analysed by HPLC analysis after concentration. The clean-up was performed using an SPE RP 18 cartridge (Phenomenex, Strata C18-E, 20 g), which was conditioned with methanol, water and an acetonitrile/water mixture beforehand. The flow-through fraction (percolate) was collected and the cartridge was rinsed with acetonitrile/water (8:2, v/v) and methanol/dichloromethane (1:1, v/v). The percolate and the acetonitrile/water fraction were combined and concentrated by rotary evaporation in vacuo prior to chromatographic analysis by HPLC. The solids remaining after the conventional extraction steps of the gin trash samples were additionally submitted two times to microwave-assisted extraction with acetonitrile/water mixtures (8:2, v/v and 1:1, v/v). After each extraction step, extracts and solids were separated by filtration. The extracts were combined and concentrated by rotary evaporation in vacuo prior to HPLC analysis.

Quantification:
Parent compound and metabolites in the extracts were analysed by reversed phase HPLC coupled to a radioactivity detector with a glass scintillator cell. The HPLC chromatograms (= metabolite profiles) were integrated for quantification of compounds.

Identification and characterisation:
Identification of parent compound and metabolites common to both radiolabels was based on the metabolite profiles obtained in the cotton metabolism study with [pyridinylmethyl-^{14}C]BYI 02960. In this study parent compound and the major metabolites were identified in at least one matrix either by LC-MS/MS after semi-preparative isolation of the compounds or by HPLC and TLC co-

chromatography with authentic reference compounds. All assignments made in the present study were confirmed by HPLC co-chromatography in gin trash using authentic reference compounds. Assignment in the other matrices was performed by comparison of HPLC profiles since the metabolite profiles of all matrices were very well comparable. No label-specific metabolite was identified.

Storage stability:

All extraction experiments and the first HPLC analyses were performed within 3 weeks (21 days) after harvest of the cotton plants. The extracts were analysed after 6 days following the start of extraction at the latest. Since the first metabolic analysis of the RACs as well as the last experiments with extracts (HPLC co-chromatography) took place within 6 months, no further stability investigations were necessary according to OECD Guidance for the Testing of Chemicals 501 (2007).

II. Results and Discussion

The metabolism of [furanone-4-^{14}C]BYI 02960 was investigated in cotton seeds, gin trash and lint following two different spray scenarios. In one experiment (single application experiment), five cotton plants were treated at an actual application rate of 209 g a.s./ha when the sixth true leaf was unfolded (BBCH 16), i.e. at an early growth stage. In another experiment, one cotton plant was treated two times, one time at an actual application rate of 209 g a.s./ha at BBCH 15 and a second time at an application rate of 176 g a.s./ha at 14 days before harvest of the cotton bolls (when more than 90% of the bolls were open and approx. 50% to 70% of the leaves were discoloured or fallen; BBCH 95 - 97). The total application rate in the double application experiment was 385 g a.s./ha.

Cotton seeds, gin trash and lint were harvested for analysis from the single application experiment at 169 days after the treatment and from the double application experiment at 14 days after the last treatment. In the single application experiment, additionally one plant was sampled at an intermediate growth stage (BBCH 22-24) to support the elucidation of metabolites.

TRR levels in the double application experiment were related to the higher application rate and the late application timing. Residues in gin trash and lint were considerably higher compared to the single application experiment. As expected, residues in seeds were not affected by the late second application. The TRR level in the cotton seeds, which represent the edible RAC, was low in the single and in the double application experiment: only 0.013 mg/kg and 0.016 mg/kg were found, respectively. As expected, high residues were detected in gin trash (2.767 mg/kg) and lint (4.993 mg/kg) in the double application experiment where the last treatment was performed 14 days before harvest when more than 90% of the cotton bolls were open. In the single application experiment, the TRR in gin trash accounted for 0.191 mg/kg and the TRR in lint was < 0.01 mg/kg. Due to the negligible residues, lint of the single application experiment was not subjected to extraction.

The main portion of radioactivity in the intermediate (single application experiment), gin trash (single and double application experiment) and lint (double application experiment) was extracted conventionally by acetonitrile/water mixtures. For gin trash, additional exhaustive extraction steps were applied to the solids of the conventional extraction. Exhaustive extraction comprised two extraction steps with acetonitrile/water mixtures at increased temperature (120 °C) under microwave

assistance. Extraction efficiencies ranged from 23.4% of the TRR for seeds (single application experiment) to 96.6% for lint (double application experiment) as shown in Table 6.2.1-31 and Table 6.2.1-32. Due to the low radioactivity level in seeds, no further attempts were performed to analyse the extracts or to release additional radioactivity. Analysis of the HPLC analysis of the conventional and exhaustive extracts of the intermediate, gin trash and lint after both spray scenarios revealed that all metabolite profiles were comparable. Additionally, it was shown that the metabolite profiles of all matrices were also nearly identical with those obtained in the cotton metabolism study conducted with [pyridinylmethyl-^{14}C]BYI 02960. Thus, identification of compounds was based in a first step on the assignments made in the study with the other radiolabel. In a second step, the assignments were confirmed in the gin trash extracts of the present study by HPLC co-chromatography using authentic reference compounds. Corresponding metabolites in other matrices were assigned by comparison with the gin trash profile.

The main compound in all cotton matrices was parent compound even in the single application experiment. The next prominent fraction comprised BYI 02960-OH-glyc and BYI 02960-acetic acid. These compounds co-eluted with the neutral HPLC method used for metabolite profiling. Therefore, the fraction was isolated from a gin trash extract and re-analysed with an acidic mobile phase. Both compounds were well separated using the acidic method and identification of both compounds became feasible by HPLC co-chromatography with authentic reference compounds. Separation of the compounds revealed that BYI 02960-acetic acid represented approx. 58% of the fraction in gin trash in the single application experiment and approx. 94% of the fraction in the double application experiment. The configuration of the hexose in BYI 02960-OH-glyc was identified as D-glucose in the corresponding apple metabolism study by the specific enzymatic treatment with ß-glucosidase.

Besides parent compound and the fraction comprising BYI 0260-OH-glyc and BYI 02960-acetic acid, one additional major compound was detected only in gin trash. It was identified as BYI 02960-OH. All other metabolites detected in gin trash, lint and as well in the intermediate were minor metabolites. They did not exceed 10% of the TRR in the single and the double application experiment. Three of the minor metabolites were identified in gin trash by HPLC co-chromatography using authentic reference compounds: BYI 02960-glyoxylic acid, BYI 02960-acetic acid-glyc and BYI 02960-bromo (probably co-eluting with small amounts of BYI 02960-chloro). Assignment of the metabolites in other matrices was completed by comparison of all metabolite profiles

Table 6.2.1-31: Distribution of radioactivity in the extracts of cotton matrices (intermediate, gin trash and seeds) after a single foliar application of [furanone-4-^{14}C]BYI 02960

	single application experiment					
	intermediate		**gin trash**		**seeds**	
TRR [mg/kg] =	12.391		0.191		0.013	
	% of TRR	mg/kg	% of TRR	mg/kg	% of TRR	mg/kg
Conventional extraction	90.3	11.194	69.4	0.133	23.4	0.003
Extract for analysis	89.9	11.141	66.9	0.128	---	---
Losses (not analysed)	0.4	0.053	2.6	0.005	23.4[#]	0.003[#]
Microwave extraction	---	---	10.9	0.021	---	---
Extract for analysis	---	---	10.9	0.021	---	---
Losses (not analysed)	---	---	n.q.	n.q.	---	---
Total extracted	**90.3**	**11.194**	**80.3**	**0.153**	**23.4**	**0.003**
Unextractable (PES*)	9.7	1.197	19.7	0.038	76.6	0.011
Accountability	**100.0**	**12.391**	**100.0**	**0.191**	**100.0**	**0.013**

* post extraction solids
[#] the conventional extract was not further analyzed by HPLC due to the low radioactivity level
n.q. not quantified (< LOQ)

Table 6.2.1-32: Distribution of radioactivity in the extracts of cotton matrices (gin trash, lint and seeds) after two foliar applications of [furanone-4-^{14}C]BYI 02960

	double application experiment					
	gin trash		**lint**		**seeds**	
TRR [mg/kg] =	2.767		4.993		0.016	
	% of TRR	mg/kg	% of TRR	mg/kg	% of TRR	mg/kg
Conventional extraction	90.2	2.496	96.6	4.822	57.8	0.009
Extract for analysis	89.7	2.482	96.6	4.822	---	---
Losses (not analysed)	0.5	0.014	n.q.	n.q.	57.8[#]	0.009[#]
Microwave extraction	5.6	0.155	---	---	---	---
Extract for analysis	3.8	0.104	---	---	---	---
Losses (not analysed)	1.8	0.051	---	---	---	---
Total extracted	**95.8**	**2.651**	**96.6**	**4.822**	**57.8**	**0.009**
Unextractable (PES*)	4.2	0.116	3.4	0.170	42.2	0.007
Accountability	**100.0**	**2.767**	**100.0**	**4.993**	**100.0**	**0.016**

* post extraction solids
[#] the conventional extract was not further analyzed by HPLC due to the low radioactivity level
n.q. not quantified (< LOQ)

The TRR and distribution of parent and metabolitesin the extracts is shown in Table 6.2.1-33 and Table 6.2.1-34. In total, 70.3% to 86.0% of the TRR were identified in the matrices of cotton after foliar application. A summary of the results is given in Table 6.2.1-35.

Table 6.2.1-33: TRR values and distribution of parent compound and metabolites in cotton matrices after a single foliar treatment of [furanone-4-^{14}C]BYI 02960

	single application experiment			
	intermediate		gin trash	
TRR [mg/kg] =	12.391		0.191	
Compound (BYI 02960-)	% TRR	mg/kg	% TRR	mg/kg
Conventional extraction				
BYI 02960 (parent compound)	42.3	5.237	37.5	0.072
glyoxylic acid	---	---	0.9	0.002
acetic acid-glyc	8.6	1.068	---	---
OH-glyc / acetic acid	24.9	3.082	12.8	0.024
OH	---	---	12.0	0.023
bromo/chloro	0.7	0.089	0.6	0.001
Subtotal identified	**76.5**	**9.476**	**63.7**	**0.122**
unknown 1	2.2	0.268	2.6	0.005
unknown 2	---	---	---	---
unknown 3	1.0	0.122	---	---
unknown 4	---	---	0.6	0.001
unknown 5	6.6	0.823	---	---
unknown 6	---	---	---	---
unknown 7	2.5	0.310	---	---
unknown 8	---	---	---	---
unknown 9	---	---	---	---
unknown 10	---	---	---	---
unknown 11	1.1	0.141	---	---
unknown 12	---	---	---	---
Subtotal characterised	13.4	1.665	3.2	0.006
Total conventional extraction	**89.9**	**11.141**	**66.9**	**0.128**
Microwave extraction				
BYI 02960 (parent compound)			2.5	0.005
acetic acid-glyc			---	---
OH-glyc / acetic acid			2.9	0.006
OH			1.2	0.002
Subtotal identified			6.6	0.013
unknown 1			4.2	0.008
unknown 3			---	---
unknown 5			---	---
unknown 7			---	---
Subtotal characterised			**4.2**	**0.008**
Total microwave extraction		**---**	**10.9**	**0.021**
Total identified	**76.5**	**9.476**	**70.3**	**0.134**
Total characterised	**13.4**	**1.665**	**7.4**	**0.014**
Analysed extracts	89.9	11.141	77.7	0.148
Not analysed/losses#	0.4	0.053	2.6	0.005
Total extracted	**90.3**	**11.194**	**80.3**	**0.153**
Unextractable (PES*)	9.7	1.197	19.7	0.038
Accountability	**100.0**	**12.391**	**100.0**	**0.191**

* post extraction solid
\# losses during clean-up, concentration, etc

Table 6.2.1-34: TRR values and distribution of parent compound and metabolites in cotton matrices after two foliar treatments of [furanone-4-^{14}C]BYI 02960

	double application experiment			
	gin trash		lint	
TRR [mg/kg] =	2.767		4.993	
Compound (BYI 02960-)	% TRR	mg/kg	% TRR	mg/kg
Conventional extraction				
BYI 02960 (parent compound)	53.3	1.476	70.3	3.512
glyoxylic acid	1.6	0.044	0.2	0.009
acetic acid-glyc	2.2	0.062	---	---
OH-glyc / acetic acid	20.0	0.553	13.9	0.694
OH	0.6	0.016	---	---
bromo/chloro	2.3	0.063	1.6	0.078
Subtotal identified	**80.0**	**2.214**	**86.0**	**4.292**
unknown 1	4.9	0.135	3.8	0.190
unknown 2	0.5	0.014	0.4	0.018
unknown 3	0.4	0.012	0.9	0.043
unknown 4	2.0	0.056	---	---
unknown 5	0.8	0.023	0.3	0.014
unknown 6	0.5	0.013	---	---
unknown 7	0.2	0.005	0.6	0.028
unknown 8	---	---	0.5	0.024
unknown 9	---	---	2.7	0.135
unknown 10	0.3	0.009	1.2	0.062
unknown 11	---	---	---	---
unknown 12	---	---	0.3	0.016
Subtotal characterised	9.7	0.268	10.6	0.530
Total conventional extraction	**89.7**	**2.482**	**96.6**	**4.822**
Microwave extraction				
BYI 02960 (parent compound)	1.1	0.029		
acetic acid-glyc	0.1	0.001		
OH-glyc / acetic acid	0.8	0.023		
OH	---	---		
Subtotal identified	**1.9**	**0.053**		
unknown 1	1.7	0.046		
unknown 3	0.1	0.001		
unknown 6	0.1	0.002		
unknown 7	0.1	0.002		
Subtotal characterised	1.8	0.051		
Total microwave extraction	**3.8**	**0.104**	**---**	**---**
Total identified	**81.9**	**2.268**	**86.0**	**4.292**
Total characterised	**11.5**	**0.319**	**10.6**	**0.530**
Analysed extracts	93.4	2.586	96.6	4.822
Not analysed/losses[#]	2.3	0.065	<0.1	<0.001
Total extracted	**95.8**	**2.651**	**96.6**	**4.822**
Unextractable (PES*)	4.2	0.116	3.4	0.170
Accountability	**100.0**	**2.767**	**100.0**	**4.993**

* post extraction solid

[#] losses during clean-up, concentration, etc

Table 6.2.1-35: Summary of characterized and identified radioactive residues in cotton matrices after one or two foliar applications of [furanone-4-^{14}C]BYI 02960

	single application exp.				double application exp.			
	intermediate		gin trash		gin trash		lint	
TRR [mg/kg] =	12.391		0.191		2.767		4.993	
Compound (BYI 02960-)	% TRR	mg/kg	% TRR	mg/kg	% TRR	mg/kg	% TRR	mg/kg
BYI 02960 (parent compound)	42.3	5.237	40.0	0.076	54.4	1.505	70.3	3.512
glyoxylic acid	---	---	0.9	0.002	1.6	0.044	0.2	0.009
acetic acid-glyc	8.6	1.068	---	---	2.3	0.063	---	---
OH-glyc / acetic acid	24.9	3.082	15.7	0.030	20.8	0.577	13.9	0.694
OH	---	---	13.1	0.025	0.6	0.016	---	---
bromo/chloro	0.7	0.089	0.6	0.001	2.3	0.063	1.6	0.078
Total identified	**76.5**	**9.476**	**70.3**	**0.134**	**81.9**	**2.268**	**86.0**	**4.292**
unknown 1	2.2	0.268	6.8	0.013	6.5	0.181	3.8	0.190
unknown 2	---	---	---	---	0.5	0.014	0.4	0.018
unknown 3	1.0	0.122	---	---	0.5	0.014	0.9	0.043
unknown 4	---	---	0.6	0.001	2.0	0.056	---	---
unknown 5	6.6	0.823	---	---	0.9	0.024	0.3	0.014
unknown 6	---	---	---	---	0.5	0.013	---	---
unknown 7	2.5	0.310	---	---	0.3	0.007	0.6	0.028
unknown 8	---	---	---	---	---	---	0.5	0.024
unknown 9	---	---	---	---	---	---	2.7	0.135
unknown 10	---	---	---	---	0.3	0.009	1.2	0.062
unknown 11	1.1	0.141	---	---	---	---	---	---
unknown 12	---	---	---	---	---	---	0.3	0.016
Total characterised	**13.4**	**1.665**	**7.4**	**0.014**	**11.5**	**0.319**	**10.6**	**0.530**
Analysed extracts	89.9	11.141	77.7	0.148	93.4	2.586	96.6	4.822
Not analysed/losses[#]	0.4	0.053	2.6	0.005	2.3	0.065	<0.1	<0.001
Total extracted	**90.3**	**11.194**	**80.3**	**0.153**	**95.8**	**2.651**	**96.6**	**4.822**
Unextractable (PES*)	9.7	1.197	19.7	0.038	4.2	0.116	3.4	0.170
Accountability	**100.0**	**12.391**	**100.0**	**0.191**	**100.0**	**2.767**	**100.0**	**4.993**

* post extraction solids
[#]. losses during clean-up, concentration, etc

III. Conclusions

Metabolism of BYI 02960 was moderate in cotton. The residues were dominated by parent compound in both the single and double application experiments. Cotton gin trash was the matrix showing the highest number of metabolites.

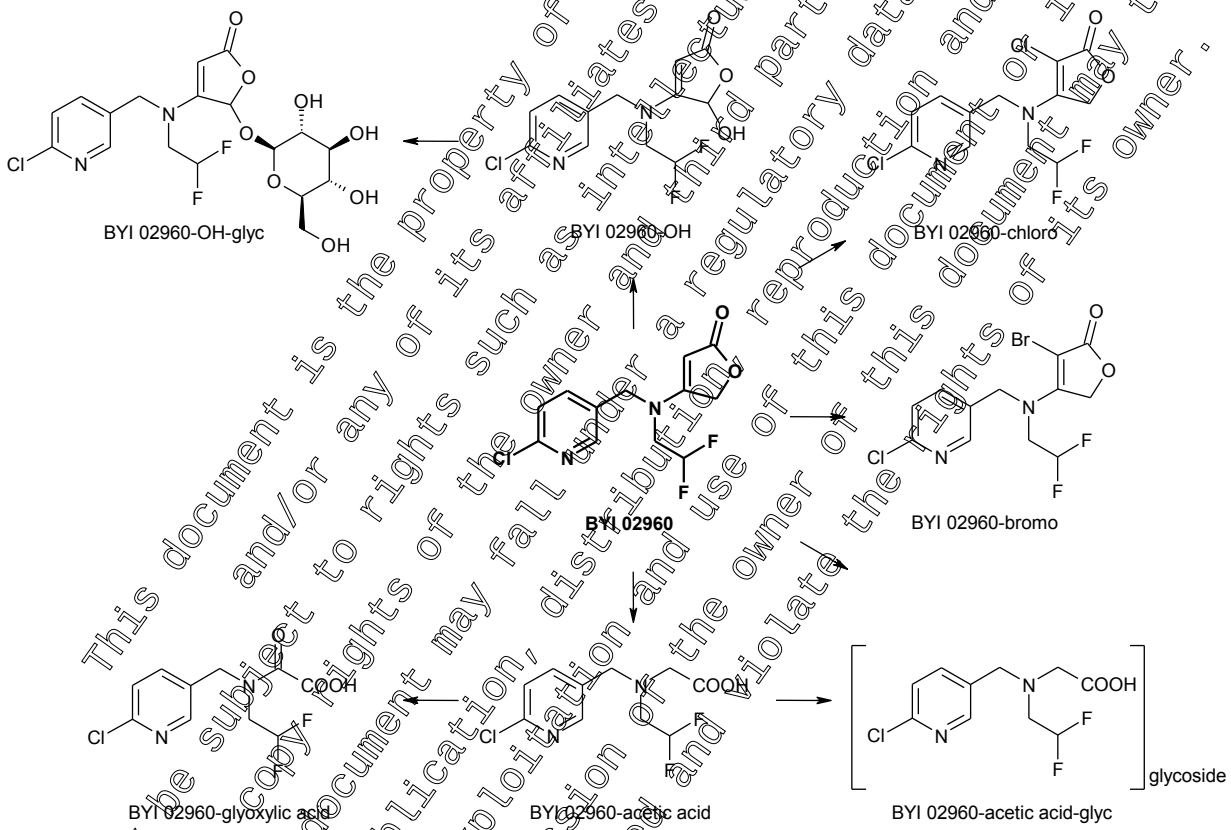
Three major metabolic routes of [furanone-4-^{14}C]BYI 02960 were observed in cotton:

- hydroxylation of the methylene group of the furanone moiety followed by conjugation (i.e. glycosylation),
- oxidative degradation of the furanone moiety to an acetic acid group followed either by conjugation with a carbohydrate or by further oxidation, and
- halogenation (bromination/chlorination) of the furanone moiety.

Halogenation of the furanone moiety of the active substance probably occurred in the soil which was supported by the fact that small amounts of halogenated parent compound were identified in the aerobic soil degradation studies. Due to the early timing of the first application, a significant portion of the parent compound could have reached the soil and can therefore been subjected to soil metabolism processes.

On the basis of the results of this study it is concluded that the metabolism of [furanone-4-^{14}C]BYI 02960 in cotton is well understood and the following metabolic pathway is proposed.

Figure 6.2.1-11: Proposed metabolic pathway of [furanone-4-^{14}C]BYI 02960 in cotton



Report:	**KIIA 6.2.1/09, [redacted], [redacted]; 2011**
Title:	Metabolism of [pyridinylmethyl-^{14}C]BYI 02960 in Cotton after Spray Application
Report No & Edition No	MEF-11/393 M-421691-01-2
Guidelines:	OECD 501 Metabolism in Crops US EPA Residue Chemistry Test Guideline OPPTS 860.1300: Nature of the Residue – Plants, Livestock PMRA Regulatory Directive Dir98-02: Residue Chemistry Guidelines Section 2: Nature of the Residue – Plants, Livestock Japanese MAFF, 12 Nousan 8147 European Parliament and Council Regulation (EC) No 1107/2009
GLP	yes

Executive Summary

The metabolism of [pyridinylmethyl-^{14}C]BYI 02960 formulated as an SL 200 was investigated in cotton after foliar spray application. In one experiment (single application experiment), five cotton plants were treated at an actual application rate of 206 g a.s./ha when the sixth true leaf was unfolded (BBCH 16), i.e. at an early growth stage. In another experiment, one cotton plant was treated two times, one time at an actual application rate of 206 g a.s./ha at BBCH 16 and a second time at an application rate of 177 g a.s./ha at 15 days before harvest of the cotton bolls (when more than 90% of the bolls were open and approx. 50% to 70% of the leaves were discoloured or fallen; BBCH 95 - 97). The total application rate in the double application experiment was 383 g a.s./ha.

Samples were taken from both experiments. Cotton seeds and gin trash were the raw agricultural commodities (RACs) harvested at maturity of the seeds in this study. Lint was collected as additional sample material at the same time. Additionally, one plant of the single application experiment was sampled at an intermediate growth stage (two to four vegetative side shoots were visible; BBCH 22 – 24) to support the identification of metabolites and to possibly obtain additional information on the metabolic degradation behaviour of BYI 02960. The TRR values of all plant matrices determined are shown in the following table:

Table 6.2.1-36: TRR values in cotton (intermediate, gin trash, lint, seeds) after foliar application of [pyridinylmethyl-^{14}C]BYI 02960

Matrix	**Timing and Application**	**PHI (days)***	**ppm (mg a.s. equiv./kg)**
intermediate	one spray application, 206 g a.s./ha (at BBCH 16)	28	14.153
gin trash		169	0.310
lint		169	0.007
seeds		169	0.045
gin trash	two spray applications, 206 g a.s./ha (at BBCH 16) 177 g a.s./ha (at BBCH 95 – 97)	15	2.344
lint		15	8.846
seeds		15	0.068

* PHI: preharvest interval (corresponds to days after last treatment (DAT) at the start of harvest/sampling)

The radioactive residues were extracted with acetonitrile/water mixtures. In the case of seeds, the samples were extracted with heptane beforehand. When necessary, additional exhaustive extraction

steps were applied to the solids of the first extraction. Extraction efficiencies ranged from 28.3% of the TRR for seeds (single application experiment) to 99.2% for lint (double application experiment). The profiles of the extracts comprising a reasonable amount of radioactivity were recorded by HPLC, and all major and several minor components were identified. Parent compound and major metabolites were isolated from a representative matrix and identified either by HPLC and TLC co-chromatography (two different chromatographic systems) using authentic reference compounds or by LC-MS/MS. Minor compounds were assigned based on the metabolite profiles obtained in the cotton metabolism study with [furanone-4-^{14}C]BYI 02960. The identification rates ranged from 16.2% of the TRR for seeds (single application experiment) to 89.7% for lint (double application experiment).

[Pyridinylmethyl-^{14}C]BYI 02960 was metabolised moderately in cotton. Parent compound was the most prominent component in all matrices, except for seeds of the single application experiment, and accounted for approx. 23% to 73% of the TRR. Additional major metabolites detected in cotton matrices were BYI 02960-acetic acid, BYI 02960-OH and the label-specific metabolite 6-CNA. All other metabolites detected represented minor or trace components.

The following metabolic routes of [pyridinylmethyl-^{14}C]BYI 02960 were observed in cotton:

- hydroxylation of the methylene group of the furanone moiety followed by conjugation,
- cleavage of the pyridinylmethylamine bond followed by oxidation of the methylene group to 6-CNA
- oxidative opening of the furanone moiety to an acetic acid group followed by further oxidation or conjugation with a carbohydrate (i.e. glycosylation), and
- halogenation (mostly bromination, to a minor extent chlorination) of the furanone moiety

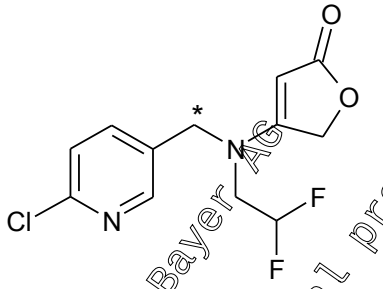
Halogenation of the furanone moiety of the active substance probably occurred in the soil which was supported by the fact that small amounts of halogenated parent compound were identified in the aerobic soil degradation studies. Due to the early timing of the first application, a significant portion of the parent compound could have reached the soil and been subjected to soil metabolism processes.

On the basis of these results, a metabolic pathway of [pyridinylmethyl-^{14}C]BYI 02960 in cotton can be proposed.

I. Materials and Methods

A. Materials

1. Test Material:

Chemical structure	 * position of the radiolabel
Radiolabelled test material	[pyridinylmethyl-^{14}C]BYI 02960
Specific radioactivity	4.37 MBq/mg (118.08 µCi/mg)
Chemical Purity	> 99% (HPLC)
Radiochemical purity	> 98% (HPLC and TLC)

The supplied radiolabelled test compound [pyridinylmethyl-^{14}C]BYI 02960 was dissolved in acetonitrile. Formulation of the test compound was performed prior to each application. For the preparation of the SL 200 formulation, an adequate portion of the stock solution was transferred into a special glass vial and evaporated to dryness. The liquid blank formulation was added to the test item using a magnetic stirrer. The mixture was diluted with water to get the aqueous application solution.

2. Soil: "Einheitserde T" (standard soil containing 2 kg/m^3 of a water soluble salt mixture), pH ($CaCl_2$) = 5.8, 85% white moor peat, 15% clay, containing NO_3^-, NH_4^+, P_2O_5, K_2O, Fe, Mn, B, Cu, Mo, Zn; distributer: [redacted]

3. Plant Cotton, variety "Carmen", Gossypium hirsutum, representative for oil seeds

B. Study Design

Experimental conditions:

Two experiments were performed with a total of six cotton plants of the variety "Carmen". The experiments represented the intended foliar spray application scenarios of BYI 02960 in cotton. In one experiment (single foliar spray application), the plants were treated at a target application rate of 200 g a.s./ha when the sixth true leaf was unfolded (BBCH 16). In the other experiment (double foliar spray application), one cotton plant was treated two times. The target application rate was 200 g a.s./ha at each application. The first application was conducted at BBCH 16 and the second at 15 days before harvest. The total target rate of 400 g a.s./ha corresponds to the anticipated maximum application rate. The cotton plants were cultivated in the standard soil "Einheitserde T" in planting pots with a surface area of approx. 0.076 m^2 and a volume of 15 L. The plants were grown in the greenhouse of the test facility which allows cultivation similar to natural temperature and light conditions. The plants were irrigated according to their needs.

Sampling:

In both experiments cotton seeds (= undelinted seeds), lint and gin trash were harvested at maturity of the seeds (BBCH 99). The cotton bolls (capsules) were cut from the plants and the seeds were separated manually from the lint. No chemical process followed to delint the seeds. The remaining plants (= gin trash) were cut off just above the soil surface and were shredded into small pieces. The gin trash chaff was combined with the empty capsules which had contained the lint and the seeds. Gin trash and seed samples were further processed using a Polytron homogenizer and liquid nitrogen. The homogenized samples were stored in a freezer (≤ -18°C) until analysis. Lint samples of the double application experiment were not submitted to any sample preparation steps and were stored in a freezer (≤ -18°C) until analysis.

In the single application experiment, additionally an intermediate was sampled at an early growth stage (two to four vegetative side shoots were visible; BBCH 22 – 24) to support the identification of metabolites. The plant was cut off just above the soil surface and was cut in small pieces prior to extraction and analysis.

C. Analytical Procedures

Extraction:

Aliquots of all sample matrices were extracted three times with a mixture of acetonitrile/water (8:2, v/v) using a high speed blender. The cotton seed samples were additionally extracted with heptane prior to the first acetonitrile/water extraction step. The radioactivity in the extracts was determined by LSC and in the solids by combustion followed by LSC. The actual TRR value of the RAC was determined by summing up the radioactivity measured in the extracts and in the remaining solids. All extracts were combined. The intermediate, gin trash and seed extracts were subjected to a clean-up step, whereas the lint extract was directly analysed by HPLC analysis after concentration. The clean-up was performed using an SPE RP 18 cartridge (Phenomenex, Strata C18-E, 20 g), which was conditioned with methanol, water and an acetonitrile/water mixture beforehand. The flow-through fraction (percolate) was collected and the cartridge was rinsed with acetonitrile/water (8:2, v/v). Elution of retained compounds was performed with methanol/dichloromethane (1:1, v/v). The percolate and the acetonitrile/water fraction (= rinse) were combined and concentrated by rotary evaporation in vacuo prior to chromatographic analysis by HPLC. The solids remaining after the conventional extraction steps of the gin trash samples were additionally submitted two times to microwave-assisted extraction with acetonitrile/water mixtures (8:2, v/v and 1:1, v/v). After each extraction step, extracts and solids were separated by filtration. The extracts were combined and concentrated by rotary evaporation in vacuo prior to HPLC analysis.

Quantification:

Parent compound and metabolites in the extracts were analysed by reversed phase HPLC coupled to a radioactivity detector with a glass scintillator cell. The HPLC chromatograms (= metabolite profiles) were integrated for quantification of compounds.

Identification and characterisation:

Identification of parent compound and all major metabolites was performed either by HPLC and TLC co-chromatography or by mass spectroscopic means after semi-preparative isolation of the compounds from a representative matrix. Minor metabolites were identified based on the assignments made in the cotton metabolism study with [furanone-4-^{14}C]BYI 02960. In that study all minor metabolites were

identified in a representative matrix by HPLC co-chromatography with an authentic reference compound. Assignment in the other matrices was performed by comparison of HPLC profiles since the metabolite profiles of all matrices were very well comparable within both cotton metabolism studies.

Storage stability:

Extraction experiments and the first HPLC analyses for quantification were performed within 9 weeks (61 days) after harvest of the cotton plants. The extracts were analysed after 6 days following the start of extraction at the latest. An exception was the analysis of the seed extracts. The extracts of the seeds of the single application experiment (1x) and the seeds of the double application experiment (2x) were analysed 35 days and 49 days following the start of extraction, respectively. The storage stability of the compounds present in the seed extracts was shown by repeating the extraction of seeds of the single application experiment after 5 months. HPLC analysis was done 8 days following the start of extraction. The metabolite profile looked very similar to the one of the first extraction. Hence, the first profile was deemed adequate for quantification despite the prolonged storage period of the extract. Additionally, stability of parent compound, 6-CNA, BYI 02960-acetic acid, BYI 02960-OH-glyc and BYI 02960-OH was proven in a grain extract for at least 21 months in the confined rotational crop study with [pyridinylmethyl-^{14}C]BYI 02960. Therefore, analysis of the extracts of seeds and quantification of the resulting metabolic profiles was deemed acceptable despite the prolonged storage period.

Since the first metabolic analysis of the RACs as well as the last experiments with extracts (HPLC co-chromatography) took place within 5.5 months, no further stability investigations were necessary according to OECD Guidance for the Testing of Chemicals 501 (2007).

II. Results and Discussion

The metabolism of [pyridinylmethyl-^{14}C]BYI 02960 was investigated in cotton seeds, gin trash and lint following two different spray scenarios. In one experiment (single application experiment), five cotton plants were treated at an actual application rate of 206 g a.s./ha when the sixth true leaf was unfolded (BBCH 16), i.e. at an early growth stage. In another experiment, one cotton plant was treated two times, one time at an actual application rate of 206 g a.s./ha at BBCH 16 and a second time at an application rate of 177 g a.s./ha at 15 days before harvest of the cotton bolls (when more than 90% of the bolls were open and approx. 50% to 70% of the leaves were discoloured or fallen; BBCH 95 - 97). The total application rate in the double application experiment was 383 g a.s./ha.

Cotton seeds, gin trash and lint were harvested for analysis from the single application experiment at 169 days after the treatment and from the double application experiment at 15 days after the last treatment. In the single application experiment, additionally one plant was sampled at an intermediate growth stage (BBCH 22-24) to support the elucidation of metabolites.

TRR levels in the double application experiment were related to the higher application rate and the late application timing. Residues in gin trash and lint were considerably higher compared to the single application experiment. As expected, residues in seeds were not strongly affected by the late second application. The TRR level in the cotton seeds, which represent the edible RAC, was low in the single

and in the double application experiment: only 0.045 mg/kg and 0.068 mg/kg were found, respectively. As expected, high residues were detected in gin trash (2.344 mg/kg) and lint (8.846 mg/kg) in the double application experiment where the last treatment was performed 15 days before harvest when more than 90% of the cotton bolls were open. In the single application experiment, the TRR in gin trash accounted for 0.310 mg/kg and the TRR in lint was < 0.01 mg/kg. Due to the negligible residues, lint of the single application experiment was not subjected to extraction.

The main portion of radioactivity in the intermediate (single application experiment), gin trash (single and double application experiment) and lint (double application experiment) was extracted conventionally by acetonitrile/water mixtures. For gin trash, additional exhaustive extraction steps were applied to the solids of the conventional extraction. Exhaustive extraction comprised two extraction steps with acetonitrile/water mixtures at increased temperature (120 °C) under microwave assistance. Extraction efficiencies ranged from 28.3% of the TRR for seeds (single application experiment) to 99.2% for lint (double application experiment) as shown in Table 6.2.1-37 and Table 6.2.1-38. HPLC analysis of the conventional and exhaustive extracts of the intermediate, gin trash, seeds and lint after both spray scenarios revealed that all metabolite profiles were very well comparable. The conventional extracts of gin trash showed the highest number of metabolites along with the extract of the intermediate. Additionally, it was shown that the metabolite profiles of all matrices were nearly identical with those obtained in the cotton metabolism study conducted with [furanone-4-^{14}C]BYI 02960.

Parent compound and major metabolites were identified either by HPLC and TLC co-chromatography (two independent chromatographic systems) or by mass spectroscopic means (LC-MS/MS) in the gin trash extracts. Minor compounds were assigned by comparison with the corresponding profiles of the cotton metabolism study with [furanone-4-^{14}C]BYI 02960.

The main compound in all cotton matrices, except for seeds of the single application experiment, was parent compound, even after only one treatment. In seeds of the single application experiment, 6-CNA was identified as main compound. This finding corresponds with the known fact that weak acids have a pronounced phloem mobility and are therefore transported selectively into the seeds as a phloem sink. Initial formation of 6-CNA took most probably place in the leaves, which is in line with the finding that 6-CNA is also the main metabolite detected in gin trash. 6-CNA was also detected in the seeds of the double application experiment, however quite high amounts of parent compound and the co-eluting compounds BYI 02960-OH-glyc and BYI 02960-acetic acid superposed the 6-CNA amounts. Most probably the high amounts of these compounds were caused by “contamination” of the seeds by lint residues. The reason contamination is proposed is that in the cotton bolls (capsules) the seeds are imbedded in the lint. Separation of seeds and lint was performed manually and resulted in undelinted seeds, i.e. the seeds were still covered by lint fibres. Thus it is not uncommon to detect the major residues of lint as well in the seed extract. Since lint showed only high residues in the double application experiment, interfering residues were only detected in seeds of this experiment.

The fraction comprising the metabolites BYI 02960-OH-glyc and BYI 02960-acetic acid represented a major portion of the TRR in all sample matrices except for seeds. These compounds co-eluted when using the neutral HPLC method for metabolite profiling. Therefore, the fraction was isolated from the

gin trash extracts and was re-analysed with an acidic mobile phase. Both compounds were very well separated using the acidic method and identification of both compounds became feasible by either HPLC co-chromatography with an authentic reference compound (BYI 02960-OH-glyc) or by LC-MS/MS (BYI 02960-acetic acid). The authentic reference compound used for co-chromatography was a metabolite isolated and identified in the apple metabolism study. The hexose in the conjugate was unambiguously assigned to D-glucose and the hydroxy group was located in the 5-position of the molecule. Separation of the compounds revealed that BYI 02960-acetic acid represented approx. 67% of the fraction in gin trash in the single application experiment and approx. 94% of the fraction in the double application experiment.

In gin trash of the first application experiment, BYI 02960-OH was identified as an additional major metabolite. All other metabolites detected in gin trash, seeds and lint and as well in the intermediate were minor, they did not exceed 10% of the TRR in either the single or the double application experiment. In a first step they were identified in gin trash by HPLC comparison. The metabolite profiles of the gin trash extracts were compared with the corresponding profiles obtained in the cotton metabolism study with [furanone-4-^{14}C]BYI 02960. Assignment of the metabolites in all other matrices was completed by comparison of the gin trash profiles with all other metabolite profiles.

Table 6.2.1-37: Distribution of radioactivity in the extracts of cotton matrices (intermediate, gin trash and seeds) after a single foliar application of [pyridinylmethyl-^{14}C]BYI 02960

	single application experiment					
	intermediate		**gin trash**		**seeds**	
TRR [mg/kg] =	14.153		0.310		0.045	
	% of TRR	mg/kg	% of TRR	mg/kg	% of TRR	mg/kg
Conventional extraction	95.0	13.449	81.5	0.253	28.3	0.013
Extract for analysis	94.2	13.336	80.6	0.250	21.9	0.010
Losses (not analysed)	0.7	0.105	0.9	0.003	6.5	0.003
Microwave extraction	---	---	10.4	0.032	---	---
Extract for analysis	---	---	10.4	0.032	---	---
Losses (not analysed)	---	---	n.q.	n.q.	---	---
Total extracted	**95.0**	**13.440**	**92.0**	**0.285**	**28.3**	**0.013**
Unextractable (PES*)	5.0	0.713	8.0	0.025	71.7	0.032
Accountability	**100.0**	**14.153**	**100.0**	**0.310**	**100.0**	**0.045**

* post extraction solids
n.q. not quantified (< LOQ)

Table 6.2.1-38: Distribution of radioactivity in the extracts of cotton matrices (gin trash, lint and seeds) after two foliar applications of [pyridinylmethyl-^{14}C]BYI 02960

	double application experiment					
	Gin trash		**Lint**		**Seeds**	
TRR [mg/kg] =	2.344		8.846		0.068	
	% of TRR	mg/kg	% of TRR	mg/kg	% of TRR	mg/kg
Conventional extraction	89.3	2.094	99.2	8.774	66.1	0.045
Extract for analysis	88.7	2.079	99.2	8.774	38.8	0.026
Losses (not analysed)	0.6	0.014	n.q.	n.q.	27.6	0.019
Microwave extraction	7.9	0.185	---	---	---	---
Extract for analysis	4.7	0.110	---	---	---	---
Losses (not analysed)	3.2	0.075	---	---	---	---
Total extracted	**97.2**	**2.279**	**99.2**	**8.774**	**66.1**	**0.045**
Unextractable (PES*)	2.8	0.065	0.8	0.073	33.9	0.023
Accountability	**100.0**	**2.344**	**100.0**	**8.846**	**100.0**	**0.068**

* post extraction solids

The TRRs and the distribution of parent and metabolites in the extracts is shown in Table 6.2.1-39 and Table 6.2.1-40. In total, 16.2% to 89.7% of the TRR were identified in the matrices of cotton after foliar application. A summary of the results is given in Table 6.2.1-41 and Table 6.2.1-42.

Table 6.2.1-39: TRR values and distribution of parent compound and metabolites in cotton matrices after a single foliar treatment of [pyridinylmethyl-^{14}C]BYI 02960

	single application experiment					
	intermediate		gin trash		seeds	
TRR [mg/kg] =	14.153		0.310		0.045	
Compound (BYI 02960-)	% TRR	mg/kg	% TRR	mg/kg	% TRR	mg/kg
Conventional extraction						
BYI 02960 (parent compound)	36.9	5.221	24.7	0.077	---	---
6-CNA	2.1	0.298	18.5	0.057	16.2	0.007
glyoxylic acid	1.5	0.209	2.1	0.007	---	---
acetic acid-glyc	6.4	0.899	---	---	---	---
OH-glyc / acetic acid	25.1	3.559	12.0	0.037	---	---
OH	1.2	0.168	13.4	0.042	---	---
bromo/chloro	0.5	0.064	---	---	---	---
Subtotal identified	73.6	10.419	70.7	0.219	16.2	0.007
unknown 1	---	---	2.5	0.008	5.7	0.003
unknown 2	---	---	0.6	0.002	---	---
unknown 3	1.4	0.196	---	---	---	---
unknown 4	2.8	0.403	3.4	0.010	---	---
unknown 5	1.1	0.159	1.0	0.003	---	---
unknown 6	6.6	0.935	0.6	0.002	---	---
unknown 7	3.9	0.551	---	---	---	---
unknown 10	1.9	0.270	---	---	---	---
unknown 11	1.9	0.273	1.2	0.004	---	---
unknown 12	0.9	0.130	0.6	0.002	---	---
Subtotal characterised	20.6	2.917	9.9	0.031	5.7	0.003
Total conventional extraction[1]	**94.2**	**13.336**	**80.6**	**0.250**	**21.9**	**0.010**
Microwave extraction						
BYI 02960 (parent compound)			1.6	0.005		
6-CNA			1.7	0.005		
OH-glyc / acetic acid			1.7	0.005		
OH			1.1	0.003		
Subtotal identified			**6.1**	**0.019**		
unknown 1			3.8	0.012		
unknown 11			0.5	0.002		
Subtotal characterised			4.3	0.013		
Total microwavee extraction	**---**	**---**	**10.4**	**0.032**	**---**	**---**
Total identified	**73.6**	**10.419**	**76.8**	**0.238**	**16.2**	**0.007**
Total characterised	**20.6**	**2.917**	**14.2**	**0.044**	**5.7**	**0.003**
Analysed extracts	94.2	13.336	91.0	0.282	21.9	0.010
Not analysed/losses[2]	0.7	0.105	0.9	0.003	6.5	0.003
Total extracted	**95.0**	**13.440**	**92.0**	**0.285**	**28.3**	**0.013**
Unextractable (PES*)	5.0	0.713	8.0	0.025	71.7	0.032
Accountability	**100.0**	**14.153**	**100.0**	**0.310**	**100.0**	**0.045**

* post extraction solid
[1] analysed extracts only
[2] losses during clean-up, concentration, etc

Table 6.2.1-40: TRR values and distribution of parent compound and metabolites in cotton matrices after two foliar treatments of [pyridinylmethyl-^{14}C]BYI 02960

	double application experiment					
	gin trash		**lint**		**seeds**	
TRR [mg/kg] =	2.344		8.846		0.068	
Compound (BYI 02960-)	% TRR	mg/kg	% TRR	mg/kg	% TRR	mg/kg
Conventional extraction						
BYI 02960 (parent compound)	51.3	1.204	73.0	6.455	23.4	0.016
6-CNA	2.0	0.047	0.4	0.031	5.0	0.003
glyoxylic acid	1.5	0.035	0.2	0.015	---	---
acetic acid-glyc	3.6	0.085	---	---	---	---
OH-glyc / acetic acid	20.6	0.484	14.6	1.295	4.9	0.003
OH	1.3	0.030	---	---	---	---
bromo/chloro	2.1	0.049	1.6	0.140	---	---
Subtotal identified	**82.6**	**1.935**	**89.7**	**7.936**	**33.3**	**0.023**
unknown 1	0.4	0.010	---	---	---	---
unknown 2	0.2	0.005	---	---	---	---
unknown 3	0.1	0.004	---	---	---	---
unknown 4	2.2	0.052	---	---	---	---
unknown 5	1.3	0.031	0.3	0.027	---	---
unknown 6	---	---	0.6	0.051	---	---
unknown 7	---	---	0.7	0.064	---	---
unknown 8	---	---	4.9	0.432	---	---
unknown 9	---	---	1.5	0.129	---	---
unknown 10	0.4	0.009	0.3	0.023	---	---
unknown 11	1.4	0.033	0.8	0.070	5.2	0.003
unknown 12	---	---	0.5	0.040	---	---
Subtotal characterised	6.1	0.144	9.5	0.837	5.2	0.003
Total conventional extraction[1]	**88.7**	**2.079**	**99.2**	**8.774**	**38.5**	**0.026**
Microwave extraction						
BYI 02960 (parent compound)	1.9	0.044				
6-CNA	0.2	0.005				
acetic acid-glyc	0.1	0.002				
OH-glyc / acetic acid	1.8	0.042				
Subtotal identified	**3.9**	**0.092**				
unknown 1	0.1	0.004				
unknown 2	0.1	0.002				
unknown 5	0.1	0.003				
unknown 6	0.2	0.004				
unknown 11	0.2	0.006				
Subtotal characterised	0.8	0.018				
Total microwave extraction	**4.7**	**0.110**	**---**	**---**	**---**	**---**
Total identified	**86.5**	**2.028**	**89.7**	**7.936**	**33.3**	**0.023**
Total characterised	**6.9**	**0.162**	**9.5**	**0.837**	**5.2**	**0.003**
Analysed extracts	93.4	2.190	99.2	8.774	38.5	0.026
Not analysed/losses[2]	3.8	0.089	<0.1	<0.001	27.6	0.019
Total extracted	**97.2**	**2.279**	**99.2**	**8.774**	**66.1**	**0.045**
Unextractable (PES*)	2.8	0.065	0.8	0.073	33.9	0.023
Accountability	**100.0**	**2.344**	**100.0**	**8.846**	**100.0**	**0.068**

* post extraction solid

[1] analysed extracts only

[2] losses during clean-up, concentration, etc

Table 6.2.1-41: Summary of characterized and identified radioactive residues in cotton matrices after one foliar application of [pyridinylmethyl-^{14}C]BYI 02960

	single application experiment					
	intermediate		**gin trash**		**seeds**	
TRR [mg/kg] =	14.153		0.310		0.045	
Compound (BYI 02960-)	% TRR	mg/kg	% TRR	mg/kg	% TRR	mg/kg
BYI 02960 (parent compound)	36.9	5.221	26.3	0.082	---	---
6-CNA	2.1	0.298	20.2	0.063	16.2	0.007
glyoxylic acid	1.5	0.209	2.1	0.007	---	---
acetic acid-glyc	6.4	0.899	---	---	---	---
OH-glyc / acetic acid	25.1	3.559	13.7	0.043	---	---
OH	1.2	0.168	14.5	0.045	---	---
bromo/chloro	0.5	0.064	---	---	---	---
Total identified	**73.6**	**10.419**	**76.8**	**0.238**	**16.2**	**0.007**
unknown 1	---	---	6.4	0.020	5.7	0.003
unknown 2	---	---	0.6	0.002	---	---
unknown 3	1.4	0.196	---	---	---	---
unknown 4	2.8	0.403	3.4	0.010	---	---
unknown 5	1.1	0.159	1.0	0.003	---	---
unknown 6	6.6	0.935	0.6	0.002	---	---
unknown 7	3.9	0.551	---	---	---	---
unknown 10	1.9	0.270	---	---	---	---
unknown 11	1.9	0.273	1.7	0.005	---	---
unknown 12	0.9	0.130	0.6	0.002	---	---
Total characterised	**20.6**	**2.917**	**14.2**	**0.044**	**5.7**	**0.003**
Analysed extracts	94.2	13.336	91.0	0.282	21.9	0.010
Not analysed/losses[#]	0.7	0.105	0.9	0.003	6.5	0.003
Total extracted	**95.0**	**13.440**	**92.0**	**0.285**	**28.3**	**0.013**
Unextractable (PES*)	5.0	0.713	8.0	0.025	71.7	0.032
Accountability	**100.0**	**14.153**	**100.0**	**0.310**	**100.0**	**0.045**

* post extraction solids
[#]. losses during clean-up, concentration, etc

Table 6.2.1-42: Summary of characterized and identified radioactive residues in cotton matrices after two foliar applications of [pyridinylmethyl-^{14}C]BYI 02960

	double application experiment					
	gin trash		**lint**		**seeds**	
TRR [mg/kg] =	2.344		8.846		0.068	
Compound (BYI 02960-)	% TRR	mg/kg	% TRR	mg/kg	% TRR	mg/kg
BYI 02960 (parent compound)	53.2	1.247	73.0	6.455	23.4	0.016
6-CNA	2.2	0.053	0.4	0.031	5.0	0.003
glyoxylic acid	1.5	0.035	0.2	0.015	---	---
acetic acid-glyc	3.7	0.087	---	---	---	---
OH-glyc / acetic acid	22.4	0.526	14.6	1.295	4.9	0.003
OH	1.3	0.030	---	---	---	---
bromo/chloro	2.1	0.049	1.6	0.140	---	---
Total identified	**86.5**	**2.028**	**89.7**	**7.936**	**33.3**	**0.023**
unknown 1	0.6	0.013	---	---	---	---
unknown 2	0.3	0.007	---	---	---	---
unknown 3	0.1	0.004	---	---	---	---
unknown 4	2.2	0.052	---	---	---	---
unknown 5	1.5	0.034	0.3	0.027	---	---
unknown 6	0.2	0.004	0.6	0.051	---	---
unknown 7	---	---	0.7	0.064	---	---
unknown 8	---	---	4.9	0.432	---	---
unknown 9	---	---	1.5	0.129	---	---
unknown 10	0.4	0.009	0.3	0.023	---	---
unknown 11	1.6	0.039	0.8	0.070	5.2	0.003
unknown 12	---	---	0.5	0.040	---	---
Total characterised	**6.9**	**0.162**	**9.5**	**0.837**	**5.2**	**0.003**
Analysed extracts	93.4	2.190	99.2	8.774	38.5	0.026
Not analysed/losses[#]	3.8	0.089	<0.1	<0.001	27.6	0.019
Total extracted	**97.2**	**2.279**	**99.2**	**8.774**	**66.1**	**0.045**
Unextractable (PES*)	2.8	0.065	0.8	0.073	33.9	0.023
Accountability	**100.0**	**2.344**	**100.0**	**8.846**	**100.0**	**0.068**

* post extraction solids
#. losses during clean-up, concentration, etc.

III. Conclusions:

[Pyridinylmethyl-^{14}C]BYI 02960 is metabolised moderately in cotton. In both the double and single application experiments, residues were dominated by parent compound except for seeds receiving a single foliar application. In total, seven different metabolites were identified in gin trash and the intermediate, which showed the most pronounced metabolite patterns.

Generally, four major metabolic routes of [pyridinylmethyl-^{14}C]BYI 02960 were observed in cotton:

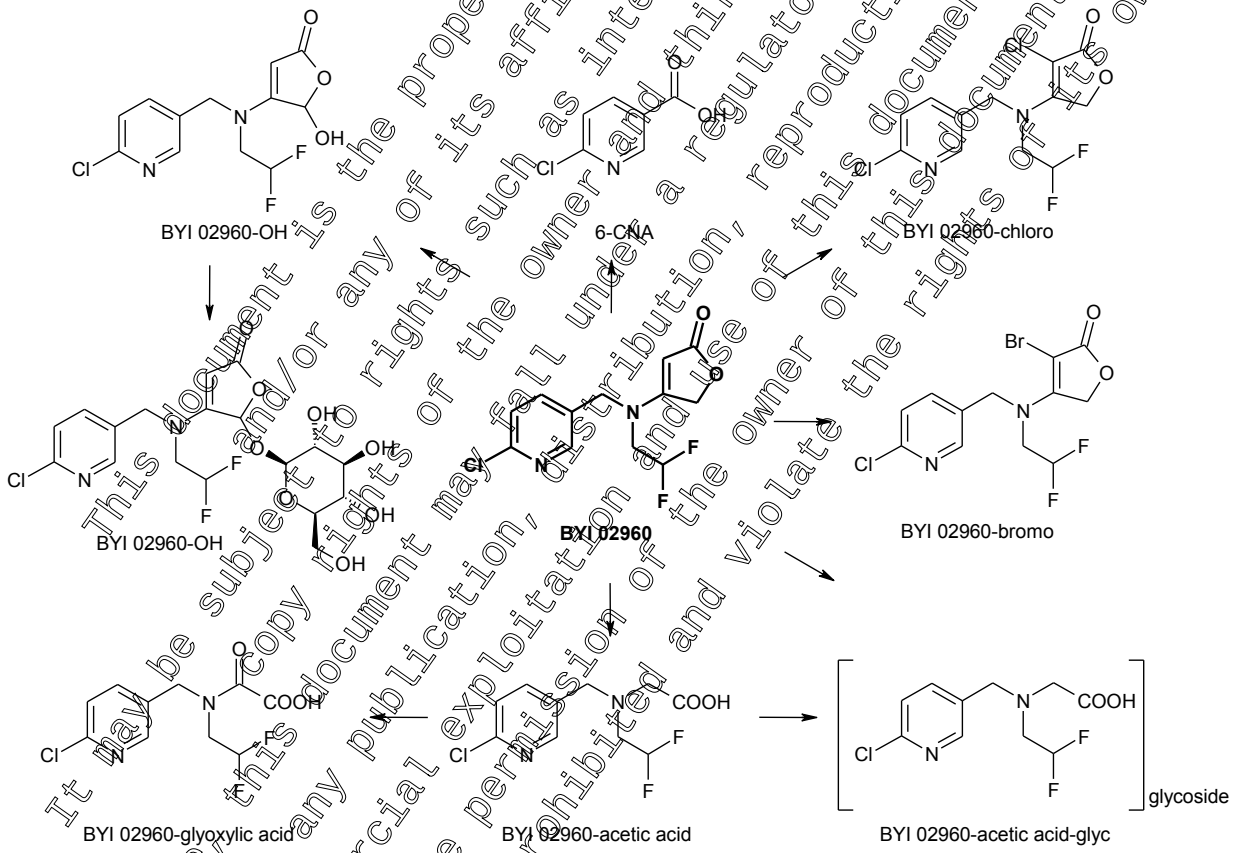
- oxidative degradation of the furanone moiety to an acetic acid group followed by conjugation with a carbohydrate or by further oxidation,
- cleavage of the pyridinylmethylamine bond followed by oxidation of the methylene group to 6-CNA,

- hydroxylation of the methylene group of the furanone moiety, followed by further conjugation steps (i.e. glycosylation), and
- halogenation (bromination and most probably chlorination, as well) of the furanone moiety.

Halogenation of the furanone moiety of the active substance probably occurred in the soil which is supported by the fact that small amounts of halogenated parent compound were identified in the aerobic soil degradation studies. Due to the early timing of the first application, a significant portion of the parent compound could have reached the soil and been subjected to soil metabolism processes.

On the basis of the results of this study it is concluded that the metabolism of [pyridinylmethyl-^{14}C]BYI 02960 in cotton is well understood and the following metabolic pathway is proposed.

Figure 6.2.1-12: Proposed metabolic pathway of [pyridinylmethyl-^{14}C]BYI 02960 in cotton



Overall Conclusions Cotton (foliar application)

The metabolism of the insecticide BYI 02960 was investigated in the raw agricultural commodities cotton seeds and gin trash and as well in lint following two different spray application scenarios of (1) [furanone-4-^{14}C]BYI 02960 or (2) [pyridinylmethyl-^{14}C]BYI 02960. In the single spray application experiment, cotton plants were treated once at an early growth stage (BBCH 16). In the double spray

application experiment, one plant was treated at the early growth stage (BBCH 15) and additionally 14 days before harvest. The target rate per application in both experiments was 200 g a.s./ha.

In order to gain information on the fate of the difluoroethane moiety of BYI 02960, the extracts obtained in the cotton metabolism study with [pyridinylmethyl-^{14}C]BYI 02960 were additionally analysed for non-radiolabelled difluoroacetic acid by LC-MS/MS according to residue method 01304 (see KIIA 6.2./12).

(1) Label 1: [furanone-4-^{14}C]BYI 02960
At harvest, the radioactive residues in cotton seeds (edible commodity) were very low in both experiments and did not exceed 0.016 mg/kg. Analysis of the extracts was not feasible and therefore, elucidation of the metabolic behaviour of BYI 02960 was based on the gin trash extracts of both experiments, an intermediate extract of the single application experiment and the lint extract of the double application experiment. As expected, the residues in the double spray application experiment were dominated by parent compound. However, even after the single application, parent compound was the main residue in all matrices analysed. One major metabolite fraction was detected in all matrices under investigation, comprising the co-eluting metabolites BYI 02960-acetic acid and BYI 02960-OH-glyc. BYI 02960-acetic acid represented the main part of the fraction in the double application experiment, whereas the dominance was less pronounced in the single application experiment. In gin trash, BYI 02960-OH was detected as additional major metabolite (12% of the TRR). All other metabolites represented less than 10% of the TRR in all matrices, with BYI 02960-acetic acid-glyc showing the highest portion (9% of the TRR) in the intermediate. Overall, three major metabolic routes were detected: (1) Hydroxylation of the methylene group of the furanone moiety followed by conjugation, (2) oxidative degradation of the furanone moiety to an acetic acid group followed either by conjugation with a carbohydrate or by further oxidation, and (3) halogenation (bromination/chlorination) of the furanone moiety. This metabolisation step probably occurred in the soil and halogenated parent compound was then taken up by the plants. No label-specific metabolites were identified. All metabolic routes were also detected in the cotton metabolism study with [pyridinylmethyl-^{14}C]BYI 02960.

(2) Label 2: [pyridinylmethyl-^{14}C]BYI 02960
As expected, the residues in the double spray application experiment were dominated by parent compound. However, even in the single spray application, parent compound was the main residue in all cotton matrices, except for seeds. In seeds, 6-CNA was identified as the only prominent compound. The metabolite patterns of all matrices were very similar in both experiments. Besides parent compound, BYI 02960-acetic acid, BYI 02960-OH-glyc, 6-CNA and BYI 02960-OH were major metabolites detected in the different matrices. Three additional minor metabolites were identified, but none of them exceeded 7% of the TRR. Overall, four major metabolic routes were detected: (1) Oxidative degradation of the furanone moiety to an acetic acid group followed by conjugation with a carbohydrate or by further oxidation, (2) cleavage of the pyridinylmethylamine bond followed by oxidation of the methylene group to 6-CNA; (3) hydroxylation of the furanone moiety of the parent compound followed by conjugation, and (4) halogenation (bromination, to a minor extent chlorination) of the furanone moiety. Only the second route led to a label-specific metabolite, all other routes were also detected in the cotton metabolism study with [furnaone-4-^{14}C]BYI 02960 which showed no label-

specific metabolites. Thus, the results of the present metabolism study in cotton are in good conformity with the results of the corresponding study performed with [furanone-4-^{14}C]BYI 02960.

When considering the results from both metabolism studies conducted on cotton, it can be concluded that BYI02960 is rather extensively metabolised in this crop. A total of 4 major and approx. 13 minor metabolites were found, and all major and 3 minor have been identified. The edible commodity cotton seed showed only very low radioactive residues and analysis of the extracts was only feasible in the study conducted with the pyridinylmethyl-label. The distribution of parent compound and metabolites in the edible commodity seeds is shown in Table 6.2.1-43; the distribution in the feed commodity gin trash is summarised in Table 6.2.1-44.

Table 6.2.1-43: TRR values and distribution of parent compound and metabolites in cotton seeds after foliar application of BYI 02960

	cotton seed			
Radiolabel	[pyridinylmethyl-^{14}C]			
	single appl.		double appl.	
TRR [mg/kg] =	0.045		0.068	
Compound (BYI 02960-)	% TRR	mg/kg	% TRR	mg/kg
BYI 02960	---	---	23.4	0.016
6-CNA	16.2	0.007	5.0	0.003
OH-glyc/ acetic acid	---	---	4.9	0.003
Total identified	**16.2**	**0.007**	**33.3**	**0.023**
Total characterised	**5.7**	**0.003**	**5.2**	**0.003**
Analysed extract(s)	21.9	0.010	38.5	0.026
Extract(s) not analysed	6.5	0.003	27.6	0.019
Total extracted	28.3	0.013	66.1	0.045
Unextractable (PES*)	71.7	0.032	33.9	0.023
Accountability	**100.0**	**0.045**	**100.0**	**0.068**

* post extraction solids

Label specific metabolites are printed in italics

Remark:
The metabolite profile of cotton seeds from the double application experiment shows most probably an interference from the lint profile since undelinted seeds were analysed.

Table 6.2.1-44: TRR values and distribution of parent compound and metabolites in gin trash after foliar application of BYI 02960

	gin trash							
Radiolabel	[furanone-4-^{14}C]				[pyridinylmethyl-^{14}C]			
	single appl.		double appl.		single appl.		double appl.	
TRR [mg/kg] =	0.191		2.767		0.310		2.344	
Compound (BYI 02960-)	% TRR	mg/kg	% TRR	mg/kg	% TRR	mg/kg	% TRR	mg/kg
BYI 02960	40.0	0.076	54.4	1.505	26.3	0.082	53.2	1.247
6-CNA					20.2	0.063	2.2	0.053
glyoxylic acid	0.9	0.002	1.6	0.044	2.1	0.007	1.5	0.035
acetic acid-glyc	---	---	2.3	0.063	---	---	3.7	0.087
OH-glyc/ acetic acid	15.7	0.030	20.8	0.577	13.7	0.043	22.4	0.526
OH	13.1	0.025	0.6	0.016	14.5	0.045	1.3	0.030
chloro/ bromo	0.6	0.001	2.3	0.063	---	---	2.1	0.049
Total identified	**70.3**	**0.134**	**81.9**	**2.268**	**76.8**	**0.238**	**86.5**	**2.028**
Total characterised	**7.4**	**0.014**	**11.5**	**0.319**	**14.2**	**0.044**	**6.9**	**0.162**
Analysed extract(s)	77.7	0.148	93.4	2.586	91.0	0.282	93.4	2.190
Extract(s) not analysed	2.6	0.005	2.3	0.065	0.9	0.003	3.8	0.089
Total extracted	80.3	0.153	95.8	2.651	92.0	0.285	97.2	2.279
Unextractable (PES*)	19.7	0.038	4.2	0.116	8.0	0.025	2.8	0.065
Accountability	**100.0**	**0.191**	**100.0**	**2.767**	**100.0**	**0.310**	**100.0**	**2.344**

* post extraction solids

Label specific metabolites are printed in italic.

Analysis of cotton seed and gin trash extracts on the non-radiolabelled metabolite difluoroacetic acid revealed that this metabolite represents a significant proportion of the residues in the single and double application experiments. Difluoroacetic acid accounted for 0.09 mg a.s. equiv./kg and 0.06 mg a.s. equiv./kg in cotton seeds in the single and the double application experiment and thus represents by far the main proportion of the residues. These findings support the assumption that difluoroacetic acid has a pronounced phloem mobility and is therefore transported selectively into the seeds as a phloem sink. In gin trash, difluoroacetic acid accounted for 0.12 mg/kg in the single application experiment and exceeded the BYI 02960 concentration. In the double application experiment, parent compound and several metabolites showed higher concentrations than difluoroacetic acid due to the short PHI of the last application.

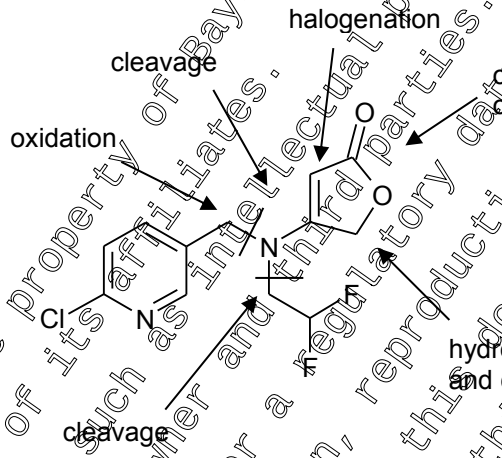
On basis of the metabolites identified, biotransformation of BYI 02960 in cotton proceeds by the following pathways:

- oxidative cleavage of the difluoroethylamine bond and formation of difluoroacetic acid
- oxidative degradation of the furanone moiety to an acetic acid group followed either by conjugation with a carbohydrate or by further oxidation to BYI 02960-glyoxylic acid
- oxidative cleavage of the pyridinylmethylamine bond and formation of 6-chloronicotinic acid (6-CNA)
- hydroxylation of the furanone moiety followed by conjugation with glucose
- halogenation (bromination and most probably chlorination, as well) of the furanone moiety

Halogenation of the furanone moiety of the active substance probably occurred in the soil which is supported by the fact that small amounts of halogenated parent compound were identified in the aerobic soil degradation studies. Due to the early timing of the first application, a significant portion of the parent compound could have reached the soil and been subjected to soil metabolism processes.

The positions involved in the metabolic degradation are summarised in the following figure.

Figure 6.2.1-13: Positions involved in metabolic degradation of BYI 02960 in cotton matrices



Metabolism, distribution and expression of residues in paddy rice (foliar or granular application)

Metabolism studies in paddy rice were conducted with [furanone-4-^{14}C]- and [pyridinylmethyl-^{14}C]BYI 02960:

Report:	**KIIA 6.2.1/10, [redacted], [redacted]; 2011**
Title:	Metabolism of [furanone-4-^{14}C]BYI 02960 in paddy rice
Report No & Edition No	MEF-11/058 M-414219-01-2
Guidelines:	OECD 501 Metabolism in Crops US EPA Residue Chemistry Test Guideline OPPTS 860.1300: Nature of the Residue – Plants, Livestock PMRA Regulatory Directive Dir98-02, Residue Chemistry Guidelines Section 2: Nature of the Residue – Plants, Livestock Japanese MAFF, 12 Nousan 8147 European Parliament and Council Regulation (EC) No 1107/2009
GLP	yes

Executive Summary

The metabolism of [furanone-4-^{14}C]BYI 02960 was investigated in paddy rice according to the maximum envisaged use pattern. Two different methods of application were covered in this study. One experiment was a granule treatment (GR) with [furanone-4-^{14}C]BYI 02960 applied during the transplanting of the rice seedlings at an actual application rate of 409 g a.s./ha. In the second experiment, [furanone-4-^{14}C]BYI 02960 was formulated as an SL 200 and applied twice as spray treatment (SP) onto plants as well as the water surface. The first spray application took place directly after transplanting of the rice seedlings at a rate of 175 g a.s./ha and the second approx. one month before harvest at a rate of 240 g a.s./ha.

At maturity the rice plants were harvested and separated into straw, kernels and husks. The TRR values of all plant matrices determined are shown in the following table:

Table 6.2.1-45: TRR values in paddy rice (kernels, husks and straw) after granular or spray treatment of [furanone-4-^{14}C]BYI 02960

Matrix	**Timing and Application**	**PHI (days)***	**ppm (mg a.s. equiv./kg)**
kernels	one granule application at transplanting of the rice seedlings (BBCH 13-15), 409 g a.s./ha	127	0.140
husks		127	1.404
straw		127	2.879
kernels	two spray applications at transplanting of the rice seedlings (BBCH 13-15) and approx. 30 days before harvest (BBCH 87-89)	29	0.659
husks		29	24.098
straw		29	19.891

* PHI: preharvest interval (corresponds to days after last treatment (DAT) at the start of harvest/sampling)

The radioactive residues were efficiently extracted with acetonitrile/water mixtures. When necessary, additional exhaustive extraction steps were applied to the solids of the first extraction. Extraction efficiencies ranged from 68.7% of the TRR for kernels (GR) to 93.6% for straw (SP) after exhaustive

extraction. The profiles of the extracts were recorded with HPLC, and all major and several minor components were identified. Identification was performed by LC-MS/MS after isolation of the compounds or by co-chromatography (HPLC or TLC) with reference compounds, as well as by comparison of HPLC profiles. The identification rates ranged from 50.1% of the TRR for kernels (GR) to 85.7% for straw (GR). [Furanone-4-^{14}C]BYI 02960 was metabolised moderately in paddy rice. Parent compound was the most prominent component and represented approx. 20 – 70% of the TRR in all matrices. Metabolite BYI 02960-chloro/bromo was detected as a major metabolite in straw after granular (GR) and spray treatment (SP) and accounted for approx. 11% of the TRR. Halogenation of the furanone moiety of the active substance probably occurred in the paddy soil. Soil contact with parent compound resulted in both experiments. Either the active substance was applied as granule in the planting holes or a significant portion was sprayed on the water surface and based on findings in aquatic metabolism studies would be expected to reach the paddy sediment. Glucose/carbohydrates deriving from BYI 02960 was also detected as major metabolite in kernels (GR) and represented 26.9% of the TRR. All other detected metabolites were minor or trace components.

The following metabolic routes of [Furanone-4-^{14}C]BYI 02960 were observed in paddy rice:

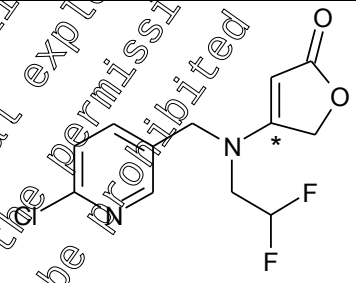
- halogenation (mostly bromination, to a minor extent chlorination) of the furanone moiety,
- hydroxylation of the methylene group of the furanone moiety,
- oxidative opening of the furanone moiety to an acetic acid group followed by further oxidation or conjugation with a carbohydrate (i.e. glycosylation), and
- complete degradation of the furanone moiety and incorporation of the carbon atoms into the natural compound pool, i.e. into glucose/carbohydrates

On the basis of these results, a metabolic pathway of [furanone-4-^{14}C]BYI 02960 in paddy rice can be proposed.

I. Materials and Methods

A. Materials

1. Test Material:

Chemical structure	 * position of the radiolabel
Radiolabelled test material	[furanone-4-^{14}C]BYI 02960
Specific radioactivity	3.94 MBq/mg (106.46 µCi/mg)
Chemical Purity	> 99% (HPLC)
Radiochemical purity	> 99% (HPLC and TLC)

The supplied radiolabelled test compound [furanone-4-^{14}C]BYI 02960 was dissolved in acetonitrile. Formulation of the test compound was performed prior to each application. For the granule

application, a GR type formulation was simulated. Therefore, an adequate portion of the stock solution was concentrated under a constant flow of nitrogen. The concentrate was then mixed with Sepiolite 30/60, a carrier granule, and the remaining solvent was evaporated yielding the ready-to-use granules. For the preparation of the SL 200 formulation, an adequate portion of the stock solution was transferred into a special glass vial and evaporated to dryness. The liquid blank formulation was added to the test item using a magnetic stirrer. The mixture was diluted with water to get the aqueous application solutions.

2. Soil: "[redacted]4" (sandy loam soil from Germany), pH ($CaCl_2$) = 6.8, 69% sand, 18% silt and 13% clay, 1.2% organic carbon, cation exchange capacity (CEC) of 8.3 meq/100 g
The soil was submerged and kept under paddy conditions. An adequate water level was maintained for optimal growing conditions.

3. Plant Rice, variety "Nihonbare", Oryza sativa L., representative for cereals

B. Study Design

Experimental conditions:

Two experiments were performed representing the intended application types in rice (granule application and spray treatment). Each experiment was conducted under paddy conditions in a planting container with a surface area of 0.5 m². The plants were cultivated in a climate chamber in a greenhouse under controlled environmental conditions.

Granule application:

The granules were distributed equally in the planting holes directly before the seedlings (BBCH 13-15) were transplanted into the holes. The target application rate was 400 g a.s./ha. After transplanting of the seedlings, the planting container was flooded with water to simulate paddy conditions. An actual amount of 80.6 MBq or 20.5 mg a.s. was applied, corresponding to 409 g a.s./ha.

Spray application:

For the spray application, a defined volume of the aqueous application solution was filled into the bottle of a hand pump sprayer. The first application was performed after transplanting of the seedlings and flooding of the planting container. The application solution was sprayed equally onto the plants and the water surface. An actual amount of 34.6 MBq or 8.8 mg a.s. was applied at the first application, corresponding to an actual application rate of 175 g a.s./ha. In the second application at 29 days before harvest, an actual amount of 240 g a.s./ha was applied (47.2 MBq or 12.0 g).

Sampling:

In both experiments, the rice was harvested at maturity (BBCH 89-92). The panicles were cut off from the plants and the rice kernels were separated from panicles and husks with an automatic husking machine. The remaining plant parts (straw) were cut off just above the soil surface with scissors. The straw was then cut into pieces and combined with the empty panicles. All plant parts were dried at room temperature for five days. The samples were further processed directly after drying using a Polytron homogenizer and liquid nitrogen. The homogenized samples (kernels, husks and straw) were stored in a freezer (≤ -18°C) until analysis.

C. Analytical Procedures

Extraction:

Prior to extraction, the homogenized sample materials were soaked in a solvent mixture of acetonitrile/water (8:2; v/v) for one night. Three extraction steps with acetonitrile/water (8:2; v/v) followed using an Ultraturrax high speed blender. The radioactivity in the extracts was determined by LSC, in the solids by combustion followed by LSC. The actual TRR values of the samples were determined by summing up the radioactivity measured in the extracts and in the remaining solids. All extracts were combined per matrix. The extracts of the sample materials husks and straw were subjected to a clean-up step using an SPE RP 18 cartridge (Phenomenex, Strata C18-E, 20 g), which was conditioned with acetonitrile/water (8:2; v/v) beforehand. The flow-through fraction (percolate) was collected and the cartridge was rinsed four times with 60 mL of acetonitrile/water (8:2; v/v). The percolate and the acetonitrile/water fraction were combined, mixed with a small amount of emulsifier and concentrated by rotary evaporation in vacuo for HPLC analysis. Non-polar contaminations on the cartridge were eluted by rinsing with methanol/dichloromethane (1:1; v/v). Volume and radioactivity of this fraction was also determined. Extracts of kernels were concentrated and analysed by HPLC without any additional purification step.

The post-extraction solids (PES) of kernels and straw of both experiments were subjected to an exhaustive extraction procedure. The solids were extracted in a first step with acetonitrile/water (8:2, v/v) and in a second step with acetonitrile/water (1:1, v/v) under microwave assistance (120 °C for 15 min.). After each extraction step, extracts and solids were separated by filtration. The radioactivity in the extracts was determined by LSC, in the solids by combustion followed by LSC. The two extracts were combined per matrix, purified by SPE and concentrated by rotary evaporation in vacuo for HPLC analysis. The solids of rice kernels of both experiments were subjected to an additional third microwave extraction step with sodium chloride/water (1:99; w/v). This exhaustive extract of the granule treatment was further purified by partitioning with dichloromethane under addition of Celite. The corresponding exhaustive extract of rice kernels from the spray treatment was subjected to a diastase treatment (pH 5, 26 °C, 20 h) before partitioning with dichloromethane and Celite. The resulting organic phases were separated from the aqueous phases, the volumes were measured and aliquots were radioassayed by LSC.

Quantification:

Parent compound and metabolites in the extracts were analysed by reversed phase HPLC coupled to a radioactivity detector with a glass scintillator cell. The HPLC chromatograms (= metabolite profiles) were integrated for quantification of compounds.

Identification and characterisation:

Identification of parent compound and metabolites common to both radiolabels was based on the metabolite profiles obtained in the rice metabolism study with [pyridinylmethyl-^{14}C]BYI 02960. In this study parent compound and all metabolites common to both radiolabels were identified by LC-MS/MS after semi-preparative isolation of the compounds or by HPLC co-chromatography with authentic reference compounds. Thus assignment of compounds in the present study was possible by comparing the metabolite profiles. Some of theses assignments were additionally confirmed by HPLC co-chromatography with reference compounds. The only label-specific metabolite detected was identified by TLC co-chromatography with the polar fraction isolated and identified in the tomato

metabolism study. Additionally, two non-polar metabolites were identified by LC-MS/MS after isolation of the compounds.

Storage stability:
All extraction experiments and the first HPLC analyses were performed within 3.5 months (104 days) after harvest of the rice plants. The extracts were analysed after 5 days following the start of extraction at the latest. Since the first metabolic analysis of the RACs as well as the last experiments with extracts (HPLC co-chromatography) took place within 6 months, no further stability investigations were necessary according to OECD Guidance for the Testing of Chemicals 501 (2007).

II. Results and Discussion

The metabolism of [furanone-4-^{14}C]BYI 02960 was investigated in rice kernels, straw and husks following two different application scenarios. In the granule application experiment, the active substance was applied on carrier granules during the transplanting of the seedlings (approx. 3 to 5 leaves unfolded, BBCH 13-15) at an application rate of 409 g a.s./ha. In the spray application experiment, rice was treated two times, one time at an actual application rate of 175 g a.s./ha directly after transplanting of the seedlings (BBCH 13-15) and a second time at an application rate of 240 g a.s./ha at 29 days before harvest. The total application rate in the double application experiment was 415 g a.s./ha. The actual single application rate of the first application was slightly below the anticipated maximum rate of 200 g a.s./ha, thus the second application was slightly overdosed to reach the intended maximum seasonal application rate of 400 g a.s./ha.

The raw agricultural commodities rice kernels and straw were collected in both experiments at maturity (BBCH 89-92), 127 and 29 days after the last application. Rice husks were sampled during the preparation of rice kernels.

The TRR levels in the samples of the granular treatment were approx. five to seventeen times lower compared to those of the spray treatment. The TRR in edible RAC rice kernels accounted for 0.140 mg/kg after granule application and for 0.659 mg/kg after spray application. Straw showed a TRR of 2.879 mg/kg after granule application, whereas the TRR amounts to 19.891 mg/kg in straw from the spray experiment.

The radioactive residues were efficiently extracted with acetonitrile/water mixtures. When necessary, additional exhaustive extraction steps were applied to the solids of the conventional extraction. Extraction efficiencies ranged from 68.7% of the TRR for kernels (GR) to 93.6% for straw (SP) after exhaustive extraction as shown in Table 6.2.1-46 and Table 6.2.1-47. Exhaustive extraction comprised two extraction steps with acetonitrile/water mixtures and for kernels one additional with an aqueous sodium chloride solution at increased temperature (120 °C) under microwave assistance.

HPLC analysis of the conventional and exhaustive extracts after both application scenarios revealed that all metabolite profiles were comparable among each other and with the profiles obtained in the rice metabolism study performed with [pyridinylmethyl-^{14}C]BYI 02960.

The main compound in all rice matrices, except for rice kernels after granule application, was parent compound. It was identified unambiguously in the extracts of straw in the rice metabolism study performed with [pyridinylmethyl-^{14}C]BYI 02960. Confirmation of the assignment was achieved in rice kernels and rice straw (RACs) in the present study by HPLC co-chromatography with a radiolabelled reference compound. The main metabolite in rice kernels after granule application was the natural compound glucose/carbohydrates. This metabolite was isolated from the conventional extract of kernels and identified by TLC co-chromatography using the glucose fraction isolated and identified in the tomato metabolism study. Since conventional solvent extraction did not release the total amount of natural compounds, an additional exhaustive extraction step with an aqueous sodium chloride solution under microwave assistance at increased temperature (120 °C) was applied. Partitioning of the extract with dichloromethane under addition of Celite showed that by far the majority of the radioactivity remained in the aqueous phase and indicated the presence of natural compounds. HPLC analysis of the aqueous phase confirmed the assumption. Nearly 90% of the extract was represented by glucose/carbohydrates. In all other matrices, glucose/carbohydrates were assigned by profile comparison.

In rice straw, BYI 02960-bromo was identified as the only major metabolite present. It co-eluted with BYI 02960-chloro, which accounted for significant lower concentrations. Both metabolites were identified by LC-MS/MS analysis in rice straw in the spray application experiment following isolation of the fraction containing both compounds. Most probably, halogenation of the furanone moiety of the active substance occurred already in the paddy soil and the metabolite was taken up by the rice plants. All other metabolites detected in the sample matrices of rice were minor metabolites. They did not exceed 10% of the TRR in both application experiments. In kernels and straw, the presence of metabolites BYI 02960-acetic acid and BYI 02960-OH was confirmed by HPLC co-chromatography and the metabolites BYI 02960-glyoxylic acid and BYI 02960-acetic acid-glyc were identified by comparing the HPLC profiles of the extracts with the corresponding ones obtained in the rice study with the other radiolabel. Co-elution of metabolite BYI 02960-OH-glyc with metabolite BYI 02960-acetic acid was not expected since HPLC-MS/MS analysis of the isolated peak in the corresponding rice metabolism study with [pyridinylmethyl-^{14}C]BYI 02960 showed only BYI 02960-acetic acid. Compounds in the extracts of husks and in the exhaustive extracts were also assigned by comparison of profiles.

Table 6.2.1-46: Distribution of radioactivity in the extracts of rice matrices after one granule application of [furanone-4-^{14}C]BYI 02960

	granule application experiment					
	kernels		husks		straw	
TRR [mg/kg] =	0.140		1.404		2.879	
	% of TRR	mg/kg	% of TRR	mg/kg	% of TRR	mg/kg
Conventionally extracted	20.5	0.029	75.6	1.062	79.7	2.294
Extract for analysis	19.2	0.027	75.6	1.062	79.0	2.275
Losses (not analysed)	1.2	0.002	n.q.	n.q.	0.7	0.020
Exhaustive solvent extr.	6.5	0.009	---	---	11.5	0.331
Extract for analysis	5.6	0.008	---	---	10.9	0.313
Losses (not analysed)	0.8	0.001	---	---	0.6	0.017
Exhaustive NaCl extraction	41.8	0.058	---	---	---	---
aqueous phase	28.6	0.040	---	---	---	---
organic phase	0.8	0.001	---	---	---	---
Losses (not analysed)	12.3	0.017	---	---	---	---
Total extracted	**68.7**	**0.096**	**75.6**	**1.062**	**91.2**	**2.625**
Unextractable (PES*)	31.3	0.044	24.4	0.342	8.8	0.254
Accountability	**100.0**	**0.140**	**100.0**	**1.404**	**100.0**	**2.879**

* post extraction solids
n.q. not quantifiable (residues < LOQ)

Table 6.2.1-47: Distribution of radioactivity in the extracts of rice matrices after two spray applications of [furanone-4-^{14}C]BYI 02960

	spray application experiment					
	kernels		husks		straw	
TRR [mg/kg] =	0.659		24.098		19.891	
	% of TRR	mg/kg	% of TRR	mg/kg	% of TRR	mg/kg
Conventionally extracted	65.8	0.433	90.9	21.917	83.7	16.643
Extract for analysis	65.8	0.433	90.4	21.790	83.3	16.561
Losses (not analysed)	n.q.	n.q.	0.5	0.127	0.4	0.081
Exhaustive solvent extr.	5.6	0.037	---	---	9.9	1.977
Extract for analysis	5.2	0.034	---	---	9.9	1.964
Losses (not analysed)	0.3	0.002	---	---	0.1	0.013
Exhaustive NaCl/ diastase extraction[1]	15.8	0.104	---	---	---	---
aqueous phase	13.7	0.090	---	---	---	---
organic phase	0.9	0.006	---	---	---	---
Losses (not analysed)	1.2	0.008	---	---	---	---
Total extracted	**87.2**	**0.574**	**90.9**	**21.917**	**93.6**	**18.620**
Unextractable (PES*)	12.8	0.085	9.1	2.182	6.4	1.271
Accountability	**100.0**	**0.659**	**100.0**	**24.098**	**100.0**	**19.891**

[1] no further HPLC analysis due to high matrix load of the extract
* post extraction solids
n.q. not quantifiable (residues < LOQ)

The TRR values and the distribution of parent compound and metabolites in the extracts is shown in Table 6.2.1-48 and Table 6.2.1-49. In total, 50.1% to 85.7% of the TRR were identified in the raw agricultural commodities of rice after granular and foliar application. A summary of the compounds identified and characterized is given in Table 6.2.1-50 and Table 6.2.1-51.

Table 6.2.1-48: TRR values and distribution of parent compound and metabolites in rice matrices after a single granule application of [furanone-4-^{14}C]BYI 02960

	granule application experiment					
	kernels		**husks**		**straw**	
TRR [mg/kg] =	0.140		1.404		2.879	
Compound (BYI 02960-)	% of TRR	mg/kg	% of TRR	mg/kg	% of TRR	mg/kg
Conventional extraction						
BYI 02960 (parent comp.)	17.5	0.024	72.3	1.016	57.6	1.658
glucose/carbohydrates	1.7	0.002	1.0	0.014	3.1	0.088
glyoxylic acid	---	---	---	---	1.7	0.050
acetic acid	---	---	0.4	0.006	1.8	0.051
OH	---	---	---	---	0.8	0.024
bromo/chloro	---	---	0.6	0.008	10.6	0.306
Subtotal identified	**19.2**	**0.027**	**74.3**	**1.043**	**75.6**	**2.176**
unknown 1	---	---	---	---	0.8	0.023
unknown 3	---	---	1.3	0.019	1.1	0.031
unknown 5	---	---	---	---	1.1	0.033
unknown 7	---	---	---	---	0.4	0.012
Subtotal characterised	---	---	1.3	0.019	3.4	0.099
Total conventional extr.	**19.2**	**0.027**	**75.6**	**1.062**	**79.0**	**2.275**
Microwave extraction						
BYI 02960 (parent comp.)	5.6	0.008			6.4	0.183
glucose/carbohydrates	---	---			2.3	0.066
glyoxylic acid	---	---			0.2	0.007
acetic acid	---	---			0.2	0.007
OH	---	---			0.2	0.005
bromo/chloro	---	---			0.8	0.022
Subtotal identified	**5.6**	**0.008**			**10.1**	**0.290**
unknown 3	---	---			0.4	0.011
unknown 4	---	---			0.3	0.007
unknown 5	---	---			0.2	0.005
Subtotal characterized	---	---			0.8	0.023
Total microwave extr.	**5.6**	**0.008**	**---**	**---**	**10.9**	**0.313**
Exhaustive NaCl extraction						
organic phase	0.8	0.001				
aqueous phase	28.6	0.040				
glucose/carbohydrates	25.2	0.035				
Subtotal identified	25.2	0.035				
unknown 2	3.4	0.005				
Subtotal characterized	4.2	0.006				
Total exh. NaCl extraction	**29.4**	**0.041**	**---**	**---**	**---**	**---**

Table continued on next page…

	granule application experiment					
	kernels		husks		straw	
Total identified	**50.1**	**0.070**	**74.3**	**1.043**	**85.7**	**2.466**
Total characterised	**4.2**	**0.006**	**1.3**	**0.019**	**4.2**	**0.122**
Analysed extract(s)	54.3	0.076	75.6	1.062	89.9	2.588
Extracts not analysed	14.4	0.020	n.q.	n.q.	1.3	0.037
Total extracted	**68.7**	**0.096**	**75.6**	**1.062**	**91.2**	**2.625**
Unextractable (PES*)	31.3	0.044	24.4	0.342	8.8	0.254
Accountability	**100.0**	**0.140**	**100.0**	**1.404**	**100.0**	**2.879**

* post extraction solids
n.q. not quantifiable (residues < LOQ)

Table 6.2.1-49: TRR values and distribution of parent compound and metabolites in rice matrices after a two spray application of [furanone-4-^{14}C]BYI 02960

	spray application experiment					
	kernels		husks		straw	
TRR [mg/kg] =	0.039		24.098		19.891	
Compound (BYI 02960-)	% of TRR	mg/kg	% of TRR	mg/kg	% of TRR	mg/kg
Conventional extraction						
BYI 02960 (parent comp.)	53.3	0.021	74.6	17.972	52.9	10.527
glucose/carbohydrates	1.9	0.012	2.1	0.497	2.0	0.401
glyoxylic acid	---	---	---	---	2.0	0.393
acetic acid-glyc	0.4	0.003	0.2	0.054	1.7	0.329
acetic acid	5.9	0.039	7.0	1.693	7.2	1.432
OH	---	---	---	---	0.4	0.081
bromo/chloro	1.7	0.011	1.6	0.389	10.1	2.016
Subtotal identified	63.1	0.416	85.5	20.605	76.3	15.179
unknown 1	---	---	0.4	0.092	0.6	0.126
unknown 2	---	---	---	---	0.3	0.053
unknown 3	0.9	0.006	0.6	0.138	0.6	0.123
unknown 4	---	---	0.1	0.033	0.1	0.025
unknown 5	---	---	---	---	0.9	0.173
unknown 6	0.9	0.006	0.4	0.103	1.2	0.229
unknown 7	0.8	0.005	2.5	0.608	0.2	0.044
unknown 9	---	---	0.9	0.211	3.1	0.609
Subtotal characterised	2.7	0.017	4.9	1.185	6.9	1.382
Total conventional extr.	**65.8**	**0.433**	**90.4**	**21.790**	**83.3**	**16.561**
Microwave extraction						
BYI 02960 (parent comp.)	3.3	0.021			3.6	0.720
glucose/carbohydrates	1.7	0.011			1.4	0.287
glyoxylic acid	---	---			0.1	0.023
acetic acid-glyc	---	---			0.7	0.132
acetic acid	0.3	0.002			0.8	0.149
OH	---	---			0.1	0.025
bromo/chloro	---	---			0.6	0.116
Subtotal identified	5.2	0.034			7.3	1.451

Table continued on next page…

	kernels		husks		straw	
unknown 1	---	---			0.3	0.062
unknown 3	---	---			0.1	0.022
unknown 5	---	---			0.2	0.033
unknown 8	---	---			0.2	0.031
unknown 9	---	---			1.8	0.359
Subtotal characterised	---	---			2.6	0.513
Total microwave extr.	**5.2**	**0.034**	**---**		**9.9**	**1.964**
Exhaustive NaCl/diastase extraction						
organic phase	0.9	0.006				
aqueous phase	13.7	0.090				
Total exhaustive extr.	**14.6**	**0.096**	**---**	**---**	**---**	
Total identified	**68.4**	**0.450**	**85.5**	**20.605**	**83.6**	**16.630**
Total characterised	**17.2**	**0.113**	**4.9**	**1.185**	**9.5**	**1.896**
Analysed extract(s)	85.6	0.564	90.4	21.790	93.1	18.526
Extracts not analysed	1.6	0.010	0.5	0.127	0.5	0.094
Total extracted	**87.2**	**0.574**	**90.9**	**21.917**	**93.6**	**18.620**
Unextractable (PES*)	12.8	0.085	9.1	2.182	6.4	1.271
Accountability	**100.0**	**0.659**	**100.0**	**24.098**	**100.0**	**19.891**

* post extraction solids

Table 6.2.1-50: Summary of characterization and identification of radioactive residues in rice matrices after one granular application of [furanone-4-^{14}C]BYI 02960

	granule application experiment					
	kernels		husks		straw	
TRR [mg/kg] =	0.140		1.404		2.879	
Compound (BYI 02960-)	% of TRR	mg/kg	% of TRR	mg/kg	% of TRR	mg/kg
Conventional extraction						
BYI 02960 (parent comp.)	23.1	0.032	72.3	1.016	64.0	1.841
glucose/carbohydrates	26.9	0.038	1.0	0.014	5.3	0.153
glyoxylic acid	---	---	---	---	2.0	0.056
acetic acid	---	---	0.4	0.006	2.0	0.058
OH	---	---	---	---	1.0	0.029
bromo/chloro	---	---	0.6	0.008	11.4	0.328
Total identified	**50.1**	**0.070**	**74.3**	**1.043**	**85.7**	**2.466**
unknown 1	---	---	---	---	0.8	0.023
unknown 2	3.4	0.005	---	---	---	---
unknown 3	---	---	1.3	0.019	1.5	0.043
unknown 4					0.3	0.007
unknown 5	---	---	---	---	1.3	0.037
unknown 7	---	---	---	---	0.4	0.012
Total characterised[1]	**4.2**	**0.006**	**1.3**	**0.019**	**4.2**	**0.122**
Analysed extract(s)	54.3	0.076	75.6	1.062	89.9	2.588
Extracts not analysed	14.4	0.020	n.q.	n.q.	1.3	0.037
Total extracted	**68.7**	**0.096**	**75.6**	**1.062**	**91.2**	**2.625**
Unextractable (PES*)	31.3	0.044	24.4	0.342	8.8	0.254
Accountability	**100.0**	**0.140**	**100.0**	**1.404**	**100.0**	**2.879**

[1] including characterization by partitioning
* post extraction solids
n.q. not quantifiable (residues < LOQ)

Table 6.2.1-51: Summary of characterization and identification of radioactive residues in rice matrices after two spray applications of [furanone-4-^{14}C]BYI 02960

	spray application experiment					
	kernels		husks		straw	
TRR [mg/kg] =	0.659		24.098		19.891	
Compound (BYI 02960-)	% of TRR	mg/kg	% of TRR	mg/kg	% of TRR	mg/kg
BYI 02960 (parent comp.)	56.6	0.373	74.6	17.972	56.5	11.247
glucose/carbohydrates	3.6	0.023	2.1	0.497	3.5	0.688
glyoxylic acid	---	---	---	---	2.1	0.416
acetic acid-glyc	0.4	0.003	0.2	0.054	2.3	0.461
acetic acid	6.1	0.040	7.0	1.693	7.9	1.581
OH	---	---	---	---	0.5	0.106
bromo/chloro	1.7	0.011	1.6	0.389	10.7	2.132
Total identified	**68.4**	**0.450**	**85.5**	**20.605**	**83.6**	**16.630**
unknown 1	---	---	0.4	0.092	0.9	0.188
unknown 2	---	---	---	---	0.3	0.053
unknown 3	0.9	0.006	0.6	0.138	0.8	0.151
unknown 4	---	---	0.1	0.033	0.1	0.025
unknown 5	---	---	---	---	1.0	0.206
unknown 6	0.9	0.006	0.4	0.103	1.2	0.229
unknown 7	0.8	0.005	2.5	0.608	0.2	0.044
unknown 8	---	---	---	---	0.2	0.031
unknown 9	---	---	0.9	0.211	4.9	0.969
Subtotal characterised	2.7	0.017	4.9	1.185	6.9	1.382
Total characterised[1]	**17.2**	**0.113**	**4.9**	**1.185**	**9.5**	**1.896**
Analysed extract(s)	85.6	0.564	90.4	21.790	93.1	18.526
Extracts not analysed	1.6	0.010	0.5	0.127	0.5	0.094
Total extracted	**87.2**	**0.574**	**90.9**	**21.917**	**93.6**	**18.620**
Unextractable (PES*)	12.8	0.085	9.1	2.182	6.4	1.271
Accountability	**100.0**	**0.659**	**100.0**	**24.098**	**100.0**	**19.891**

1 including characterization by partitioning
* post extraction solids
n.q. not quantifiable (residues < LOQ)

III. Conclusions

[Furanone-4-^{14}C]BYI 02960 was moderately metabolised in rice. Residues were dominated by parent compound in the foliar application experiment. Parent compound was also the main or the second major compound in all matrices of rice after granule application. Nevertheless, complete degradation of the furanone moiety and incorporation of carbon atoms into the natural compound pool, e.g. into glucose/carbohydrates was observed.

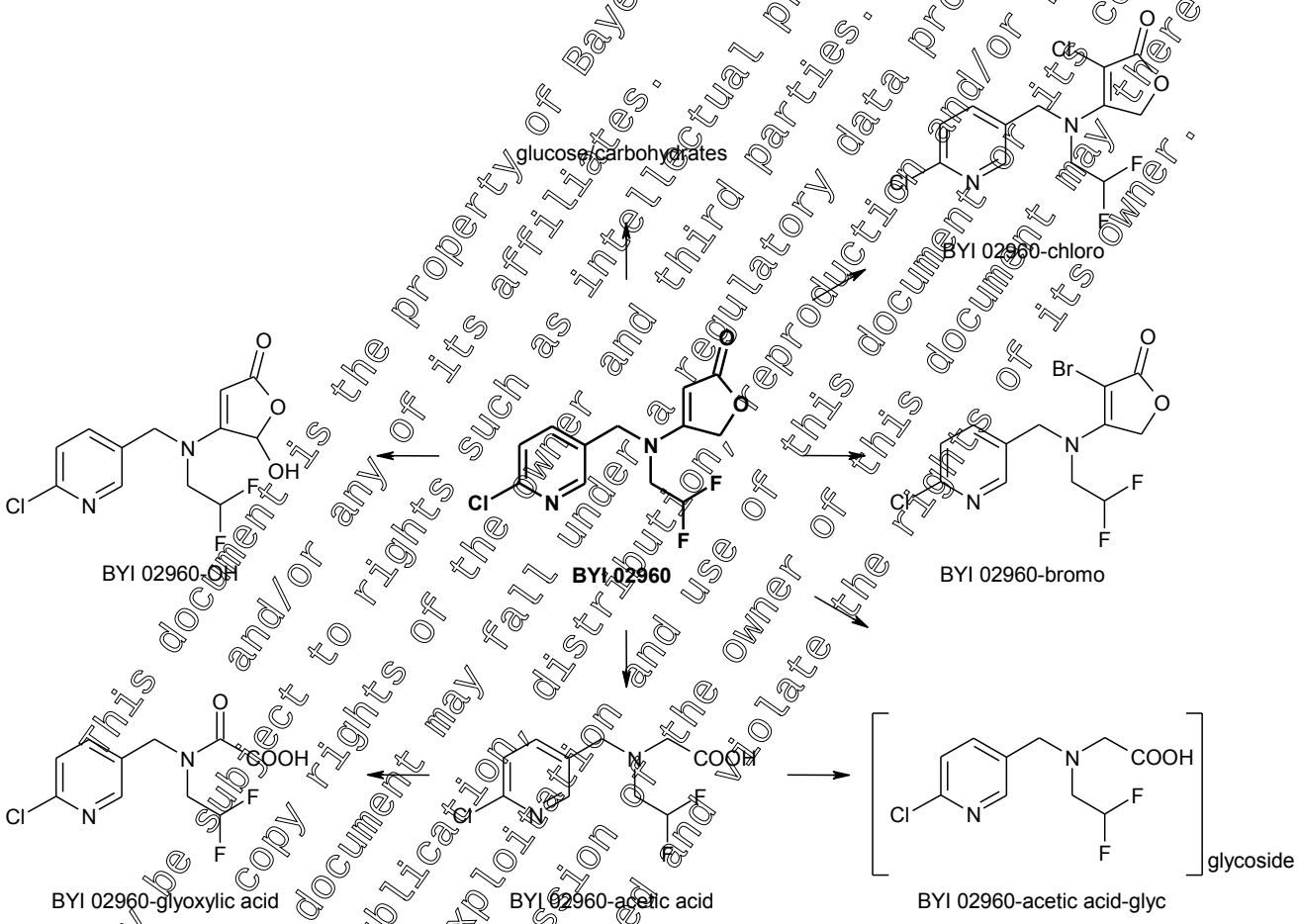
Generally, four major metabolic routes of [furanone-4-^{14}C]BYI 02960 were observed in rice:

- hydroxylation of the methylene group of the furanone moiety,
- complete degradation of the furanone moiety and incorporation of carbon atoms into the natural compound pool,
- oxidative degradation of the furanone moiety to an acetic acid group followed by conjugation with a carbohydrate or further oxidation, and
- halogenation (mostly bromination, to a minor extent chlorination) of the furanone moiety.

Halogenation of the furanone moiety of the active substance probably occurred in the paddy soil. Soil contact with parent compound resulted in both experiments.

On the basis of the results of this study it is concluded that the metabolism of [furanone-4-^{14}C]BYI 02960 in rice is well understood and the following metabolic pathway is proposed.

Figure 6.2.1-14: Proposed metabolic pathway of [furanone-4-^{14}C]BYI 02960 in rice



Report:	**KIIA 6.2.1/11, [redacted], [redacted]; 2011**
Title:	Metabolism of [pyridinylmethyl-^{14}C]BYI 02960 in paddy rice
Report No & Edition No	MEF-11/059 M-414328-01-2
Guidelines:	OECD 501 Metabolism in Crops US EPA Residue Chemistry Test Guideline OPPTS 860.1300: Nature of the Residue – Plants, Livestock PMRA Regulatory Directive Dir98-02: Residue Chemistry Guidelines Section 2: Nature of the Residue – Plants, Livestock Japanese MAFF, 12 Nousan 8147 European Parliament and Council Regulation (EC) No 1107/2009
GLP	yes

Executive Summary

The metabolism of [pyridinylmethyl-^{14}C]BYI 02960 was investigated in paddy rice according to the maximum envisaged use pattern. Two different methods of application were covered in this study. One experiment was a granule treatment (GR) with [pyridinylmethyl-^{14}C]BYI 02960 during the transplanting of the rice seedlings at an application rate of 434 g a.s./ha. In the other experiment, [pyridinylmethyl-^{14}C]BYI 02960 was formulated as an SL 200 and applied twice as spray treatment (SP) onto plants and the water surface. The first application took place directly after transplanting of the rice seedlings at a rate of 178 g a.s./ha and the second approx. one month before harvest at a rate of 236 g a.s./ha.

At maturity the rice plants were harvested and separated into straw, kernels and husks. The TRR values of all plant matrices determined are shown in the following table:

Table 6.2.1-52: TRR values in paddy rice (kernels, husks and straw) after granular or spray treatment of [pyridinylmethyl-^{14}C]BYI 02960

Matrix	Timing and Application	PHI (days)*	ppm (mg a.s. equiv./kg)
kernels	one granule application at transplanting of the rice seedlings (BBCH 13-15), 434 g a.s./ha	127	0.050
husks		127	1.602
straw		127	3.280
kernels	two spray applications at transplanting of the rice seedlings (BBCH 13-15) and approx. 30 days before harvest (BBCH 87-89)	29	0.620
husks		29	23.957
straw		29	24.731

* PHI: preharvest interval (corresponds to days after last treatment (DAT) at the start of harvest/sampling)

The radioactive residues were efficiently extracted with acetonitrile/water mixtures. When necessary, additional exhaustive extraction steps were applied to the solids of the first extraction. Extraction efficiencies ranged from 74.3% of the TRR for kernels (GR) to 97.3% for kernel (SP) after exhaustive extraction. The profiles of the extracts were recorded with HPLC and all major and several minor components were identified. Identification was performed by LC-MS/MS after isolation of the compounds or by co-chromatography (HPLC or TLC) with reference compounds, as well as by comparison of HPLC profiles. The identification rates ranged from 74.3% of the TRR for kernels (GR) to 88.9% for kernels (GR). [Pyridinylmethyl-^{14}C]BYI 02960 was metabolised moderately in

paddy rice. Parent compound was the most prominent component and represented ≥ 60% of the TRR in all matrices. BYI 02960-chloro/bromo was detected as a major metabolite in straw after granular (GR) treatment and represented 12.3% of the TRR. Halogenated parent compound was likely formed in the paddy soil and then taken up by the rice plants. Minor or trace metabolites identified were BYI 02960-6-CNA, BYI 02960-glyoxylic acid, BYI 02960-acetic acid-glyc, BYI 02960-acetic acid and BYI 02960-OH.

The following metabolic routes of [pyridinylmethyl-^{14}C]BYI 02960 were observed in paddy rice:

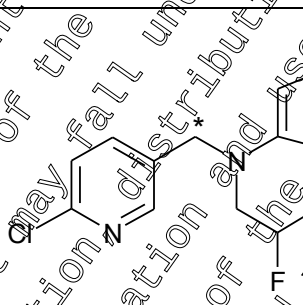
- halogenation (mostly bromination, to a minor extent chlorination) of the furanone moiety,
- hydroxylation of the methylene group of the furanone moiety,
- oxidative opening of the furanone moiety to an acetic acid group followed by further oxidation or conjugation with a carbohydrate (e.g. glycosylation), and
- cleavage of the pyridinylmethylamine bond followed by oxidation of the methylene group to chloronicotinic acid

On the basis of these results, a metabolic pathway of [pyridinylmethyl-^{14}C]BYI 02960 in paddy rice can be proposed.

I. Materials and Methods

A. Materials

1. Test Material:

Chemical structure	 * position of the radiolabel
Radiolabelled test material	[pyridinylmethyl-^{14}C]BYI 02960
Specific radioactivity	4.37 MBq/mg (118.08 µCi/mg)
Chemical Purity	> 99% (HPLC)
Radiochemical purity	> 98% (HPLC and TLC)

The supplied radiolabelled test compound [pyridinylmethyl-^{14}C]BYI 02960 was dissolved in acetonitrile. Formulation of the test compound was performed prior to each application. For the granule application, a GR type formulation was simulated. Therefore, an adequate portion of the stock solution was concentrated under a constant flow of nitrogen. The concentrate was then mixed with Sepiolite 30/60, a carrier granule, and the remaining solvent was evaporated yielding the ready-to-use granules. For the preparation of the SL 200 formulation, an adequate portion of the stock solution was transferred into a special glass vial and evaporated to dryness. The liquid blank formulation was added to the test item and the mixture was homogenized by ultrasonication. Thereafter, the mixture was diluted with water to get the aqueous application solution.

2. Soil: “[redacted]4” (sandy loam soil from Germany), pH ($CaCl_2$) = 6.8, 69% sand, 18% silt and 13% clay, 1.2% organic carbon, cation exchange capacity (CEC) of 8.3 meq/100 g
The soil was submerged and kept under paddy conditions. An adequate water level was maintained for optimal growing conditions.

3. Plant Rice, variety “Nihonbare”, Oryza sativa L., representative for cereals

B. Study Design

Experimental conditions:

Two experiments were performed representing the intended application types in rice (granule application and spray treatment). Each experiment was conducted under paddy conditions in a planting container with a surface area of 0.5 m^2. The plants were cultivated in a climate chamber in a greenhouse under controlled environmental conditions.

<u>Granule application:</u>
The granules were distributed equally in the planting holes directly before the seedlings (BBCH 13-15) were transplanted into the holes. The target application rate was 400 g a.s./ha. After transplanting of the seedlings, the planting container was flooded with water to simulate paddy conditions. An actual amount of 94.9 MBq or 21.7 mg a.s. was applied, corresponding to 434 g a.s./ha.
<u>Spray application:</u>
For the spray application, a defined volume of the aqueous application solution was filled into the bottle of a hand pump sprayer. The first application was performed after transplanting of the seedlings and flooding of the planting container. The application solution was sprayed equally onto the plants and the water surface. An actual amount of 39.0 MBq or 8.9 mg a.s. was applied at the first application, corresponding to an actual application rate of 178 g a.s./ha. In the second application at 29 days before harvest, an actual amount of 236 g a.s./ha was applied (51.5 MBq or 11.8 g).

Sampling:

In both experiments, the rice was harvested at maturity (BBCH 89-92). The panicles were cut off from the plants and the rice kernels were separated from panicles and husks with an automatic husking machine. The remaining plant parts (straw) were cut off just above the soil surface with scissors. The straw was then cut into pieces and combined with the empty panicles. All plant parts were dried at room temperature for five days. The samples were further processed directly after drying using a Polytron homogenizer and liquid nitrogen. The homogenized samples (kernels, husks and straw) were stored in a freezer (≤ -18°C) until analysis.

C. Analytical Procedures

Extraction:

Prior to extraction, the homogenized sample materials were soaked in a solvent mixture of acetonitrile/water (8:2; v/v) for one night. Three extraction steps with acetonitrile/water (8:2; v/v) followed using an Ultraturrax high speed blender. The radioactivity in the extracts was determined by LSC, in the solids by combustion followed by LSC. The actual TRR values of the samples were determined by summing up the radioactivity measured in the extracts and in the remaining solids. All extracts were combined per matrix. The extracts of the sample materials husks and straw were subjected to a clean-up step using an SPE RP 18 cartridge (Phenomenex, Strata C18-E, 20 g), which

was conditioned with acetonitrile/water (8:2; v/v) beforehand. The flow-through fraction (percolate) was collected and the cartridge was rinsed four times with 60 mL of acetonitrile/water (8:2; v/v). The percolate and the acetonitrile/water fraction were combined and concentrated by rotary evaporation in vacuo for HPLC analysis. Non-polar contaminations on the cartridge were eluted by rinsing with methanol/dichloromethane (1:1; v/v). Volume and radioactivity of this fraction was also determined. Conventional extracts of kernels were concentrated and analysed by HPLC without any additional purification step.

The post-extraction solids (PES) of kernels and straw of both experiments were subjected to an exhaustive extraction procedure. The solids were extracted in a first step with acetonitrile/water (8:2, v/v) and in a second step with acetonitrile/water (1:1, v/v) under microwave assistance (120 °C for 15 min.). After each extraction step, extracts and solids were separated by filtration. The radioactivity in the extracts was determined by LSC, in the solids by combustion followed by LSC. The two extracts were combined per matrix, purified by SPE and concentrated by rotary evaporation in vacuo for HPLC analysis. The solids of rice kernels from the spray application experiments were subjected to an additional third microwave extraction step with sodium chloride/water (1:99; w/v). Volume and radioactivity of the resulting extract was determined but it was not combined with the other microwave extracts. It was further subjected to a diastase treatment to characterize the radioactivity incorporated into starch components. Therefore, the extract was buffered with sodium acetate to maintain pH 5.0 and diastase was added. The solution was incubated for 20 h at 26°C while stirring. Afterwards, the pH of the mixture was adjusted to pH 7.0 and the supernatant was separated from the solids by centrifugation. Partitioning with acetonitrile followed after addition of sodium chloride. A second partitioning step with acetonitrile followed after adjusting the aqueous phase to pH 3. HPLC analysis of the aqueous and the organic extracts was not possible due to low radioactivity levels.

Quantification:
Parent compound and metabolites in the extracts were analysed by reversed phase HPLC coupled to a radioactivity detector with a glass scintillator cell. The HPLC chromatograms (= metabolite profiles) were integrated for quantification of compounds.

Identification and characterisation:
Identification of parent compound, the only major metabolite, and the next most prominent metabolite was performed by mass spectrometry after isolation of the compounds from the rice straw extract after spray application. Assignment of the compounds in other matrices was performed by HPLC co-chromatography or by comparison of the HPLC profiles. Additionally, four minor metabolites were identified in rice straw by HPLC co-chromatography using authentic reference compounds. Confirmation of the assignments by a second chromatographic method was not necessary due to the low residue levels. The presence of the only label-specific metabolite was confirmed in rice kernels by HPLC co-chromatography. Assignment of the other minor compounds in additional matrices was performed by comparison of the HPLC profiles.

Storage stability:
All extraction experiments and the first HPLC analyses were performed within 3.5 months (104 days) after harvest of the rice plants. The extracts were analysed after 4 days following the start of extraction at the latest. Since the first metabolic analysis of the RACs, as well as the last experiments with the

extracts (HPLC co-chromatography) took place within 6 months, no further stability investigations were necessary according to OECD Guidance for the Testing of Chemicals 501 (2007).

II. Results and Discussion

The metabolism of [pyridinylmethyl-^{14}C]BYI 02960 was investigated in rice kernels, straw and husks following two different application scenarios. In the granule application experiment, carrier granules with the active substance were placed in the planting holes during the transplanting of the seedlings (approx. 3 to 5 leaves unfolded, BBCH 13-15). A total amount of 434 g a.s./ha was applied. In the spray application experiment, rice was treated two times, one time at an actual application rate of 178 g a.s./ha directly after transplanting of the seedlings (BBCH 13-15) and a second time at an application rate of 236 g a.s./ha at 29 days before harvest for a total application rate of 414 g a.s./ha. Since the actual single application rate of the first application was below the anticipated maximum rate of 200 g a.s./ha, the second application was slightly overdosed to achieve the intended maximum seasonal application rate of 400 g a.s./ha.

The raw agricultural commodities rice kernels and straw were collected in both experiments at maturity (BBCH 89-92), 127 and 29 days after the last application. Rice husks were sampled during the preparation of rice kernels.

The TRR levels in the samples of the granular treatment were approx. seven to fifteen times lower compared to those of the spray treatment. The TRR in edible RAC rice kernels accounted for 0.050 mg/kg after granule application and for 0.620 mg/kg after spray application. Straw showed a TRR of 3.280 mg/kg after granule application, whereas the TRR amounted to 24.731 mg/kg in straw from the spray experiment.

The radioactive residues were efficiently extracted with acetonitrile/water mixtures. When necessary, additional exhaustive extraction steps were applied to the solids after conventional extraction. Extraction efficiencies ranged from 74.3% of the TRR for kernels (GR) to 97.3% for kernels (SP) after exhaustive extraction as shown in Table 6.2.1-53 and Table 6.2.1-54. Exhaustive extraction comprised two extraction steps with acetonitrile/water mixtures and for kernels (SP) one additional with an aqueous sodium chloride solution at increased temperature (120 °C) under microwave assistance. The sodium chloride extract was additionally subjected to a diastase digestion step.

HPLC analysis of the conventional and exhaustive extracts after both application scenarios revealed that all metabolite profiles were comparable among each other and with the profiles obtained in the rice metabolism study performed with [furanone-4-^{14}C]BYI 02960.

By far, the main compound in all rice matrices was parent compound. It was identified unambiguously in the conventional extract of straw (SP) by LC-MS/MS and in the exhaustive extract of straw (SP) by HPLC co-chromatography. The compound was isolated from the respective extracts and purified prior to identification. In all other matrices, parent was assigned by comparison of the metabolite profiles. In rice kernel (edible RAC), the assignment was additionally confirmed by HPLC co-chromatography with an authentic reference compound.

One major metabolite (>10% of the TRR) was detected only in rice straw from the granular experiment. The metabolite was identified as BYI 02960-bromo by LC-MS/MS. Co-elution of trace amounts of BYI 02960-chloro are possible since BYI 02960-chloro was identified in the corresponding rice metabolism study with [furanone-4-^{14}C]BYI 02960 by mass spectrometric means. Halogenation of the furanone moiety of the active substance probably occurred in the paddy soil. Soil contact with parent compound resulted in both experiments. Either the active substance was applied as granule in the planting holes or a significant portion was sprayed on the water surface and based on findings in aquatic metabolism studies BYI 02960 would be expected to reach the paddy sediment.

The most prominent metabolite in rice kernels, straw and husks after spray application was BYI 02960-acetic acid, representing between 6.5% and 7.8% of the TRR. It was isolated from straw and identified by LC-MS/MS. In all other matrices assignment of the metabolite was achieved by comparison of metabolite profiles. Additional minor metabolites identified by HPLC co-chromatography were BYI 02960-acetic acid-glyc, BYI 02960-glyoxylic acid, BYI 02960-OH and the label-specific metabolite 6-CNA

Table 6.2.1-53: Distribution of radioactivity in the extracts of rice matrices after one granule application of [pyridinylmethyl-^{14}C]BYI 02960

	granule application experiment					
	kernels		**husks**		**straw**	
TRR [mg/kg] =	0.050		1.602		3.280	
	% of TRR	mg/kg	% of TRR	mg/kg	% of TRR	mg/kg
Conventionally extracted	62.8	0.032	79.6	1.275	81.6	2.677
Extract for analysis	62.8	0.032	79.6	1.275	80.7	2.648
Losses (not analysed)	n.q.	n.q.	n.q.	n.q.	0.9	0.030
Exhaustive solvent extr.	11.5	0.006	---	---	7.7	0.252
Extract for analysis	11.5	0.006	---	---	7.2	0.237
Losses (not analysed)	n.q.	n.q.	---	---	0.5	0.016
Total extracted	**74.3**	**0.037**	**79.6**	**1.275**	**89.3**	**2.930**
Unextractable (PES*)	25.7	0.013	20.4	0.327	10.7	0.350
Accountability	**100.0**	**0.050**	**100.0**	**1.602**	**100.0**	**3.280**

* post extraction solids
n.q. not quantified (= losses were < LOQ)

Table 6.2.1-54: Distribution of radioactivity in the extracts of rice matrices after two spray applications of [pyridinylmethyl-^{14}C]BYI 02960

	spray application experiment					
	kernels		husks		straw	
TRR [mg/kg] =	0.620		23.957		24.731	
	% of TRR	mg/kg	% of TRR	mg/kg	% of TRR	mg/kg
Conventionally extracted	88.4	0.548	90.3	21.625	84.0	20.764
Extract for analysis	88.4	0.548	89.8	21.514	82.7	20.708
Losses (not analysed)	n.q.	n.q.	0.5	0.111	0.2	0.056
Exhaustive solvent extr.	5.6	0.035	---	---	9.7	2.393
Extract for analysis	4.9	0.031	---	---	9.6	2.374
Losses (not analysed)	0.7	0.004	---	---	0.1	0.020
Exhaustive NaCl/ diastase extraction[1]	3.3	0.021	---	---	---	---
aqueous phase	1.8	0.011	---	---	---	---
organic phase	1.2	0.008	---	---	---	---
Losses (not analysed)	0.3	0.003	---	---	---	---
Total extracted	**97.3**	**0.604**	**90.3**	**21.625**	**93.6**	**23.157**
Unextractable (PES*)	2.7	0.017	9.7	2.332	6.4	1.573
Accountability	**100.0**	**0.620**	**100.0**	**23.957**	**100.0**	**24.731**

[1] no further HPLC analysis due to high matrix load of the extract
* post extraction solids

The TRR values and the distribution of parent compound and metabolites in the extracts is shown in Table 6.2.1-55 and Table 6.2.1-56. A summary of the compounds identified and characterized is given in Table 6.2.1-57 and Table 6.2.1-58.

Table 6.2.1-55: TRR values and distribution of parent compound and metabolites in rice matrices after a single granule application of [pyridinylmethyl-^{14}C]BYI 02960

	granule application experiment					
	kernels		husks		straw	
TRR [mg/kg] =	0.050		1.602		3.280	
Compound (BYI 02960-)	% of TRR	mg/kg	% of TRR	mg/kg	% of TRR	mg/kg
Conventional extraction						
BYI 02960 (parent comp.)	62.8	0.032	77.7	1.244	56.2	1.842
6-CNA	---	---	0.5	0.009	2.9	0.093
glyoxylic acid	---	---	---	---	0.2	0.007
acetic acid	---	---	---	---	1.8	0.058
OH	---	---	---	---	1.1	0.037
bromo/chloro	---	---	0.6	0.010	11.7	0.385
Subtotal identified	62.8	0.032	78.8	1.263	73.9	2.423
unknown 3	---	---	---	---	0.7	0.023
unknown 4	---	---	0.8	0.012	0.7	0.022
unknown 6	---	---	---	---	0.5	0.016
unknown 7	---	---	---	---	1.4	0.047
unknown 9	---	---	---	---	0.3	0.011
unknown 11	---	---	---	---	0.4	0.015
unknown 12	---	---	---	---	0.8	0.027
unknown 13	---	---	---	---	0.9	0.031
unknown 14	---	---	---	---	1.0	0.033
Subtotal characterised	---	---	0.8	0.012	6.9	0.225
Total conventional extr.	**62.8**	**0.032**	**79.6**	**1.275**	**80.7**	**2.648**
Microwave extraction						
BYI 02960 (parent comp.)	6.8	0.003			3.7	0.122
6-CNA	4.7	0.002			1.0	0.031
acetic acid	---	---			0.2	0.006
OH	---	---			0.1	0.004
bromo/chloro	---	---			0.5	0.018
Subtotal identified	11.5	0.006			5.5	0.181
unknown 2	---	---			0.2	0.006
unknown 3	---	---			0.1	0.003
unknown 5	---	---			0.2	0.005
unknown 6	---	---			0.1	0.003
unknown 12	---	---			0.1	0.005
unknown 13	---	---			0.8	0.025
unknown 14	---	---			0.3	0.010
Subtotal characterised	---	---			1.7	0.056
Total microwave extraction	**11.5**	**0.006**	**---**	**---**	**7.2**	**0.237**
Total identified	**74.3**	**0.037**	**78.8**	**1.263**	**79.4**	**2.603**
Total characterised	**---**	**---**	**0.8**	**0.012**	**8.6**	**0.281**
Analysed extracts	74.3	0.037	79.6	1.275	87.9	2.884
Losses extracts not analysed	n.q.	n.q.	n.q.	n.q.	1.4	0.046
Total extracted	**74.3**	**0.037**	**79.6**	**1.275**	**89.3**	**2.930**
Unextractable (PES*)	25.7	0.013	20.4	0.327	10.7	0.350
Accountability	**100.0**	**0.050**	**100.0**	**1.602**	**100.0**	**3.280**

* post extraction solids

n.q. not quantified (= losses were < LOQ)

Table 6.2.1-56: TRR values and distribution of parent compound and metabolites in rice matrices after two spray applications of [pyridinylmethyl-^{14}C]BYI 02960

	spray application experiment					
	kernels		husks		straw	
TRR [mg/kg] =	0.620		23.957		24.731	
Compound (BYI 02960-)	% of TRR	mg/kg	% of TRR	mg/kg	% of TRR	mg/kg
Conventional extraction						
BYI 02960 (parent comp.)	72.0	0.447	77.3	18.526	56.9	14.071
6-CNA	2.1	0.013	0.4	0.107	0.9	0.227
glyoxylic acid	0.4	0.003	---	---	2.0	0.505
acetic acid-glyc	0.6	0.003	0.2	0.048	1.2	0.308
acetic acid	7.8	0.048	6.5	1.548	6.6	1.631
OH	0.4	0.002	---	---	0.6	0.146
bromo/chloro	1.5	0.009	1.2	0.295	7.9	1.955
Subtotal identified	84.8	0.526	85.7	20.524	76.2	18.844
unknown 1	---	---	---	---	0.1	0.033
unknown 2	---	---	---	---	0.1	0.031
unknown 3	---	---	---	---	0.3	0.079
unknown 4	---	---	0.4	0.085	0.3	0.068
unknown 5	---	---	---	---	0.2	0.047
unknown 6	---	---	0.2	0.044	0.2	0.049
unknown 7	---	---	---	---	0.1	0.022
unknown 8	---	---	---	---	0.2	0.042
unknown 9	0.5	0.003	0.4	0.090	0.8	0.188
unknown 10	1.2	0.008	1.7	0.401	0.3	0.068
unknown 11	---	---	---	---	0.3	0.070
unknown 12	---	---	0.2	0.044	0.7	0.171
unknown 13	1.1	0.007	0.4	0.106	1.1	0.279
unknown 14	0.8	0.005	0.9	0.221	2.9	0.718
Subtotal characterised	3.6	0.022	4.1	0.990	7.5	1.864
Total conventional extr.	**88.4**	**0.548**	**89.8**	**21.514**	**83.7**	**20.708**
Microwave extraction						
BYI 02960 (parent comp.)	3.2	0.020			3.9	0.958
6-CNA	0.9	0.006			0.3	0.073
glyoxylic acid	---	---			0.2	0.052
acetic acid-glyc	---	---			0.7	0.166
acetic acid	---	---			0.7	0.183
OH	---	---			0.2	0.043
bromo/chloro	---	---			0.6	0.139
Subtotal identified	4.1	0.026			6.5	1.615
unknown 1	---	---			<0.1	0.011
unknown 2	---	---			0.1	0.036
unknown 13	0.8	0.005			1.1	0.276
unknown 14	---	---			1.8	0.435
Subtotal characterized	0.8	0.005			3.1	0.758
Total microwave extraction	**4.9**	**0.031**	**---**	**---**	**9.6**	**2.374**

Table continued on next page…

	kernels		husks		straw	
Exhaustive NaCl / diastase extraction						
organic phase	1.8	0.011				
aqueous phase	1.2	0.007				
Total exhaust. NaCl / diastase extraction	**3.1**	**0.021**	**---**	**---**	**---**	**---**
Total identified	**88.9**	**0.552**	**85.7**	**20.524**	**82.7**	**20.460**
Total characterised	**7.4**	**0.045**	**4.1**	**0.990**	**10.6**	**2.622**
Analysed extract(s)	96.4	0.597	89.8	21.514	93.3	23.082
Extracts not analysed	0.9	0.007	0.5	0.111	0.3	0.075
Total extracted	**97.3**	**0.604**	**90.3**	**21.625**	**93.6**	**23.157**
Unextractable (PES*)	2.7	0.017	9.7	2.332	6.4	1.573
Accountability	**100.0**	**0.620**	**100.0**	**23.957**	**100.0**	**24.731**

* post extraction solids

Table 6.2.1-57: Summary of characterization and identification of radioactive residues in rice matrices after one granular application of [pyridinylmethyl-^{14}C]BYI 02960

	granule application experiment					
	kernels		husks		straw	
TRR [mg/kg] =	0.050		1.602		3.280	
Compound (BYI 02960-)	% of TRR	mg/kg	% of TRR	mg/kg	% of TRR	mg/kg
BYI 02960 (parent comp.)	69.6	0.035	77.7	1.244	59.9	1.964
6-CNA	4.7	0.002	0.5	0.009	3.8	0.125
glyoxylic acid	---	---	---	---	0.2	0.007
acetic acid	---	---	---	---	1.9	0.063
OH	---	---	---	---	1.2	0.040
bromo/chloro	---	---	0.6	0.010	12.3	0.403
Total identified	**74.3**	**0.037**	**78.8**	**1.263**	**79.4**	**2.603**
unknown 2	---	---	---	---	0.2	0.006
unknown 3	---	---	---	---	0.8	0.026
unknown 4	---	---	0.08	0.012	0.7	0.022
unknown 5	---	---	---	---	0.2	0.005
unknown 6	---	---	---	---	0.6	0.019
unknown 7	---	---	---	---	1.4	0.047
unknown 9	---	---	---	---	0.3	0.011
unknown 11	---	---	---	---	0.4	0.015
unknown 12	---	---	---	---	1.0	0.032
unknown 13	---	---	---	---	1.7	0.056
unknown 14	---	---	---	---	1.3	0.044
Total characterised	**---**	**---**	**0.8**	**0.012**	**8.6**	**0.281**
Analysed extract(s)	74.3	0.037	79.6	1.275	87.9	2.884
Losses/extracts not analysed	n.q.	n.q.	n.q.	n.q.	1.4	0.046
Total extracted	**74.3**	**0.037**	**79.6**	**1.275**	**89.3**	**2.930**
Unextractable (PES*)	25.7	0.013	20.4	0.327	10.7	0.350
Accountability	**100.0**	**0.050**	**100.0**	**1.602**	**100.0**	**3.280**

* post extraction solids
n.q. not quantified (< LOQ)

Table 6.2.1-58: Summary of characterization and identification of radioactive residues in rice matrices after two spray applications of [pyridinylmethyl-^{14}C]BYI 02960

	spray application experiment					
	kernels		husks		straw	
TRR [mg/kg] =	0.620		23.957		24.731	
Compound (BYI 02960-)	% of TRR	mg/kg	% of TRR	mg/kg	% of TRR	mg/kg
BYI 02960 (parent comp.)	75.2	0.467	77.3	18.526	60.8	15.029
6-CNA	3.1	0.019	0.4	0.107	1.2	0.301
glyoxylic acid	0.4	0.003	---	---	2.3	0.557
acetic acid-glyc	0.6	0.003	0.2	0.048	1.9	0.475
acetic acid	7.8	0.048	6.5	1.548	7.3	1.804
OH	0.4	0.002	---	---	0.8	0.189
bromo/chloro	1.5	0.009	1.2	0.295	8.5	2.094
Total identified	**88.9**	**0.552**	**85.7**	**20.524**	**82.7**	**20.460**
unknown 1	---	---	---	---	0.2	0.043
unknown 2	---	---	---	---	0.3	0.068
unknown 3	---	---	---	---	0.3	0.079
unknown 4	---	---	0.4	0.085	0.3	0.068
unknown 5	---	---	---	---	0.2	0.047
unknown 6	---	---	0.2	0.044	0.2	0.049
unknown 7	---	---	---	---	0.1	0.022
unknown 8	---	---	---	---	0.2	0.042
unknown 9	0.3	0.003	0.4	0.090	0.8	0.188
unknown 10	1.2	0.008	1.7	0.401	0.3	0.068
unknown 11	---	---	---	---	0.3	0.070
unknown 12	---	---	0.2	0.044	0.7	0.171
unknown 13	1.9	0.012	0.4	0.106	2.2	0.555
unknown 14	0.8	0.005	0.9	0.221	4.7	1.154
Total characterised	**7.4**	**0.045**	**4.1**	**0.990**	**10.6**	**2.622**
Analysed extract(s)	96.4	0.597	89.8	21.514	93.3	23.082
Losses/extracts not analysed	0.9	0.007	0.5	0.111	0.3	0.075
Total extracted	**97.3**	**0.604**	**90.3**	**21.625**	**93.6**	**23.157**
Unextractable (PES*)	2.7	0.017	9.7	2.332	6.4	1.573
Accountability	**100.0**	**0.620**	**100.0**	**23.957**	**100.0**	**24.731**

III. Conclusions

[Pyridinylmethyl-^{14}C]BYI 02960 is metabolised moderately in rice. Residues were dominated by parent compound in the foliar application experiment, and parent compound was also the main compound in all matrices of rice after granule application. In total, seven different metabolites were identified in rice straw, which shows the most pronounced metabolite pattern. Only one of these metabolites (the halogenated parent compound) represented more than 10% of the TRR (approx. 12%).

Generally, four major metabolic routes of [pyridinylmethyl-^{14}C]BYI 02960 were observed in rice:

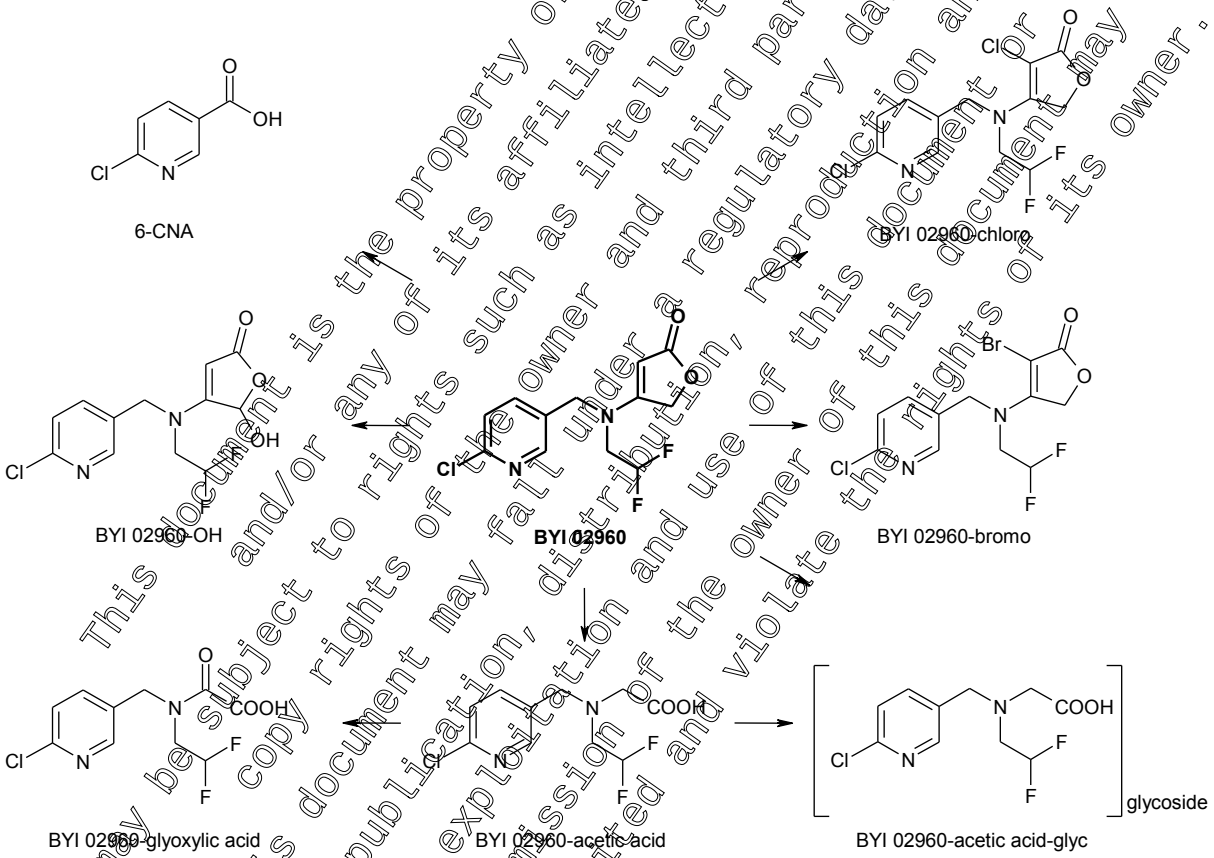
- hydroxylation of the methylene group of the furanone moiety,
- cleavage of the pyridinylmethylamine bond followed by oxidation of the methylene group to 6-CNA,

- oxidative degradation of the furanone moiety to an acetic acid group followed by either conjugation with a carbohydrate or further oxidation, and
- halogenation (mostly bromination, to a minor extent chlorination) of the furanone moiety,

It is considered likely that halogenation of the furanone moiety of the active substance occurred in the paddy soil/sediment.

On the basis of the results of this study it is concluded that the metabolism of [pyridinylmethyl-^{14}C]BYI 02960 in rice is well understood and the following metabolic pathway is proposed.

Figure 6.2.1-15: Proposed metabolic pathway of [pyridinylmethyl-^{14}C]BYI 02960 in rice



Overall Conclusions Rice (foliar and granule application)

The metabolism of the insecticide BYI 02960 was investigated in rice kernels, straw and husks following two different application scenarios of (1) [furanone-4-^{14}C]BYI 02960 or (2) [pyridinylmethyl-^{14}C]BYI 02960. In a granule application experiment, rice was treated once at transplanting of the plants (BBCH 13-15), and in a spray application experiment, the plants were treated at transplanting and additionally 30 days before harvest. The total target application rate in both experiments was 400 g a.s./ha. Regardless of the application rate, the TRR values in all rice matrices were significant lower after the granule application compared to the foliar application. As

expected, the residues in the spray application experiments were dominated by parent compound in both studies. But, even after the early granule application, parent compound was the main residue in rice husks and straw and, if not the main, a prominent residue in kernels. Subsequent analysis of the extracts on the non-radiolabelled metabolite difluoroacetic acid – which cannot be detected with the radiolabels used – confirmed that parent compound represented always the highest proportion of the residue, except for rice kernels after granular application, where difluoroacetic acid was the main constituent.

(1) Label 1: [furanone-4-^{14}C]BYI 02960

The lowest residue levels were detected in rice kernels, irrespective from the application scenario. After granule application, the main residue in rice kernels was represented by a natural compound (glucose/carbohydrates), indicating a quite intense degradation of the furanone moiety, whereas parent compound was the main compound after spray application.

The residue levels in straw and husks were significantly higher (by a factor of 10 to 36), but the metabolite patterns were very comparable for all matrices in both experiments. Besides parent compound or glucose, an additional major metabolite (>10% of the TRR) was detected only in rice straw: BYI 02960-bromo, co-eluting with small amounts of BYI 02960-chloro. In total, four additional minor metabolites were identified, but none of them exceeded 8% of the TRR. Overall, four major metabolic routes were detected: (1) Oxidative degradation of the furanone moiety to an acetic acid group followed by further oxidation or conjugation with a carbohydrate, (2) complete degradation of the furanone moiety and incorporation of carbon atoms into the natural compound pool, i.e. into glucose/carbohydrates; (3) hydroxylation of the furanone moiety of the parent compound and (4) halogenation (bromination, to a minor extent chlorination) of the furanone moiety. However, it is considered likely that the halogenation of the furanone moiety of BYI 02960 occurred in the paddy soil/sediment and was not a transformation path in the plant.

Only the second route led to a label-specific metabolite. A corresponding counterpart was detected in the rice study performed with [pyridinylmethyl-^{14}C]BYI 02960. The concentrations of the metabolites common to both radiolabels tested correspond very well when comparing the two metabolism studies conducted in rice. Thus, the results of the present metabolism study are in good conformity with the results of the corresponding study performed with [pyridinylmethyl-^{14}C]BYI 02960.

(2) Label 2: [pyridinylmethyl-^{14}C]BYI 02960

As for the study with the furanone-label, the lowest residue levels were detected in rice kernels, irrespective from the application scenario. The residue levels in straw and husks were significantly higher (by a factor of 32 to 66), but the metabolite patterns were very comparable for all matrices in both experiments. Besides parent compound, only in rice straw one additional major metabolite (>10% of the TRR) was detected: BYI 02960-bromo, most probably co-eluting with small amounts of BYI 02960-chloro. These halogenated metabolites were most likely not formed in the plants, but in the paddy soil/sediment and taken up by the plants. Overall, five additional minor metabolites were identified, but none of them exceeded 8% of the TRR. Based on the metabolites identified, four major metabolic routes were detected: (1) Oxidative degradation of the furanone moiety to an acetic acid group followed by either conjugation with a carbohydrate or further oxidation, (2) cleavage of the pyridinylmethylamine bond followed by oxidation of the methylene group to 6-CNA; (3)

hydroxylation of the furanone moiety of the parent compound and (4) halogenation (bromination, to a minor extent chlorination) of the furanone moiety. The halogenated parent compound was probably formed in the paddy soil and was taken up by the rice plants.

Only the second route led to a label-specific metabolite, all other routes were also detected in the rice metabolism study with [furnaone-4-^{14}C]BYI 02960. The concentrations of the metabolites common to both radiolabels tested correspond very well. Thus, the results of the present metabolism study in rice are in good conformity with the results of the corresponding study performed with [furanone-4-^{14}C]BYI 02960.

When considering the results from both metabolism studies conducted in rice, it can be concluded that BYI02960 is rather extensively metabolised in this crop. A total of 3 major and approx. 25 minor metabolites were found, and all major and 5 minor have been identified. The edible commodity rice kernel showed low radioactive residues, especially after granule application. The distribution of parent compound and metabolites in the edible commodity rice kernels is shown in Table 6.2.1-59.

Table 6.2.1-59: TRR values and distribution of parent compound and metabolites in rice kernels after granule and foliar application of BYI 02960

	rice kernels							
Radiolabel	[furanone-4-^{14}C]				[pyridinylmethyl-^{14}C]			
	granule application		foliar application		granule application		foliar application	
TRR [mg/kg] =	0.140		0.659		0.050		0.620	
Compound (BYI 02960-)	% TRR	mg/kg	% TRR	mg/kg	% TRR	mg/kg	% TRR	mg/kg
BYI 02960	23.1	0.032	56.6	0.373	69.6	0.035	75.2	0.467
glucose/carbohydrates	*26.9*	*0.038*	*3.6*	*0.023*				
6-CNA					*4.7*	*0.002*	*3.1*	*0.019*
glyoxylic acid	---	---	---	---	---	---	0.4	0.003
acetic acid-glyc	---	---	0.4	0.003	---	---	0.6	0.003
acetic acid	---	---	6.1	0.040	---	---	7.8	0.048
OH	---	---	---	---	---	---	0.4	0.002
chloro/ bromo	---	---	1.7	0.011	---	---	1.5	0.009
Total identified	**50.1**	**0.070**	**68.4**	**0.450**	**74.3**	**0.037**	**88.9**	**0.552**
Total characterised	**4.2**	**0.006**	**17.2**	**0.113**	**---**	**---**	**7.4**	**0.045**
Analysed extract(s)	54.3	0.076	85.6	0.564	74.3	0.037	96.4	0.597
Extract(s) not analysed	14.4	0.020	1.6	0.010	---	---	0.9	0.007
Total extracted	68.7	0.096	87.2	0.574	74.3	0.037	97.3	0.604
Unextractable (PES*)	31.3	0.044	12.8	0.085	25.7	0.013	2.7	0.017
Accountability	**100.0**	**0.140**	**100.0**	**0.659**	**100.0**	**0.050**	**100.0**	**0.620**

* post extraction solids

Label specific metabolites are printed in italic.

Analysis of the extracts of rice kernels and the feed items husk and straw on the non-radiolabelled metabolite difluoroacetic acid revealed that this metabolite represents a significant proportion of the residues in rice kernels after granule and spray application and in husks and straw after granule application. It is also detected in rice husks and straw after spray application, but only as minor metabolite. Difluoroacetic acid accounted for 0.06 mg a.s. equiv./kg and 0.24 mg a.s. equiv./kg in rice

kernel after granule application and after spray application, respectively. These findings support the assumption that difluoroacetic acid has a pronounced phloem mobility and is therefore transported selectively into the seeds as a phloem sink. In rice straw and husks, the difluoroacetic acid concentrations ranged between 0.36 mg/kg and 1.38 mg/kg. Generally, higher concentrations of difluoroacetic acid were detected after spray application.

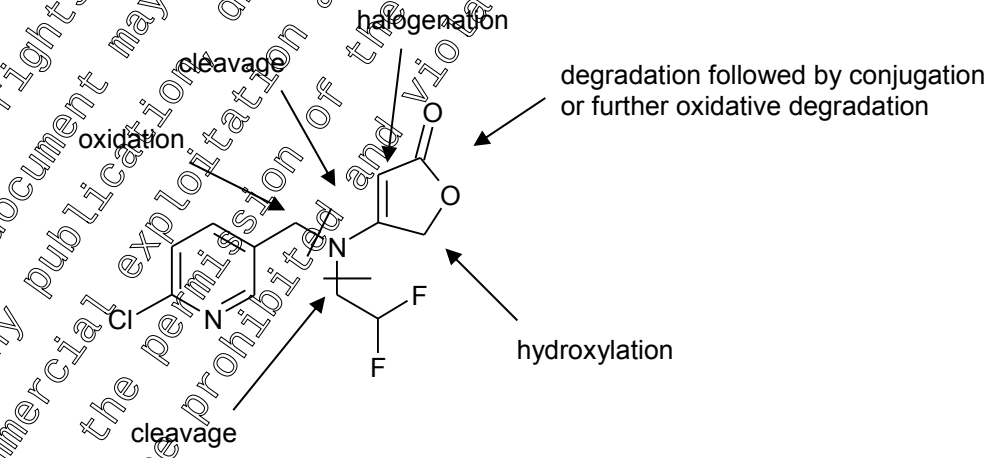
On basis of the metabolites identified, biotransformation of BYI 02960 in rice proceeds by the following pathways:

- oxidative cleavage of the difluoroethylamine bond and formation of difluoroacetic acid
- oxidative degradation of the furanone moiety to an acetic acid group followed either by conjugation with a carbohydrate or by further oxidation to BYI 02960-glyoxylic acid
- complete degradation of the furanone moiety and incorporation of carbon atoms into the natural compound pool, e.g. into glucose/carbohydrates
- oxidative cleavage of the pyridinylmethylamine bond and formation of 6-chloronicotinic acid (6-CNA)
- hydroxylation of the furanone moiety
- halogenation (bromination and most probably chlorination, as well) of the furanone moiety

It is considered likely that halogenation of the furanone moiety of the active substance occurred in the paddy soil/sediment. Uptake of the soil metabolite results in plant residues.

The positions involved in the metabolic degradation are summarised in the following figure.

Figure 6.2.1-16: Positions involved in metabolic degradation of BYI 02960 in rice matrices



Analysis on difluoroacetic acid to get information on the fate of the difluoroethane moiety of parent compound BYI 02960

Extracts of all target and confined rotational crops were analysed for difluoroacetic acid. The extracts originated from the metabolism studies conducted with either [furanone-4-^{14}C]- or [pyridinylmethyl-^{14}C]BYI 02960:

Report:	**KIIA 6.2.1/12, [redacted], [redacted]; 2012**
Title:	Determination of residues of difluoroacetic acid in extracts of samples from plant metabolism and confined rotational crops studies after application of BYI 02960
Report No & Edition No	MR-11/050 M-422550-01-1
Guidelines:	OECD 501 Metabolism in Crops US EPA Residue Chemistry Test Guideline OPPTS 860.1300: Nature of the Residue – Plants, Livestock PMRA Regulatory Directive Dir98-02; Residue Chemistry Guidelines Section 2: Nature of the Residue – Plants, Livestock Japanese MAFF, 12 Nousan 8147 European Parliament and Council Regulation (EC) No 1107/2009
GLP	yes

Executive Summary

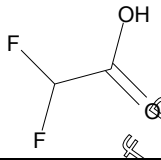
Due to the fact that only one tomato metabolism study was performed with [ethyl-1-^{14}C]BYI 02960, additional information on the fate of the difluoroethane moiety of BYI 02960 was provided in the present report. Residues of non-radiolabelled difluoroacetic acid, the most plausible metabolite that can be formed from the difluoroethane moiety due to cleavage of BYI 02960, were determined in extracts of samples of apples, potatoes, cotton, rice, wheat, Swiss chard and turnips originating from plant metabolism and confined rotational crops studies after application of [pyridinylmethyl-^{14}C]BYI 02960 or [furanone-4-^{14}C]BYI 02960. To estimate the residue levels of difluoroacetic acid in these crop samples, non-radiolabelled difluoroacetic acid was analyzed according to the provisions of residue analytical method 01304. As expected from the results of the tomato metabolism study performed with [ethyl-1-^{14}C]BYI 02960, difluoroacetic acid was detected in all crops as a major metabolite accounting for a significant proportion of the BYI 02960 residue.

Material and Methods

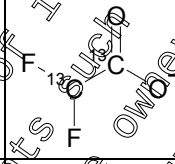
Samples of apples, potatoes, cotton, rice, wheat, Swiss chard and turnips were harvested and extracted in different plant metabolism and confined rotational crop studies following application of either [furanone-4-^{14}C]BYI 02960 or [pyridinylmethyl-^{14}C]BYI 02960. Aliquots of the crude extracts of all RACs were diluted for analysis of the non-radiolabelled soil and plant metabolite difluoroacetic acid. After dilution an isotopically labelled internal standard solution was added and the extract was analysed by LC-MS/MS according to residue analytical method 01304. No further sample work up was performed.

The quantification was done by external standardisation in pure solvent using an internal stable labelled standard. During each set of analyses, a calibration curve was established with two measurements on at least five concentration levels for each sample material. The Limit of Quantification (LOQ), defined as the lowest validated fortification level of recovery experiments, was set at 0.01 mg/kg. Difluoroacetic acid was determined as difluoroacetic acid and residues were calculated as difluoroacetic acid.

1. Reference item:

Chemical structure	
Name of Compound	Difluoroacetic acid (BCS-AA56716, DFA)
Certificate of Analysis	AZ 16523, dated 2010-03-31
Chemical name	Difluoroacetic acid
Purity	98.3% (w/w)

2. Internal Standard:

Chemical structure	
Name of Compound	Difluoroacetic acid $^{13}C_2$ (BCS-AB60481-ISTD, DFA-ISTD)
Certificate of Analysis	KATH 15199-1-4, dated 2010-06-14
Chemical name	Sodium difluoro($^{13}C_2$)acetate
Purity	> 99%

Findings

The residue levels determined in the samples from the different metabolism studies are summarized in the following tables. The residues are expressed as difluoroacetic acid equivalents.

Table 6.2.1-60: Summary of difluoroacetic acid residues in crop matrices after spray application of BYI 02960

Metabolism study	Crop	Sample material	Use pattern	Residues [mg/kg]	Reference in dossier
Metabolism of [furanone-4-^{14}C]-BYI 02960 in apples	apple	fruits	one foliar spray application at BBCH 69, 86 g a.s./(ha x m CH)	0.23	KIIA 6.2.1/06
		leaves		0.62	
		fruits	two foliar spray applications, at BBCH 69 and 14 days PHI, 2 x 86 g a.s./(ha x m CH)	0.04	
		leaves		0.45	
Metabolism of [pyridinylmethyl-^{14}C]-BYI 02960 in potatoes	potato	tuber	tuber treatment at planting (BBCH 03), 10.0 g a.s./dt	0.13	KIIA 6.2.1/05
			in-furrow spray application at planting (BBCH 03), 626 g a.s./ha	0.18	
Metabolism of [pyridinylmethyl-^{14}C]-BYI 02960 in Cotton after Spray Application	cotton	gin trash	one spray application, 206 g a.s./ha (at BBCH 16)	0.04	KIIA 6.2.1/09
		seeds		0.03	
		gin trash	two spray applications, 206 g a.s./ha (at BBCH 16), 177 g a.s./ha (at BBCH 95 - 97)	0.02	
		seeds		0.02	
Metabolism of [pyridinylmethyl-^{14}C]-BYI 02960 in paddy rice	rice	straw	two spray applications onto the plants at different growth stages, 178 g a.s./ha (at BBCH 13 - 15) 236 g a.s./ha (at BBCH 87 - 89	0.39	KIIA 6.2.1/11
		husk		0.46	
		grains		0.08	
		straw	one granular application at the time of transplanting, 434 g a.s./ha (at BBCH 13 - 15)	0.12	
		husk		0.20	
		grains		0.02	

Table 6.2.1-61 Summary of difluoroacetic acid residues in rotational crop matrices after soil application of BYI 02960

Metabolism study	Rotation	Crop	Sample material	Residues [mg/kg]	Reference in dossier
Metabolism of [furanone-4-^{14}C]BYI 02960 in Confined Rotational Crops	1st rotation	wheat	forage	0.09	KIIA 6.2.2/01
			hay	0.32	
			straw	0.20	
			grain	1.15	
		Swiss chard	intermediate	0.08	
			mature	0.16	
		turnip	leaves	0.08	
			roots	0.02	
	2nd rotation	wheat	forage	0.02	
			hay	0.14	
			straw	0.06	
			grain	0.26	
		Swiss chard	intermediate	0.04	
			mature	0.05	
		turnip	leaves	0.03	
			roots	0.01	
	3rd rotation	wheat	forage	< 0.01	
			hay	0.01	
			straw	0.02	
			grain	0.05	
		Swiss chard	intermediate	< 0.01	
			mature	0.01	
		turnip	leaves	< 0.01	
			roots	< 0.01	

Conclusions

Significant levels of difluoroacetic acid (DFA) were detected in most of the primary and confined rotational crops under investigation irrespective of the application technique. High difluoroacetic acid concentrations after foliar spray application indicate that this metabolite is also formed in plants and not only in soil.

Overall Conclusions considering radiolabelled and non-radiolabelled metabolites

Difluoroacetic acid represents the main - or at least a major - proportion of the residue in all edible matrices of primary crops when considering the results of the studies conducted with [^{14}C]BYI 02960 as shown in Table 6.2.1-62 and Table 6.2.1-63 (see also KIIA 6.11.1).

Table 6.2.1-62: Residues [mg/kg] of BYI 02960 and major metabolites in edible matrices after one application

Compound (BYI 02960-)	**potato tubers**				**apple fruits**		**rice kernels**		**cotton seeds**	
	tuber treatment		in-furrow appl.		foliar appl.		granule appl.		foliar appl.	
Label	F	P	F	P	F	P	F	P	F	P
TRR	**0.078**	**0.076**	**0.171**	**0.115**	**0.280**	**0.079**	**0.140**	**0.050**	**0.013**[2]	**0.045**
BYI 02960	0.031	0.031	0.097	0.051	0.021	0.034	0.032	0.035		
DFA		0.39[1]		0.54[1]	0.69[1]			0.06[1]		0.09[1]
glucose	---		---		0.201		0.038			
6-CNA		0.016		0.021		0.004		0.002		0.007
CHPM-di-glyc		0.003		0.006		---		---		
CHMP-glyc		0.003		0.003		0.004		---		
difluoroethyl-amino-furanone	0.003		0.005		0.009		---			
OH-glyc	0.005	0.005	0.007	0.005	0.001	0.004	---	---		

[1] LC-MS/MS analysis of non-radiolabelled DFA in the extract obtained in the metabolism study
[2] analysis of the extracts was not feasible due low extraction efficiency and high matrix load in the extracts
F = [furanone-4-^{14}C]-label P = [pyridinylmethyl-^{14}C]-label

Table 6.2.1-63: Residues [mg/kg] of BYI 02960 and major metabolites in edible matrices after two applications

Compound (BYI 02960-)	**tomato fruits**			**apple fruits - with surface wash**		**apple fruits - w/o surface wash**		**rice kernels**		**cotton seeds**	
	drench application			spray application				spray appl.		spray appl.	
Label	F	P	E	F	P	F	P	F	P	F	P
TRR	**0.096**	**0.130**	**0.201**	**1.133**	**1.868**	**1.286**	**0.545**	**0.659**	**0.620**	**0.016**[2]	**0.068**
BYI 02960	0.034	0.031	0.020	0.809	1.652	0.946	0.467	0.373	0.467		0.016
DFA			0.174				0.12[1]		0.24[1]		0.06[1]
glucose	0.026			0.193		0.182		0.023			
6-CNA		0.017			0.009		0.008		0.019		0.003
CHPM-di-glyc		0.048			---		---		---		
CHMP-glyc		0.007			0.010		0.005		---		
difluoroethyl-amino-furanone	0.010		0.004	0.007		0.003		---			
OH-glyc	0.005	0.004	0.001	0.014	0.024	0.014	0.009	---	---		0.003

[1] LC-MS/MS analysis of non-radiolabelled DFA in the extract obtained in the metabolism study
[2] analysis of the extracts was not feasible due low extraction efficiency and high matrix load in the extracts
F = [furanone-4-^{14}C]-label E = [ethyl-1-^{14}C] P = [pyridinylmethyl-^{14}C]-label

In most of the non-edible matrices difluoroacetic acid was also detected at high concentrations, however in lower proportions of the total residue. For example, the non-radioactive DFA residue

accounted for 0.23 mg/kg in apple fruits after one application of [furanone-4-^{14}C]BYI 02960, corresponding to 0.69 mg a.s. equiv./kg. Comparing this residue value with the total radioactive residue determined in the fruits of the study (0.28 mg a.s. equiv./kg), it can be estimated that DFA would represent the main portion of the TRR (approx. 90%) in a hypothetical apple metabolism study performed with [ethyl-1-^{14}C]BYI 02960. The proportion of DFA in apple leaves was much lower: Only approx. 5% of the hypothetical TRR would be represented by DFA, although the absolute residue level was high (0.62 mg/kg, corresponding to 1.86 mg a.s. equiv./kg).

Thus, based on the DFA residue values determined in primary crops, high DFA proportions can be expected in all edible matrices: For apple fruits (single spray treatment), potato tubers (tuber treatment and in-furrow application), cotton seeds (single and double spray experiment) and rice kernels (granular application) it can be estimated that DFA accounts for a higher proportion than parent compound or the main compound detected in the studies with radiolabelled BYI 02960. Thus based upon these findings it can be concluded that DFA is a major plant metabolite in edible crops and should be part of the residue definition for data collection and enforcement.

The difluoroacetic acid residues determined in matrices of confined rotational crops revealed the same picture. For the edible crops wheat grains, Swiss chard and turnip roots, difluoroacetic acid accounted also for a high proportion of the residue, especially in the early rotations. In wheat grains, difluoroacetic acid represented by far the main proportion of a hypothetical TRR (for more details please refer to KIIA 6.11.1) in all three rotations as shown in th following tables.

Table 6.2.1-64: Residues [mg/kg] of BYI 02960 and major metabolites in the matrices of rotational crops (1st rotation)

Compound (BYI 02960-)	**wheat forage**		**wheat hay**		**wheat straw**		**wheat grain**	
Label	F	P	F	P	F	P	F	P
TRR	**0.783**	**1.407**	**2.003**	**2.409**	**6.290**	**9.015**	**0.478**	**0.177**
BYI 02960	0.365	0.640	0.672	0.676	2.459	3.261	0.002	0.015
DFA	0.27[1]		0.96[1]		0.60[1]		3.45[1]	
glucose	---		---		---		0.338	
bromo-amino-furanone	0.016		0.033		0.172			---
difluoroethyl-amino-furanone	0.077		0.205		0.374		---	
glyoxylic acid	0.124	0.172	0.227	0.176	0.965	0.615	0.024	0.011
OH-glyc	0.028	0.048	0.067	0.135	0.242	0.296	0.007	0.009
6-CNA-glycerol-gluA (2 + 3)		0.199		0.569		1.900		0.036
OH	0.010	0.019	0.038	0.052	0.161	0.239	0.011	0.019

(2 + 3) isomer 2 and/or isomer 3
F = [furanone-4-^{14}C]-label P = [pyridinylmethyl-^{14}C]-label

Compound (BYI 02960-)	**Swiss chard intermediate**		**Swiss chard mature**		**turnip leaves**		**turnip roots**	
Label	F	P	F	P	F	P	F	P
TRR	**0.848**	**1.358**	**0.871**	**1.483**	**0.679**	**0.815**	**0.074**	**0.072**
BYI 02960	0.460	0.779	0.371	0.687	0.437	0.508	0.041	0.042
DFA	0.24[1]		0.48[1]		0.24[1]		0.06[1]	
glucose	---		---		---		0.003	
bromo-amino-furanone	---		---		---		---	
difluoroethyl-amino-furanone	---		0.010		---		---	
glyoxylic acid	0.031	0.021	0.041	0.039	0.045	0.021	0.009	0.006
OH-glyc	0.072	0.101	0.119	0.162	0.076	0.076	<0.001	0.002
6-CNA-glycerol-gluA (2 + 3)		---		---		---		---
OH	0.017	0.024	0.017	0.023	0.012	0.011	<0.001	<0.001

[1] LC-MS/MS analysis of non-radiolabelled DFA in the extract obtained in the confined rotation crop study performed with [furanone-4-^{14}C]BYI 02960
(2 + 3) isomer 2 and/or isomer 3
F = [furanone-4-^{14}C]-label P = [pyridinylmethyl-^{14}C]-label

Table 6.2.1-65: Residues [mg/kg] of BYI 02960 and major metabolites in the matrices of rotational crops (2nd rotation)

Compound (BYI 02960-)	**wheat forage**		**wheat hay**		**wheat straw**		**wheat grain**	
Label	F	P	F	P	F	P	F	P
TRR	**0.193**	**0.308**	**1.081**	**1.009**	**1.519**	**2.148**	**0.103**	**0.057**
BYI 02960	0.124	0.183	0.314	0.283	0.538	0.804	0.001	0.001
DFA	0.06[1]		0.42[1]		0.18[1]		0.78[1]	
glucose	---		---		---		---	
bromo-amino-furanone	0.006		0.107		0.093		---	
difluoroethyl-amino-furanone	0.016		0.075		0.090		---	
glyoxylic acid	<0.001	---	0.020	0.010	0.018	0.036	0.001	0.001
OH-glyc	0.007	0.008	0.037	0.045	0.08	0.112	0.002	0.003
6-CNA-glycerol-gluA (2 + 3)	---	0.044	---	0.242	---	0.472	---	0.007
OH	0.003	0.005	0.021	0.019	0.047	0.079	0.003	0.004

(2 + 3) isomer 2 and/or isomer 3
F = [furanone-4-^{14}C]-label P = [pyridinylmethyl-^{14}C]-label

Compound (BYI 02960-)	**Swiss chard intermediate**		**Swiss chard mature**		**turnip leaves**		**turnip roots**	
Label	F	P	F	P	F	P	F	P
TRR	**0.311**	**0.332**	**0.263**	**0.438**	**0.158**	**0.230**	**0.014**	**0.022**
BYI 02960	0.171	0.170	0.072	0.108	0.108	0.153	0.004	0.011
DFA	0.12[1]		0.15[1]		0.09[1]		<0.003[1]	
glucose	---		---		---		0.002	
bromo-amino-furanone	---		---		---		---	
difluoroethyl-amino-furanone	0.032		0.046		0.002		---	
glyoxylic acid	---	---	---	---	0.002	---	<0.001	---
OH-glyc	0.036	0.058	0.047	0.111	0.020	0.025	<0.001	0.001
6-CNA-glycerol-gluA (2 + 3)		---		---		---		---
OH	0.006	0.013	0.006	0.016	0.002	0.004	---	---

[1] LC-MS/MS analysis of non-radiolabelled DFA in the extract obtained in the confined rotation crop study performed with [furanone-4-^{14}C]BYI 02960
(2 + 3) isomer 2 and/or isomer 3
F = [furanone-4-^{14}C]-label P = [pyridinylmethyl-^{14}C]-label

Table 6.2.1-66: Residues [mg/kg] of BYI 02960 and major metabolites in the matrices of rotational crops (3rd rotation)

Compound (BYI 02960-)	**wheat forage**		**wheat hay**		**wheat straw**		**wheat grain**	
Label	F	P	F	P	F	P	F	P
TRR	**0.111**	**0.117**	**0.254**	**0.321**	**0.462**	**0.491**	**0.047**	**0.017**
BYI 02960	0.048	0.053	0.047	0.063	0.096	0.129	0.001	0.002
DFA	<0.03[1]		0.03[1]		0.06[1]		0.15[1]	
glucose	---		---		---		---	
bromo-amino-furanone	0.006		0.027		0.034		---	
difluoroethyl-amino-furanone	0.013		0.021		0.024		---	
glyoxylic acid	---	0.001	0.001	---	---	0.013	---	---
OH-glyc	0.003	0.004	0.006	0.010	0.016	0.014	0.001	0.001
6-CNA-glycerol-gluA (2 + 3)		0.022		0.095		0.140		0.002
OH	0.001	0.001	0.004	0.005	0.010	0.010	0.001	0.001

(2 + 3) isomer 2 and/or isomer 3
F = [furanone-4-^{14}C]-label P = [pyridinylmethyl-^{14}C]-label

Compound (BYI 02960-)	**Swiss chard intermediate**		**Swiss chard mature**		**turnip leaves**		**turnip roots**	
Label	F	P	F	P	F	P	F	P
TRR	**0.180**	**0.135**	**0.152**	**0.130**	**0.090**	**0.083**	**0.008**	**0.008**
BYI 02960	0.066	0.042	0.051	0.036	0.065	0.058	0.006	0.005
DFA	<0.03[1]		0.03[1]		<0.03[1]		<0.03[1]	
glucose	---		---		---		<0.001	
bromo-amino-furanone	---		---		---		---	
difluoroethyl-amino-furanone	0.025		0.024		0.001		---	
glyoxylic acid	<0.001	0.003	---	0.001	0.002	---	---	---
OH-glyc	0.040	0.033	0.033	0.036	0.009	0.008	<0.001	<0.001
6-CNA-glycerol-gluA (2 + 3)		---		---		---		---
OH	0.007	0.005	0.005	0.004	0.001	---	---	---

[1] LC-MS/MS analysis of non-radiolabelled DFA in the extract obtained in the confined rotation crop study performed with [furanone-4-^{14}C]BYI 02960
(2 + 3) isomer 2 and/or isomer 3
F = [furanone-4-^{14}C]-label P = [pyridinylmethyl-^{14}C]-label

High difluoroacetic acid concentrations were also detected for the non-edible RACs, but parent compound represented generally the main or at least a comparable residue. In corresponding crop samples from different plant back intervals, the DFA levels generally decreased significantly from the first to the third rotation, showing DFA levels slightly above or below the limit of quantification in all crops of the third rotation. The highest DFA proportion in crops of the third rotation was detected in wheat grains (0.05 mg/kg).

Thus, based on the difluoroacetic acid residue values determined, high DFA proportions can be expected in confined rotational crops, as well. Based upon these findings it can be concluded that difluoroacetic acid is a major metabolite in edible matrices of rotational crops and should be part of the residue definition for data collection and enforcement.

The analyses on non-radiolabelled difluoroacetic acid has shown that this metabolite can be found at significant residue levels/proportions in fruits, tubers, roots and in seeds. On basis of these results, difluoroacetic acid is expected to have a pronounced phloem mobility and will therefore be transported in these repository parts of plants known as phloem sinks.

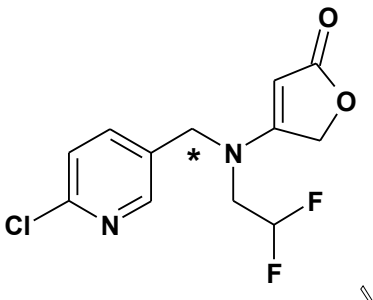
Additional non-GLP studies -initiated to gain information on the systemicity of DFA - confirmed this assumption. In one study BYI 02960 was applied on cucumber leaf sheaths (leaf 1 to 5) and the residue levels of BYI 02960, difluoroacetic acid, BYI 02960-difluoroethyl-amino-furanone and 6-CNA were determined in mature fruits of lower and higher plant parts at different time points (3, 7, 14, 21 and 28 days after application). Only difluoroacetic acid was detected in significant concentrations in fruits of the upper and lower plant parts indicating phloem mobility of this metabolite. Highest DFA levels (approx. 0.5 mg/kg) were found in cucumbers sampled 14 and 21 days after application. BYI 02960-difluoroethyl-amino-furanone was not detected in cucumber fruits, and parent compound BYI 02960 and 6-CNA were detected in trace levels only in some fruit samples. This experiment shows clearly that difluoroacetic acid is phloem mobile and can be transported within the plant.

With this information, an additional experiment was conducted. [Ethyl-1-^{14}C]BYI 02960 was applied on cucumber leaves (scenario 1: two single droplets on either side of the midrib of leaf 4 and scenario 2: one single droplet on the axil of leaf 4) and the phloem translocation of the radioactivity in lower and upper plant parts was detected at three different time points by employing a Fuji BAS 5000® phosphor-imaging system. The visualized distribution of the radioactivity confirmed phloem mobile compounds. With the radiolabel chosen, it is very likely that metabolite difluoroacetic acid is the phloem mobile compound detected.

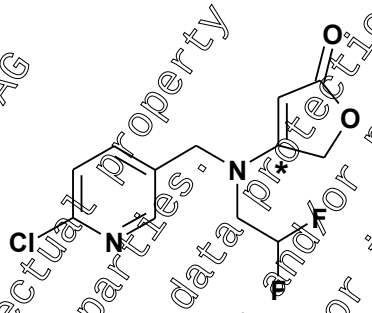
The report of these non-GLP studies is summarized in chapter 6.10.

IIA 6.2.2 Poultry

Two studies on the metabolism of BYI02960 in laying hens were conducted with the test compound labelled either in the [pyridinylmethyl-^{14}C]- or the [furanone-4-^{14}C]-position as shown by the following structural formulas (* denotes the label position):



[pyridinylmethyl-^{14}C]



[furanone-4-^{14}C]

Report:	**KIIA 6.2.2/01, Authors: [redacted], [redacted], 2012**
Title:	[Pyridinylmethyl-^{14}C]BYI02960: Metabolism in the Laying Hen
Report No & Document No	MEF-11/199 Date: 10.1.2012 M-422162-01-2
Guidelines:	OECD-Guideline for the Testing of Chemicals No. 503, Metabolism in Livestock US-EPA, Residue Chemistry OPPTS 860.1300; EU-Regulation 1107/2009 amended by Directive 96/68/EC, 7030/VI/95/rev.3 Appendix F
GLP	Yes, according to Japan MAFF GLP standard / Nousan 6283; US-EPA – FIFRA GLP (40CFR Part 160); Principles of GLP, German Chemical Law, current version of Annex 1
Testing Facility and Dates:	[redacted] Germany Experimental work: 5.3.2010 – 11.4.2011

Executive Summary

The metabolism and excretion of [pyridinylmethyl-^{14}C]BYI 02960 (common name: flupyradifurone) were investigated in laying hens as a model for poultry. Six hens were orally dosed once daily in the morning for 14 consecutive days with an aqueous 0.5% Tragacanth® suspension of 1.02 mg/kg body weight which corresponded to 10.98 mg a.s. /kg dry feed/day. The animals were sacrificed six hours after the last administration. Total radioactive residues (TRR) were determined daily in the eggs and excreta, and at sacrifice in the dissected organs and tissues (muscle, fat, liver, kidney, skin and eggs from ovary/oviduct). Eggs, muscle, fat, liver and excreta were extracted and analysed for parent compound and metabolites.

Recovery and Elimination of Radioactivity

The overall recovery rate was 96.11% of the total dose. The remaining radioactivity was expected to still be present in the gastro-intestinal tract at sacrifice, due to the short period between the last administration and sacrifice. On average, only 0.24% of the total dose was measured in the eggs. At sacrifice, the radioactive residues in the organs and tissues were calculated or estimated to be about

0.37% of the total dose, half of which was detected in the skeletal muscle (0.19%). Until sacrifice, the excretion of radioactivity accounted for 95.51% of the total dose.

Total Radioactive Residues in Eggs, Organs and Tissues

The concentration of radioactivity in **eggs** ranged from 0.016 mg/kg at day one to 0.119 mg/kg at sacrifice. Following a rather linear increase, a plateau level of approx. 0.08 mg/kg was reached six days after the first administration.

In the **organs** and **tissues**, the highest radioactivity concentrations were determined in kidney (1.073 mg/kg) and liver (0.435 mg/kg) indicating the significance of these organs for excretion and metabolism. These values corresponded to 0.05% and 0.08% of the total dose, respectively. The residue level of the eggs collected from the ovary and oviduct at sacrifice (0.147 mg/kg) was insignificantly higher than the levels of the laid eggs collected at sacrifice (0.119 mg/kg). This indicated that the egg yolk was not a preferential site for secretion. The residue levels of liver and kidney were followed in decreasing order by those found in the skin (0.094 mg/kg), muscle (0.070 mg/kg) and subcutaneous fat (0.021 mg/kg). The residue level of the total muscle corresponded to about 0.19% of the total dose assuming a value of 40% of the body weight for this tissue. Assuming values of 12% and 4% of the body weight for fat and skin, the residues in both tissues accounted for about 0.04% of the total dose.

Metabolism

For the determination of parent compound and metabolites, eggs, muscle, fat, liver and excreta from day 13 were pooled from all six animals. Eggs, muscle and liver as well as excreta were extracted with mixtures of acetonitrile/water and pure acetonitrile. Fat was extracted with acetonitrile and n-heptane followed by solvent partition. Post-extraction solids of liver were exhaustively extracted using microwave assistance. The resulting extracts of eggs, muscle and liver contained more than 92% of the total radioactive residue. Approx. 80% of the radioactivity was extractable from fat. Unextractable residues in fat were very low and amounted to only 0.004 mg/kg. After purification and concentration, the extracts were analysed using HPLC with radiometric detection.

The metabolic pattern determined in the excreta was similar to those in the eggs, organs and tissues. Therefore, parent compound and metabolites were isolated from excreta and identified by LC-MS/MS or NMR spectroscopy. The identified metabolites were used as reference compounds in extracts of eggs, muscle, fat and liver.

The identification rate was approx. 86% in eggs, 84% in muscle, 78% in fat and 59 % in liver. The remaining residues were characterised by their extraction- and chromatographic behaviour. Generally, the concentrations of parent compound and metabolites were very low. The parent compound was the major compound in eggs, muscle and fat (approx. 10 - 20%) and amounted to less than 0.017 mg/kg. Major metabolites were BYI 02960-acetyl-AMCP in eggs, muscle and fat and BYI 02960-OH-SA in fat and liver. Other metabolites were BYI 02960-lactato-mercaptyl-nicotinic acid, BYI 02960-6-CNA, BYI 02960-des-difluoroethyl and BYI 02960-OH. Minor residues were identified as BYI 02960-acetyl-cysteinyl-nicotinic acid, BYI 02960-des-difluoroethyl-OH-SA and BYI 02960-AMCP-difluoro-ethanamine-SA. In addition, BYI 02960-acetic acid was detected in the excreta.

The metabolite pattern of the extracts of the current hen study and the hen study with the ^{14}C-label in the furanone ring of the molecule (KIIA 6.2.2/02) were comparable, except the label specific metabolites.

The concentration of the total radioactivity (expressed as mg a.s. equiv. /kg), as well as the distribution of the parent compound and metabolites and the identification rates in eggs, liver, muscle and fat are summarised in the following table:

	Eggs (day 3 to 13.25)		Muscle		Fat		Liver	
TRR [mg/kg]	0.084		0.070		0.021		0.435	
Sample/Report name BYI 02960-	% of TRR	mg/kg	% of TRR	mg/kg	% of TRR	mg/kg	% of TRR	mg/kg
Conventional extraction	96.1	0.081	92.6	0.064	79.7	0.017	74.6	0.324
lactato-mercaptyl-nicotinic acid	4.0	0.003	3.6	0.002	---	---	15.5	0.068
acetyl-cysteinyl-nicotinic acid	---	---	---	---	---	---	0.2	0.001
6-CNA	7.2	0.006	8.8	0.006	1.8	<0.001	6.4	0.028
des-difluoroethyl-OH-SA	--	--	2.1	0.001	5.6	0.001	3.1	0.014
acetyl-AMCP	23.1	0.019	40.2	0.028	28.5	0.006	6.3	0.027
des-difluoroethyl	8.9	0.007	9.9	0.007	5.0	0.001	1.8	0.008
AMCP-difluoroethanamine-SA	--	--	---	---	---	---	0.3	0.001
OH-SA	5.1	0.004	1.8	0.001	16.2	0.003	22.5	0.098
OH	18.0	0.015	8.1	0.006	5.5	0.001	1.5	0.007
parent compound	19.8	0.017	9.8	0.007	15.3	0.003	0.9	0.004
Identified in conventional extract	86.2	0.072	84.2	0.059	77.9	0.016	58.6	0.255
Characterised in conventional extract	9.9	0.008	8.4	0.006	1.8	<0.001	16.0	0.070
Exhaustive extraction	n.a.		n.a.		n.a.		19.8	0.086
Total identified	86.2	0.072	84.2	0.059	77.9	0.016	58.6	0.255
Total characterised	9.9	0.008	8.4	0.006	1.8	<0.001	35.8	0.156
Total extracted	96.1	0.081	92.6	0.064	79.7	0.017	94.5	0.411
Solids	3.9	0.003	7.4	0.005	20.3	0.004	5.5	0.024
Accountability	100.0	0.084	100.0	0.070	100.0	0.021	100.0	0.435

The main metabolic reactions of [pyridinylmethyl-^{14}C]BYI 02960 in the laying hen are:

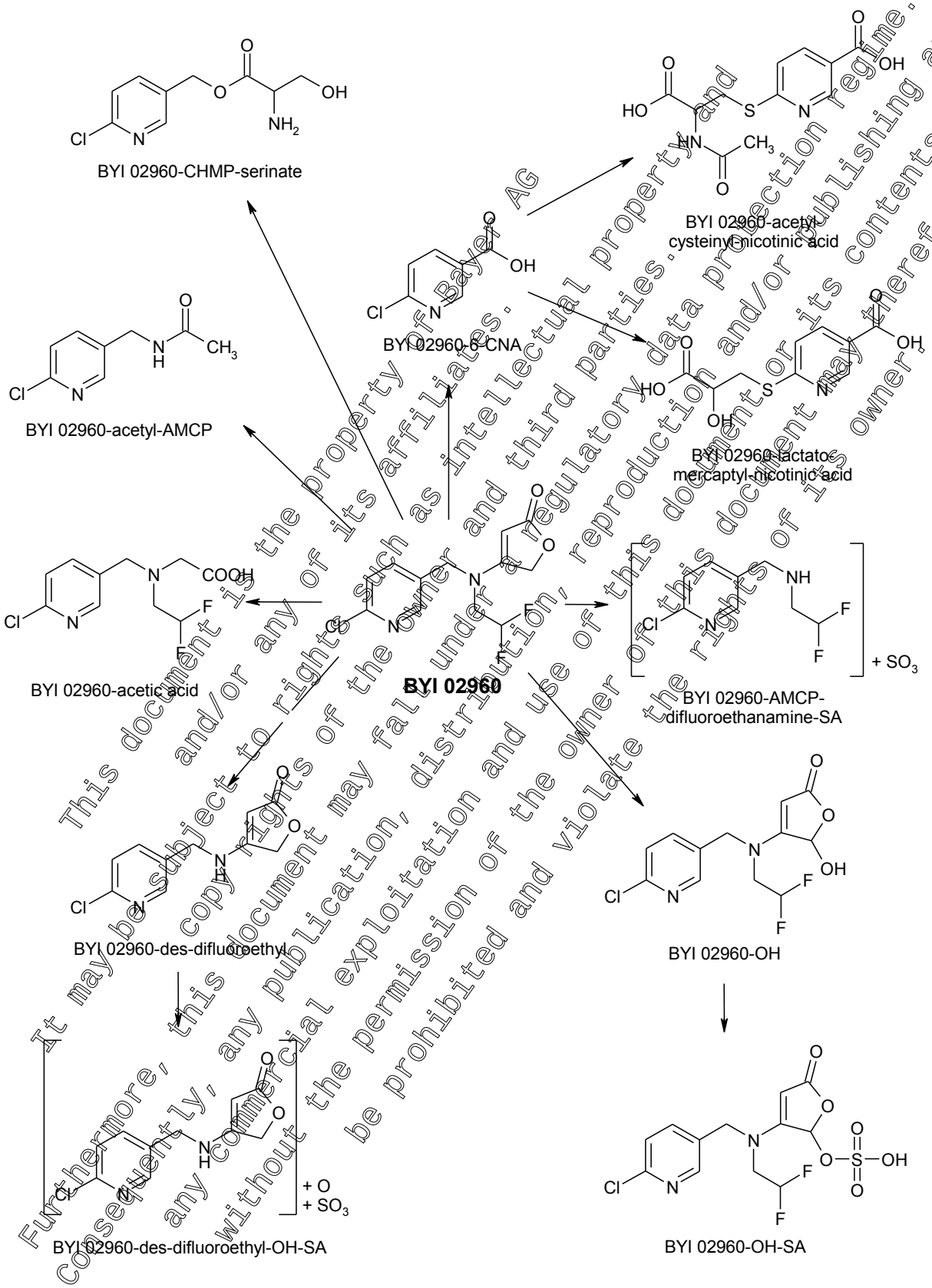
Hydroxylation in position 5 of the furanone ring forming BYI 02960-hydroxy followed by conjugation with sulfuric acid to BYI 02960-OH-SA

- Oxidative cleavage of the pyridinylmethyl moiety forming BYI 02960-6-CNA
- Substitution of the chloro group of BYI 02960-6-CNA with glutathione followed by degradation resulting in the conjugates BYI 02960-acetyl-cysteinyl-nicotinic acid and BYI 02960-lactato-mercaptyl-nicotinic acid
- Cleavage of the difluoroethyl group forming BYI 02960-des-difluoroethyl followed by hydroxylation and conjugation with sulfuric acid to BYI 02960-des-difluoroethyl-OH-SA
- Cleavage of the furanone ring and conjugation with sulfonic acid forming BYI 02960-AMCP-difluoroethanamine-SA

- Oxidative degradation of the furanone ring forming BYI 02960-acetic acid
- Cleavage of the pyridinylmethyl moiety forming an alcohol conjugated with serine (BYI 02960-CHMP-serinate)
- Cleavage of the furanone ring and the difluoroethyl group forming an amine followed by acetylation to BYI 02960-acetyl-AMCP

Based on these results a metabolic pathway of [pyridinylmethyl-^{14}C]BYI 02960 in the laying hen is proposed as shown on the next page.

Proposed metabolic Pathway of [pyridinylmethyl-^{14}C]BYI 02960 in laying hens:



I. Materials and Methods

A. Materials

1. Test Material

IUPAC Name	4-{[(6-chloropyridin-3-yl)methyl](2,2-difluoroethyl)amino}furan-2(5H)-one
Code name	BYI02960
Common name	flupyradifurone (proposed ISO)
Empirical formula	$C_{12}H_{11}ClF_2N_2O_2$
Molar mass	288.68 g/mol
Labelling position	[pyridinylmethyl-^{14}C]
Specific radioactivity	4.37 MBq/mg = 118.08 µCi/mg (delivered sample before radiodilution) 3.50 MBq/mg = 2.10 x 10^8 dpm/mg = 94.59 µCi/mg = 27.31 Ci/mol (sample after radiodilution)
Radiochemical purity	> 99 % by radio-HPLC and > 98 % by radio-TLC
Nonradioactive test substance	Batch BYI 02960-PU-02
Chemical purity	99.4%
Dose level	14 oral doses of 1.02 mg/kg bw/day by gavage
Vehicle	0.5% aqueous Tragacanth® suspension

2. Test Animals

Species	Laying hen (*Gallus gallus domesticus*)
Strain	"White Leghorn"
Breeding facility	[redacted]
Sex and numbers involved	6 out of 18 hens were selected by maximum egg production
Age	6 – 8 months
Body weight	1.63 kg at first administration, 1.60 kg at sacrifice
Acclimatization	35 days
Identification	Cage labelling and wing tags
Housing	Individually in stainless steel metabolism cages for laying hens allowing almost quantitative collection of eggs and excreta (supplier: [redacted] room temperature 22 - 30°C, relative humidity 30 - 57%. 16 h light, 8 h dark cycle, air change 10 – 15 times per hour
Feed and water	Hens were fed with "RWZ-LegeGold Mehl", a pulverised chicken feed. This feed was not a certified diet, i.e. it was not checked for contamination according to current standards. The feed was supplemented by eggshells and crushed marine shells during the acclimation period. The feed consumption was recorded by weighing during the experiment (mean consumption 103 g/day/hen), tap water, ad libitum

B. Study Design

<u>Dosing</u>

The radiolabelled test compound was mixed with the non-radiolabelled test compound in order to reduce the specific radioactivity from 4.37 MBq/mg to 3.5 MBq/mg. For the 14 administrations, 4 dosing suspensions in 0.5% aqueous Tragacanth® were prepared and each suspension was applied for three or four administrations. The radiolabelled test substance proved to be stable until the last dose.

The oral administration was carried out with a knob cannula attached to a glass syringe. Immediately after dosage, the swallowing was supported by a gentle massage of the throat towards the crop. The hens received a mean dose of 1.67 mg (3.51 x 10^8 dpm) per animal and day at a mean body weight of 1.63 kg, corresponding to an actual dose of 1.02 mg a.s./kg bw. The amount of radioactivity of the actually administered amounts served as reference for the calculation of total radioactivity in the biological samples. The administration volume was 1.0 mL/kg body weight. The dose level was tolerated without any observable toxicological symptoms.

<u>Sampling of eggs and excreta</u>

During the test, the cages were inspected for egg production once daily (in the morning before administration) and the number of eggs was recorded for all hens. After sampling, the eggshells were discarded, and the egg white and yolk were weighed and then thoroughly mixed. An aliquot sample of each egg mix was taken for the determination of the total radioactivity in triplicate by LSC. The residual amount of the egg-mix from all animals was stored at ca. – 18°C until metabolite analysis.

The excreta of each hen were collected once daily from the collecting tins as quantitatively as possible. The individual samples were homogenised after the addition of water, before recording the total weights. An aliquot of each fraction was processed for radioactivity measurement by combustion/LSC. The remaining samples were stored in a freezer until metabolite analysis.

<u>Sacrifice and sampling of organs and tissues</u>

The treated hens were weighed and sacrificed ca. 6 hours after the last (14th) dosage. The animals were anaesthetised using carbon dioxide, sacrificed by decapitation and exsanguinated. The organs and tissues were prepared immediately after sacrifice. Liver without the gall bladder, kidneys, leg and breast muscle, skin without subcutaneous fat, subcutaneous fat and eggs from the ovary and oviduct were sampled immediately after sacrifice and their fresh weights were recorded. The gall bladders were punctured for the collection of the bile fluid which was then stored frozen for an optional metabolite analysis.

<u>Sample preparation</u>

After dissection, the organs or tissue samples were transferred into ice-cooled vessels. Liver, kidneys, muscle samples and subcutaneous fat as well as eggs dissected from the ovary and oviduct were thoroughly homogenised in half-frozen state. An aliquot of each resulting tissue pulp was weighed, freeze-dried, weighed again and homogenised. The radioactivity was then determined in triplicate using combustion/LSC. Aliquots of subcutaneous fat and skin without fat were weighed and solubilised with tissue solubiliser (BTS-450®) and measured for radioactivity by LSC. All samples were divided into equal portions and stored at ca. -18°C until metabolite analysis.

To prepare representative samples of eggs, organs or tissues, corresponding samples of all individual animals were pooled before extraction. An egg pool was prepared from day 3 to sacrifice. All egg samples from this collection period were used. Composite samples of muscle (leg and breast), subcutaneous fat, liver and excreta (day 13) from all animals were prepared. The individual samples were thoroughly homogenised and kept frozen until extraction.

The total radioactive residue of each pool was determined by combustion/LSC (solid samples) or directly by LSC (e.g. combined eggs and extracts). Eggs, muscle, liver and excreta were extracted 3 times with acetonitrile/water 80:20 (v/v) and finally with pure acetonitrile. For eggs, muscle and liver, the combined conventional extracts were subjected to a clean-up step using an SPE-cartridge. Post extraction solids of liver were exhaustively extracted twice with acetonitrile/water (1:1, v/v) under microwave assistance. Fat was extracted twice with n-heptane and acetonitrile followed by a solvent partition procedure yielding an acetonitrile - and an n-heptane phase. All extracts were used for quantification of parent compound and metabolites by HPLC.

Radioactivity measurement

Solid samples were combusted prior to radioactivity determination and the formed $^{14}CO_2$ absorbed in an alkaline trapping solvent. The determination of radioactivity of liquid samples was conducted by liquid scintillation counting (LSC) using sub-samples (1 - 3 replicates). Quenching effects were automatically corrected using an external standard and a quenching library. The instrument background was automatically subtracted. For all samples, the limit of detection (LOD) was established at approximately 10 dpm per aliquot after instrument background correction. The limit of quantification (LOQ) was established as 2 times the background radioactivity (dpm) of each instrument/method.

Metabolite analysis

The prepared extracts were subjected to HPLC using a reversed phase column (C18) and the eluting solvents water/formic acid 99:1 (v/v) and acetonitrile/water/formic acid 97:2:1 (v/v/v) in the gradient mode. Detection was performed by a UV- (254 nm) and a radioisotope detector with a glass bead scintillator. In order to check the completeness of the elution, representative samples of egg, muscle and liver extract were injected, re-collected, and radioassayed by LSC. The recoveries were between 99.1 and 100.5% of the injected radioactivity. Radiolabelled and non-labelled reference compounds were used in co-chromatography for identification of metabolites.

Metabolites were isolated from the excreta, identified by LC-MS/MS or NMR-spectroscopy and used as reference compounds for the identification of metabolites in eggs and liver by HPLC co-chromatography. Other reference compounds were taken from the goat metabolism study also using the pyridinylmethyl-^{14}C - labelled test compound [KIIA 6.2.3/01] or provided as non-radiolabelled reference compounds. Metabolites in muscle and fat were assigned by comparison of the metabolic patterns and retention times.

II. Results and Discussion

A. Recovery and Elimination of Radioactivity

The recovery of radioactivity in laying hens after administration of an average daily dose of 1.02 mg [pyridinylmethyl-^{14}C]BYI 02960 per kg bw/day on 14 consecutive days is presented in Table 6.2.2-1. The overall recovery accounted for 96.11% of the totally administered dose. The remaining amount of radioactivity (approx. 4%) was expected to still be present in the gastro-intestinal tract at sacrifice, due to the short period between the last administration and sacrifice.

Table 6.2.2-1: Distribution of residues in eggs, muscle, fat, liver and kidney of laying hens following oral administration of 14 daily doses of [pyridinylmethyl-^{14}C] BYI02960 at a dose rate of 1.02 mg/kg

Sample	Percent of total dose administered	Concentration of total radioactivity [mg/kg]
Liver	0.08	0.435
Kidney	0.05	1.073
Eggs from ovary/oviduct	0.01	0.147
Muscle, total	0.09	0.070
Skin, total	0.02	0.094
Fat, total	0.02	0.021
Organs/tissues, total	0.37	-----
Eggs	0.24	0.081
Excreta, total	95.51	-----
Total Recovery	96.11	-----

B. Levels and Time Course of Total Radioactive Residues in Eggs

The radioactivity levels measured in the egg samples from all animals are presented in Table 6.2.2-2. The concentration in eggs ranged from 0.016 mg/kg at day one to 0.119 mg/kg at sacrifice. Following a rather linear increase a plateau level of approx. 0.08 mg/kg was reached at day six.

Table 6.2.2-2: Time course of total radioactivity in eggs following oral administration of 14 daily doses of [pyridinylmethyl-^{14}C] BYI02960 at a dose rate of 1.02 mg/kg

Time after the first admin. [d]	Admin. no.	Cumulative secretion [% of total dose admin.] (mean)	Concentration of total radioactivity [mg/kg]
0	1	----- #	----- #
1	2	0.01	0.016
2	3	0.01	0.044
3	4	0.03	0.061
4	5	0.04	0.068
5	6	0.06	0.075
6	7	0.08	0.080
7	8	0.09	0.083
8	9	0.11	0.083
9	10	0.13	0.087
10	11	0.15	0.090
11	12	0.17	0.087
12	13	0.19	0.085
13	14	0.21	0.088
13.25	--	0.24	0.119

----- # no egg collected

C. Total Radioactive Residues in Dissected Organs and Tissues

The concentration of the total radioactivity in the dissected organs and tissues at sacrifice is shown in Table 6.2.2-1 (last column). The highest concentrations were determined in kidney (1.073 mg/kg) and liver (0.435 mg/kg) reflecting the significance of these organs for excretion and metabolism. In relation to the dose totally administered, these values corresponded to 0.05% and 0.08%, respectively. The residue level of the eggs collected from the ovary and oviduct at sacrifice (0.147 mg/kg) was insignificantly higher (factor 1.2) than the levels of the laid eggs collected at sacrifice (0.119 mg/kg). This indicated that the egg yolk was not a preferential site for secretion of the radioactivity. The residue levels of liver and kidney were followed in decreasing order by those determined in the skin (0.094 mg/kg), muscle (0.070 mg/kg) and subcutaneous fat (0.021 mg/kg). The residue level of the total skeletal muscle corresponded to about 0.19% of the total dose assuming a 40% contribution to the body weight. Assuming values of 12% and 4% of the body weight for fat and skin, the residues in both tissues accounted for about 0.04% of the dose totally administered.

D. Extraction Efficiency of Residues

Eggs (day 3 to sacrifice), muscle and liver pools as well as excreta (day 13) were extracted with acetonitrile/water (8:2; v/v) followed by pure acetonitrile. Additionally, solids of liver were exhaustively extracted with acetonitrile/water (1:1, v/v) under microwave assistance. Fat was extracted with acetonitrile and n-heptane followed by a solvent partition into an acetonitrile- and an n-heptane phase. After purification and concentration, the resulting extracts represented 96.1% of the total

radioactivity for eggs (based on the 1st extraction), 92.6% for muscle, 79.7% for fat (based on the 2nd extraction), 94.5% for liver and 97.7% for excreta.

The radioactivity concentrations in the post-extraction solids amounted to 0.003 mg/kg (3.9%) for eggs (based on the 1st extraction), 0.005 mg/kg (7.4%) for muscle, 0.004 mg/kg (20.3%) for fat (based on the 2nd extraction) and 0.024 mg/kg (5.5%) for liver.

A summary of the extraction efficiency is shown in Table 6.2.2-3.

Table 6.2.2-3: Extraction efficiency of eggs, muscle, fat and liver samples following oral administration of 14 daily doses of [pyridinylmethyl-^{14}C]BYI02960 at a dose rate of 1.02 mg/kg

	Eggs (1st extraction)		Muscle		Fat (2nd extraction)		Liver	
TRR [mg/kg]	0.084		0.070		0.021		0.435	
	% of TRR	mg/kg	% of TRR	mg/kg	% of TRR	mg/kg	% of TRR	mg/kg
Conventional extraction	96.1	0.081	92.6	0.064	79.7	0.017	74.6	0.324
- acetonitrile/water (8/2, v/v)	96.1	0.081	92.6	0.064	---		74.6	0.324
- acetonitrile phase	---		---		79.7	0.017	---	
- n-heptane phase	---		---		n.d.	n.d.	---	
Exhaustive extract:	---		---		---		19.8	0.086
Total extracted	96.1	0.081	92.6	0.064	79.7	0.017	94.5	0.411
Post-extraction solids (PES)	3.9	0.003	7.4	0.005	20.3	0.004	5.5	0.024
Accountability	100.0	0.084	100.0	0.070	100.0	0.021	100.0	0.435

n.d.: not detected

To demonstrate storage stability, a second extraction and sample preparation of eggs and fat were performed approx. 7 months after the first extraction followed by profiling of the metabolites.

E. Quantification, Identification and Characterisation of Residues

Isolation and Identification of Parent Compound and Metabolites in Excreta

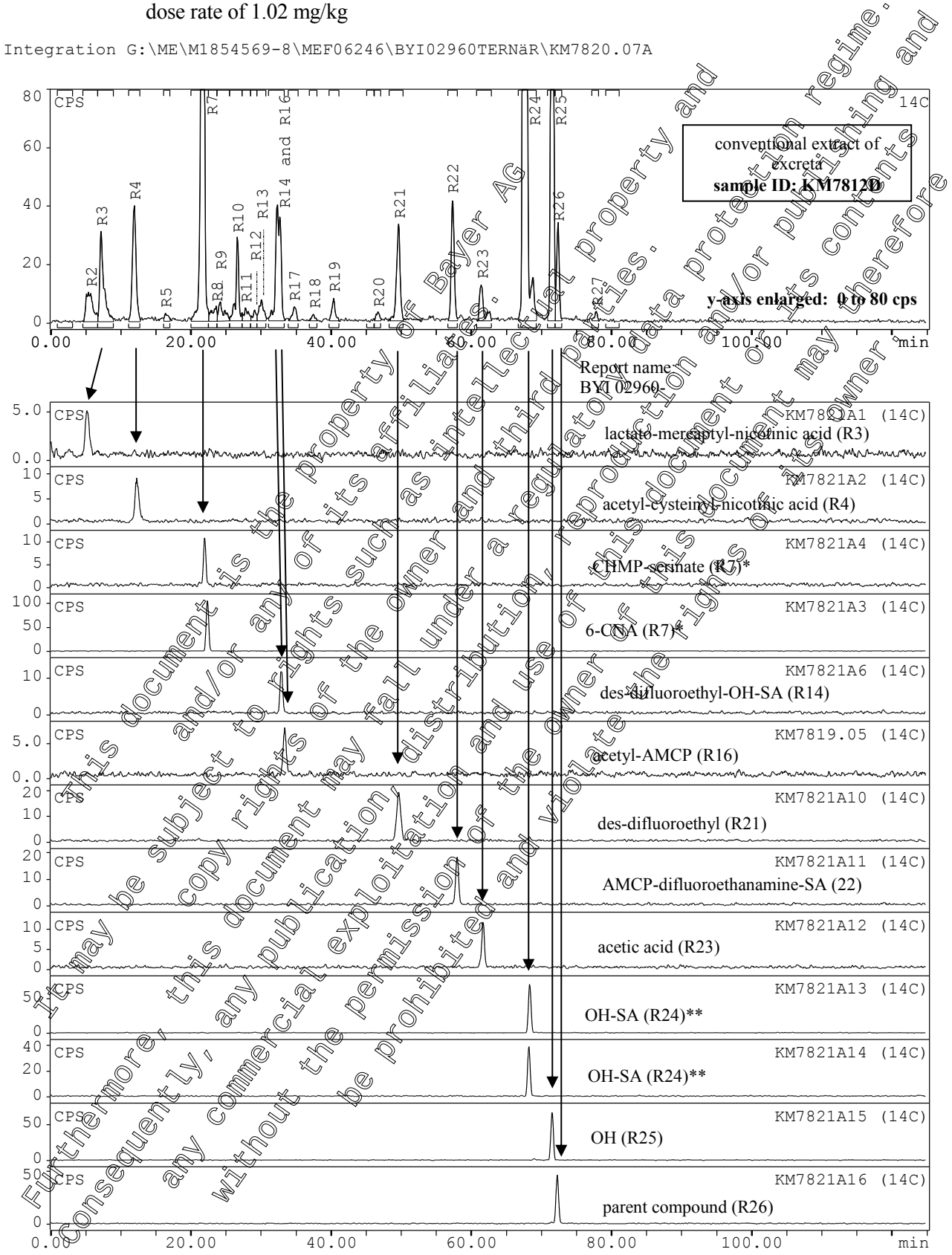
The extract of excreta was used to isolate the metabolites using a ternary reversed phase HPLC system. The three eluents employed were:

A: 1L water + 1.54 mL ammonium hydroxide solution (25%) + 0.8 mL formic acid, adjusted to pH 7
B: acetonitrile / eluent A (99:1, v/v)
C: methanol / tetrahydrofurane (1:1, v/v)

Detailed information can be found in the report.

The identification of the metabolites was achieved by LC-MS/MS. The position of the hydroxy-sulfate group of metabolite BYI 02960-OH-SA was assigned by NMR spectroscopy after multiple purification steps. In case of BYI 02960-OH, the position of the hydroxy group was identified by HPLC co-chromatography with the non-radiolabelled reference compound. An assignment of all identified metabolites to the metabolite profile of excreta is shown in Figure 6.2.2-1.

Figure 6.2.2-1: Assignment of isolated and identified metabolites in the excreta of laying hens following oral administration of 14 daily doses of [pyridinylmethyl-^{14}C] BYI02960 at a dose rate of 1.02 mg/kg



* both metabolites can be separated using an acidic RP HPLC system

** BYI 02960-OH-SA was isolated and identified in two neighbouring regions due to a matrix effect

Identification and Assignment of Metabolites in Eggs, Organs and Tissues

Identified metabolites from excreta were used as reference compounds for the identification or assignment of metabolites in eggs and liver. Metabolites in other organs and tissues were assigned by comparison of the metabolic profiles and retention times.

F. Distribution of Parent Compound and Metabolites in Eggs, Organs and Tissues

The distribution of the parent compound and metabolites in eggs, organs and tissues is summarised in Table 6.2.2-4. Of the total radioactivity, ca. 86% was identified in eggs, 84% in muscle, 78% in fat and 59% in liver. All other residues were characterised by their extraction and chromatographic behaviour.

Table 6.2.2-4: Radioactive residues of parent compound and metabolites in eggs and edible organs and tissues of laying hens following oral administration of 14 daily doses of [pyridinylmethyl-^{14}C] BYI02960 at a dose rate of 1.02 mg/kg

	Eggs (day 3 to 13,25)		Muscle		Fat		Liver	
TRR [mg/kg]	0.084		0.070		0.021		0.435	
Sample/Report name BYI 02960-	% of TRR	mg/kg	% of TRR	mg/kg	% of TRR	mg/kg	% of TRR	mg/kg
Conventional extraction	96.1	0.081	92.6	0.064	79.7	0.017	74.6	0.324
lactato-mercaptyl-nicotinic acid	4.0	0.003	3.6	0.002	---	---	15.5	0.068
acetyl-cysteinyl-nicotinic acid	---	---	---	---	---	---	0.3	0.001
6-CNA*	7.2	0.006	8.8	0.006	1.8	<0.001	6.4	0.028
des-difluoroethyl-OH-SA	---	---	2.1	0.001	5.6	0.001	3.1	0.014
acetyl-AMCP	23.1	0.019	40.2	0.028	28.5	0.006	6.3	0.027
des-difluoroethyl	8.9	0.007	9.9	0.007	5.0	0.001	1.8	0.008
AMCP-difluoroethanamine-SA	---	---	---	---	---	---	0.3	0.001
OH-SA	5.1	0.004	1.8	0.001	16.2	0.003	22.5	0.098
OH	18.0	0.015	8.1	0.006	5.5	0.001	1.5	0.007
parent compound	19.8	0.017	9.8	0.007	15.3	0.003	0.9	0.004
identified in the conventional extract	86.2	0.072	84.2	0.059	77.9	0.016	58.6	0.255
characterised in the conventional extract	9.9	0.008	8.4	0.006	1.8	<0.001	16.0	0.070
Exhaustive extraction	n.a.		n.a.		n.a.		19.8	0.086
Total identified	86.2	0.072	84.2	0.059	77.9	0.016	58.6	0.255
Total characterised	9.9	0.008	8.4	0.006	1.8	<0.001	35.8	0.156
Total extracted	96.1	0.081	92.6	0.064	79.7	0.017	94.5	0.411
Solids	3.9	0.003	7.4	0.005	20.3	0.004	5.5	0.024
Accountability	100.0	0.084	100.0	0.070	100.0	0.021	100.0	0.435

* co-elution with BYI 02960-CHMP-serinate was excluded for eggs, muscle and liver

Metabolites in Eggs

Besides the parent compound (0.017 mg/kg; 19.8%), major metabolites in eggs were BYI 02960-acetyl-AMCP (0.019 mg/kg; 23.1%) and BYI 02960-OH (0.015 mg/kg, 18.0%). Further metabolites

were BYI 02960-lactato-mercaptyl-nicotinic acid, BYI 02960-6-CNA, BYI 02960-des-difluoroethyl, BYI 02960-OH-SA and two unknown metabolites. They were detected in low amounts ≤0.007 mg/kg.

Metabolites in Muscle

The main metabolite in muscle was BYI 02960-acetyl-AMCP, which amounted to 0.028 mg/kg (40.2%). Parent compound, BYI 02960-6-CNA, BYI 02960-des-difluoroethyl and BYI 02960-OH were quantified in amounts between 0.006 to 0.007 mg/kg (approx. 9%). Minor identified metabolites were BYI 02960-lactato-mercaptyl-nicotinic acid, BYI 02960-des-difluoroethyl-OH-SA and BYI 02960-OH-SA (each ≤0.002 mg/kg).

Metabolites in Fat

No residues were detected in the n-heptane phase. The main metabolites in the polar phase of fat were parent compound (0.003 mg/kg, 15.3%), BYI 02960-acetyl-AMCP (0.006 mg/kg, 28.5%) and BYI02960-OH-SA (0.003 mg/kg, 16.2%). All other metabolites were detected in amounts ≤0.001 mg/kg.

Metabolites in Liver

The majority of the radioactive residue (approx. 75%) was extracted with acetonitrile/water. The main metabolites in this extract were BYI 02960-OH-SA (0.098 mg/kg, 22.5%), BYI 02960-lactato-mercaptyl-nicotinic acid (0.068 mg/kg, 15.5%), BYI 02960-6-CNA (0.028 mg/kg, 6.4%) and BYI 02960-acetyl-AMCP (0.027 mg/kg, 6.3%). The parent compound was only detected in negligible amounts of 0.004 mg/kg. All other metabolites amounted to ≤0.014 mg/kg, except one unknown metabolite (0.027 mg/kg).

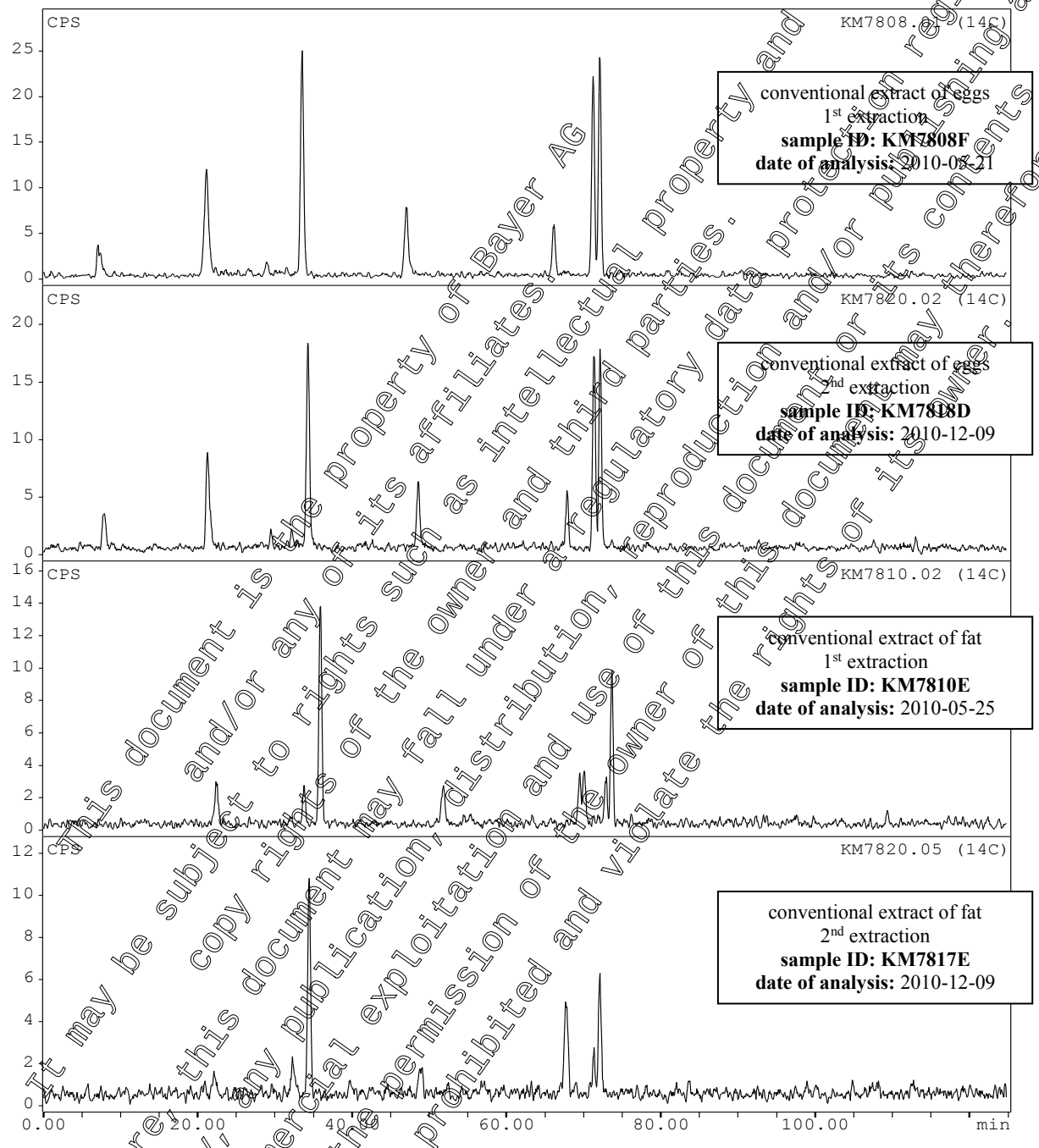
Metabolites in the exhaustive extract of liver ranged from 0.002 to 0.027 mg/kg. They were not identical with the metabolites in the acetonitrile/water extract. All unknown metabolites in the exhaustive extract of liver were not further investigated, due to their low amount and the high concentration of matrix. Therefore, they were characterised by their extraction and chromatographic behaviour.

G. Storage Stability of Residues

During the study, all samples and extracts were stored below -18°C or for a short time in a refrigerator. All egg samples and edible organs or tissues were extracted within approx. 2 months after sample collection. The first metabolic profile was recorded within six days after the start of the first extraction and sample preparation.

The storage stability of residues in the extracts of eggs and fat was demonstrated for a period of approx. 7 months by a repeated extraction and HPLC profiling (Figure 6.2.2-2).

Figure 6.2.2-2: Storage stability of aged sample material of eggs and fat of laying hens following oral administration of 14 daily doses of [pyridinylmethyl-14C] BYI 02960 at a dose rate of 1.02 mg/kg



The stability of the extracts of muscle and liver was concluded in analogy to eggs. The pattern of metabolites (fingerprint) in the first profile of the extracts of muscle and liver was similar to the pattern analysed approx. 6 months later. In particular, the distribution of the peak areas was very similar for both analyses. Therefore, it was concluded that as for eggs, also the extracts of muscle and liver were stable for a period of approx. 6 months after extraction.

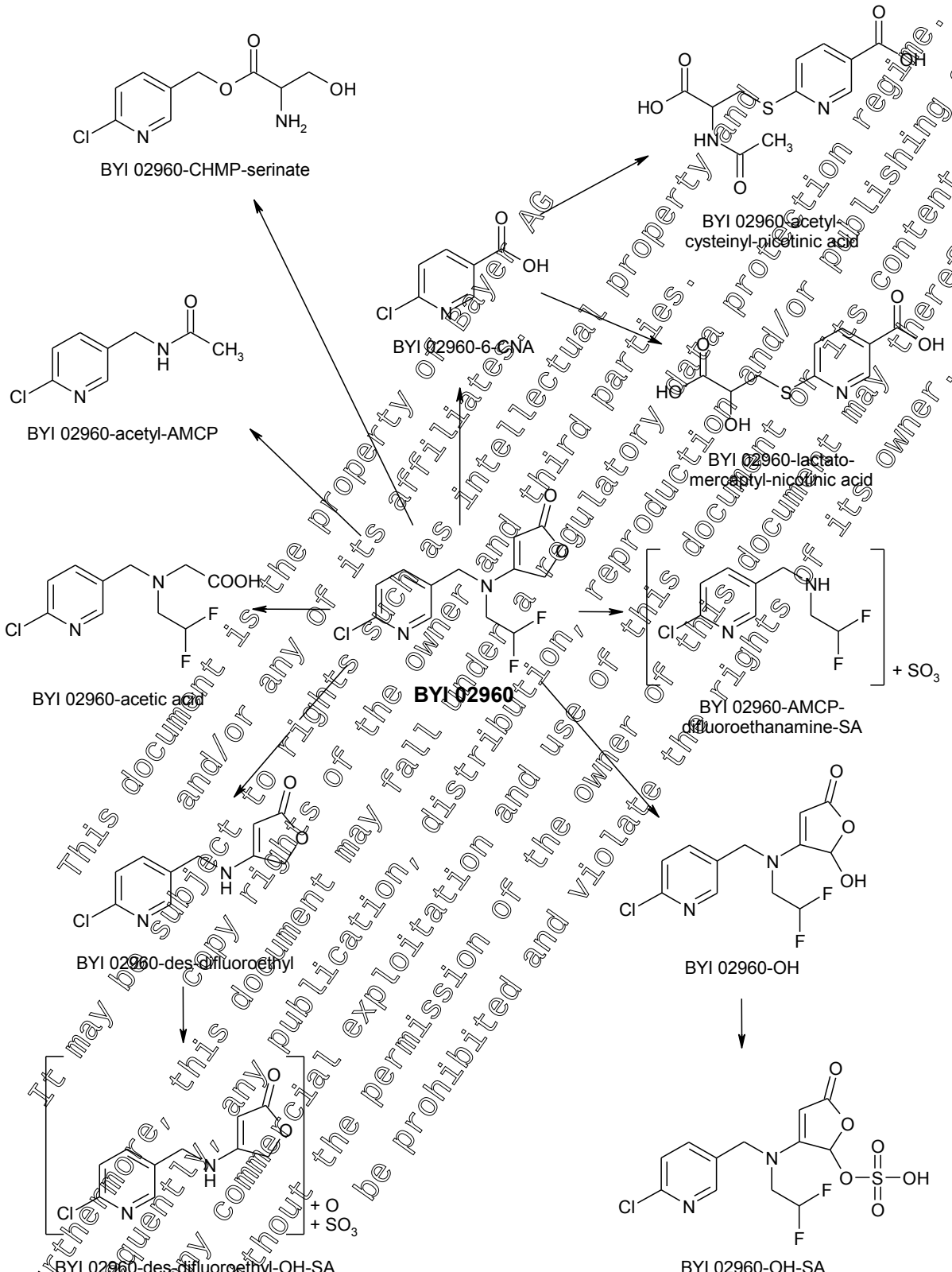
III. Conclusion

The metabolic and excretion behaviour of [pyridinylmethyl-^{14}C]BYI 02960 in laying hens can be characterised as follows:

- The concentrations of radioactivity in eggs and edible tissues were relatively low if compared to the dose level and the dosing period of 14 days.
- The evaluation of these results should consider the fact that an exaggerated dose level of 16.18 mg/kg feed/day was administered. Furthermore, a significant amount of radioactivity was detected in the excreta and the relatively high radioactivity in kidney and liver at sacrifice 6 hours after the last administration indicate that the residues are further metabolised and finally eliminated.
- A residue plateau level in whole eggs was reached within the test period at the 6th day after the first administration.
- The main portion of residues (ca. 80 to 96%, depending on the matrix) was efficiently extracted from eggs, muscle, fat and liver.
- Only low amounts of parent compound were detected in eggs, muscle, fat and liver.
- Non label specific metabolites are BYI 02960-hydroxy, BYI 02960-OH-SA, BYI 02960-des-difluoroethyl, BYI 02960-des-difluoroethyl-OH-SA and BYI 02960-acetic acid. They were detected in approximately the same low amounts as in the study with the furanone label.
- The cleavage of the molecule was a significant reaction in the metabolism. A prominent portion of label specific metabolites was detected in the edible tissues. Major label specific metabolites were BYI 02960-6-CNA and BYI 02960-acetyl-AMCP.
- The main metabolic reactions in the laying hen are:
    - Hydroxylation in position 5 of the furanone ring forming BYI 02960-OH followed by conjugation with sulfuric acid to BYI 02960-OH-SA
    - Oxidative cleavage of the pyridinylmethyl-bridge forming BYI 02960-6-CNA
    - Substitution of the chloro group of BYI 02960-6-CNA with glutathione followed by degradation resulting in two conjugates BYI 02960-acetyl-cysteinyl-nicotinic acid and BYI 02960-lactato-mercaptyl-nicotinic acid
    - Cleavage of the difluoroethyl group forming BYI 02960-des-difluoroethyl followed by hydroxylation and conjugation with sulfuric acid to BYI 02960-des-difluoroethyl-OH-SA
    - Cleavage of the furanone ring and conjugation with sulfonic acid forming BYI 02960-AMCP-difluoroethanamine-SA
    - Oxidative degradation of the furanone ring forming BYI 02960-acetic acid
    - Cleavage of the pyridinylmethyl-bridge forming an alcohol conjugated with serine (BYI 02960-CHMP-serinate)
    - Cleavage of the furanone ring and the difluoroethyl group forming an amine followed by acetylation (BYI 02960-acetyl-AMCP)

Based on these results a metabolic pathway of [pyridinylmethyl-^{14}C] BYI02960 in the laying hen is proposed in Figure 6.2.2-3.

Figure 6.2.2-3: Proposed metabolic pathway of [pyridinylmethyl-^{14}C] BYI02960 in laying hens



Report:	**KIIA 6.2.2/02, Authors: [redacted], [redacted], 2012**
Title:	[Furanone-4-^{14}C]BYI02960: Metabolism in the Laying Hen
Report No & Document No	MEF-11/200 Date: 10.1.2012 M-422263-01-2
Guidelines:	OECD-Guideline for the Testing of Chemicals No. 503, Metabolism in Livestock US-EPA, Residue Chemistry OPPTS 860.1300; EU Regulation 1107/2009 amended by Directive 96/68/EC, 7030/VI/95/rev.3 Appendix F
GLP	Yes, according to Japan MAFF GLP standard 11 Nousan 6283; US EPA – FIFRA GLP (40CFR Part 160); Principles of GLP, German Chemical Law, current version of Annex 1
Testing Facility and Dates	[redacted], Germany Experimental work: 12.2.2010 - 9.12.2010

Executive Summary

The metabolism and excretion of [furanone-4-^{14}C]BYI 02960 (common name: flupyradifurone) were investigated in laying hens as a model for poultry. Six hens were orally dosed once daily in the morning for 14 consecutive days with an aqueous 0.5% Tragacanth® suspension of 1.05 mg of the active substance per kg body weight which corresponded to 17.13 mg a.s./kg dry feed/day. The animals were sacrificed six hours after the last administration. Total radioactive residues were determined daily in the eggs and excreta, and at sacrifice in the dissected organs and tissues (muscle, fat, liver, kidney, skin and eggs from ovary/oviduct). Eggs, muscle, fat, liver and excreta were extracted and analysed for parent compound and metabolites.

Recovery and Elimination of Radioactivity

The overall recovery rate was 82.16% of the total dose. A part of the remaining radioactivity was expected to still be present in the gastro-intestinal tract at sacrifice, due to the short period between the last administration and sacrifice. Another part is probably exhaled as $^{14}CO_2$. The formation of carbon dioxide was also shown in a rat quantitative whole-body autoradiography study using the test compound with the same labelling position (KIIA 5.1.2/02). An average amount of 2.35% of the total dose was determined in the eggs. At sacrifice, the residues in the organs and tissues were calculated or estimated to be about 1.8% of the total dose from which about 28% was detected in the skeletal muscle (0.5%). Until sacrifice, the excretion products accounted for 78.01% of the total dose.
Total Radioactive Residues in Eggs and in Organs and Tissues
The concentration of radioactivity in eggs ranged from 0.024 mg/kg at day one to 1.198 mg/kg at sacrifice. Following a linear increase a plateau level of 1.035 mg/kg was reached nine days after the first administration.

In the organs and tissues, the highest radioactivity concentrations were determined in liver (2.178 mg/kg) and kidney (1.083 mg/kg) indicating the significance of these organs for metabolism and excretion. These values corresponded to 0.37% and 0.05% of the total dose, respectively. The residue level of the eggs collected from the ovary and oviduct at sacrifice (2.774 mg/kg) was by a factor of 2.3 higher than the levels of the laid eggs collected at sacrifice (1.198 mg/kg). This indicated that the egg yolk was a preferential site for secretion of the radioactivity. The residue levels of liver and kidney were followed in decreasing order by those found in the subcutaneous fat (0.427 mg/kg), skin

(0.257 mg/kg) and muscle (0.183 mg/kg). The residue level of the total muscle corresponded to about 0.50% of the total dose assuming a value of 40% of the body weight for this tissue. Assuming values of 12% and 4% of the body weight for fat and skin, the residues in both tissues accounted for about 0.41% of the total dose.

Metabolism

For metabolism investigations, eggs of day 2 to 7 and 8 to sacrifice, muscle, fat, liver and excreta from day 13 were pooled from all six animals. Eggs, muscle, fat and liver were extracted with acetonitrile and n-heptane followed by solvent partition. In case of eggs, muscle and liver the extraction procedure was continued with a mixture of acetonitrile/water. Excreta were extracted with acetonitrile/water followed by pure acetonitrile. Post-extraction solids of eggs, muscle and liver were exhaustively extracted with mixtures of acetonitrile/water followed by acetonitrile/water/formic acid using microwave assistance. The extraction efficiencies were above 81% of the total radioctivity in all samples. Unextractable residues were low and ranged from 0.006 (eggs day 2 to 7) to 0.036 mg/kg (liver).

The major part of residues was detected in the n-heptane phases of eggs (days 2 to7 ca. 52% and days 8 to sacrifice ca. 58%), fat (ca. 96%) and liver (ca. 52%). The residues in the n-heptane phase of muscle only amounted to ca. 8%. Residues in the acetonitrile/water extracts of eggs, muscle, fat and liver were detected at the same level as analysed in the laying hen study using the pyridinylmethyl label. The residues ranged from 0.011 mg/kg (2.6%) for fat to 0.450 mg/kg (20.7%) for liver. A significant part of the residues was found in the exhaustive extracted using microwave assistance. Between 4 and 10% of the radioactivity was detected in the neutral exhaustive extracts and between 8 and 40% in the acidic exhaustive extracts of eggs, muscle and liver.

The radioactive residues in the n-heptane phases of eggs, muscle, fat and liver showed the same behaviour in thin layer chromatography. They were identified as fatty acids after saponification. These residues in the n-heptane phases were specific for the furanone label and were caused by the cleavage and subsequent total degradation of the furanone ring to smaller carbon units (C-1- or C-2-fragments), entering the carbon pool of endogenous compounds. A part of these fragments was probably also converted to $^{14}CO_2$ as can be suggested from the lower overall recovery if compared with the study using the metabolically stable pyridinylmethyl label.

Residues in the acetonitrile/water- and exhaustive extracts were analysed by HPLC with radiometric detection. The metabolite patterns of the current hen study and the corresponding extracts of the hen study with the pyridinylmethyl-label were comparable, except the label specific metabolites. Parent compound, BYI 02960-OH, BYI 02960-OH-SA, BYI 02960-des-difluoroethyl, BYI 02960-des-difluoroethyl-OH-SA were identified in the acetonitrile/water extracts by comparing the retention times and the metabolite patterns found in the current study with those of the laying hen study with the pyridinylmethyl label [KIIA 6.2.2/01]. The metabolites were quantified at almost the same low level as detected in the extracts of the study with the pyridinylmethyl label. Additionally, BYI 02960-acetic acid was detected in excreta.

Metabolites in the polar region of the acetonitrile/water extracts were specific for the furanone label, they ranged from 0.001 to 0.012 mg/kg for muscle and from 0.009 to 0.050 mg/kg for liver. These

polar metabolites showed the same behaviour in thin layer chromatography as the metabolites in the corresponding polar regions of urine from the rat (KIIA 5.1.2/01) and urine, extracts of liver and kidney of the goat (KIIA 6.2.3/02). Metabolites in the neutral and acidic exhaustive extracts were characterised by their extraction behaviour and in some cases by HPLC, depending on the amount of matrix content.

The identification rate, including the parent compound, metabolites and residues identified as fatty acids after saponification, accounted for approx. 59% for eggs day 2 to 7, 63% for eggs day 8 to sacrifice, 17% for muscle, 96% for fat and 59% for liver. All other residues as well as the losses during the concentration procedures were characterised by their extraction- or chromatographic behaviour.

The concentration of the total radioactivity (expressed as mg a.s. equiv. /kg) as well as the distribution of the parent compound and metabolites and the identification rates in all edible samples are summarised in the following table:

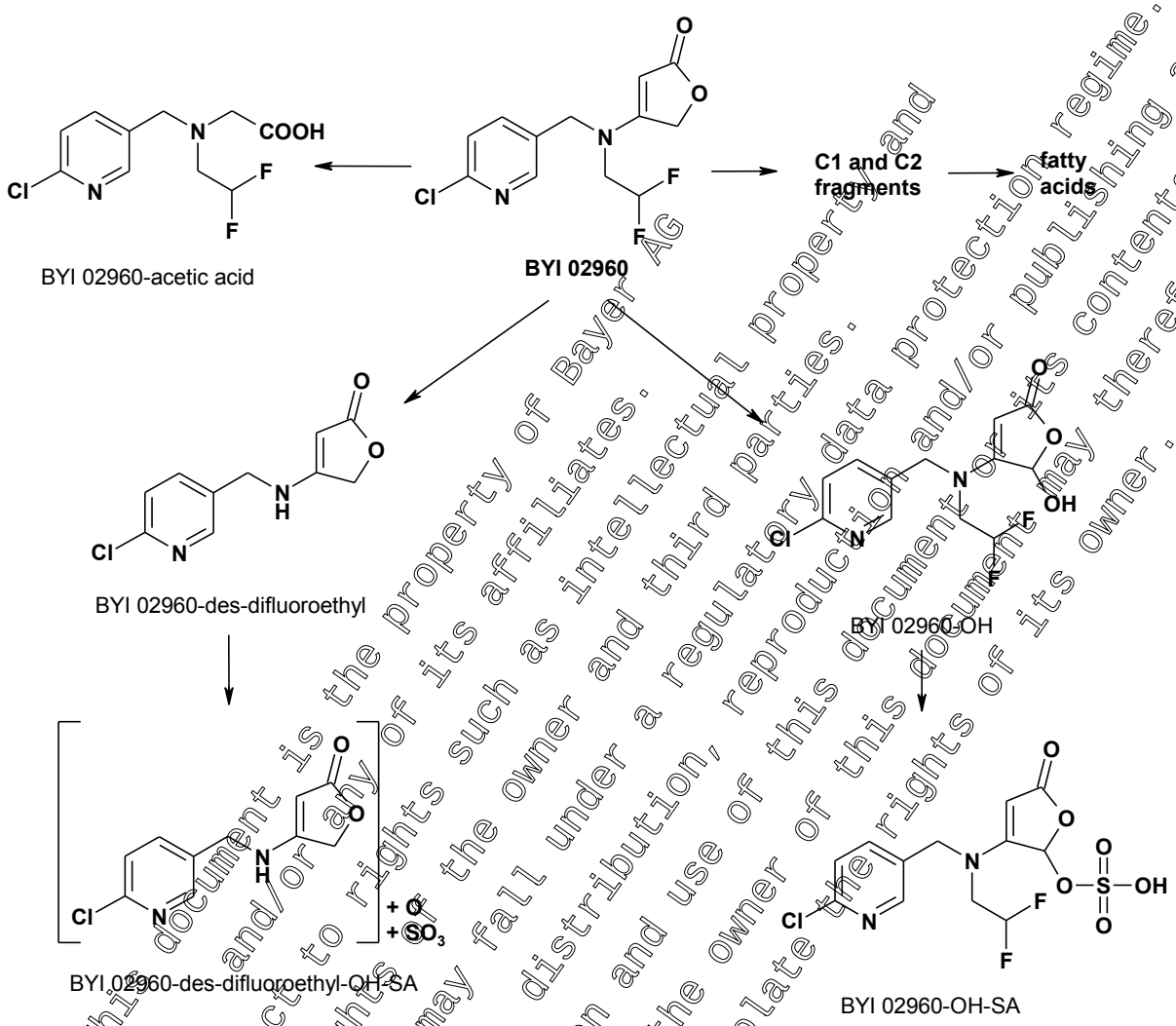
	Eggs (day 2 to 7)		Eggs (day 8 to 13.25)		Muscle		Fat		Liver	
TRR [mg/kg]	0.540		1.048		0.183		0.427		2.178	
Sample/Report name BYI 02960-	% of TRR	mg/kg	% of TRR	mg/kg	% of TRR	mg/kg	% of TRR	mg/kg	% of TRR	mg/kg
Conventional extraction	65.0	0.351	66.8	0.701	38.6	0.071	98.5	0.421	72.1	1.571
n-heptane phase (identified as fatty acids)	52.0	0.281	58.3	0.611	8.1	0.015	95.9	0.410	51.5	1.121
des-difluoroethyl-OH-SA	0.1	0.001	n.d.	n.d.	0.5	0.001			0.2	0.004
des-difluoroethyl	1.2	0.006	0.6	0.007	2.6	0.005			0.8	0.017
OH-SA	0.6	0.003	0.5	0.005	n.d.	n.d.			5.1	0.112
OH	2.3	0.013	1.6	0.016	2.4	0.004			0.8	0.018
Parent compound	2.3	0.013	1.6	0.016	2.9	0.005			0.5	0.010
identified in the conventional extract	58.5	0.316	62.5	0.656	16.5	0.030	95.9	0.410	58.9	1.282
characterised in the conventional extract	6.4	0.035	4.3	0.045	22.0	0.040	2.6	0.011	13.2	0.288
losses during the conventional extraction	10.7	0.058	10.7	0.112	10.3	0.019	n.d.		n.d.	
Exhaustive extraction										
- neutral ACN/water extract	7.0	0.038	3.6	0.038	8.1	0.015	n.a.		9.6	0.209
- 1st acidic ACN/water extract	8.3	0.045	10.0	0.105	39.5	0.072	n.a.		12.1	0.264
- 2nd acidic ACN/water extract	1.1	0.006	3.3	0.035	see**		n.a.		n.a.	
characterised in the exhaustive extracts	16.5	0.089	16.9	0.177	47.7	0.087	n.a.		21.7	0.473
Total identified	58.5	0.316	62.5	0.656	16.5	0.030	95.9	0.410	58.9	1.282
Total characterised	22.9	0.124	21.2	0.223	69.7	0.128	2.6	0.011	34.9	0.761
Total extracted	81.5	0.440	83.8	0.878	86.2	0.158	98.5	0.421	93.8	2.044
Total losses	17.5	0.094	14.7	0.155	10.3	0.019	n.d.	n.d.	4.5	0.098
Solids	1.1	0.006	1.5	0.016	3.5	0.006	1.5	0.006	1.7	0.036
Accountability	100.0	0.540	100.0	1.048	100.0	0.183	100.0	0.427	100.0	2.178

** Both acidic ACN/water extracts of muscle were combined before concentration.

The metabolic and excretion behaviour of [furanone-4-^{14}C]BYI 02960 in the laying hen can be characterised by the following observations:

- The concentration of the radioactivity in eggs and edible tissues was slightly elevated when compared to the dose level and the dosing period of 14 days. This was mainly caused by the partial instability of the radiolabel used. For a small part of the total dose the furanone ring of the molecule obviously underwent extensive biotransformation to C-1- and C-2-fragments resulting in an accumulation of radioactivity in biomolecules. Some of these fragments were probably also converted to the terminal product $^{14}CO_2$ as can be derived from the lower overall recovery in comparison to the study with the metabolically stable pyridinylmethyl label.
- The relatively high values in liver and kidney at sacrifice 6 hours after the last dose indicated that metabolism and excretion are still ongoing processes.
- A residue plateau level in whole eggs was reached at day nine after the first administration.
- The extraction rates were above 80% for eggs and edible tissues. Unextracted residues were quite low and amounted to ≤0.036 mg/kg.
- Parent compound and non label specific metabolites, such as BYI 02960-OH, BYI 02960-OH-SA, BYI 02960-des-difluoroethyl and BYI 02960-des-difluoroethyl-OH-SA were identified in similar amounts as in the study using the pyridinylmethyl label. Additionally, BYI 02960-acetic acid was detected in excreta.
- The main metabolic reactions of [furanone-4-^{14}C]BYI 02960 in the laying hen are:
    - Cleavage and subsequent total degradation of the furanone ring forming smaller carbon units (C-1- or C-2-fragments), entering the carbon pool of endogenous compounds and then being used for the biosynthesis of fatty acids
    - Hydroxylation in position 5 of the furanone ring forming BYI 02960-OH followed by conjugation with sulphuric acid to BYI 02960-OH-SA
    - Cleavage of the difluoroethyl group forming BYI 02960-des-difluoroethyl followed by hydroxylation and conjugation with sulfuric acid to BYI 02960-des-difluoroethyl-OH-SA
    - Oxidative degradation of the furanone ring forming BYI 02960-acetic acid

Based on these results the following metabolic pathway is proposed:



I. Materials and Methods

A. Materials

1. Test Material

IUPAC Name	4-{[(6-chloropyridin-3-yl)methyl](2,2-difluoroethyl)amino}furan-2(5H)-one
Code name	BYI02960
Common name	flupyradifurone (proposed ISO)
Empirical formula	$C_{12}H_{11}ClF_2N_2O_2$
Molar mass	288.68 g/mol
Labelling position	[furanone-4-^{14}C]
Specific radioactivity	4.24 MBq/mg = 114.50 µCi/mg (delivered sample before radiodilution) 3.50 MBq/mg = 2.10 x 10^8 dpm/mg = 94.59 µCi/mg = 27.31 GCi/mol (sample after radiodilution)
Radiochemical purity	> 98.1 % by radio-HPLC and > 99 % by radio-TLC
Nonradioactive test substance	Batch BYI 02960-PU-02
Chemical purity	99.4%
Dose level	14 oral doses of 1.05 mg/kg bw/day by gavage
Vehicle	0.5 % aqueous Tragacanth® suspension

2. Test Animals

Species	Laying hen (*Gallus gallus domesticus*)
Strain	"White Leghorn"
Breeding facility	[redacted]
Sex and numbers involved	6 out of 18 hens were selected by maximum egg production
Age	6 – 8 months
Body weight	1.56 kg at first administration, 1.56 kg at sacrifice
Acclimatization	14 days
Identification	Cage labelling and wing tags
Housing	Individually in stainless steel metabolism cages for laying hens allowing almost quantitative collection of eggs and excreta (supplier: [redacted] room temperature 21 - 26°C, relative humidity 39 - 56%. 16 h light / 8 h dark cycle, air change 10 – 15 times per hour
Feed and water	The hens were fed with "RWZ-LegeGold Mehl", a pulverised chicken feed. This feed was not a certified diet, i.e. it was not checked for contamination according to current standards. The feed was supplemented by eggshells and crushed marine shells during the acclimation period. The feed consumption was recorded by weighing during the experiment (mean consumption 103 g/day/hen), tap water, ad libitum

B. Study Design

Dosing

The radiolabelled test compound was mixed with the non-radiolabelled test compound in order to reduce the specific radioactivity from 4.24 MBq/mg to 3.5 MBq/mg. For the 14 administrations, 4 dosing suspensions in 0.5% aqueous Tragacanth® were prepared and each suspension was applied for three or four administrations. The radiolabelled test substance proved to be stable until the last dose.

The oral administration was carried out with a knob cannula attached to a glass syringe. Immediately after dosage, the swallowing was supported by a gentle massage of the throat towards the crop. The hens received a mean dose of 1.63 mg (3.42 x 10^8 dpm) per animal and day at a mean body weight of 1.56 kg, corresponding to an actual dose of 1.05 mg a.s./kg bw. The amount of radioactivity of the actually administered amounts served as reference for the calculation of total radioactivity in the biological samples. The administration volume was 1.0 mL/kg body weight. The dose level was tolerated without any observable toxicological symptoms.

Sampling of eggs and excreta

During the test, the cages were inspected for egg production once daily (in the morning before administration) and the number of eggs was recorded for all hens. After sampling, the eggshells were discarded, and the egg white and yolk were weighed and then thoroughly mixed. An aliquot of each egg mix was taken for the determination of the total radioactivity in triplicate by LSC. The residual amount of the egg-mix from all animals was stored at ca. – 18°C until metabolite analysis.

The excreta of each hen were collected once daily from the collecting tins as quantitatively as possible. The individual samples were homogenised after the addition of water, before recording the total weights. An aliquot of each fraction was processed for radioactivity measurement by combustion/LSC. The remaining samples were stored in a freezer until metabolite analysis.

Sacrifice and sampling of organs and tissues

The treated hens were weighed and sacrificed ca. 6 hours after the last (14th) dosage. The animals were anaesthetised using carbon dioxide, sacrificed by decapitation and exsanguinated. The organs and tissues were prepared immediately after sacrifice. Liver without the gall bladder, kidneys, leg and breast muscle, skin without subcutaneous fat, subcutaneous fat, eggs from the ovary and oviduct, were sampled immediately after sacrifice and their fresh weights were recorded. The gall bladders were punctured for the collection of the bile fluid which was then stored frozen for an optional metabolite analysis.

Sample preparation

After dissection, the organs or tissue samples were transferred into ice-cooled vessels. Liver, kidneys, muscle samples, and subcutaneous fat as well as eggs dissected from the ovary and oviduct were thoroughly homogenised in half-frozen state. An aliquot of each resulting tissue pulp was weighed, freeze-dried, weighed again and homogenised. The radioactivity was then determined in triplicate using combustion/LSC. Aliquots of subcutaneous fat and skin without fat were weighed and solubilised with tissue solubiliser (BTS-450®) and measured for radioactivity by LSC. All samples were divided into equal portions and stored at ca. -18°C until metabolite analysis.

To prepare representative samples of eggs, organs or tissues, corresponding samples of all individual animals were pooled before extraction. Two egg pools were prepared (day 2 - day 7 and day 3 - sacrifice). All egg samples from these collection periods were used. Composite samples of muscle (leg and breast), subcutaneous fat, liver and excreta (day 13) from all animals were prepared. The individual samples were thoroughly homogenised and kept frozen until extraction.

The total radioactive residues of each pool were determined by combustion/LSC (solid samples) or direct LSC (e.g. combined eggs and extracts). Eggs, muscle, fat and liver were successively extracted with acetonitrile and n-heptane followed by a solvent partition procedure. In case of eggs, muscle and liver this extraction procedure was continued using a mixture of acetonitrile/water (7:3; v/v). An aliquot of the excreta of day 13 was extracted with acetonitrile/water (8:2, v/v) followed by pure acetonitrile. All extracts were used for quantification of parent compound and metabolites by HPLC.

Radioactivity measurement

Solid samples were combusted prior to radioactivity determination and the formed $^{14}CO_2$ absorbed in an alkaline trapping solvent. The determination of radioactivity of liquid samples was conducted by liquid scintillation counting (LSC) using sub-samples (1 - 3 replicates). Quenching effects were automatically corrected using an external standard and a quenching library. The instrument background was automatically subtracted. For all samples, the limit of detection (LOD) was established at approximately 10 dpm per aliquot after instrument background correction. The limit of quantification (LOQ) was established as 2 times the background radioactivity (dpm) of each instrument/method.

Metabolite analysis

The n-heptane phases of eggs, muscle, fat and liver were concentrated and analysed by thin layer chromatography on silica gel plates and radioluminography for detection of radioactive spots. The combined acetonitrile and acetonitrile/water extracts were concentrated and used for the quantification of parent compound and metabolites by HPLC. Although the acetonitrile/water phase was partitioned against n-heptane, a portion of the fatty matrix remained in the acetonitrile/water phase and caused incomplete elution of the rarioactivity from the HPLC column and interferences during detection due to chemiluminescence. Therefore a washing step of the HPLC column was implemented and the HPLC run as well as the washing step were quantified by LSC. The eluent of subregion 1 (= analytical run: 0 to 80 min.) and subregion 2 (= washing step: 80 to 120 min.) was collected and the radioactivity was determined by LSC. The sum of both subregions was related to the origin percentage and ppm-value. The completeness of the chromatographic elution for the adapted HPLC profiling method (including the washing step) was shown for extracts from eggs, muscle and liver. The recovery ranged from 97.6 to 104.3% of the injected radioactivity. Parent compound and metabolites were detected and quantified in subregion 1. In case of muscle and liver, the polar region R1 of the subregion 1 was isolated and further investigated by TLC. Post extraction solids of eggs, muscle and liver were exhaustively extracted twice with acetonitrile/water (1:1; v/v) followed by 2 extractions with acetonitrile/water/formic acid (50:50:2.5; v/v/v) using microwave assistance. The concentrated exhaustive extracts were analysed by HPLC, depending on the matrix content.

Basically, the isolation and identification of parent compound and metabolites were performed in the study using the pyridinylmethyl label (KIIA 6.2.2/01). An additional identification of BYI 02960-des-

difluoroethyl in the extract of muscle was performed by HPLC co-chromatography with the radiolabelled reference compound. The reference compound was isolated and identified in the laying hen study with the pyridinylmethyl label. Metabolites in the polar region R1 of the acetonitrile/water extracts of muscle and liver were quantified and further characterised by thin layer chromatography. Their commonality with polar metabolites in urine and organs of rat and goat was demonstrated by comparison of the TLC profiles (KIIA 5.1.2/01; KIIA 6.2.3/02). Radioactive residues in the n-heptane phases were analysed by TLC and visualised in an iodine vapour chamber. Residues in the n-heptane phase of fat were further identified by investigation of their partition behaviour after saponification and subsequent acidification.

II. Results and Discussion

A. Recovery and Elimination of Radioactivity

The recovery of radioactivity in laying hens after administration of an average daily dose of 1.05 mg [furanone-4-^{14}C]BYI 02960 per kg bw/day on 14 consecutive days is presented in Table 6.2.2-5. The overall recovery accounted for 82.16% of the totally administered dose. A part of the remaining radioactivity was expected to still be present in the gastro-intestinal tract at sacrifice due to the short period between the last administration and sacrifice. Another part is probably exhaled as $^{14}CO_2$. The formation of carbon dioxide was also shown in a rat quantitative whole-body autoradiography study using the same labelling position (KIIA 5.1.2/02).

Table 6.2.2-5: Distribution of residues in eggs, muscle, fat, liver and kidney of laying hens following oral administration of 14 daily doses of furanone-4-^{14}C] BYI02960 at a dose rate of 1.05 mg/kg

Sample	Percent of total dose administered	Concentration of total radioactivity [mg/kg]
Liver	0.37	2.178
Kidney	0.05	1.083
Eggs from ovary/oviduct	0.46	2.774
Muscle, total	0.50	0.183
Skin, total	0.07	0.257
Fat, total	0.34	0.427
Organs/tissues, total	1.80	-----
Eggs	2.35	0.757
Excreta, total	78.01	-----
Total Recovery	82.16	-----

An average amount of 2.35% of the total dose was measured in the eggs. At sacrifice, the residues in the organs and tissues were calculated or estimated to be about 1.8% of the total dose from which about 28% was detected in the skeletal muscle (0.5%). Until sacrifice, the excreted radioactivity accounted for 78.01% of the total dose.

B. Levels and Time Course of Total Radioactivity in Eggs

The radioactivity levels measured in the egg samples from all animals are presented in Table 6.2.2-6. The concentrations in eggs ranged from 0.024 mg/kg at day one to 1.198 mg/kg at sacrifice. Following

a linear increase a residue plateau level of 1.035 mg/kg was reached at day nine after the first administration.

Table 6.2.2-6: Time course of total radioactivity in eggs following oral administration of 14 daily doses of [furanone-4-^{14}C] BYI02960 at a dose rate of 1.05 mg/kg

Time after the first admin. [d]	Admin. no.	Cumulative secretion [% of total dose admin.] (mean)	Concentration of total radioactivity [mg/kg]
0	1	----- #	----- #
1	2	0.01	0.024
2	3	0.04	0.146
3	4	0.10	0.284
4	5	0.20	0.438
5	6	0.40	0.647
6	7	0.56	0.789
7	8	0.72	0.923
8	9	0.94	1.002
9	10	1.20	1.035
10	11	1.44	1.035
11	12	1.74	1.100
12	13	1.97	1.036
13	14	2.04	1.005
13.25	---	2.35	1.198

----- # no egg collected

C. Total Radioactive Residues in Dissected Organs and Tissues

The concentration of the total radioactivity in the dissected organs and tissues at sacrifice is shown in Table 6.2.2-5 (last column). The highest concentrations were determined in liver (2.178 mg/kg) and kidney (1.083 mg/kg) reflecting the significance of these organs for metabolism and excretion. In relation to the dose totally administered, these values corresponded to 0.37% and 0.05%, respectively. The residue level of the eggs collected from the ovary and oviduct at sacrifice (2.774 mg/kg) was higher by a factor of 2.3 than the levels of the laid eggs collected at sacrifice (1.198 mg/kg). This indicated that the egg yolk was a preferential site for the secretion of radioactivity. The residue levels of liver and kidney were followed in decreasing order by those determined in the subcutaneous fat (0.427 mg/kg), skin (0.257 mg/kg) and muscle (0.183 mg/kg). The residue level of the total skeletal muscle corresponded to about 0.50% of the total dose assuming a value of 40% contribution to the body weight. Assuming values of 12% and 4% of the body weight for fat and skin, the residues in both tissues accounted for about 0.41% of the dose totally administered.

D. Extraction Efficiency of Residues

Egg pools (day 2 to 7 and 8 to sacrifice), muscle-, fat- and liver pools were extracted with acetonitrile and n-heptane followed by solvent partition. In case of eggs, muscle and liver the extraction procedure was continued with a mixture of acetonitrile/water (7:3; v/v). Excreta (day 13) were extracted with

acetonitrile/water (8:2, v/v) and pure acetonitrile. Post-extraction solids of eggs, muscle and liver were extracted with neutral (acetonitrile/water (1:1, v/v)) and acidic (acetonitrile/water/formic acid (50:50:2.5, v/v/v)) solvent mixtures using microwave assistance. Radioactivity concentrations in the post-extraction solids amounted to 0.006 mg/kg (1.1%) for the egg pool 1 (day 2 to 7), 0.016 mg/kg (1.5%) for the egg pool 2 (day 8 to sacrifice), 0.006 mg/kg (3.5%) for muscle, 0.006 mg/kg (1.5%) for fat and 0.036 mg/kg (1.7%) for liver.

The major part of residues was detected in the n-heptane phases of eggs (ca. 52 and 58%), fat (ca. 96%) and liver (ca. 52%). The residues in the n-heptane phase of muscle amounted to only ca. 8%.

Residues in the acetonitrile/water extract of eggs, muscle, fat and liver were detected at the same residue level as analysed in the laying hen study with the pyridinylmethyl label (KIIA 6.2.2/01). The residue concentrationss ranged from 0.011 mg/kg for fat to 0.450 mg/kg for liver. Because of the low residue concentration the acetonitrile phase of fat was not further investigated.

A significant part of residues was exhaustively extracted using microwave assistance. Between 4 and 10% of the total radioactivity was detected in the neutral extracts and between 8 and 40% in the acidic extracts of eggs, muscle and liver. Losses of radioactivity during the extraction procedure of eggs, muscle and liver were between 5 and 18% of the total radioactivity. During the concentration procedure of the extracts losses were between 1.7 and 4.5%. Losses of radioactivity during the extraction and concentration procedures of eggs, muscle and liver were not further investigated. Most probably, the losses of radioactivity were caused by adhesion of radioactive fat or non-polar matrix on the equipment (e.g. Polytron homogenizer, filter etc.). A summary of the extraction efficiency of the samples is presented in Table 6.2.2.7.

Table 6.2.2-7: Extraction efficiency of eggs, muscle, fat and liver samples following oral administration of 14 daily doses of [furanone-4-^{14}C] BYI02960 at a dose rate of 1.05 mg/kg

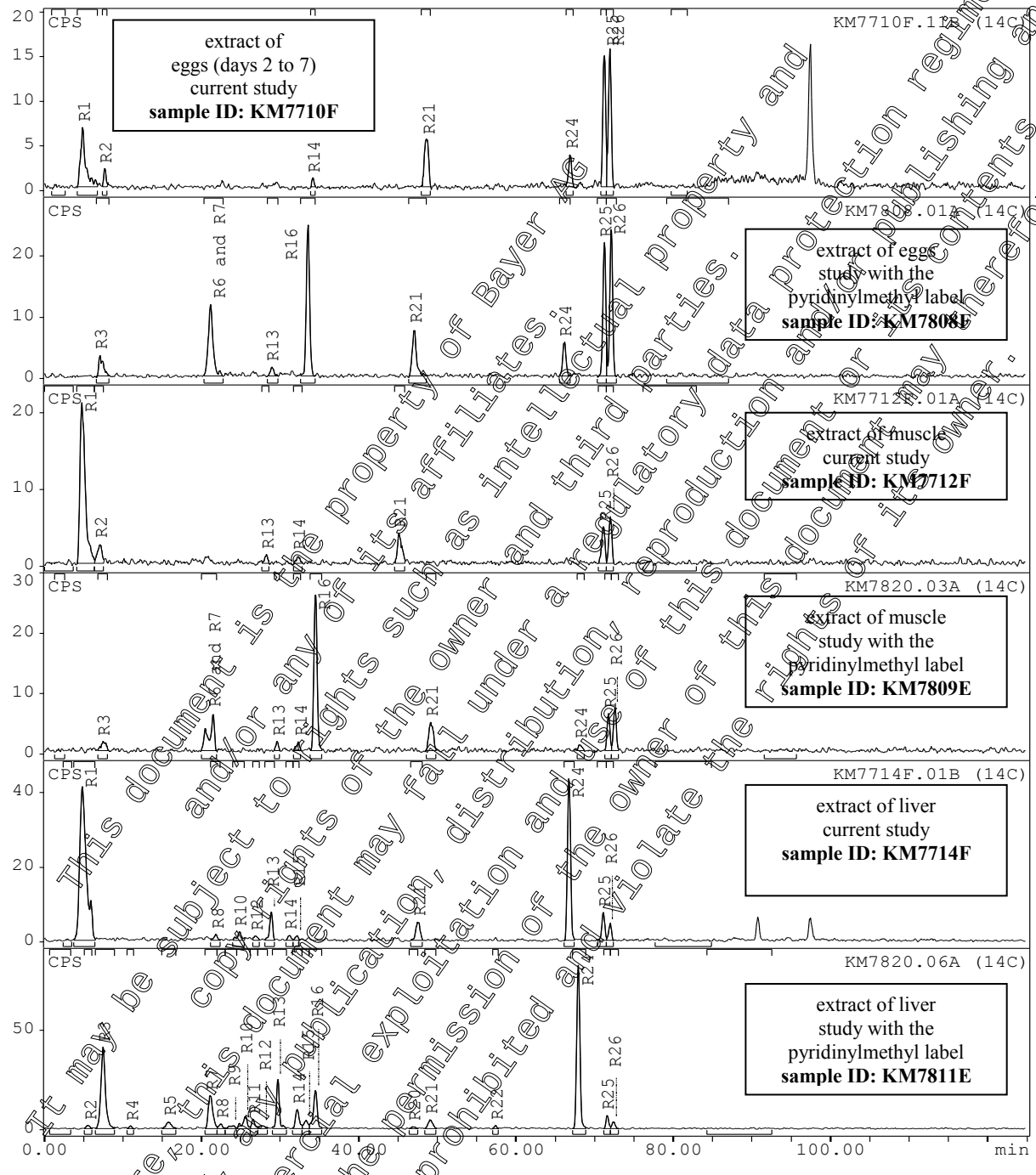
	Eggs (days 2 to 7)		Eggs (days 8 to 13.25)		Muscle		Fat		Liver	
TRR [mg/kg]	0.540		1.048		0.183		0.427		2.178	
	% of TRR	mg/kg	% of TRR	mg/kg	% of TRR	mg/kg	% of TRR	mg/kg	% of TRR	mg/kg
Conventional extraction	65.0	0.351	66.8	0.701	38.6	0.071	98.5	0.421	72.1	1.571
- n-heptane phase	52.0	0.281	58.3	0.611	8.1	0.015	95.9	0.410	51.5	1.121
- ACN/water extract	13.0	0.070	8.5	0.089	30.4	0.056	2.6	0.011	20.7	0.450
- subregion 1	8.6	0.046	4.9	0.052	27.4	0.050	n.a.		19.6	0.426
- subregion 2	2.7	0.015	3.6	0.038	n.d.	n.d.	n.a.		1.1	0.024
- losses during the conventional extraction	10.7	0.058	10.7	0.112	10.3	0.019	n.d.	n.d.	n.d.	n.d.
Exhaustive extraction										
- combined neutral ACN/water extract	7.0	0.038	3.6	0.038	8.1	0.015	n.a.	9.6	0.209	
- 1st acidic ACN/water extract	8.3	0.045	10.0	0.105	39.5	0.072	n.a.	12.1	0.264	
- 2nd acidic ACN/water extract	1.1	0.006	3.3	0.035	see**	n.a.	n.a.			
- losses during the exhaustive extraction	6.8	0.037	4.1	0.043	n.d.	n.d.	n.a.	4.5	0.098	
Total extracted	81.5	0.440	83.8	0.878	86.2	0.158	98.5	0.421	93.8	2.044
Total losses of the extraction procedures	17.5	0.094	14.7	0.155	10.3	0.019	n.d.	n.d.	4.5	0.098
Solids	1.1	0.006	1.5	0.016	3.5	0.006	1.5	0.006	1.7	0.036
Accountability	100.0	0.540	100.0	1.048	100.0	0.183	100.0	0.427	100.0	2.178

** Both acidic ACN/water extracts of muscle were combined before concentration.

E. Quantification, Identification and Characterisation of Residues

The identification and assignment of parent compound and metabolites in the acetonitrile/water extracts was based on the comparison of the metabolite profiles of the current study with the profiles of the study with the pyridinylmethyl label (KIIA 6.2.2/01) using the HPLC system described there. Additionally, BYI 02960-des-difluoroethyl was identified in the extract of muscle by HPLC co-chromatography with the radiolabelled reference compound. As an example of such a comparison the metabolic profiles in extracts of egg, muscle and liver is shown in Figure 6.2.2-4.

Figure 6.2.2-4: Comparison of HPLC profiles of the acetonitrile/water extracts from eggs, musle and liver of the current study and the study with the pyridinylmethyl ^{14}C-label



Metabolites in the Polar Region (R1) of the Conventional Extracts

Metabolites in the polar region R1 of the acetonitrile/water extract of muscle and liver (see Figure 6.2.2-4 above) were further characterised by thin layer chromatography. These metabolites ranged from 0.001 to 0.012 mg/kg for muscle (six metabolites) and from 0.009 to 0.050 mg/kg for liver (nine metabolites). These polar metabolites were specific for the furanone label and were caused by the degradation of the furanone ring. These metabolites were also detected in the corresponding polar region of urine from the rat- (KIIA 5.1.2/01) and urine, liver and kidney from the goat-metabolism

studies ([KIIA 6.2.3/02). The polar region R1 of eggs was not investigated due to its low residue level (ca. 0.01 mg/kg) and the high matrix content.

Identification of Residues in the n-Heptane Phases

The thin-layer chromatographic behaviour of the residues in the n-heptane phases of eggs, muscle, fat and liver was identical. As an example, the partition behaviour of the radioactive residues in the n-heptane phase of fat was investigated after saponification and subsequent acidification. The radioactive residues were cleaved by saponification yielding glycerol and salts of fatty acids. After solvent partition, the entire radioactivity was detected in the aqueous phase, where the salts of the fatty acids were dissolved. The radioactivity was transferred back into the n-heptane phase after acidification of the aqueous phase. This partition behaviour is typical for fatty acids, which are building blocks of natural lipids.

The residues in the n-heptane phases were specific for the furanone label and were caused by the cleavage and subsequent degradation of the furanone ring to smaller carbon units (C-1- or C-2-fragments), entering the carbon pool of endogenous compounds.

Distribution of Parent Compound and Metabolites in Eggs, Organs and Tissues

The distribution of the parent compound and metabolites in eggs, organs and tissues is summarised in Table 6.2.2-8. The identification rate was 59% in eggs (days 2 to 7), 62% in eggs (days 8 to sacrifice), 17% in muscle, 96% in fat and 59% in liver. All other residues were characterised by their extraction or chromatographic behaviour.

Table 6.2.2-8: Radioactive residues in eggs, muscle, fat and liver samples following oral administration of 14 daily doses of [furanone-4-^{14}C] BYI02960 at a dose rate of 1.05 mg/kg

	Eggs (day 2 to 7)		Eggs (day 8 to 13.25)		Muscle		Fat		Liver	
TRR [mg/kg]	0.540		1.048		0.183		0.427		2.178	
Sample/Report name BYI 02960-	% of TRR	mg/kg	% of TRR	mg/kg	% of TRR	mg/kg	% of TRR	mg/kg	% of TRR	mg/kg
Conventional extraction	65.0	0.351	66.8	0.701	38.6	0.071	98.5	0.421	72.4	1.577
n-heptane phase (identified as fatty acids)	52.0	0.281	58.3	0.611	8.1	0.015	95.9	0.410	51.5	1.121
des-difluoroethyl-OH-SA	0.1	0.001	n.d.	n.d.	0.5	0.001			0.2	0.004
des-difluoroethyl	1.2	0.006	0.6	0.007	2.6	0.005			0.8	0.017
OH-SA	0.6	0.003	0.5	0.005	n.d.	n.d.			5.1	0.112
OH	2.3	0.013	1.6	0.016	2.4	0.004			0.8	0.018
Parent compound	2.3	0.013	1.6	0.016	2.9	0.005			0.5	0.010
identified in the conventional extract	58.5	0.316	62.5	0.656	16.5	0.030	95.9	0.410	58.9	1.282
characterised in the conventional extract	6.4	0.035	4.3	0.045	22.0	0.040	2.6	0.011	13.2	0.288
losses during the conventional extraction	10.7	0.058	10.7	0.112	10.3	0.019	n.d.		n.d.	
Exhaustive extraction										
- neutral ACN/water extract	7.0	0.038	3.6	0.038	8.1	0.015	n.a.		9.6	0.209
- 1st acidic ACN/water extract	8.3	0.045	10.0	0.105	39.5	0.072	n.a.		12.1	0.264
- 2nd acidic ACN/water extract	1.1	0.006	3.3	0.035	see **		n.a.		n.a.	
characterised in the exhaustive extracts	16.5	0.089	16.9	0.177	47.7	0.087	n.a.		21.7	0.473
Total identified	58.5	0.316	62.5	0.656	16.5	0.030	95.9	0.410	58.9	1.282
Total characterised	22.9	0.124	21.2	0.223	69.7	0.128	2.6	0.011	34.9	0.761
Total extracted	81.5	0.440	83.8	0.878	86.2	0.158	98.5	0.421	93.8	2.044
Total losses	17.5	0.094	14.7	0.155	10.3	0.019	n.d.	n.d.	4.5	0.098
Solids	1.1	0.006	1.5	0.016	3.5	0.006	1.5	0.006	1.7	0.036
Accountability	100.0	0.540	100.0	1.048	100.0	0.183	100.0	0.427	100.0	2.178

** Both acidic ACN/water extracts of muscle were combined before concentration.

Metabolites in Eggs

The main part of the radioactivity in eggs (days 2 to 7 0.281 mg/kg, 52.0%; days 8 to sacrifice 0.611 mg/kg, 58.3%) was detected in the n-heptane phase and identified as natural fats. The parent compound, BYI 02960-OH, BYI 02960-OH-SA, BYI 02960-des-difluoroethyl and BYI 02960-des-difluoroethyl-OH-SA were identified in low amounts (≤ 0.016 mg/kg). Metabolites in the polar region R1 (see Fig. 6.2.2-4) were not further quantified due to the low concentration (≤ 0.010 mg/kg).

Metabolites in Muscle

Residues in the n-heptane phase of muscle were identified as natural fats and amounted to 0.015 mg/kg (8.1%), only. Very low amounts (≤ 0.005 mg/kg) of parent compound, BYI 02960-OH, BYI 02960-des-difluoroethyl and BYI 02960-des-difluoroethyl-OH-SA were identified in the acetonitrile/water extract. Metabolites in the polar region R1 of the acetonitrile/water extract (see Fig. 6.2.2-4) were further analysed and subquantified by thin layer chromatography. The concentrations ranged from 0.001 to 0.012 mg/kg. The commonality of these metabolites with polar metabolites, which were detected in the urine of rats (KIIA 5.1.2/01) and in the urine, liver and kidney of the goat (KIIA 6.2.3/02) was demonstrated by comparison of the thin layer chromatograms.

Metabolites in the acidic exhaustive extract represented the major portion of residues in the muscle sample (0.072 mg/kg, 39.5%). They were characterised by their extraction behaviour. An HPLC analysis could not be performed, due to the high matrix burden in the extract.

Metabolites in Fat

The dominating part of the radioactive residues in fat (0.410 mg/kg, 95.9%) was identified as fatty acids after saponification. The fatty acids were formed from smaller carbon units (C-1- or C-2-fragments), which entered the carbon pool of endogenous compounds after cleavage and subsequent degradation of the furanone ring. A small part (0.011 mg/kg, 2.6%) was detected in the acetonitrile phase and was not further investigated.

Metabolites in Liver

More than half of the residues in liver (1.121 mg/kg, 51.5%) was identified as natural fats. Parent compound, BYI 02960-OH, BYI 02960-OH-SA, BYI 02960-des-difluoroethyl and BYI 02960-des-difluoroethyl-OH-SA were identified in the acetonitrile/water extract. The most prominent metabolite was BYI 02960-OH-SA which amounted to 0.112 mg/kg (5.1%). All other identified metabolites were detected in low amounts between 0.004 and 0.018 mg/kg.

Metabolites in the polar region R1 of the acetonitrile/water extract (see Fig. 6.2.2-4) were subquantified by thin layer chromatography. Their concentrations ranged from 0.009 to 0.050 mg/kg. The commonality of these metabolites with polar metabolites, which were detected in the urine of rats (KIIA 5.1.2/01) and in the urine, liver and kidney of the lactating goat, (KIIA 6.2.3/02) was demonstrated by comparison of the thin layer chromatograms.

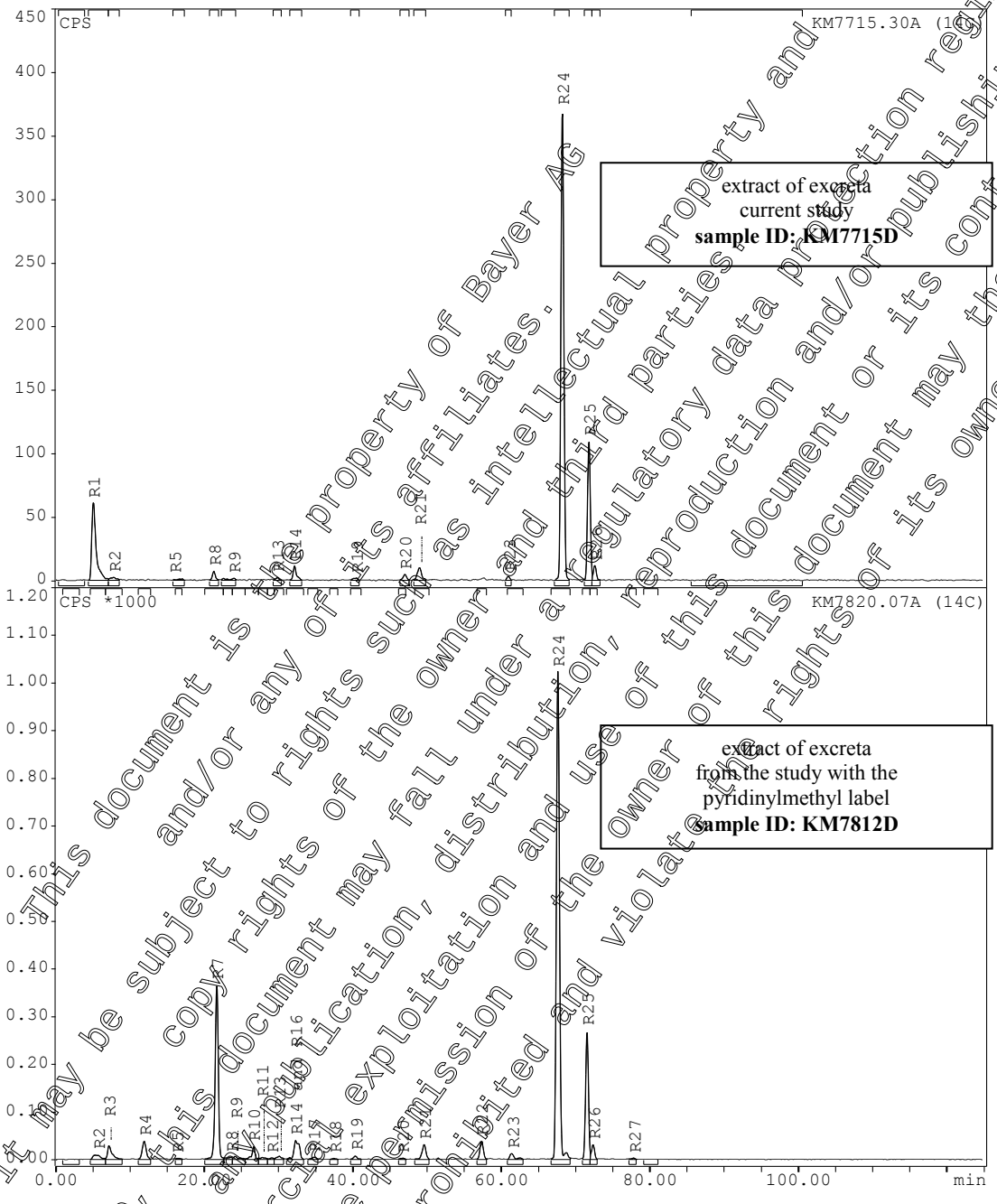
Metabolites in Excreta

The metabolite profiles of excreta of the current study and the study using the pyridinylmethyl label were very similar, except for the label specific compounds - e.g. in the region of polar metabolites (R1). A comparison of the profiles from both studies is shown in Figure 6.2.2-5. The quantification of metabolites is given in Table 6.2.2-9.

Table 6.2.2-9: Identified metabolites in the excreta following oral administration of 14 daily doses of [furanone-4-^{14}C] BYI02960 at a dose rate of 1.05 mg/kg

Report name BYI 02960-	Rt [min]	Area [%]	% of RA in sample
Polar metabolites	5.0	14.28	13.5
des-difluoroethyl-OH-SA	32.1	2.05	1.9
des-difluoroethyl	48.9	2.27	2.2
acetic acid	61.0	0.46	0.4
OH-SA	68.2	58.10	55.1
OH	71.8	16.45	15.6
Parent compound	72.6	1.78	1.7
Total			94.8
Sum identified (w/o polar metabolites)			76.9

Figure 6.2.2-5: Comparison of HPLC profiles of the extracts from the excreta of laying hens of the current study and the study with the pyridinylmethyl ^{14}C-label



The dominating metabolite was BYI 02960-OH-SA (55.1%) followed by the free aglycone BYI 02960-OH (15.6%). Only traces of parent compound were identified (1.7%). All other identified metabolites were detected in amounts between 0.4 and 2.2%. The label-specific metabolites in the polar region accounted for 13.5% of the radioactivity in the excreta.

F. Storage Stability of Residues

All samples of eggs, and edible organs and tissues were extracted within approx. 2 months after sample collection. The first metabolite profile was recorded latest within about 3 weeks after sample

preparation and extraction. The stability of the extract of muscle and liver was demonstrated for a period of ca. 7 months by comparison of the HPLC profiles. Further details are provided in the report.

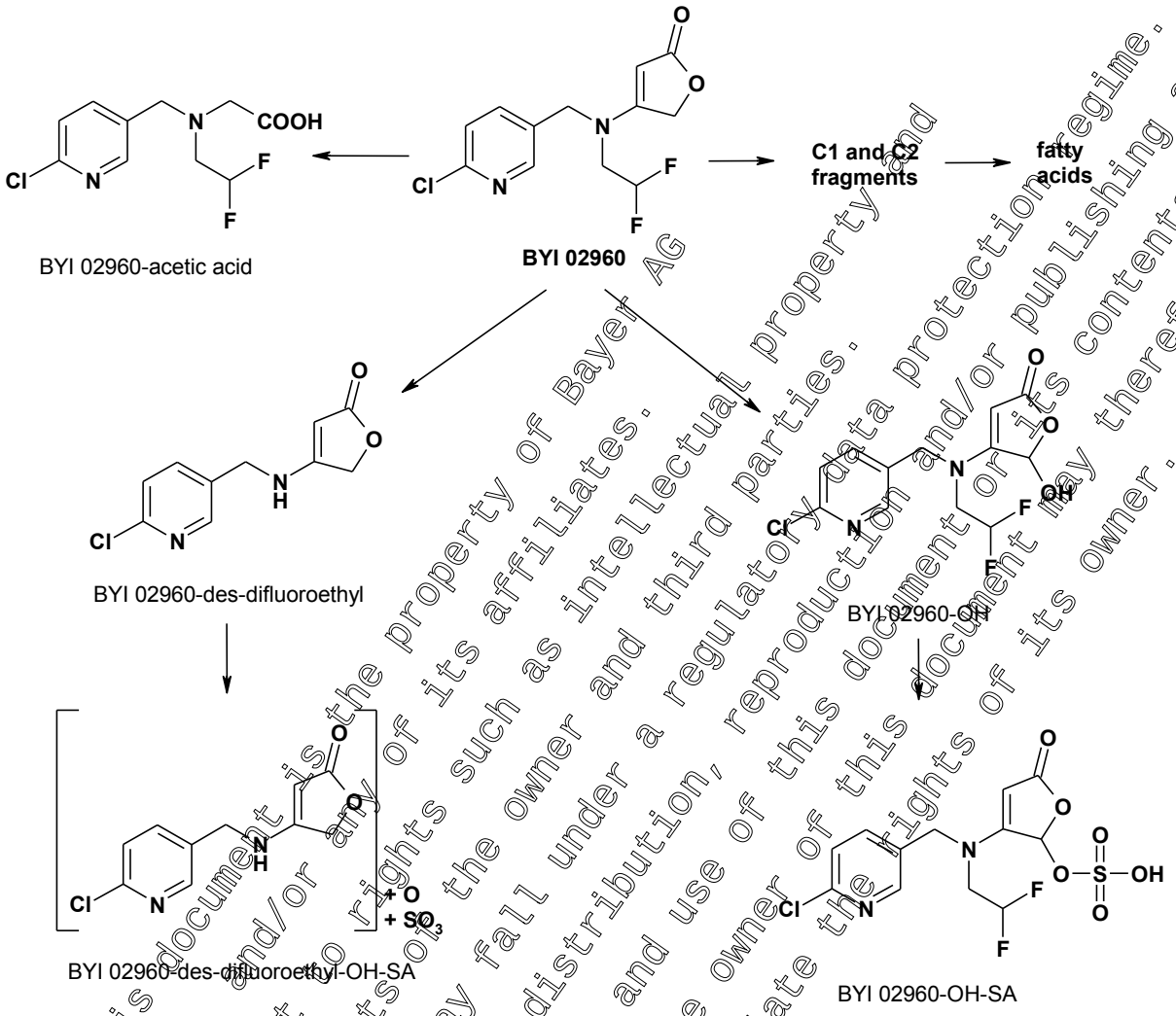
III. Conclusion

The metabolic and excretion behaviour of [furanone-4-^{14}C]BYI 02960 in the laying hen can be characterised by the following observations:

- The concentration of the radioactivity in eggs and edible tissues was slightly elevated when compared to the dose level and the dosing period of 14 days. This was mainly caused by the partial instability of the radiolabel used. For a small part of the total dose the furanone ring of the molecule obviously underwent extensive biotransformation to C-1- and C-2-fragments resulting in an accumulation of radioactivity in biomolecules. Some of these fragments were probably also converted to the terminal product $^{14}CO_2$ as can be derived from the lower overall recovery in comparison to the study with the metabolically stable pyridinylmethyl label.
- The relatively high values in liver and kidney at sacrifice 6 hours after the last dose indicated that metabolism and excretion are still ongoing processes.
- A residue plateau level in whole eggs was reached at day nine after the first administration.
- The extraction rates were above 80% for eggs and edible tissues. Unextracted residues were quite low and amounted to ≤0.036 mg/kg.
- Parent compound and non-label specific metabolites, such as BYI 02960-OH, BYI 02960-OH-SA, BYI 02960-des-difluoroethyl and BYI 02960-des-difluoroethyl-OH-SA were identified in similar amounts as in the study using the pyridinylmethyl label. Additionally, BYI 02960-acetic acid was detected in excreta.
- The main metabolic reactions of [furanone-4-^{14}C]BYI 02960 in the laying hen are:
  - Cleavage and subsequent total degradation of the furanone ring forming smaller carbon units (C-1- or C-2-fragments), entering the carbon pool of endogenous compounds and then being used for the biosynthesis of fatty acids
  - Hydroxylation in position 5 of the furanone ring forming BYI 02960-OH followed by conjugation with sulphuric acid to BYI 02960-OH-SA
  - Cleavage of the difluoroethyl group forming BYI 02960-des-difluoroethyl followed by hydroxylation and conjugation with sulfuric acid to BYI 02960-des-difluoroethyl-OH-SA
  - Oxidative degradation of the furanone ring forming BYI 02960-acetic acid

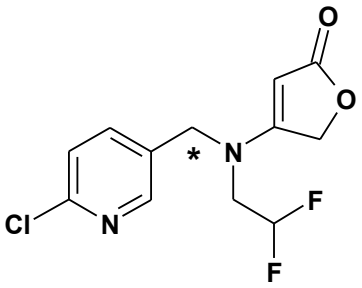
Based on these results the metabolic pathway shown in Figure 6.2.2-6 is proposed.

Figure 6.2.2-6: Proposed metabolic pathway of [furanone-4-^{14}C] BYI02960 in laying hens

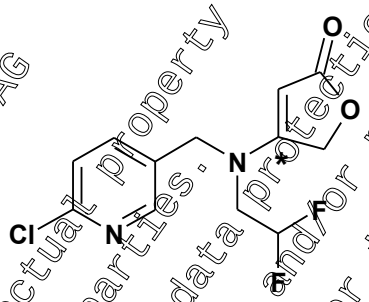


IIA 6.2.3 Lactating ruminants (goat or cow)

Two studies on the metabolism of BYI 02960 in lactating goats were conducted with the test compound labelled either in the [pyridinylmethyl-^{14}C]- or the [furanone-4-^{14}C]-position as shown by the following structural formulas (* denotes the label position):



[pyridinylmethyl-^{14}C]



[furanone-4-^{14}C]

Report:	**KIIA 6.2.3/01, Authors: [redacted], [redacted], (2011)**
Title:	[Pyridinylmethyl-^{14}C]BYI02960: Metabolism in the lactating goat
Report No & Document No:	MEF-11/269 Date: 7.12.2011 M-419701-01-2
Guidelines:	OECD-Guideline for the Testing of Chemicals No. 503, Metabolism in Livestock US EPA Residue Chemistry Test Guideline OPPTS 860.1300 Nature of the Residue – Plants, Livestock European Parliament and Council Regulation (EC) No 1107/2009
GLP:	Yes, according to Japan MAFF GLP standard 14 Nousan 6283 US EPA – FIFRA GLP (40CFR Part 160); Principles of GLP, German Chemical Law, current version of Annex 1
Testing Facility and Dates:	[redacted] Germany Experimental work: 9.6.2010 – 6.5.2011

Executive Summary

The metabolism and excretion of [pyridinylmethyl-^{14}C]BYI 02960 (common name: flupyradifurone) were investigated in the lactating goat as a model for ruminants. The goat was orally dosed once daily for five consecutive days in the morning after milking with 1.0 mg of the active substance per kg body weight which corresponded to 24.4 mg a.s. /kg dry feed/day. The animal was sacrificed about six hours after the last administration. Total radioactive residues (TRR) were determined in milk and excreta at various sampling intervals, and in muscle, fat, kidney and liver at sacrifice. Milk, edible organs and tissues as well as urine were analysed for the unchanged parent compound and metabolites.

Recovery and Elimination of Radioactivity

The overall recovery amounted to 88.75% of the total dose. Much of the remaining radioactivity (ca. 11%) was expected to be still present in the gastro-intestinal tract at sacrifice, due to the short period between the last administration and sacrifice. An amount of 0.78% of the total dose was secreted with

the milk. At sacrifice, the residues in the organs and tissues were calculated or estimated to be 2.94% of the total dose from which about 71% was detected in the skeletal muscle.

Until sacrifice, the excretion of radioactivity accounted for 85.02% of the total dose. A portion of 71.74% was found in the urine and 13.28% in the faeces. The urinary and faecal excretion started immediately after the first administration.

Total Radioactive Residues in Milk, Organs and Tissues

The concentration of radioactivity in **milk** samples ranged from 0.053 mg/kg at 96 hours to 1.345 mg/kg at sacrifice, 102 hours after the first administration. The time course in the evening and morning milk pool samples showed a pronounced diurnal pattern. The radioactive residues increased significantly during the eight hour period after each administration followed by a decrease to a very low level of about 0.05 mg/kg measured prior to the next dosing. A stable plateau level of about 0.3 mg/kg was reached already at 8 hours after the first administration.

In the **organs and tissues**, the highest concentrations were determined in kidney (1.869 mg/kg) and liver (1.215 mg/kg) indicating the significance of these organs for excretion and metabolism. These values corresponded to 0.10% and 0.50% of the total dose, respectively. For muscle and fat, 0.356 mg/kg and 0.106 mg/kg, respectively, were determined. The radioactivity concentration of the muscle corresponded to 2.10% and that of fat to 0.25% of the total dose assuming a value of 30% and 12% of the body weight for these tissues.

Metabolism

For analysis of parent compound and metabolites, milk, muscle, kidney and liver were extracted with mixtures of acetonitrile/water. For milk an additional extraction step with acetone was performed. Fat was extracted with mixtures of n-heptane and acetonitrile/water followed by a solvent partitioning step yielding an n-heptane and an acetonitrile/water phase. No residues were found in the n-heptane phase of fat. The resulting extracts of milk, muscle, fat, kidney and liver represented between 92.9% and 99.8% of the total radioactive residue. Unextractable residues in milk, muscle and fat were below 0.002 mg/kg. For kidney and liver they were 0.021 mg/kg and 0.086 mg/kg, respectively.
After purification and concentration, the extracts were analysed by HPLC with radiometric detection. The HPLC profiling method was based on the use of a reversed phase column and a neutral water/acetonitrile gradient. The metabolite pattern of the extracts of the current goat study and the goat study with the furanone-label (KIIA 6.2.3/02) were comparable with the exception of the label specific metabolites.
All prominent metabolites of the extracts of milk, muscle, fat, kidney and liver were also detected in urine. Therefore, the isolation and identification of metabolites were performed using the urine samples. Isolated metabolites were identified by LC-MS/MS and used as radiolabelled reference compounds for the identification in milk, muscle, fat, kidney and liver by HPLC co-chromatography. The identification of the minor metabolite BYI 02960-AMCP-difluoroethanamine was performed in the liver by HPLC co-chromatography with the non-radiolabelled reference compound.
Approximately 99% of the total radioactivity in milk, muscle, fat and kidney and ca. 93% in liver was identified. All other residues were characterised by their extraction behaviour. The unchanged parent compound was by far the major constituent of the residue in milk, organs and tissues and amounted to

0.165 mg/kg (88.8%) for milk, 0.349 mg/kg (98.0%) for muscle, 0.105 mg/kg (99.2%) for fat, 0.650 mg/kg (34.8%) for kidney and 1.028 mg/kg (84.6%) for liver.

Extensive metabolism was detected in the kidney, where the main portion of metabolites was found. The major metabolite in the kidney was BYI 02960-OH, with a concentration of 0.299 mg/kg (16.0%). Four prominent glucuronic acid conjugates of the hydroxylated parent compound were detected in the kidney as well as in the urine. The four isomers were named BYI 02960-OH-gluA (isomer 1 to isomer 4); their concentration ranged from 0.112 mg/kg (6.0%) to 0.175 mg/kg (9.3%). BYI 02960-OH-gluA (isomer 2) and BYI 02960-OH-gluA (isomer 3) are diastereomers, which are hydroxylated and conjugated in the 5-position of the furanone ring. BYI 02960-OH-gluA (isomer 4) is hydroxylated and conjugated in the difluoroethyl side chain of the molecule and BYI 02960-OH-gluA (isomer 1) in an unknown position.

A further metabolite was BYI 02960-hippuric acid, which was detected in milk (0.017 mg/kg, 9.1%) and kidney (0.178 mg/kg, 9.5%). Traces of BYI 02960-hippuric acid were also found in the liver (0.010 mg/kg, 0.8%). BYI 02960-cysteinylnicotinic acid was identified in the kidney (0.114 mg/kg, 6.1%) and in the liver (0.058 mg/kg, 4.8%). Minor identified metabolites were BYI 02960-methylthio-glyoxylic acid in milk and muscle and BYI 02960-AMCP-difluoroethanamine in kidney and liver. The concentration of each was below 0.020 mg/kg.

The concentration of the total radioactivity (expressed as mg a.s. equiv./kg) as well as the distribution of the parent compound and metabolites and the identification rates in milk and edible tissues are summarised in the following table:

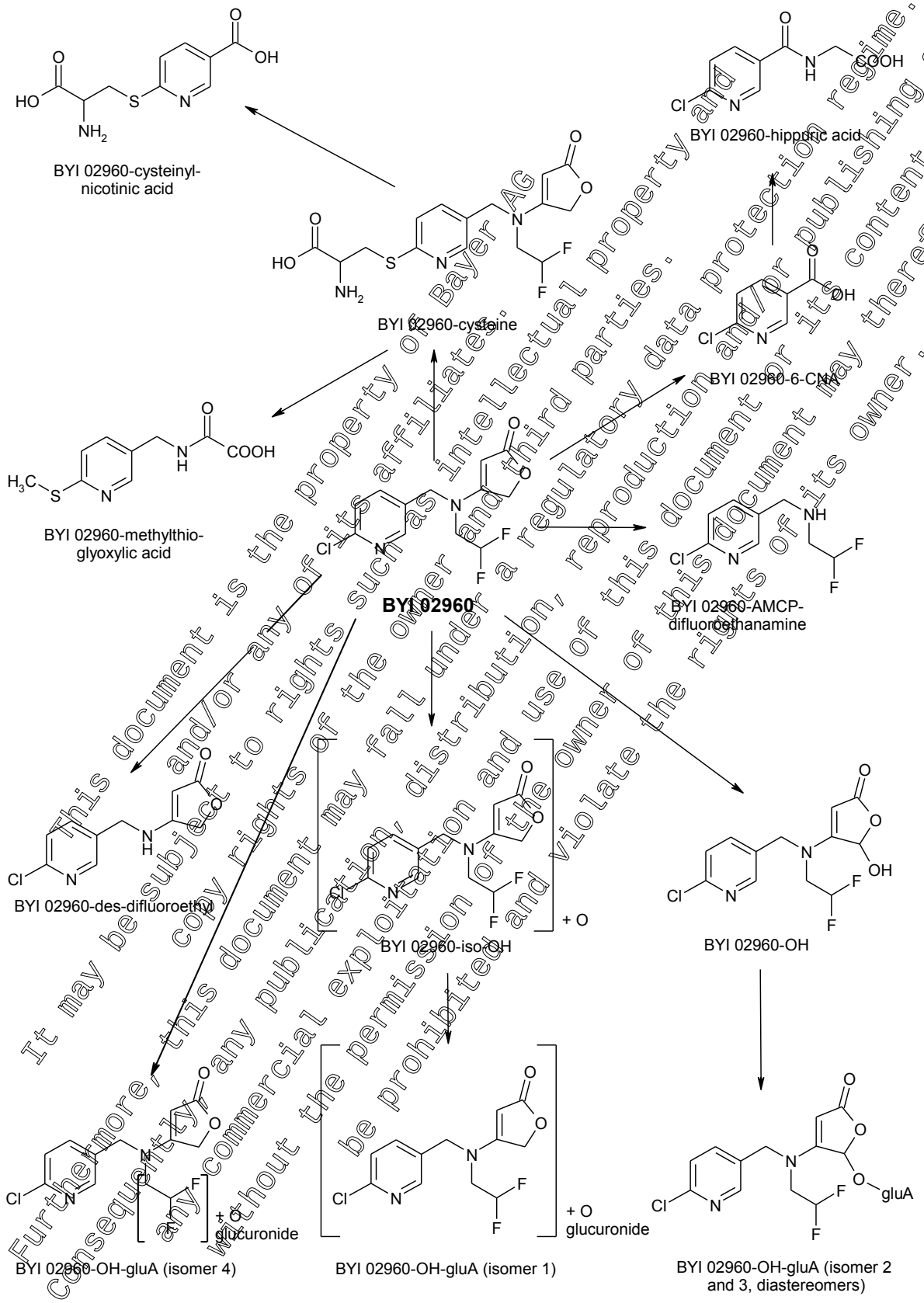
	Milk (24 to 192 h)		Muscle		Fat		Kidney		Liver	
TRR [mg/kg]	0.186		0.356		0.106		1.869		1.215	
Sample/ Report name BYI 02960-	% of TRR	mg/kg	% of TRR	mg/kg	% of TRR	mg/kg	% of TRR	mg/kg	% of TRR	mg/kg
ACN/water extract	99.3	0.184	99.4	0.353	99.2	0.105	98.8	1.847	92.8	1.128
cysteinyl-nicotinic acid	---	---	---	---	---	---	6.1	0.114	4.8	0.058
hippuric acid	9.1	0.017	---	---	---	---	9.5	0.178	0.8	0.010
methylthio-glyoxylic acid	1.5	0.003	1.3	0.005	---	---	---	---	---	---
OH-gluA (isomer 1)	---	---	---	---	---	---	6.0	0.112	---	---
OH-gluA (isomer 2)	---	---	---	---	---	---	9.3	0.175	1.4	0.016
OH-gluA (isomer 3)	---	---	---	---	---	---	8.4	0.158	---	---
OH-gluA (isomer 4)	---	---	---	---	---	---	7.5	0.141	---	---
AMCP-difluoroethanamine	---	---	---	---	---	---	1.1	0.020	1.2	0.015
OH	---	---	---	---	---	---	16.0	0.299	---	---
Parent compound	88.8	0.165	98.0	0.349	99.2	0.105	34.8	0.650	84.6	1.028
Total identified	99.3	0.184	99.4	0.353	99.2	0.105	98.8	1.847	92.8	1.128
Total extracted	99.5	0.185	99.5	0.354	99.8	0.106	98.9	1.848	92.9	1.129
Solids	0.5	0.001	0.5	0.002	0.2	<0.001	1.1	0.021	7.1	0.086
Accountability	100.0	0.186	100.0	0.356	100.0	0.106	100.0	1.869	100.0	1.215

The metabolic reactions of [pyridinylmethyl-^{14}C-]BYI 02960 in the lactating goat are:

- Hydroxylation followed by conjugation with glucuronic acid forming two diastereomeric conjugates of BYI 02960-OH (BYI 02960-OH-gluA, isomer 2 and 3), the hydroxylation and conjugation being in the 5-position of the furanone ring. One isomer (BYI 02960-OH-gluA, isomer 4) with hydroxylation and conjugation in the difluoroethyl side chain and one isomer (BYI 02960-OH-gluA, isomer 1) with hydroxylation and conjugation in an unknown position
- Oxidative cleavage of the pyridinylmethyl bridge forming BYI 02960-6-CNA followed by conjugation with glycine to form BYI 02960-hippuric acid
- Substitution of the chlorine atom of BYI 02960 with glutathione followed by degradation resulting in the conjugate BYI 02960-cysteine
- Oxidative cleavage of the pyridinylmethyl bridge of BYI 02960-cysteine forming BYI 02960-cysteinyl-nicotinic acid
- Further degradation of BYI 02960-cysteine in the cysteine group and the furanone ring forming BYI 02960-methylthio-glyoxylic acid
- Cleavage of the furanone ring forming BYI 02960-AMCP-difluoroethanamine
- Cleavage of the difluoroethyl group forming BYI 02960-des-difluoroethyl

Based on these results the metabolic pathway of [pyridinylmethyl-^{14}C-]BYI 02960 in the lactating goat shown on the next page is proposed.

Proposed metabolic pathway of [pyridinylmethyl-^{14}C]BYI 02960 in the lactating goat:



I. Materials and Methods

A. Materials

1. Test Material

IUPAC Name	4-{[(6-chloropyridin-3-yl)methyl](2,2-difluoroethyl)amino}furan-2(5H)-one
Code name	BYI02960
Common name	Flupyradifurone (proposed ISO)
Empirical formula	$C_{12}H_{11}ClF_2N_2O_2$
Molar mass	288.68
Labelling	[pyridinylmethyl-^{14}C]
Specific radioactivity used for administration	3.92 MBq/mg = 105.93 µCi/mg (delivered sample before radiodilution) 3.425 MBq/mg = 2.06×10^8 dpm/mg = 92.57 µCi/mg = 26.73 Ci/mol (sample after radiodilution)
Radiochemical purity	> 99 % (HPLC)
Dose level	5 daily oral doses of 1 mg/kg bw, by gavage
Vehicle	Capsule

2. Test Animals

Species	Lactating goat (*Capra hircus*)
Strain	"Weiße Deutsche Edelziege"
Breeding facility	[redacted] Member of the Landesverband [redacted]
Sex and numbers involved	1 female animal
Age	ca. 36 months
Body weight	60 kg at first administration, 59 kg at sacrifice
Acclimatization	7 days
Identification	skin marking
Housing	During acclimatization period: raised stall with a metal grid as base and straw and hay as bedding. During the test period: electro-polished stainless steel metabolism cage for farm animals (goat, sheep, and pig), supplied by [redacted]. The cage was equipped with a variable-restraining device. room temperature 17 - 24°C, relative humidity 48-93% 12 h light / 12 h dark cycle, air change 10 – 15 times per hour.
Feed and water	The goat was fed *ad libitum* with hay, hay pellets, apples and supplementary ruminant feed ("Raiffeisen LammGold", supplied by [redacted] During the test period, the average feed consumption was 2.48 kg/day, tap water was offered a*d libitum*

B. Study Design

Dosing

The test compound was orally dosed once daily for five consecutive days in the morning after milking. Based on the daily feed consumption during the test of 4.11% of the body weight, the dose of 1.0 mg a.s. /kg bw corresponded to 24.36 mg a.s. /kg dry feed per day in the diet.

The radiolabelled test compound was delivered with a specific radioactivity of 3.92 MBq/mg. It was diluted with the non-radiolabelled test compound to a specific radioactivity of 3.425 MBq/mg. In total, five gelatine capsules containing the test compound were prepared. They were stored at ≤ -18°C until administration. The remaining test compound was stored in solid form together with the capsules. An aliquot of this sample was dissolved in water (pH 7) and analysed using HPLC after the first administration and after a storage period of about three months in order to demonstrate the stability of the test compound during the administration phase of the study.

The administrations were performed using a capsule applicator. The goat received on each day one gelatine capsule containing on average an amount of 60.41 mg. The totally administered amount and radioactivity accounted for 302.03 mg and 62,067,165,000 dpm, respectively. The total amount of radioactivity administered to the animal served as reference value (A_0 = 100%) for the percentage calculation of the total radioactivity in the biological samples.

Sampling of milk, urine and faeces during the in-life phase

The goat was milked in the morning immediately prior to each administration, about 8 hours later in the afternoon, and directly before sacrifice (8, 24, 32, 48, 56, 72, 80, 96 and 102 hours after the first administration). The milk weights were recorded.

Urine and faeces samples were collected in plastic vessels as quantitatively as possible under dry ice cooling in intervals of 24 hours after the administrations 1 to 4 and 6 hours after the 5th administration. The vessels were changed immediately before the next administration. The collection funnel was rinsed with deionised water into the vessel of the respective collection period. An aliquot of each milk and urine fraction was taken and processed for LSC. The remaining samples were stored at about –18 °C for metabolite analysis. For collection of faeces, the collecting grid was cleaned prior to each administration. No samples of the rinsing water were taken for radioactivity measurement. Each faeces fraction was homogenised after addition of water to obtain a wet paste before the total weight was recorded. Aliquots of each sample were weighed and prepared for radioactivity measurement by combustion/LSC. The remainder was stored at room temperature for metabolite analysis.

Sacrifice and dissection of organs and tissues

The animal was sacrificed approx. 6 hours after the last administration. The animal was anaesthetised by an intravenous dose of about 40 mg/kg bw Pentobarbital-Na (Narcoren®), exsanguinated by cannulating the jugular vein and finally terminated by intracardiac injection with approx. 10 mL of the veterinary drug "T 61®". Following sacrifice, the following organs and tissues were sampled: liver without gall bladder, kidneys, two different types of muscle (round and loin), and two different types of fat (perirenal and omental).

Sample preparation

The organs or tissue samples were weighed and transferred into ice-cooled vessels. Liver, kidneys, muscle, and fat samples were thoroughly homogenised in half-frozen state for several times in a mincing machine. Each resulting tissue pulp was weighed and aliquots were prepared for radioactivity

measurement by combustion/LSC. All samples were divided into portions and stored in a freezer until metabolite analysis. The remaining samples of each organ or tissue were stored at ≤ -18 °C.
For metabolism investigations, a pooled sample of milk collected from 24 h until 102 h after the first administration, composite samples of muscle (loin and round) and fat (omental and perirenal), and samples of kidneys and liver were prepared. The samples were homogenised and kept frozen until extraction.
Aliquot samples of milk, muscle, kidney and liver were extracted with acetonitrile/water (8:2; v/v) using an Ultra Turrax homogeniser. In case of milk an additional extraction step with acetone was performed. The fat sample was treated three times with mixtures of n-heptane and acetonitrile/water (8:2; v/v) also using an Ultra Turrax homogeniser followed by a solvent partition yielding single acetonitrile/water phases and a combined n-heptane phase. All acetonitrile/water extracts were subjected to an SPE clean-up step followed by concentration. The final extracts were used for the profiling, quantification and identification of parent compound and metabolites by HPLC.

Radioactivity measurement

The measurement of the radioactivity in the liquid samples was carried out by liquid scintillation counting (LSC). Quenching effects were automatically corrected using an external standard and quenching library. The instrument background of 10 - 32 dpm was subtracted automatically. For all samples, the limit of detection (LOD) was established at approximately 10 dpm measured per aliquot after instrument background correction. The limit of quantification (LOQ) was established as 2 times of the background radioactivity (dpm) of each instrument/method. All solid samples were combusted in an oxygen atmosphere using an oxidiser. The released $^{14}CO_2$ was trapped in an alkaline scintillation cocktail and the radioactivity was determined by LSC.

Metabolite analysis

The purified extracts of milk and the tissues, and samples of urine were subjected to HPLC using a reversed phase column (C_{18}) and the eluting solvents water/formic acid 99:1 (v/v) and acetonitrile/water/formic acid 97:2:1 (v/v/v) in the gradient mode. Detection was performed by a UV- (254 nm) and a radioisotope detector with a glass bead scintillator. In order to check the completeness of the elution for the HPLC profile, representative samples of milk, muscle, liver, and kidney extract were injected, re-collected, and radioassayed by LSC. The recoveries were between 94.4 and 106.5% of the injected radioactivity. Radiolabelled and non-labelled reference compounds were used in co-chromatography for identification of metabolites.
As a second chromatographic method, thin layer chromatography (TLC) was employed on silica gel plates and radioluminography for detection of radioactive spots. As a solvent system a mixture of acetonitrile/water/formic acid (70:25:5, v/v/v) was used.
The electrospray ionisation mass spectra (ESI) were obtained with an LTQ Orbitrap XL mass spectrometer (Thermo, San Jose, CA, U.S.A.). The chromatographic conditions for the MS experiments are given in the report. The HPLC instrument used for chromatography was an Agilent HP1100 (Agilent, Waldbronn, Germany). The flow from the HPLC column was split between UV-detector followed by a radioactivity detector (Ramona Star, Raytest, Straubenhardt, Germany) and MS spectrometer. ^{1}H-NMR spectra were obtained using a 600 MHz NMR-spectrometer (BRUKER AV 600, Bruker, Karlsruhe, Germany).
Basically, for the lactating goat studies with the pyridinylmethyl-^{14}C- and the furanone-4-^{14}C label (KIIA 6.2.3/02), the isolation and identification of parent compound and metabolites were

performed in the current study (pyridinylmethyl label). Metabolites were isolated from urine (24 to 48 h) by liquid/liquid partitioning via Extrelut® cartridge followed by HPLC. The isolated metabolites were identified by LC-MS/MS and served as reference compounds for the identification by HPLC co-chromatography. A further reference compound was BYI 02960-AMCP-difluoroethanamine, which was provided as a non-radiolabelled compound by Bayer CropScience AG, Product Technology-Analytics Frankfurt.

II. Results and Discussion

A. Recovery and Elimination of Radioactivity

The recovery of radioactivity after administration of a daily dose of 1.0 mg [pyridinylmethyl-^{14}C]BYI 02960 per kg body weight on five consecutive days is presented in Table 6.2.3-1. The overall recovery accounted for 88.75% of the total dose. Much of the remaining radioactivity (ca. 11%) was expected to be still present in the gastro-intestinal tract at sacrifice, due to the short period between the last administration and sacrifice. An amount of 0.78% of the total dose was secreted with the milk. At sacrifice, the residues in the organs and tissues dissected from the body were calculated or estimated to be 2.94% of the total dose from which about 71% was detected in skeletal muscle.
Until sacrifice, the excretion of radioactivity accounted for 85.02% of the total dose. A portion of 71.74% was found in the urine and 13.28% in the faeces. The urinary and faecal excretion started immediately after the first administration.

Table 6.2.3-1: Distribution of residues in milk, muscle, fat, liver and kidney of lactating goats following oral administration of 5 daily doses of [pyridinylmethyl-^{14}C] BYI02960 at a dose rate of 1.0 mg/kg

Sample	Percent of total dose administered	Concentration of total radioactivity [mg/kg]
Liver	0.50	1.215
Kidney	0.10	1.869
Muscle, total	2.10	0.356
Fat, total	0.25	0.106
Total of organs/tissues	2.94	----
Milk, 0 – 102 h	0.78	0.186*
Urine, 0 – 102 h	71.74	----
Faeces, 0 – 102 h	13.28	----
Total excreted	85.02	----
Total Recovery	88.75	----

* : Milk 24 – 102 h

B. Levels and Time Course of Total Radioactive Residues in Milk

The radioactivity levels and concentrations measured in the milk are presented in Table 6.2.3-2. The concentrations ranged from 0.053 mg/kg at 96 hours to 1.345 mg/kg at sacrifice. The concentrations found in the evening and morning milk samples showed a distinct diurnal pattern. The radioactive

residues increased significantly during the eight hour period after each administration followed by a decrease to a very low level of about 0.05 mg/kg measured prior to the administration of the next dose. A stable plateau level of about 0.3 mg/kg was reached already at 8 hours after the first administration.

Table 6.2.3-2: Time course of total radioactivity in the milk of lactating goats following oral administration of 5 daily doses of [pyridinylmethyl-14C] BYI02960 at a dose rate of 1.0 mg/kg

Time after the first admin. [h]	Admin. no.	Cumulative secretion [% of total dose admin.]	Secretion per day [% of total dose admin.]	TRR [mg/kg]
0	1	-----	-----	-----
8		0.12		0.292
24		0.46	0.16	0.060
24	2	-----	-----	-----
32		0.30		0.336
48		0.34	0.18	0.055
48	3	-----	-----	-----
56		0.47		0.318
72		0.50	0.17	0.055
72	4	-----	-----	-----
80		0.64		0.318
96		0.67	0.17	0.053
96	5	-----	-----	-----
102		0.78	0.10	1.345

C. Total Radioactive Residues in the Dissected Organs and Tissues

The concentration of the total radioactivity measured in the dissected organs and tissues collected at sacrifice are presented in Table 6.2.3-1 (last column). The highest concentrations were determined in kidney (1.869 mg/kg) and liver (1.215 mg/kg) indicating the significance of these organs for excretion and metabolism. In relation to the total dose administered, these values corresponded to 0.10% and 0.50%, respectively. For muscle and fat 0.356 mg/kg and 0.106 mg/kg, respectively were determined. The radioactivity concentration of the total body muscle corresponded to 2.10% and of fat to 0.25% of the total dose assuming a value of 30% and 12% of the body weight for these tissues.

D. Extraction Efficiency of Residues

Milk (24 to 102 h), muscle, kidney and liver pools were extracted with a mixture of acetonitrile/water (8:2; v/v). In case of milk an additional extraction step with acetone was performed. Fat was extracted with a mixture of acetonitrile/water (8:2; v/v) and n-heptane followed by a solvent partition yielding an acetonitrile/water phase and a n-heptane phase. Residues were not detected in the n-heptane phase. After purification and concentration steps, the resulting extracts represented 99.3% of the total radioactivity for milk (24 to 102 h), 99.4% for muscle, 99.2% for fat, 98.8% for kidney and 92.8% for liver. Losses were not detected during the sample preparation. There was also no radioactivity in the distillates. Unextractable residues amounted to 0.001 mg/kg (0.5%) for milk, 0.002 mg/kg (0.5%) for

muscle, <0.001 mg/kg (0.2%) for fat, 0.021 mg/kg (1.1%) for kidney and 0.086 mg/kg (7.1%) for liver.

E. Quantification, Identification and Characterisation of Residues

Quantification of Parent Compound and Metabolites

Parent compound and metabolites were quantified in the extracts as well as in the urine (24 to 48 h) using a ternary reversed phase HPL system. The three eluents employed were:

A: 1L water + 1.54 mL ammonium hydroxide solution (25%) + 0.8 mL formic acid, adjusted to pH 7
B: acetonitrile / eluent A (99:1; v/v)
C: methanol / tetrahydrofurane (1:1; v/v)

Detailed information can be found in the report.

Metabolites in the extracts as well as in the urine (24 to 48 h) were assigned by comparison of the metabolite profiles and their retention times. A summary of the quantification of parent compound and metabolites in milk, muscle, fat, kidney and liver is presented in Table 6.2.3-3.

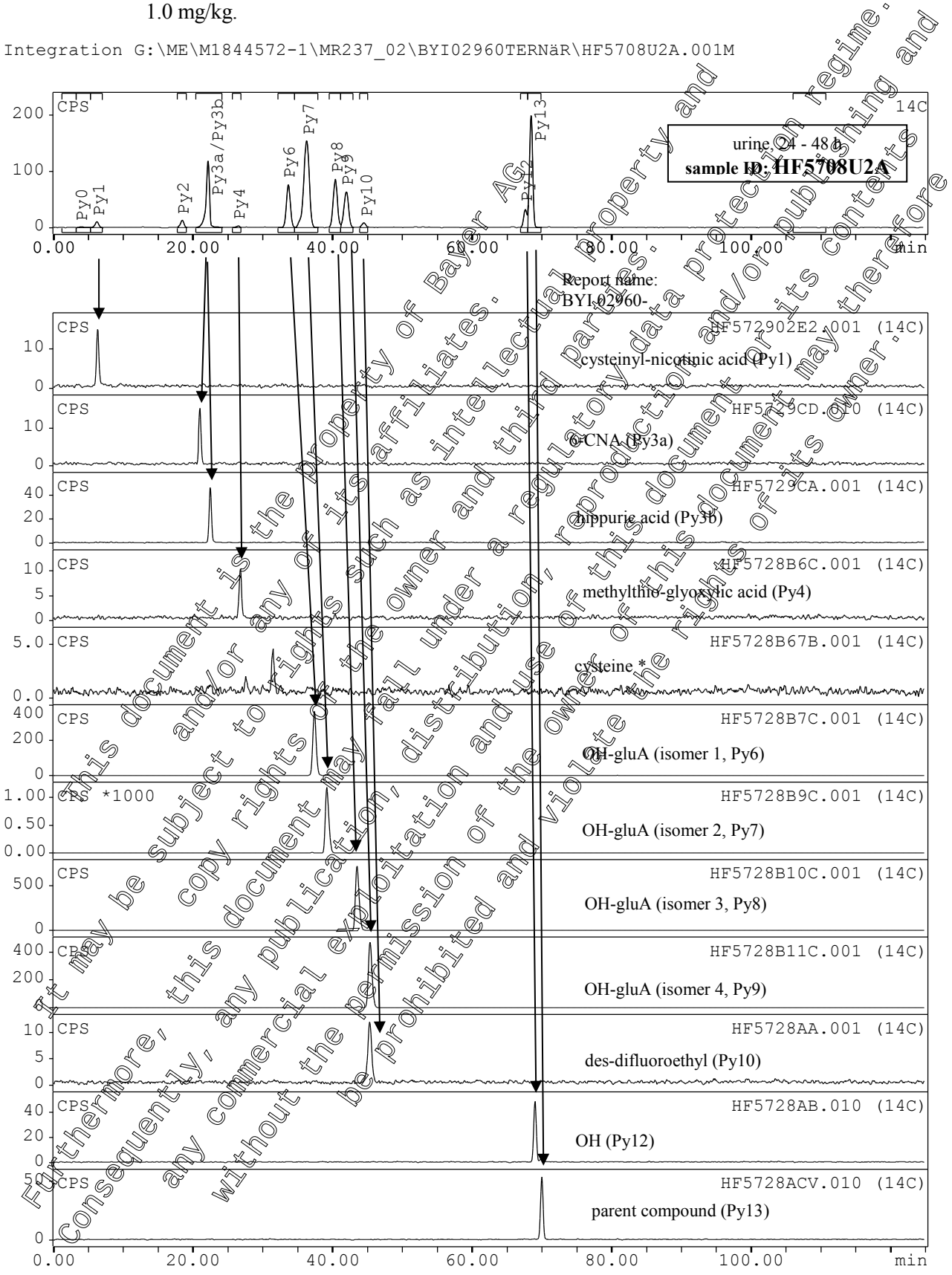
Isolation and Identification of Parent Compound and Metabolites in Urine

Metabolites were isolated from urine (24 to 48 h) and identified by LC-MS/MS investigations. An assignment of all identified metabolites to the metabolic profile of urine is presented in Figure 6.2.3-1. BYI 02960-OH-gluA (isomer 1, Py6) to BYI 02960-OH-gluA (isomer 4, Py9) were identified as glucuronic acid conjugates of hydroxylated BYI 02960 by LC-MS/MS. The LC-MS/MS fragments of BYI 02960-OH-gluA (isomer 1) and BYI 02960-OH-gluA (isomer 4) were different from each other and were different from BYI 02960-OH-gluA (isomer 2) and BYI 02960-OH-gluA (isomer 3). Most probably BYI 02960-OH-gluA (isomer 2) and BYI 02960-OH-gluA (isomer 3) are two diastereomers because of their identical LC-MS/MS fragments. Therefore, it was concluded that BYI 02960 was hydroxylated and conjugated with glucuronic acid in three different positions of the molecule.

The hydroxylation of the two diastereomers (isomer 2 and 3) was assigned to the 5-position of the furanone ring based on the similarity of the mass spectra with those of BYI 02960-OH-SA. The position of the hydroxylation of BYI 02960-OH-SA was clearly identified by NMR-spectroscopy in the laying hen study with the pyridinylmethyl label)KIIA 6.2.2/01). In the case of isomer 4, the position of the hydroxylation was assigned to the difluoroethyl side chain based on the fragments 225 (ESI+) and 223 (ESI-) proving the presence of the unchanged pyridinylmethyl and furanone moieties. For isomer 1, the hydroxylated position could not be derived from the mass spectra.

Further evidence for the assignment of the four isomers of BYI 02960-OH-gluA to their corresponding aglycones and decomposition product is presented in the lactating goat study with the furanone label.)KIIA 6.2.3/02).

Figure 6.2.3-1: Assignment of isolated and identified metabolites in urine of lactating goats following oral administration of 5 daily doses of [pyridinylmethyl-^{14}C] BYI02960 at a dose rate of 1.0 mg/kg.



Note: * BYI 02960-cysteine was isolated from concentrated urine; it was not detected in native urine.

Identification, Assignment and Quantification of Metabolites in Milk, Organs and Tissues

Identified metabolites from urine were used as reference compounds to identify the metabolites in the extracts of milk, muscle, fat, kidney and liver by HPLC or TLC co-chromatography. The identification of BYI 02960-AMCP-difluoroethanamine in the liver was performed with the non-radiolabelled reference compound by HPLC co-chromatography. All other metabolites in milk, organs and tissues were assigned by comparison of the metabolite profiles and retention times based on the profiling method.

The distribution of the parent compound and metabolites in milk, organs and tissues is summarised in Table 6.2.3-3. Approx. 99% of the total radioactivity in milk, muscle, fat and kidney and approx. 93% in liver were identified.

Table 6.2.3-3: Radioactive residues of parnt compound and metabolites in milk, urine and edible organs of lactating goats following oral administration of 5 daily doses of [pyridinylmethyl-^{14}C] BYI02960 at a dose rate of 1.0 mg/kg

	Milk (24 to 102 h)		Muscle		Fat		Kidney		Liver	
TRR [mg/kg]	0.186		0.356		0.106		1.869		1.215	
Sample/ Report name BYI 02960-	% of TRR	mg/kg	% of TRR	mg/kg	% of TRR	mg/kg	% of TRR	mg/kg	% of TRR	mg/kg
- ACN/water extract	99.3	0.184	99.4	0.353	99.2	0.105	98.8	1.847	92.8	1.128
cysteinyl-nicotinic acid	---	---	---	---	---	---	6.1	0.114	4.8	0.058
hippuric acid	9.1	0.017	---	---	---	---	9.5	0.178	0.8	0.010
methylthio-glyoxylic acid	1.5	0.003	1.3	0.005	---	---	---	---	---	---
OH-gluA (isomer 1)	---	---	---	---	---	---	6.0	0.112	---	---
OH-gluA (isomer 2)	---	---	---	---	---	---	9.3	0.175	1.4	0.016
OH-gluA (isomer 3)	---	---	---	---	---	---	8.4	0.158	---	---
OH-gluA (isomer 4)	---	---	---	---	---	---	7.5	0.141	---	---
AMCP-difluoroethanamine	---	---	---	---	---	---	1.1	0.020	1.2	0.015
OH	---	---	---	---	---	---	16.0	0.299	---	---
Parent compound	88.8	0.165	98.0	0.349	99.2	0.105	34.8	0.650	84.6	1.028
Total identified	99.3	0.184	99.4	0.353	99.2	0.105	98.8	1.847	92.8	1.128
Total extracted	99.5	0.185	99.5	0.354	99.8	0.106	98.9	1.848	92.9	1.129
Solids	0.5	0.001	0.5	0.002	0.2	<0.001	1.1	0.021	7.1	0.086
Accountability	100.0	0.186	100.0	0.356	100.0	0.106	100.0	1.869	100.0	1.215

Parent compound was by far the dominating constituent of the residue in milk, organs and tissues. Its concentration amounted to 0.165 mg/kg (88.8%) for milk, 0.349 mg/kg (98.0%) for muscle, 0.105 mg/kg (99.2%) for fat, 0.650 mg/kg (34.8%) for kidney and 1.028 mg/kg (84.6%) for liver.

The highest number of metabolites was found in the kidney. The major metabolite was BYI 02960-OH (hydroxylation in position 5 of the furanone ring), which amounted to 0.299 mg/kg (16.0%). Four prominent glucuronic acid conjugates of hydroxylated parent compound were detected in the kidney. The four conjugates were named BYI 02960-OH-gluA (isomer 1 to isomer 4), their concentration

ranged from 0.112 mg/kg (6.0%) to 0.175 mg/kg (9.3%). The hydroxylation of BYI 02960 and conjugation with glucuronic acid resulted into two diastereomers of conjugated BYI 02960-OH (BYI 02960-OH-gluA, isomer 2 and 3) with hydroxylation and conjugation in the 5-position of the furanone ring), one isomer (BYI 02960-OH-gluA, isomer 4) with the hydroxylation and conjugation in the difluoroethyl side chain and one isomer (BYI 02960-OH-gluA, isomer 1) with the hydroxylation and conjugation in an unknown position.
A further major metabolite was BYI 02960-hippuric acid, which was detected in the milk (0.012 mg/kg, 9.1%) and kidney (0.178 mg/kg, 9.5%). Traces of BYI 02960-hippuric acid were also found in liver (0.010 mg/kg,). BYI 02960-cysteinyl-nicotinic acid was identified in kidney (0.114 mg/kg, 6.1%) and in liver (0.058 mg/kg, 4.8%). Minor metabolites were BYI 02960-methylthioglyoxylic acid in milk and muscle and BYI 02960-AMCP-difluoroethanamine in kidney and liver. Their concentration was below 0.020 mg/kg.

F. Storage Stability of Residues

During the study, all samples and extracts were stored at ≤ -18 °C or for a short time in a refrigerator. All samples of milk, and edible organs and tissues were extracted within approx. six weeks after sample collection. The first metabolite profile was recorded one day after the start of the extraction and sample preparation. Hence, investigations on storage stability of the residues in the samples were not necessary and it can be concluded that the metabolic profiles represent the residues in the matrices at sacrifice.

III. Conclusion

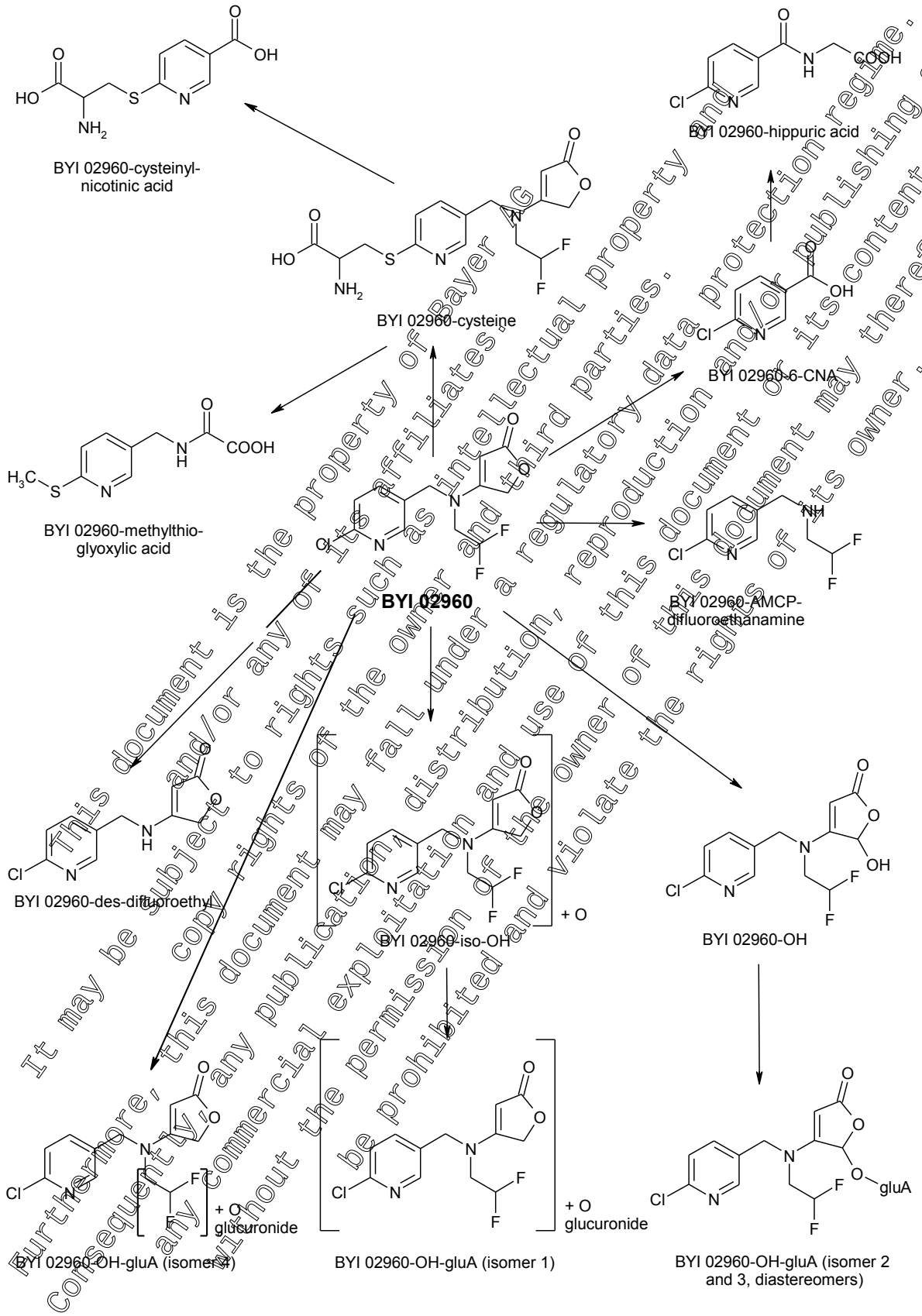
The metabolic and excretion behaviour of [pyridinylmethyl-^{14}C]BYI 02960 in the lactating goat can be characterised by the following observations:

- The concentration of radioactivity in milk and edible tissues was rather low compared to the dose level and the dosing period of five days.
- The evaluation of these concentrations should moreover consider the fact that an exaggerated dose level of 24.36 mg/kg feed/day was administered. Furthermore, the significant amount of radioactivity detected in urine and faeces and the relatively high concentration in liver and kidney at sacrifice six hours after the last administration indicate that the residues are further metabolised and finally eliminated.
- The residue level in milk showed a pronounced diurnal pattern after the first administration as they declined to a very low level prior to the next dose. A stable residue plateau level was reached already at 8 hours after the first administration.
- The radioactive residues were efficiently extracted from milk as well as from edible organs and tissues; extraction rates were between 92.9% and 99.8%.
- Parent compound was the dominating constituent of the residues in milk (ca. 89%), muscle (ca. 98%), fat (ca. 99%), kidney (ca. 35%) and liver (ca. 85%).
- The biggest number of metabolites was found in the kidney. BYI 02960-OH, four glucuronic acid conjugates of hydroxylated BYI 02960, BYI 02960-hippuric acid and BYI 02960-cysteinyl-nicotinic acid were identified.

- The cleavage of the molecule was not very pronounced (< 17%). Label specific metabolites were BYI 02960-hippuric acid, BYI 02960-cysteinyl-nicotinic acid, BYI 02960-methylthio-glyoxylic acid and BYI 02960-AMCP-difluoroethanamine. BYI 02960-6-CNA was only found in urine.
- The main metabolic reactions in the lactating goat are:
  - Hydroxylation of BYI 02960 followed by conjugation with glucuronic acid forming two diastereomeric conjugates of BYI 02960-OH (BYI 02960-OH-gluA, isomer 2 and 3) with hydroxylation and conjugation in the 5-position of the furanone ring, one isomer (BYI 02960-OH-gluA, isomer 4) with the hydroxylation and conjugation in the difluoroethyl side chain and one isomer (BYI 02960-OH-gluA, isomer 1) with the hydroxylation and conjugation in an unknown position
  - Oxidative cleavage of the pyridinylmethyl bridge forming BYI 02960-6-CNA followed by conjugation with glycine to BYI 02960-hippuric acid
  - Substitution of the chlorine atom of BYI 02960 with glutathione followed by degradation resulting in BYI 02960-cysteine
  - Oxidative cleavage of the pyridinylmethyl bridge of BYI 02960-cysteine forming BYI 02960-cysteinyl-nicotinic acid
  - Degradation of BYI 02960-cysteine in the cysteine group and furanone ring forming BYI 02960-methylthio-glyoxylic acid
  - Cleavage of the furanone ring forming BYI 02960-AMCP-difluoroethanamine
  - Cleavage of the difluoroethyl group forming BYI 02960-des-difluoroethyl

Based on these results the metabolic pathway of [pyridinylmethyl-^{14}C] BYI02960 in the lactating goat shown in Figure 6.2.3-2 is proposed.

Figure 6.2.3-2: Proposed metabolic pathway of [pyridinylmethyl-14C] BYI02960 in the lactating goat



Report:	**KIIA 6.2.3/02, Authors: [redacted], [redacted], (2011)**
Title:	[Furanone-4-^{14}C]BYI02960: Metabolism in the lactating goat
Report No & Document No:	MEF-11/268 Date: 16.12.2011 M-421995-01-2
Guidelines:	OECD-Guideline for the Testing of Chemicals No. 503, Metabolism in Livestock US EPA Residue Chemistry Test Guideline OPPTS 860.1300 Nature of the Residue – Plants, Livestock European Parliament and Council Regulation (EC) No 1107/2009
GLP:	Yes, according to Japan MAFF GLP standard 11 Nousan 6283; US EPA – FIFRA GLP (40CFR Part 160); Principles of GLP, German Chemical Law, current version of Annex 1
Testing Facility and Dates:	[redacted], Germany Experimental work: 19.4.2010 – 20.4.2011

Executive Summary

The metabolism and excretion of [furanone-4-^{14}C]BYI 02960 (common name: flupyradifurone) were investigated in the lactating goat as a model for ruminants. The goat was orally dosed once daily for five consecutive days in the morning after milking with 1.0 mg of the active substance per kg body weight which corresponded to 28.82 mg a.s. /kg dry feed/day. The animal was sacrificed about six hours after the last administration. Total radioactive residues (TRR) were determined in milk and excreta at various sampling intervals, and in muscle, fat, kidney and liver at sacrifice. Milk, edible organs and tissues and urine were analysed for the unchanged parent compound and metabolites.

Recovery and Elimination of Radioactivity

The overall recovery amounted to 78.94% of the total dose. Much of the remaining radioactivity (ca. 21%) was expected to still be present in the gastro-intestinal tract at sacrifice, due to the short period between the last administration and sacrifice. A significant proportion is probably exhaled as $^{14}CO_2$ since the formation of carbon dioxide had been clearly shown in a rat quantitative whole body autoradiography study using the test compound labelled at the same position (KIIA 5.1.2/02). An amount of 2.58% of the total dose was secreted with the milk. At sacrifice, the residues in the organs and tissues were calculated or estimated to be 4.22% of the total dose from which about 69% was detected in the skeletal muscle.

Until sacrifice, the excretion of radioactivity accounted for 72.14% of the total dose. A portion of 69.15% was found in the urine and 3.0% in the faeces. The urinary and faecal excretion started immediately after the first administration.

Total Radioactive Residues in Milk, Organs and Tissues

The concentration of radioactivity in **milk** samples ranged from 0.755 mg/kg at 24 hours to 1.213 mg/kg at 56 hours after the first administration. At sacrifice, the residue concentration was 1.165 mg/kg. The time course in the evening and morning milk pool samples showed a pronounced diurnal pattern. The residues increased significantly during the eight hour period after the second and the fifth administration followed by a decrease measured prior to the delivery of the next dose. A plateau level of about 1.1 mg/kg was reached ca. 50 hours after the first administration.

In the **organs and tissues**, the highest concentrations were determined in liver (1.746 mg/kg) and kidney (1.472 mg/kg) indicating the significance of these organs for excretion and metabolism. These

values corresponded to 0.65% and 0.09% of the total dose, respectively. For muscle and fat 0.539 mg/kg and 0.265 mg/kg, respectively, were determined. The radioactivity concentration of the muscle corresponded to 2.91% and of fat to 0.57% of the total dose assuming a value of 30% and 12% of the body weight for these tissues.

Metabolism

For analysis of parent compound and metabolites, milk, muscle, kidney and liver were extracted with mixtures of acetonitrile/water. Fat was extracted with mixtures of n-heptane and acetonitrile/water followed by a solvent partition yielding an n-heptane and acetonitrile/water phase. Residues in the n-heptane phase of fat amounted to only 0.014 mg/kg (5.4%). The resulting acetonitrile/water extracts of milk, muscle, fat, kidney and liver represented between 73.3% and 95.0% of the total radioactive residue. Solids of kidney and liver were exhaustively extracted with mixtures of acetonitrile/water, 0.1 N HCl and 0.1 N NaOH using microwave assistance. The exhaustive extracts were not further investigated, due to their low amount of residues and/or their high matrix content. Unextractable residues in milk, muscle and fat were low and amounted to ≤0.090 mg/kg. The extraction residues of kidney and liver were completely solubilised after the exhaustive extraction with sodium hydroxide. After purification and concentration, the conventional acetonitrile/water extracts were analysed by HPLC with radiometric detection. The HPLC profiling method was based on the use of a reversed phase column and a neutral water/acetonitrile gradient. The metabolite pattern of the extracts of the current goat study and the goat study with the pyridinylmethyl label (KIIA 6.2.3/01) were comparable with the exception of the label specific metabolites.

All prominent metabolites of the extracts of milk, muscle, fat, kidney and liver were also detected in urine, except lactose in the milk. Therefore, the assignment of metabolites in the profiles of the extracts was performed based on the comparison with the urine profiles of the current study and the study with the pyridinylmethyl label. Four isomers of BYI 02960-OH-gluA were isolated and identified by comparison of the LC-MS/MS data. Further identification of the BYI 02960-OH-gluA isomers was achieved by comparison of the degradation rates of isomers with the increasing rates of their aglycones and decomposition product, based on the observed decomposition of the isomers in two urine samples. The radioactive lactose in the milk was identified after isolation and acetylation by HPLC co-chromatography with the radiolabelled reference compound.

Approximately 91% of the total residue in milk, ca. 90% in muscle, 83% in fat, 79% in kidney and ca. 60% in liver was identified. All other residues were characterised by their extraction behaviour. The unchanged parent compound was the main constituent of the residue in organs and tissues and amounted to 0.475 mg/kg (88.1%) for muscle, 0.213 mg/kg (80.5%) for fat, 0.744 mg/kg (50.5%) for kidney and 1.045 mg/kg (59.8%) for liver. Lower amounts of parent compound (0.250 mg/kg, 23.9%) were found in the milk. The major part of residues in the milk was radioactive lactose and amounted to 0.698 mg/kg (66.8%). The lactose was specific for the furanone label and was formed by cleavage and subsequent degradation of the furanone ring to smaller carbon units (C-1- or C-2-fragments), which entered the carbon pool of endogenous compounds.

Extensive metabolism was detected in the kidney, where the main portion of metabolites was found. The main metabolite in kidney was BYI 02960-OH (hydroxylation in the 5-position of the furanone ring), which amounted to 0.215 mg/kg (14.6%). Four prominent glucuronic acid conjugates of hydroxylated parent compound were detected in the kidney and in the urine. The four isomers were named BYI 02960-OH-gluA (isomer 1 to isomer 4). Their concentrations were between 0.032 mg/kg (2.2%) and 0.069 mg/kg (4.7%) in the kidney. BYI 02960-OH-gluA (isomer 2) and BYI 02960-OH-

gluA (isomer 3) were diastereomers, which were hydroxylated and conjugated in 5-position of the furanone ring. BYI 02960-OH-gluA (isomer 4) was hydroxylated and conjugated in the difluoroethyl side chain and BYI 02960-OH-gluA (isomer 1) in an unknown position. A minor metabolite was BYI 02960-des-difluoroethyl which amounted to 0.019 mg/kg (1.3%) in the kidney.

Metabolites in the polar region of the acetonitrile/water extracts (≤ 5 min) were specific for the furanone label. Their concentrations ranged from 0.021 to 0.037 mg/kg for kidney and from 0.019 to 0.059 mg/kg for liver. These polar metabolites showed the same thin layer chromatographic behaviour as the metabolites, which were detected in the corresponding polar region of rat urine (KIIA 5.1.2/01) and the extracts of muscle and liver of laying hen (KIIA 6.2.2/02). The polar region of muscle and fat was not further analysed, due to the low amount of radioactivity (≤ 0.024 mg/kg).

The formation of radioactive lactose and polar metabolites was mainly caused by the partial instability of the radiolabel used. For a small portion of the total dose the furanone ring of the molecule obviously underwent extensive biotransformation to C-1- and C-2-fragments resulting in an accumulation of radioactivity in biomolecules during the dosing for five days. A part of these fragments was probably also converted to the terminal product $^{14}CO_2$, which might explain the lower overall recovery as compared to the study with the metabolically stable pyridinylmethyl label.

The concentration of the total radioactivty (expressed as mg a.s. equiv./kg) as well as the distribution of the parent compound and metabolites and the identification rates in milk and edible tissues are summarised in the following table:

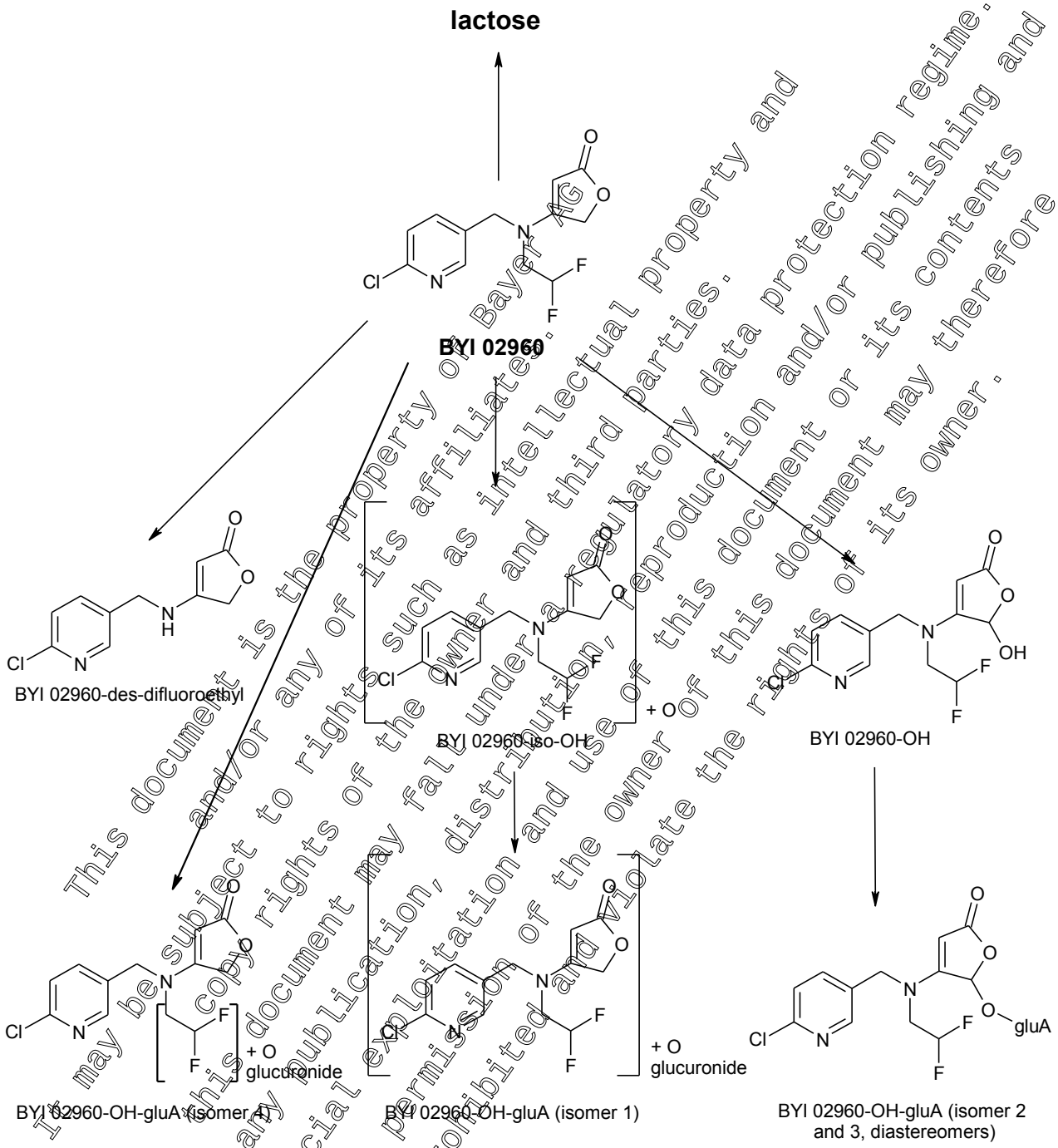
	Milk (24 to 102 h)		Muscle		Fat		Kidney		Liver	
TRR [mg/kg]	1.046		0.539		0.265		1.472		1.746	
Sample/ Report name BYI 02960-	% of TRR	mg/kg	% of TRR	mg/kg	% of TRR	mg/kg	% of TRR	mg/kg	% of TRR	mg/kg
ACN/water extract	90.7	0.948	95.0	0.512	88.4	0.234	89.1	1.311	73.3	1.280
Lactose	66.8	0.698	---	---	---	---	---	---	---	---
Polar metabolites	---	---	4.4	0.024	5.0	0.013	10.0	0.148	12.1	0.211
OH-gluA (isomer 1)	---	---	---	---	---	---	2.2	0.032	---	---
OH-gluA (isomer 2)	---	---	---	---	---	---	2.2	0.032	---	---
OH-gluA (isomer 3)	---	---	---	---	---	---	4.7	0.069	---	---
OH-gluA (isomer 4)	---	---	---	---	---	---	3.5	0.052	---	---
Des-difluoroethyl	---	---	---	---	---	---	1.3	0.019	---	---
OH	---	---	1.8	0.010	2.9	0.008	14.6	0.215	---	---
Parent compound	23.9	0.250	88.1	0.475	80.5	0.213	50.5	0.744	59.8	1.045
Total identified	90.7	0.948	89.9	0.484	83.4	0.221	79.0	1.163	59.8	1.045
Total extracted	91.4	0.956	95.2	0.513	94.1	0.249	100.0	1.472	100.0	1.746
Solids	8.6	0.090	4.8	0.026	5.9	0.016	n.d.	n.d.	n.d.	n.d.
Accountability	100.0	1.046	100.0	0.539	100.0	0.265	100.0	1.472	100.0	1.746

The metabolic and excretion behaviour of [furanone-4-^{14}C]BYI 02960 in the lactating goat can be characterised by the following observations:

- The concentration of radioactivity in milk and edible tissues were relatively high compared to the dose level and the dosing period of five days. This was mainly caused by the partial instability of the radiolabel used. For a small portion of the total dose the furanone ring obviously underwent extensive biotransformation to C1- and C2-fragments that resulted in an accumulation of radioactivity in biomolecules during dosing for five days.
  A part of these fragments was probably also converted to the terminal product $^{14}CO_2$ as can be derived from the lower overall recovery compared to the study with the metabolically stable pyridinylmethyl label.
- However, the fact should be considered that an exaggerated dose level of 28.82 mg/kg feed/day was administered. Furthermore, the significant amount of radioactivity detected in urine and faeces and the relatively high radioactivity in liver and kidney at sacrifice indicate that the residues are further metabolised and finally eliminated.
- The total radioactive residues in milk showed a diurnal pattern after the second administration as they declined significantly prior to the delivery of the next dose. A residue plateau level was reached after the third administration.
- The radioactive residues were efficiently extracted from milk as well as from edible organs and tissues; extraction rates were between 91.4% and 100.0%.
- Parent compound was the main constituent of the residues in muscle (ca. 88%), fat (ca. 81%), kidney (ca. 51%) and liver (ca. 60%). Approximately 24% of the total radioactivity in the milk was identified as parent compound.
- The main residue in the milk was radioactive lactose (approx. 67%)
- Extensive metabolism was detected in the kidney. BYI 02960-OH, four glucuronic acid conjugates of hydroxylated BYI 02960 and BYI 02960-des-difluoroethyl were identified.
- The main metabolic reactions in the lactating goat are:
  - Cleavage and subsequent degradation of the furanone ring forming small carbon units (C-1- or C-2-fragments), entering the carbon pool of endogenous compounds and finally being used for example for the biosynthesis of lactose
  - Hydroxylation followed by conjugation with glucuronic acid forming two diastereomeric conjugates of BYI 02960-OH (BYI 02960-OH-gluA, isomer 2 and 3) with hydroxylation and conjugation in the 5-position of the furanone ring, one isomer (BYI 02960-OH-gluA, isomer 4) with the hydroxylation and conjugation in the difluoroethyl side chain and one isomer (BYI 02960-OH-gluA, isomer 1) with the hydroxylation and conjugation in an unknown position
  - Cleavage of the difluoroethyl group forming BYI 02960-des-difluoroethyl

Based on these results the metabolism of [furanone-4-^{14}C]BYI 02960 in the lactating goat can be described by the metabolic pathway shown on the next page.

Proposed metabolic pathway of [furanone-4-^{14}C]BYI 02960 in the lactating goat:



I. Materials and Methods

A. Materials

1. Test Material

IUPAC Name	4-{[(6-chloropyridin-3-yl)methyl](2,2-difluoroethyl)amino}furan-2(5H)-one
Code name	BYI02960
Common name	Flupyradifurone (proposed ISO)
Empirical formula	$C_{12}H_{11}ClF_2N_2O_2$
Molar mass	288.68
Labelling	[furanone-4-^{14}C]
Specific radioactivity used for administration	4.24 MBq/mg = 114.50 µCi/mg (delivered sample before radiodilution) 3.50 MBq/mg = 2.10 x 10^8 dpm/mg = 94.59 µCi/mg = 27.31 Ci/mol (sample after radiodilution)
Radiochemical purity	> 99 % (HPLC)
Dose level	5 daily oral doses of 1 mg/kg bw by gavage
Vehicle	Capsule

2. Test Animals

Species	Lactating goat (*Capra hircus*)
Strain	"Weiße Deutsche Edelziege"
Breeding facility	[redacted]
Sex and numbers involved	1 female animal
Age	24 months
Body weight	47 kg at first administration, 42 kg at sacrifice
Acclimatization	7 days
Identification	skin marking
Housing	During acclimatization period: raised stall with a metal grid as base and straw and hay as bedding. During the test period: electro-polished stainless steel metabolism cage for farm animals (goat, sheep, and pig), supplied [redacted] The cage was equipped with a variable-restraining device. room temperature 20 - 25°C, relative humidity 40-55% 12 h light / 12 h dark cycle, air change 10 – 15 times per hour.
Feed and water	The goat was fed *ad libitum* with hay, hay pellets, apples and supplementary ruminant feed ("Raiffeisen LammGold", supplied by [redacted] During the test period, the average feed consumption was 1.63 kg/day, tap water was offered a*d libitum*

B. Study Design

Dosing

The test compound was orally dosed once daily for five consecutive days in the morning after milking. Based on the daily feed consumption during the test of 3.47% of the body weight, the dose of 1.0 mg a.s. /kg bw corresponded to 28.82 mg a.s. /kg dry feed per day in the diet.

The radiolabelled test compound was delivered with a specific radioactivity of 4.24 MBq/mg. It was diluted with the non-radiolabelled test compound to a specific radioactivity of 3.50 MBq/mg. In total, five gelatine capsules containing the test compound were prepared. They were stored at ≤ -18°C until administration. The remaining test compound was stored in solid form together with the capsules. An aliquot of this sample was dissolved in an eluent of the HPLC methode (1 L water + 1.54 mL ammonium hydroxide solution (25%) + 0.8 mL formic acid, adjusted to pH 7) and analysed by HPLC after the last administration in order to demonstrate the stability of the test compound during the administration phase.

The administrations were performed using a capsule applicator. The goat received on each day one gelatine capsule containing on average an amount of 47.01 mg. The totally administered amount and radioactivity accounted for 235.07 mg and 49,364,700,000 dpm, respectively. The total amount of radioactivity administered to the animal served as reference-value for the percentage calculation of the total radioactivity in the biological samples.

Sampling of milk, urine and faeces during the in-life phase

The goat was milked in the morning immediately prior to each administration, about 8 hours later in the afternoon, and directly before sacrifice (8, 24, 32, 48, 56, 72, 80, 96 and 102 hours after the first administration). The milk weights were recorded.

Urine and faeces samples were collected in plastic vessels as quantitatively as possible under dry ice cooling in intervals of 24 hours after the administrations 1 to 4 and 6 hours after 5th administration. The vessels were changed immediately before the next administration. The collection funnel was rinsed with deionised water into the vessel of the respective collection period. An aliquot of each milk and urine fraction was taken and processed for LSC. The remaining samples were stored at about –18 °C for metabolite analysis. For collection of faeces, the collecting grid was cleaned prior to each administration. No samples of the rinsing water were taken for radioactivity measurement. Each faeces fraction was homogenised after addition of water to obtain a wet paste before the total weight was recorded. Aliquots of each sample were weighed and prepared for radioactivity measurement by combustion/LSC. The remainder was stored at room temperature for metabolite analysis.

Sacrifice and dissection of organs and tissues

The animal was sacrificed approx. 6 hours after the last administration. The animal was anaesthetised by an intravenous dose of about 40 mg/kg bw Pentobarbital-Na (Narcoren®), exsanguinated by cannulating the jugular vein and finally terminated by intracardiac injection with approx. 10 mL of the veterinary drug "T 61®". Following sacrifice, the following organs and tissues were sampled: liver without gall bladder, kidneys, two different types of muscle (round and loin), and two different types of fat (perirenal and omental,). The gall bladder was punctured for the collection of the bile fluid that was then stored in frozen for an optional metabolite analysis.

Sample preparation

The organs or tissue samples were weighed and transferred into ice-cooled vessels. Liver, kidneys, muscle, and fat samples were thoroughly homogenised in half-frozen state for several times in a mincing machine. Each resulting tissue pulp was weighed and aliquots were prepared for radioactivity measurement by combustion/LSC. All samples were divided into portions and stored in a freezer until start of metabolite analysis. The remaining samples of each organ or tissue were stored at -18 °C.

For metabolism investigations, a pooled sample of milk collected from 24 h until 102 h after the first administration and composite samples of muscle (loin and round) and fat (omental and perirenal), as well as samples of kidneys and liver were prepared. The samples were homogenised and kept frozen until extraction.

Aliquot samples of milk, muscle, kidney and liver were extracted with acetonitrile/water (8:2; v/v) using an Ultra Turrax homogeniser. The fat sample was extracted with mixtures of n-heptane and acetonitrile/water (8:2; v/v) also using an Ultra Turrax homogeniser followed by a solvent partition yielding single acetonitrile/water phases and a combined n-heptane phase. Solids of kidney and liver were in addition exhaustively extracted with mixtures of acetonitrile/water, 0.1 N HCl and 0.1 N NaOH using microwave assistance. All acetonitrile/water extracts were subjected to an SPE clean-up step followed by concentration. The final extracts were used for the profiling, quantification and identification of parent compound and metabolites by HPLC.

Radioactivity measurement

The measurement of the radioactivity in the liquid samples was carried out by liquid scintillation counting (LSC). Quenching effects were automatically corrected using an external standard and quenching library. The instrument background of 10 - 32 dpm was subtracted automatically. For all samples, the limit of detection (LOD) was established at approximately 10 dpm measured per aliquot after instrument background correction. The limit of quantification (LOQ) was established as 2 times of the background radioactivity (dpm) of each instrument/method. All solid samples were combusted in an oxygen atmosphere using an oxidiser. The released $^{14}CO_2$ was trapped in an alkaline scintillation cocktail and the radioactivity was determined by LSC.

Metabolite analysis

The purified extracts and samples of urine were subjected to HPLC using a reversed phase column (C18) and a ternary elution gradient. Detection was performed by a UV- (254 nm) and a radioisotope detector with a glass bead scintillator. In order to check the completeness of the elution for the HPLC profile, representative samples of milk, muscle, liver, and kidney extract were injected, re-collected, and radioassayed by LSC. The recoveries were between 95.3 and 105.9% of the injected radioactivity. Radiolabelled and non-labelled reference compounds were used for co-chromatography for identification of metabolites.

As a second chromatographic method thin layer chromatography (TLC) was employed on silica gel plates and radioluminography for detection of radioactive spots. As a solvent system a mixture of acetonitrile/water/formic acid (70:25:5, v/v/v) was used:

The electrospray ionisation mass spectra (ESI) were obtained with an LTQ Orbitrap XL mass spectrometer (Thermo, San Jose, CA, U.S.A.). The chromatographic conditions for the MS experiments are given in the report. The HPLC instrument used for chromatography was an Agilent HP1100 (Agilent, Waldbronn, Germany). The flow from the HPLC column was split between UV-detector followed by a radioactivity detector (Ramona Star, Raytest, Straubenhardt, Germany) and MS

spectrometer. ^{1}H-NMR spectra were obtained using a 600 MHz NMR-spectrometer (BRUKER AV 600, Bruker, Karlsruhe, Germany).

The identification of parent compound, BYI 02960-OH and BYI 02960-des-difluoroethyl was performed in isolated fractions of a urine sample of the goat study with the pyridinylmethyl label (KIIA 6.2.3/01) by spectroscopic methods. The metabolites were assigned in the extracts of milk, organs and tissues of the current study based on the comparison of the metabolite pattern of a representative urine of the current study and of the study with the pyridinylmethyl label.

Four isomers of the glucuronic acid conjugate of hydroxylated BYI 02960 were isolated from the urine (24 to 48 h) of the current study by liquid/liquid partitioning via Extrelut® cartridge followed by HPLC. The isolated metabolites were identified by LC-MS/MS. The position of hydroxylation and conjugation for three isomers was assigned based on the LC-MS/MS fragments. Confirmation of the identification was based on the degradation to aglycones and other metabolites observed in two urine samples by comparison of the decrease of glucuronic acid conjugates with the increase of the degradation products in relation to non-degraded urine samples.

Metabolites in the polar region of the acetonitrile/water extracts of kidney and liver were quantified and further characterised by TLC (solvent system: acetonitrile/water/formic acid, 70:25:5; v/v/v). Their commonality with polar metabolites in urine of rat and organs of hen was demonstrated by comparison of the TLC profiles. The polar regions of muscle and fat were not investigated, due to their low amount of residues and high matrix content.

The polar residue in the milk was isolated and identified after acetylation. The identification was performed by HPLC co-chromatography with acetylated radioactive lactose as reference compound. Acetylated radioactive lactose was synthesised from radioactive lactose.

II. Results and Discussion

A. Recovery and Elimination of Radioactivity

The recovery of radioactivity after administration of a daily dose of 1.0 mg [furanone-4-^{14}C]BYI 02960 per kg body weight on five consecutive days is presented in Table 6.2.3-4. The overall recovery accounted for 78.94% of the total dose. Much of the remaining radioactivity (ca. 21%) was expected to still be present in the gastro-intestinal tract at sacrifice, due to the short period between the last administration and sacrifice. An amount of 2.58% of the total dose was secreted with the milk. A significant proportion is probably exhaled as $^{14}CO_2$ since the formation of carbon dioxide had been clearly shown in a rat quantitative whole body autoradiography study using the test compound labelled at the same position (KIIA 5.1.2/02). At sacrifice, the residues in the organs and tissues dissected from the body were calculated or estimated to be 4.22% of the total dose from which about 69% was detected in skeletal muscle.

Until sacrifice, the excretion of radioactivity accounted for 72.14% of the total dose. A portion of 69.15% was found in the urine and 3.0% in the faeces. The urinary and faecal excretion started immediately after the first administration.

Table 6.2.3-4: Distribution of residues in milk, muscle, fat, liver and kidney of lactating goats following oral administration of 5 daily doses of [furanone-4-14C] BYI02960 at a dose rate of 1.0 mg/kg

Sample	Percent of total dose administered	Concentration of total radioactivity [mg/kg]
Liver	0.65	1.746
Kidney	0.09	1.473
Muscle, total	2.91	0.539
Fat, total	0.57	0.265
Total of organs/tissues	4.22	---
Milk, 0 – 102 h	2.58	0.961
Urine, 0 – 102 h	69.15	----
Faeces, 0 – 102 h	3.00	---
Total excreted	72.14	----
Total Recovery	78.94	----

B. Levels and Time Course of Total Radioactive Residues in Milk

The radioactivity levels and concentrations measured in the milk are presented in Table 6.2.3-5. The concentrations ranged from 0.755 mg/kg at 24 hours to 1.213 mg/kg at 56 hours after the first administration. At sacrifice, the residue concentration was 1.165 mg/kg. The time course in the evening and morning milk pool samples showed a pronounced diurnal pattern. The residues increased significantly during the eight hour period after the second and the fifth administration followed by a decrease measured prior to the delivery of the next dose. A plateau level of about 1.1 mg/kg was reached ca. 50 hours after the first administration.

Table 6.2.3-5: Time course of total radioactivity in the milk of lactating goats following oral administration of 5 daily doses of [furanone-4-14C] BYI02960 at a dose rate of 1.0 mg/kg

Time after the first admin. [h]	Admin. no.	Cumulative secretion [% of total dose admin.]	Secretion per day [% of total dose admin.]	TRR [mg/kg]
0	1	-----	-----	-----
8		0.30		0.820
24		0.68	0.68	0.755
24	2	-----	-----	-----
32		1.10		1.130
48		1.40	0.72	0.814
48	3	-----	-----	-----
56		1.67		1.213
72		1.91	0.51	0.997
72	4	-----	-----	-----
80		2.14		1.205
96		2.44	0.53	0.992
96	5	-----	-----	-----
102		2.58	0.14	1.165

C. Total Radioactive Residues in the Dissected Organs and Tissues

The concentration of the total radioactivity measured in the dissected organs and tissues collected at sacrifice are presented in Table 6.2.3-4 (last column). The highest concentrations were determined in liver (1.746 mg/kg) and kidney (1.472 mg/kg) indicating the significance of these organs for excretion and metabolism. In relation to the total dose administered, these values corresponded to 0.65% and 0.09%, respectively. For muscle and fat 0.539 mg/kg and 0.265 mg/kg, respectively were determined. The radioactivity concentration of the total body muscle corresponded to 2.91% and of fat to 0.57% of the total dose assuming a value of 30% and 12% of the body weight for these tissues.

D. Extraction Efficiency of Residues

Milk, muscle, kidney and liver pools were extracted with a mixture of acetonitrile/water (8:2; v/v). In case of milk an additional extraction step with acetone was performed. Fat was extracted with a mixture of acetonitrile/water (8:2,v/v) and n-heptane followed by solvent partition yielding an acetonitrile/water phase and an n-heptane phase. Residues in the n-heptane phase amounted to only 0.014 mg/kg (5.4%).

After purification and concentration steps, the resulting extracts represented 91.4% of the total radioactivity for milk (24 to 102 h), 95.2% for muscle, 94.1% for fat, 89.8% for kidney and 73.9% for liver. Post-extraction residues of kidney and liver were extracted exhaustively with mixtures of acetonitrile/water, 0.1 N HCl and 0.1 N NaOH using microwave assistance. The residues in the exhaustive extracts ranged from 0.039 mg/kg to 0.059 mg/kg for the kidney and from 0.053 mg/kg to 0.209 mg/kg for the liver. The exhaustive extracts were not investigated, due to their low amount of residues or their high matrix content. Negligible amounts of radioactivity were found in the distillates. Unextractable residues amounted to 0.0901 mg/kg (8.6%) for milk, 0.026 mg/kg (4.8%) for muscle

and 0.016 mg/kg (5.9%) for fat. Solids of kidney and liver were completely solubilised after exhaustive extraction with sodium hydroxide

E. Quantification, Identification and Characterisation of Residues

Quantification of Parent Compound and Metabolites

Parent compound and metabolites were quantified in the extracts as well as in the urine (24 to 102 h) using a ternary reversed phase HPL system. The three eluents employed were:

A: 1L water + 1.54 mL ammonium hydroxide solution (25%) + 0.8 mL formic acid, adjusted to pH 7

B: acetonitrile / eluent A (99:1; v/v)

C: methanol / tetrahydrofurane (1:1; v/v)

Detailed information can be found in the report.

Metabolites in the extracts as well as in the urine (24 to 102 h) were assigned by comparison of the metabolite profiles and their retention times. A summary of the quantification of parent compound and metabolites in milk, muscle, fat, kidney and liver is presented in Table 6.2.3-6.

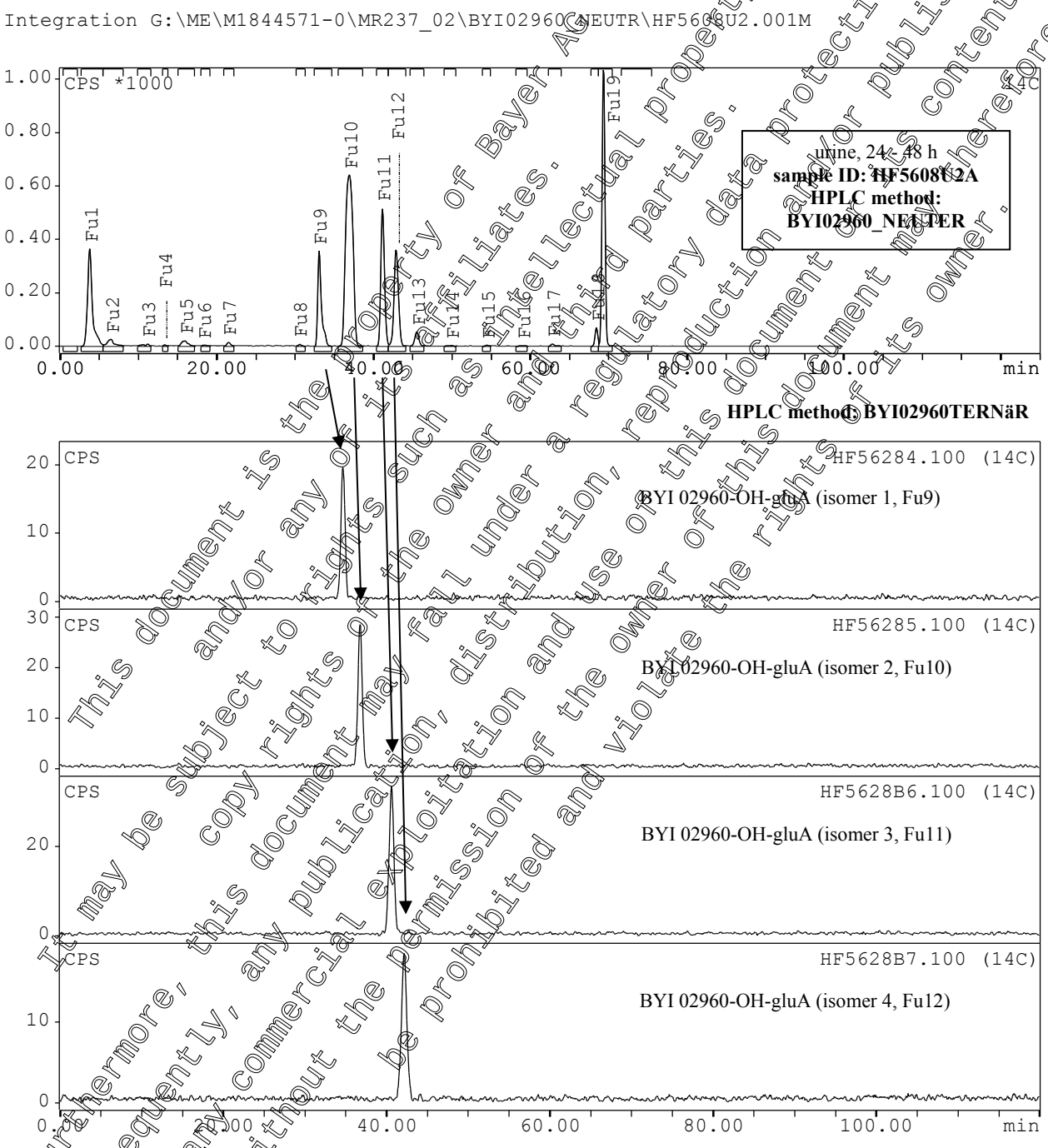
Isolation and Identification of Parent Compound and Metabolites in Urine

The identification of parent compound, BYI 02960-OH (hydroxylation in 5-position of the furanone ring) and BYI 02960-des-difluoroethyl was performed in the urine of the goat study with the pyridinylmethyl label (KIIA 6.2.3/01) by spectroscopic methods. Four isomers of the glucuronic acid conjugate of hydroxylated BYI 02960 were isolated and identified in the urine of the current study and the study with the pyridinylmethyl label. Spectra of the corresponding isomers of both studies were identical. The assignment of the four isomers to the metabolic profile of urine is presented in Figure 6.2.3-3. Identification of the four BYI 02960-OH-gluA isomers was achieved by comparison of the LC-MS/MS spectra and by comparison of the decomposition rates of the four single isomers with the increasing rates of their aglycones and a decomposition product based on the observations in the urine 72 to 96 h.

BYI 02960-OH-gluA (isomer 1) to BYI 02960-OH-gluA (isomer 4) were identified as glucuronic acid conjugates of hydroxylated BYI 02960 by LC-MS/MS. The LC-MS/MS fragments of BYI 02960-OH-gluA (isomer 1) and BYI 02960-OH-gluA (isomer 4) were different from each other and were different from BYI 02960-OH-gluA (isomer 2) and BYI 02960-OH-gluA (isomer 3). Most probably BYI 02960-OH-gluA (isomer 2) and BYI 02960-OH-gluA (isomer 3) are two diastereomers, due to their identical LC-MS/MS spectra. Therefore, it was concluded that BYI 02960 was hydroxylated and conjugated with glucuronic acid in three different positions of the molecule. The hydroxylation of the two diastereomers (isomer 2 and 3) was assigned to the 5-position of the furanone ring based on the similarity of the mass spectra with those of BYI 02960-OH-SA. The position of the hydroxylation of BYI 02960-OH-SA was clearly identified by NMR-spectroscopy in the laying hen study with the pyridinylmethyl label (KIIA 6.2.2/01). For isomer 4, the position of the hydroxylation was assigned to the difluoroethyl side chain based on the fragments 225 (ESI+) and 223 (ESI-) which prove the presence of the unchanged pyridinylmethyl and furanone moieties. For isomer 1, the position of the hydroxylation could not be derived from the mass spectra, therefore the position remains unknown. The assignment of the BYI 02960-OH-gluA isomers could be further confirmed based on the observed decomposition in two urine samples, mainly by comparison of the profiles of the urine samples collected between 72 and 96 h and between 96 and 102 h. Basically, the amounts of the BYI 02960-OH-gluA isomers as well as of the other metabolites were almost the same during the entire test period. This was observed for the urine samples of the goat study with the pyridinylmethyl label (KIIA

6.2.3/01) and also for urine samples 0 to 24 h, 24h to 48 h and 96 to 102 h of the current study. By comparison of the decrease of the conjugates with the increase of other compounds, the aglycones of the isomers could be identified.

Figure 6.2.3-3: Assignment of isolated and identified glucoronic acid conjugates in urine of lactating goats following oral administration of 5 daily doses of [furanone-4-^{14}C] BYI02960 at a dose rate of 1.0 mg/kg



Identification, Assignment and Quantification of Metabolites in Milk, Organs and Tissues

Metabolites in the extracts of milk, organs and tissues of the current study were identified based on the comparison of the metabolite pattern in the extracts with the urines of the current study and the study

with the pyridinylmethyl label. A comparison of the urine profiles from both labels is shown in Figure 6.2.3-4.

The distribution of the parent compound and metabolites in milk, organs and tissues is summarised in Table 6.2.3-6. Approximately 91% of the total radioactivity in milk, ca. 90% in muscle, ca. 83% in fat, ca. 79% in kidney and ca. 60% in liver were identified. All other residues were characterised by their extraction and chromatographic behaviour.

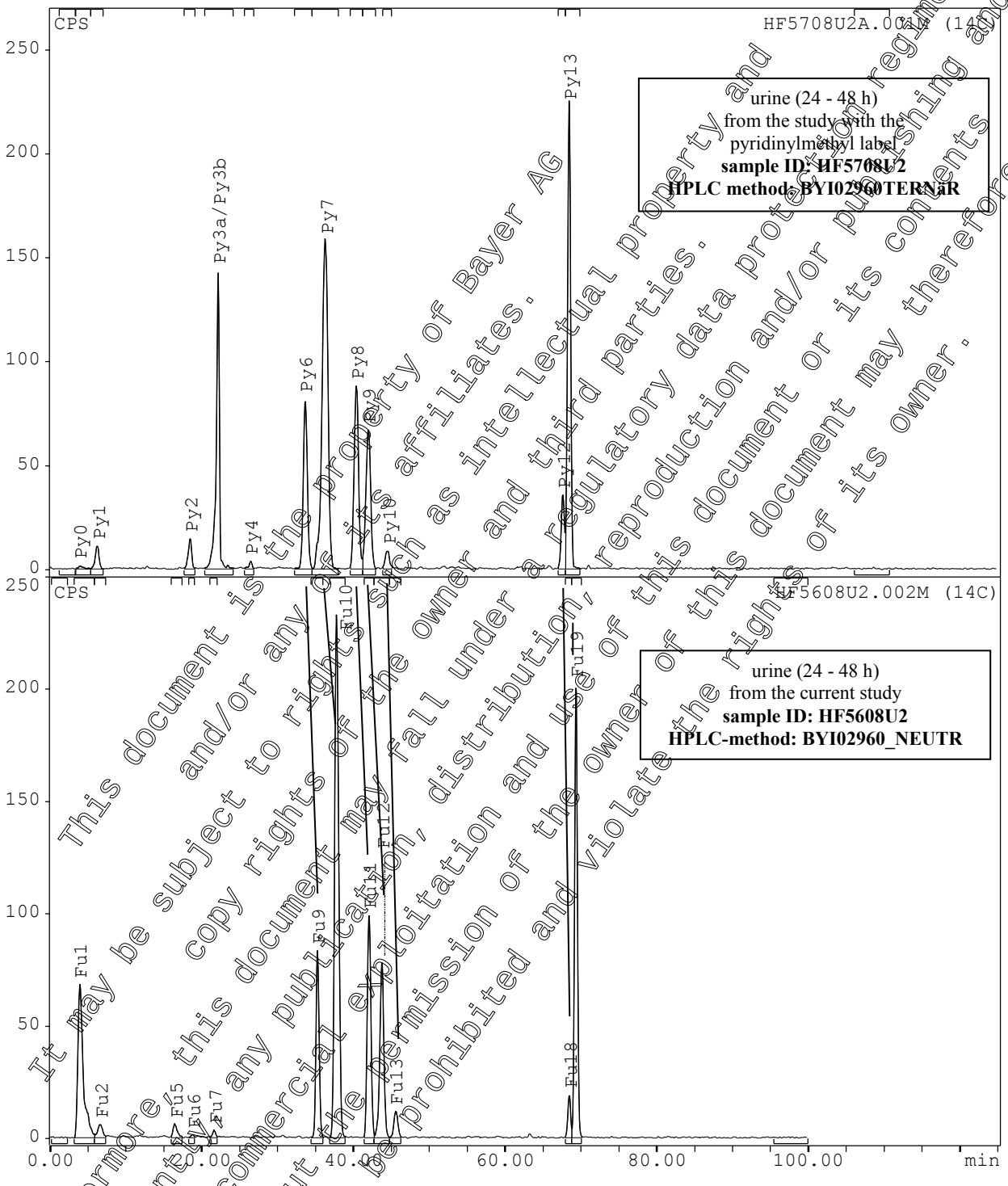
The unchanged parent compound was a major compound in milk (0.250 mg/kg, 23.9%). The main part of residues in milk was radioactive lactose and amounted to 0.698 mg/kg (66.8%). Parent compound was also the major constituent of the residue in organs and tissues and amounted to 0.475 mg/kg (88.1%) for muscle, 0.213 mg/kg (80.5%) for fat, 0.744 mg/kg (50.5%) for kidney and 1.045 mg/kg (59.8%) for liver.

Extensive metabolism was detected in the kidney, where the main portion of metabolites was found. The main metabolite in kidney was BYI 02960-OH (hydroxylation in position 5 of the furanone ring), which amounted to 0.215 mg/kg (14.6%). BYI 02960-OH was detected in lower amounts (≤ 0.010 mg/kg) in muscle and fat. Four prominent glucuronic acid conjugates of hydroxylated parent compound were detected in the kidney. The four conjugates were named BYI 02960-OH-gluA (isomer 1 to 4) and amounted between 0.032 mg/kg (2.2%) and 0.069 mg/kg (4.7%). The hydroxylation of BYI 02960 and conjugation with glucuronic acid resulted in two diastereomers (BYI 02960-OH-gluA, isomer 2 and 3) with hydroxylation and conjugation in the 5-position of the furanone ring, one isomer (BYI 02960-OH-gluA, isomer 4) with the hydroxylation and conjugation in the difluoroethyl side chain and one isomer (BYI 02960-OH-gluA, isomer 1) with the hydroxylation and conjugation in an unknown position of the molecule. Metabolites in the exhaustive extracts of kidney and liver were characterised by their extraction behaviour. They were not further investigated due to the high matrix content.

Table 6.2.3-6: Radioactive residues of parnt compound and metabolites in milk, urine and edible organs of lactating goats following oral administration of 5 daily doses of [furanone-4-^{14}C] BYI02960 at a dose rate of 1.0 mg/kg

	Milk (24 to 102 h)		Muscle		Fat		Kidney		Liver	
TRR [mg/kg]	1.046		0.539		0.265		1.472		1.746	
Sample/ Report name BYI 02960-	% of TRR	mg/kg	% of TRR	mg/kg	% of TRR	mg/kg	% of TRR	mg/kg	% of TRR	mg/kg
- ACN/water extract	90.7	0.948	95.0	0.512	88.4	0.234	89.1	1.311	73.3	1.280
Lactose	66.8	0.698	---	---	---	---	---	---	---	---
Polar metabolites	---	---	4.4	0.024	5.0	0.013	10.0	0.148	12.1	0.211
OH-gluA (isomer 1)	---	---	---	---	---	---	2.2	0.032	---	---
OH-gluA (isomer 2)	---	---	---	---	---	---	2.2	0.032	---	---
OH-gluA (isomer 3)	---	---	---	---	---	---	4.7	0.069	---	---
OH-gluA (isomer 4)	---	---	---	---	---	---	3.5	0.052	---	---
Des-difluoroethyl	---	---	---	---	---	---	1.3	0.019	---	---
OH	---	---	1.8	0.010	2.9	0.008	14.6	0.215	---	---
Parent compound	23.9	0.250	88.1	0.475	80.5	0.213	50.5	0.744	59.8	1.045
Total identified	90.7	0.948	89.9	0.484	83.4	0.221	79.0	1.163	59.8	1.045
Total extracted	91.4	0.956	95.2	0.513	94.1	0.249	100.0	1.472	100.0	1.746
Solids	8.6	0.090	4.8	0.026	5.9	0.016	n.d.	n.d.	n.d.	n.d.
Accountability	100.0	1.046	100.0	0.539	100.0	0.265	100.0	1.472	100.0	1.746

Figure 6.2.3-4: Comparison of the HPLC profiles of urine of lactating goats with the pyridinylmethyl ^{14}C- (top) and the furanone-4-^{14}C-label (bottom)



Metabolites in the polar region (Fu1 in Figure 6.2.3-4) of the acetonitrile/water extract of kidney, liver and urine were further characterised by thin-layer chromatography. Their concentration ranged from 0.021 to 0.037 mg/kg for kidney and from 0.019 to 0.059 mg/kg for liver. These polar metabolites were specific for the furanone label and were formed by the degradation of the furanone ring. The metabolites showed the same retention in TLC as the metabolites, which were detected in the corresponding polar region of urine from the rat (KIIA 5.1.2/01) and muscle and liver from the hen

(KIIA 6.2.2/02). The corresponding polar regions of muscle and fat were not investigated, due to their low amount of residues (≤ 0.013 mg/kg) and the high matrix content.
The residues in the corresponding polar region of milk were identified as radioactive lactose. The presence of the radioactive lactose in the milk was specific for the furanone label and was caused by the cleavage and subsequent degradation of the furanone ring to smaller carbon units (C-1- or C-2-fragments), entering the carbon pool of endogenous compounds. The identification was performed after isolation and acetylation by HPLC co-chromatography with acetylated radioactive lactose as reference compound. The acetylated radioactive lactose was synthesised from the radioactive lactose. The identity of the radioactive lactose and the acetylated radioactive lactose was confirmed by LC-MS/MS.

F. Storage Stability of Residues

During the study, all samples and extracts were stored in at ≤ -18 °C or for a short time in a refrigerator. All samples of milk, and edible organs and tissues were extracted within approx. seven weeks after sample collection. The first metabolite profile was recorded not later than two days after the start of the extraction and sample preparation. The first metabolic profiles were used for the quantification of metabolites, except for the profile of milk. The quantification of metabolites in the extract of milk was performed approx. four weeks later. The stability of the extract of milk was demonstrated by comparison of the first profile with the profile after storage of the extract.
For these reasons investigations on storage stability of the residues in the sample extracts were rendered unnecessary. It can be concluded that the metabolic profiles represent the residues in the matrices at sacrifice.

III. Conclusion

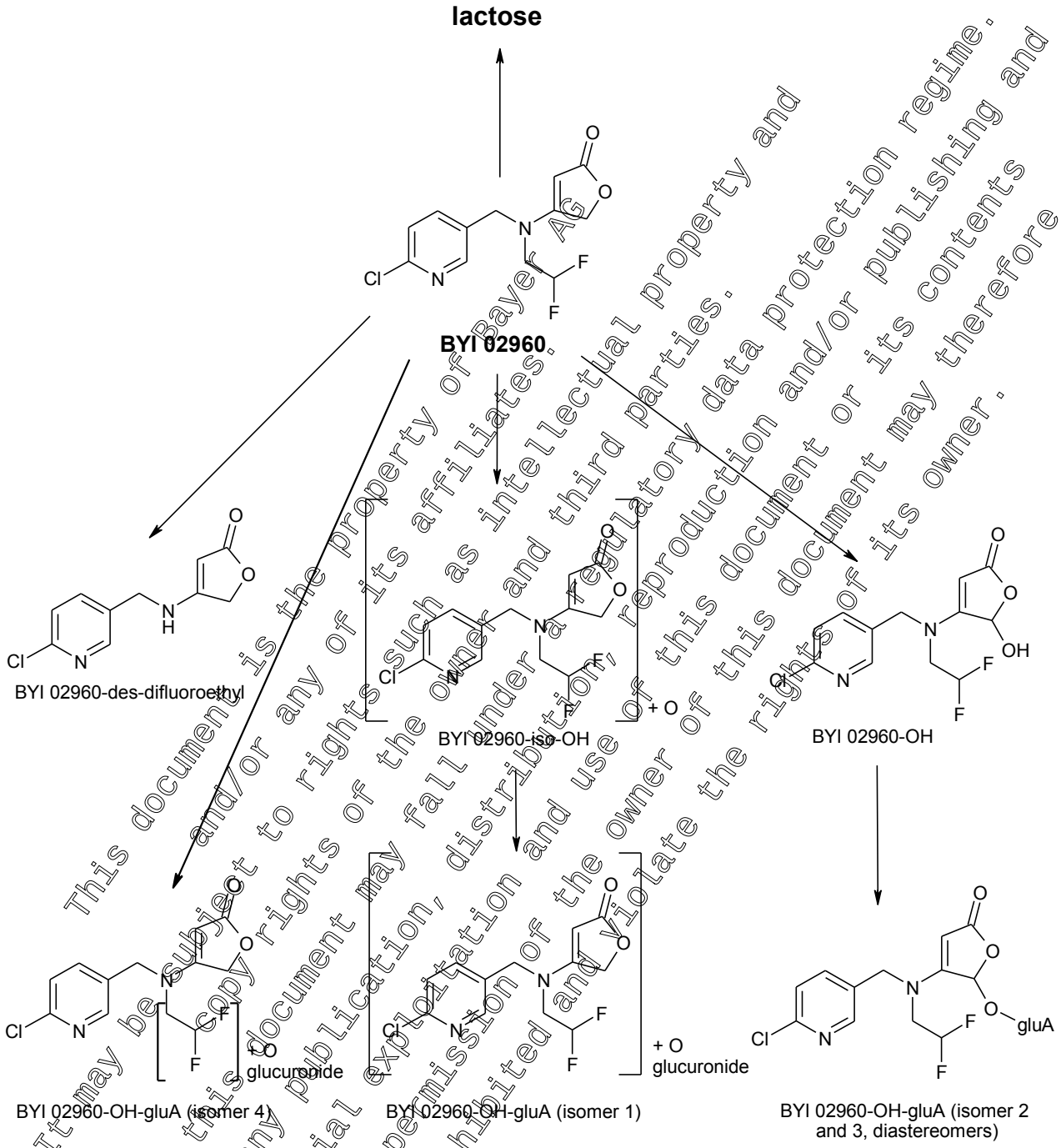
The metabolic and excretion behaviour of [furanone-4-^{14}C]BYI 02960 in the lactating goat can be characterised by the following observations:

- The concentration of radioactivity in milk and edible tissues were relatively high compared to the dose level and the dosing period of five days. This was mainly caused by the partial instability of the radiolabel used. For a small portion of the total dose the furanone ring obviously underwent extensive biotransformation to C-1- and C-2-fragments that resulted in an accumulation of radioactivity in biomolecules during dosing for five days.
  A part of these fragments was probably also converted to the terminal product $^{14}CO_2$ as can be derived from the lower overall recovery compared to the study with the metabolically stable pyridinylmethyl label.
- However, the fact should be considered that an exaggerated dose level of 28.82 mg/kg feed/day was administered. Furthermore, the significant amount of radioactivity detected in urine and faeces and the relatively high radioactivity in liver and kidney at sacrifice indicate that the residues are further metabolised and finally eliminated.
- The total radioactive residues in milk showed a diurnal pattern after the second administration as they declined significantly prior to the delivery of the next dose. A residue plateau level was reached after the third administration.
- The radioactive residues were efficiently extracted from milk as well as from edible organs and tissues; extraction rates were between 91.4% and 100.0%.

- Parent compound was the main constituent of the residues in muscle (ca. 88%), fat (ca. 81%), kidney (ca. 51%) and liver (ca. 60%). Approximately 24% of the total radioactivity in the milk was identified as parent compound.
- The main residue in the milk was radioactive lactose (approx. 67%)
- Extensive metabolism was detected in the kidney. BYI 02960-OH, four glucuronic acid conjugates of hydroxylated BYI 02960 and BYI 02960-des-difluoroethyl were identified.
- The main metabolic reactions in the lactating goat are:
  - Cleavage and subsequent degradation of the furanone ring forming small carbon units (C-1- or C-2-fragments), entering the carbon pool of endogenous compounds and finally being used for example for the biosynthesis of lactose
  - Hydroxylation followed by conjugation with glucuronic acid forming two diastereomeric conjugates of BYI 02960-OH (BYI 02960-OH-gluA, isomer 2 and 3) with hydroxylation and conjugation in the 5-position of the furanone ring, one isomer (BYI 02960-OH-gluA, isomer 4) with the hydroxylation and conjugation in the difluoroethyl side chain and one isomer (BYI 02960-OH-gluA, isomer 1) with the hydroxylation and conjugation in an unknown position
  - Cleavage of the difluoroethyl group forming BYI 02960-des-difluoroethyl

Based on these results the metabolism of [furanone-4-^{14}C] BYI 02960 in the lactating goat can be described by the metabolic pathway shown in Figure 6.2.3-5.

Figure 6.2.3-5: Proposed metabolic pathway of [furanone-4-14C] BYI02960 in the lactating goat



IIA 6.2.4 Pigs

The draft European data requirements (SANCO/11802/2010 Rev. 3) state that "metabolism studies on pigs shall be provided where the plant protection product is used in crops whose parts or products, also after processing, are fed to pigs and where it becomes apparent that metabolic pathways differ significantly in the rat as compared to ruminants." The OECD Test Guideline 503 (Metabolism in Livestock) further specifies that these significant differences may occur in the extent of the metabolism or the nature of the observed residue or the appearance of metabolites with sub-structures, which are of known potential toxicological concern.
The dominating constituent of the residue in the milk and the edible organs and tissues of the goat is the unchanged parent compound. As the main metabolite, the hydroxylated derivative (BYI 02960-OH) was identified mainly in the kidney. Further metabolites in the kidney and partly also in the liver were determined as glucuronide conjugates of BYI 02960-OH and as BYI 02960 hippuric acid which is the glycine conjugate of 6-chloronicotinic acid. All these metabolites also do occur in the rat in significant amounts (KIIA 5.1). A few minor metabolites were found exclusively in the goat. These are BYI 02960 methylthio-glyoxylic acid which occurred in milk and muscle at concentrations below 0.005 mg/kg and BYI 02960 AMCP-difluoroethanamine which was found in kindey and liver also at low concentrations (< 0.020 mg/kg).
The only major goat metabolite which was not found in the rat is BYI 02960 cysteinyl-nicotinic acid. This was found in the kidney (0.114 mg/kg) and the liver (0.058 mg/kg). This compound is formed as a product of a typical detoxification reaction by conjugation of 6-chloronicotinic acid with cysteine under loss of the chlorine atom. The compound is very polar and easily excretable via urine which is supported by the fact that it was only found in kidney and liver but not in the peripheral compartments of muscle or fat. In this context it should also be noted that the lactating goat was dosed at an exaggerated dose level of ca. 24 mg/kg dry feed which is approximately 6 times higher as the 1X dose level in the cattle feeding study.
Taking all these results into consideration, it can be concluded that the ruminant and rat metabolism follow the same metabolic pathway and thus are neither different in extent nor in nature. Furthermore, no sub-structures of known potential toxicological concern became apparent. Thus a pig metabolism study was rendered unnecessary.

IIA 6.2.5 Nature of residue in fish

The draft European data requirements (SANCO/11802/2010 Rev. 3) state that "metabolism studies on freshwater fish shall be provided where the plant protection product is used in crops whose parts or products, also after processing, are fed to fish and where significant residues in feed may occur from the intended applications. Taking into account that, according to the experience gained so far, uptake, metabolism and residues in fish are more likely associated with fat-soluble residues, tests shall be provided for residues where the log Pow of the active substance or the residue of concern, that is to say all components of the residue definition, is greater than or equal to three."

According to EPA Pesticide Assessment Guideline §165-4, a fish bioaccumulation study including investigation of metabolism will not normally required if a compound has a relatively low potential for

accumulation in fish as indicated by an octanol/water partition coefficient less than approximately 1000.

BYI 02960 has a log P_{OW} of 1.2 in the range of pH 4 - 9 which is clearly below this threshold. Furthermore, all results of ADME and livestock studies demonstrate that BYI 02960 and its metabolites are quickly excreted and not retained or accumulated in the body of animals. Therefore, a fish metabolism study is not required for BYI 02960.

IIA 6.2.6 Chemical identity

Not required by Regulation 1107/2009.