**Document Title**

# Tier 2 Summary of the Analytical Methods and Validation for

# Flupyradifurone (BYI 02960)

**Data Requirements**

## Regulation (EC) No 1107/2009

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

## Annex IIA
## Section 2, Point 4
## Document M

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

**Date**

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**Author(s)**

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M-429305-02-1

# OWNERSHIP STATEMENT

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# IIA 4 Analytical Methods and Validation

## IIA 4.1 Analytical standards and samples

### IIA 4.1.1 Analytical standards for pure active substance

Analytical standards for pure active substance are available and can be provided on request.

### IIA 4.1.2 Samples of the active substance as manufactured

Samples of the active substance as manufactured are available and can be provided on request.

### IIA 4.1.3 Analytical standards for relevant metabolites and other components

If relevant, analytical standards for relevant metabolites and other components included in the residue definition are available and can be provided on request.

### IIA 4.1.4 Samples of reference substances for relevant impurities

If relevant, samples of reference substances for relevant impurities are available and can be provided on request.

## IIA 4.2 Methods for the analysis of the active substance as manufactured

### IIA 4.2.1 Methods for the analysis of the active substance as manufactured

| **Report:** | **KIIA 4.2.1/01, zur Mühlen, U., Wagner, S.; 2011;** |
|---|---|
| Title: | Flupyradifurone (BYI 02960) Determination of technical grade active substance HPLC - ISTD |
| Report No & Document No | AM008809MP1<br>M-407218-01-1 |
| **Guidelines:** | **EC Directive 1107/2009; OPPTS 830.1800** |
| **GLP** | **Non-GLP** |

The active substance Flupyradifurone is determined in the technical material as manufactured by reversed-phase liquid chromatography (HPLC) using Diethylphthalate as internal standard (ISTD). The quantitative determination is performed with a specific UV detector (DAD detector) at 225 nm.

| | |
|---|---|
| **Report:** | **KIIA 4.2.1/02, Wagner, S.; 2011;** |
| Title: | Validation of AM008809MP1 Flupyradifurone (BYI 02960) Determination of technical grade active substance HPLC - ISTD |
| Report No & Document No | VB1-AM008809MP1<br>M-409002-01-1 |
| **Guidelines:** | **EC Directive 1107/2009; OPPTS 830.1800** |
| **GLP** | **GLP** |

The HPLC-method AM008809MP1for the determination of Flupyradifurone (BYI 02960) in technical grade active substance has been completely validated by checking the parameters linearity, precision, accuracy, specificity and interference.

| | |
|---|---|
| Linearity | 5 concentrations with double measurements; range 84.3 – 120.4%<br>(corresponding to 84.3 mg/100 mL to 120.4 mg/100 mL);<br>correlation coefficient $r^2$: 0.9999; regression equation and chromatograms are given in the report; the function is linear in the operating range. |
| Precision<br>Repeatability | 5 synthetic samples with double measurements;<br>no outliers are reported, RSD: 0.30 %;<br>acceptable according to the Horwitz equation. |
| Accuracy | 5 synthetic samples with double measurements;<br>mean recovery: 99.87 %; RSD: 0.23 %. |
| Specificity/Interference | Retention times and UV-spectra from reference substance, sample and spiked sample were compared. The UV-spectra show no spectral differences, the corresponding retention times are identical.<br>Chromatograms of reference substances, sample and spiked sample were checked and found to be free of interfering compounds. |

The HPLC-method AM008809MP1 for the determination of Flupyradifurone (BYI 02960) in technical grade active substance is found to be valid.

Linearity: results from the individual measurements

Excerpt of the GLP raw data of the validation (VB1-AM008809MP1), GLP raw data archived:

BYI 02960

| No. | Name | Sample Type | Pos | Ret.Time min BYI 02960 UV_VIS_1 | Amount BYI 02960 UV_VIS_1 | Dil.Fac. | Amount Ref. | Area mAU*min BYI 02960 UV_VIS_1 | Faktor BYI 02960 UV_VIS_1 |
|---|---|---|---|---|---|---|---|---|---|
| 2 | AZ 16895 Linearity 1 | standard | 11 | 9.05 | 1 | 1.0 | 1 | 19.27191 | 0.0111 |
| 3 | AZ 16895 Linearity 1 | standard | 11 | 9.05 | 1 | 1.0 | 1 | 19.49370 | 0.0112 |
| 4 | AZ 16895 Linearity 2 | standard | 12 | 9.05 | 1 | 1.0 | 1 | 21.86848 | 0.0111 |
| 5 | AZ 16895 Linearity 2 | standard | 12 | 9.05 | 1 | 1.0 | 1 | 22.13324 | 0.0111 |
| 6 | AZ 16895 Linearity 3 | standard | 13 | 9.05 | 1 | 1.0 | 1 | 23.10004 | 0.0111 |
| 7 | AZ 16895 Linearity 3 | standard | 13 | 9.05 | 1 | 1.0 | 1 | 23.22262 | 0.0111 |
| 8 | AZ 16895 Linearity 4 | standard | 14 | 9.05 | 1 | 1.0 | 1 | 26.30458 | 0.0111 |
| 9 | AZ 16895 Linearity 4 | standard | 14 | 9.05 | 1 | 1.0 | 1 | 26.32761 | 0.0111 |
| 10 | AZ 16895 Linearity 5 | standard | 15 | 9.05 | 1 | 1.0 | 1 | 27.79764 | 0.0111 |
| 11 | AZ 16895 Linearity 5 | standard | 15 | 9.05 | 1 | 1.0 | 1 | 27.81411 | 0.0111 |
| Average: | | | | | | | | | 0.0111 |
| RSD: | | | | | | | | | 0.112% |

Diethylphthalate

| No. | Name | Sample Type | Pos | Ret.Time min Diethylphthalate UV_VIS_1 | Amount Diethylphthalate UV_VIS_1 | Dil.Fac. | Amount Ref. | Area mAU*min Diethylphthalate UV_VIS_1 | Faktor Diethylphthalate UV_VIS_1 |
|---|---|---|---|---|---|---|---|---|---|
| 2 | AZ 16895 Linearity 1 | standard | 11 | 12.32 | 1 | 1.0 | 1 | 20.52711 | 1.0000 |
| 3 | AZ 16895 Linearity 1 | standard | 11 | 12.32 | 1 | 1.0 | 1 | 20.73443 | 1.0000 |
| 4 | AZ 16895 Linearity 2 | standard | 12 | 12.32 | 1 | 1.0 | 1 | 20.54462 | 1.0000 |
| 5 | AZ 16895 Linearity 2 | standard | 12 | 12.32 | 1 | 1.0 | 1 | 20.78160 | 1.0000 |
| 6 | AZ 16895 Linearity 3 | standard | 13 | 12.32 | 1 | 1.0 | 1 | 20.63230 | 1.0000 |
| 7 | AZ 16895 Linearity 3 | standard | 13 | 12.32 | 1 | 1.0 | 1 | 20.72506 | 1.0000 |
| 8 | AZ 16895 Linearity 4 | standard | 14 | 12.32 | 1 | 1.0 | 1 | 20.90123 | 1.0000 |
| 9 | AZ 16895 Linearity 4 | standard | 14 | 12.32 | 1 | 1.0 | 1 | 20.90567 | 1.0000 |
| 10 | AZ 16895 Linearity 5 | standard | 15 | 12.32 | 1 | 1.0 | 1 | 20.78953 | 1.0000 |
| 11 | AZ 16895 Linearity 5 | standard | 15 | 12.32 | 1 | 1.0 | 1 | 20.78473 | 1.0000 |
| Average: | | | | | | | | | 1.0000 |
| RSD: | | | | | | | | | 0.000% |

Repeatability: results from the individual measurements
Excerpt of the GLP raw data of the validation (VB1-AM008809MP1), GLP raw data archived:

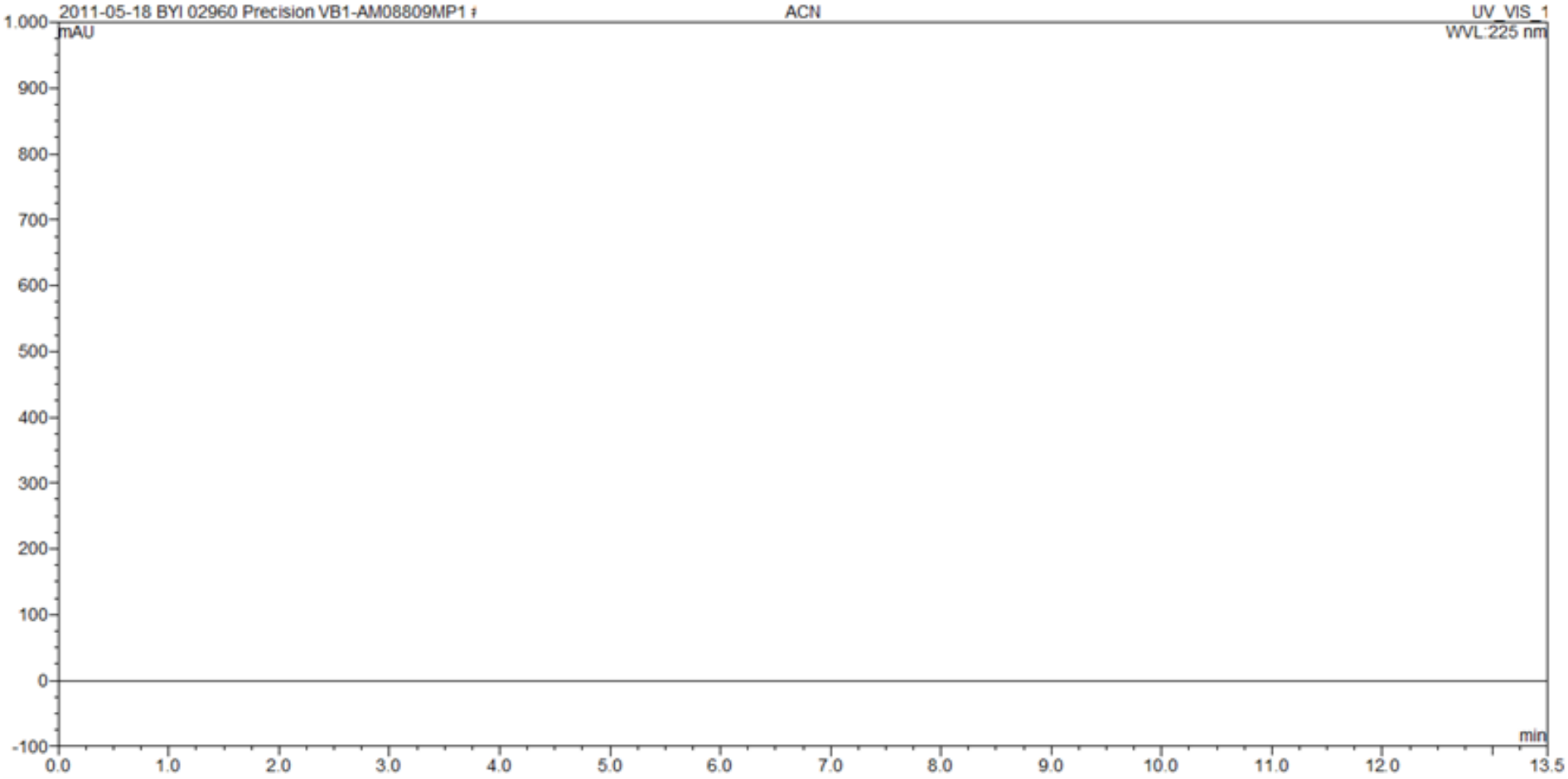
BYI 02960

| No. | Name | Replicate ID | Type<br>BYI 02960<br>UV_VIS_1 | Weight | Ret.Time<br>min<br>BYI 02960<br>UV_VIS_1 | Area<br>mAU*min<br>BYI 02960<br>UV_VIS_1 | Height<br>mAU<br>BYI 02960<br>UV_VIS_1 | Amount<br>g/kg<br>BYI 02960<br>UV_VIS_1 | Average Amount | Rel.Std.Dev.<br>% |
|---|---|---|---|---|---|---|---|---|---|---|
| 10 | Precision 1 | | BMB | 103.35 | 9.04 | 23.53196 | 379.549 | 980.3263 | | |
| 11 | Precision 1 | | BMB | 103.35 | 9.04 | 23.58201 | 380.994 | 979.8183 | | |
| 12 | Precision 2 | | BMB | 107.46 | 9.04 | 24.29565 | 391.924 | 973.1823 | | |
| 13 | Precision 2 | | BMB | 107.46 | 9.04 | 24.41577 | 394.000 | 973.7105 | | |
| 14 | Precision 3 | | BMB | 104.27 | 9.04 | 23.76318 | 383.612 | 980.0756 | | |
| 15 | Precision 3 | | BMB | 104.27 | 9.04 | 23.86380 | 385.496 | 980.2377 | | |
| 16 | Precision 4 | | BMB | 101.30 | 9.04 | 23.28641 | 376.035 | 980.8087 | | |
| 17 | Precision 4 | | BMB | 101.30 | 9.04 | 23.28014 | 375.922 | 980.5606 | | |
| 18 | Precision 5 | | BMB | 99.46 | 9.04 | 22.90803 | 370.260 | 980.6019 | | |
| 19 | Precision 5 | | BMB | 99.46 | 9.04 | 22.77664 | 368.302 | 981.1460 | 979.0468 | 0.304 |

Diethylphthalate

| No. | Name | Replicate ID | Type<br>Diethylphthalate<br>UV_VIS_1 | Weight | Ret.Time<br>min<br>Diethylphthalate<br>UV_VIS_1 | Area<br>mAU*min<br>Diethylphthalate<br>UV_VIS_1 | Height<br>mAU<br>Diethylphthalate<br>UV_VIS_1 | Amount<br>g/kg<br>Diethylphthalate<br>UV_VIS_1 | Average Amount | Rel.Std.Dev.<br>% |
|---|---|---|---|---|---|---|---|---|---|---|
| 10 | Precision 1 | | BMB | 103.35 | 12.32 | 20.88665 | 874.958 | 1.0000 | | |
| 11 | Precision 1 | | BMB | 103.35 | 12.32 | 20.94192 | 876.475 | 1.0000 | | |
| 12 | Precision 2 | | BMB | 107.46 | 12.32 | 20.89196 | 874.699 | 1.0000 | | |
| 13 | Precision 2 | | BMB | 107.46 | 12.32 | 20.98386 | 878.466 | 1.0000 | | |
| 14 | Precision 3 | | BMB | 104.27 | 12.32 | 20.91113 | 875.092 | 1.0000 | | |
| 15 | Precision 3 | | BMB | 104.27 | 12.32 | 20.99619 | 879.093 | 1.0000 | | |
| 16 | Precision 4 | | BMB | 101.30 | 12.32 | 21.07660 | 882.810 | 1.0000 | | |
| 17 | Precision 4 | | BMB | 101.30 | 12.32 | 21.07625 | 882.952 | 1.0000 | | |
| 18 | Precision 5 | | BMB | 99.46 | 12.32 | 21.12215 | 884.514 | 1.0000 | | |
| 19 | Precision 5 | | BMB | 99.46 | 12.32 | 20.98937 | 879.087 | 1.0000 | 1.0000 | 0.000 |

Repeatability: Chromatogram of a sample blank (GLP raw data, archived)



### IIA 4.2.2 Applicability of existing CIPAC methods

Up to now there is no CIPAC method available for the determination of BYI 02960 (Flupyradifurone) in technical grade active substance

### IIA 4.2.3 Description of analytical methods for the determination of impurities

Refer to file of confidential information in Document JII.

### IIA 4.2.4 Description of analytical methods for the determination of additives

Refer to file of confidential information in Document JII.

### IIA 4.2.5 Enforcement analytical methodology

Not required for the methods for the analysis of the active substance as manufactured.

### IIA 4.2.6 Inter-Laboratory analytical methodology validaton1

Not required for the methods for the analysis of the active substance as manufactured.

### IIA 4.2.7 Storage stability of working solutions in analytical methodology

Not required for the methods for the analysis of the active substance as manufactured.

## IIA 4.3 Description of analytical methods for the determination of residues in plant and animal matrices

**General remark:**

In this summary section (KIIA 4.3), the following name will be used for the metabolite BYI 02960-difluoroethyl-amino-furanone, which is relevant to the plant residue analytical methods:

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

*Plant matrices:*

For the determination of the relevant residues of BYI 02960 (common name: flupyradifurone) in plant matrices, three methods were developed.

Method 01330 was developed as an *enforcement method*. It determines BYI 02960 and its metabolite DFA by HPLC-MS/MS. Matrix-matched standards were used for quantification. The LOQs were generally 0.01 mg/kg and 0.02 mg/kg for BYI 02960 and DFA, respectively, except for hop matrices, for which the respective LOQs were 0.05 and 0.10 mg/kg. Results of an ILV demonstrated inter-laboratory reproducibility of the method.

The European multi-residue methods DFG S 19 and QuEChERS are unsuitable for the enforcement of this compound because the determination of the metabolite DFA is not possible – none of the extraction processes described in either of the methods would allow appropriate extraction of this molecule.

Two internal-standard methods were used for *data collection*. Both allow the determination of BYI 02960 and three metabolites (DFA, DFEAF, 6-CNA) in plant matrices via HPLC-MS/MS. In the first, method RARVP013 (a.k.a. method no. 01304), the LOQs in the primary study were generally 0.01 mg/kg for BYI 02960, DFEAF, and 6-CNA (all expressed in parent equivalents) and 0.02 mg/kg for DFA in crops high in acid and water content (e.g. oranges, tomatoes), or 0.05 mg/kg in dry and protein-rich matrices, fodder materials, and soybeans (such as dried bean seeds, wheat grain, wheat fodder, and soybean seeds). In the second method, 01212, the LOQs for all analytes were the same as in 01304 except for DFA, for which the LOQ was 0.02 mg/kg in all matrix types.

*Animal matrices:*

For the determination of the relevant residues of BYI 02960 in animal matrices, two methods were developed.

Method 01214 was developed as an *enforcement method*. It determines BYI 02960 and its metabolite DFA by HPLC-MS/MS. For quantification, matrix-matched standards were used. The LOQs were 0.01 mg/kg and 0.02 mg/kg for BYI 02960 and DFA, respectively, in all matrices. Results of an ILV demonstrated inter-laboratory reproducibility of the method.

Method RV-004-A11-04 was developed for *data collection* in the feeding studies. It allows the determination of BYI 02960 and three metabolites (DFA, BYI 02960-acetyl-AMCP, BYI 02960-OH) in animal matrices via HPLC-MS/MS. Internal standards were used for quantification. The LOQs were generally 0.01 mg/kg for BYI 02960, BYI 02960-acetyl-AMCP, BYI 02960-OH in all matrices (all expressed in parent equivalents), as well as for DFA in poultry tissues, and 0.02 mg/kg for DFA in bovine matrices.

Table 4.3-1: Summary of residue analytical methods for BYI 02960 in plant and animal matrices

| Matrix | Analyte | Method No. | Method principle | LOQ* | Reference |
|---|---|---|---|---|---|
| **Plant** | BYI 02960<br>DFA | 01330† | HPLC-MS/MS | BYI 02960: 0.01 or 0.05 mg/kg<br>DFA: 0.02 or 0.10 mg/kg | IIA 4.3/01<br>IIA 4.3/02 |
| **Plant** | BYI 02960<br>-DFEAF<br>DFA<br>6-CNA** | RARVP 013 (01304) | HPLC-MS/MS | BYI 02960: 0.01 mg/kg<br>-DFEAF: 0.01 mg/kg<br>DFA: 0.02 or 0.05 mg/kg<br>6-CNA: 0.01 mg/kg** | IIA 4.3/03<br>IIA 4.3/04 |
| **Plant** | BYI 02960<br>-DFEAF<br>DFA<br>6-CNA** | 01212 | HPLC-MS/MS | BYI 02960: 0.01 mg/kg<br>-DFEAF: 0.01 mg/kg<br>DFA: 0.02 mg/kg<br>6-CNA: 0.01 mg/kg** | IIA 4.3/05 |
| **Animal** | BYI 02960<br>DFA | 01214† | HPLC-MS/MS | BYI 02960: 0.01 mg/kg<br>DFA: 0.02 mg/kg | IIA 4.3/06<br>IIA 4.3/07 |
| **Animal** | BYI 02960<br>DFA<br>-acetyl-AMCP<br>-OH | (RV-004-A11-04) | HPLC-MS/MS | BYI 02960: 0.01 mg/kg<br>DFA: 0.01 or 0.02 mg/kg<br>-acetyl-AMCP: 0.01 mg/kg<br>-OH: 0.01 mg/kg | IIA 4.3/08<br>IIA 4.3/09 |

* concentrations of all metabolites given as BYI 02960 equivalents
** these methods are also capable of determining 6-CNA; however, as this compound is not part of any residue definitions, it will not be summarized further below.
† these methods are the proposed EU enforcement methods

*Plant matrices*

| **Report:** | **KIIA 4.3/01, Schulte, G., & Bauer, J.; 2012** |
|---|---|
| Title: | Analytical method 01330 for the determination of residues of BYI 02960 and its metabolite difluoroacetic acid in/on plant matrix by HPLC-MS/MS - Enforcement method plant |
| Report No. & Edition No. | Method no. 01330, report no. MR-011/096<br>M-425848-01-1 |
| **Guidelines:** | – EU Council Directive 91/414/EEC amended by Commission Directive 96/68/EC<br>– EU Guidance Document for residue analytical methods SANCO/825/00 rev. 7 |
| **GLP:** | yes (certified laboratory) |

| **Report:** | **KIIA 4.3/02, Konrad, S.; 2012** |
|---|---|
| Title: | Independent lab validation of BCS method 01330 for the determination of residues of BYI 02960 and its metabolite difluoroacetic acid in/on plant matrices by HPLC-MS/MS |
| Report No. & Edition No. | 2011/0134/01<br>M-427133-01-1 |
| **Guidelines:** | – EU Regulation (EC) No. 1107/2009<br>– EU Guidance Document for residue analytical methods SANCO/825/00 rev. 8.1<br>– EU Guidance Document for residue analytical methods SANCO/3029/99<br>– OECD Guidance Document on pesticide residue analytical methods ENV/JM/Mono (2007)<br>– US EPA Residue Chemistry Test Guideline OPPTS 860.1340, residue analytical methods |
| **GLP:** | yes (certified laboratory) |

Principle of the method

Residue analytical method 01330 (Schulte & Bauer, 2012; KIIA 4.3/01) was developed as an *EU enforcement method* for the determination of the residues of BYI 02960 (parent compound), and its metabolite DFA in/on plant materials.

The residues were extracted twice from 5 g of plant material with acetonitrile/water (4/1, v/v) with 2.2 mL/L formic acid. The materials tested included lettuce (head), rape (seed), orange (fruit), wheat (grain), and hop (cone), representing a wide variety of crops/crop types as requested by EU guidance. After dilution, an aliquot of the raw extract was filtered for measurement. The solution was analyzed by HPLC-MS/MS; residues were quantified against matrix-matched standards.

Specificity

Apparent residues in control samples were below 30% of the LOQ. Two MRM transitions for quantitation and confirmation were monitored for BYI 02960 (m/z 289/126 or 90) in each matrix tested. Using this procedure, the HPLC-MS/MS method is highly specific, thus an additional confirmatory method based on another principle is not necessary. For DFA, no second MRM transition is available. Thus, a Hypercarb column was employed as a different separation system (as opposed to HILIC for the primary determination). The confirmatory methods were fully validated, hence the quantitation and confirmation methods can be used interchangeably if so desired.

Accuracy (recovery findings)

The accuracy of the method was assessed on the basis of the determined recovery rates. Samples were fortified with BYI 02960 at concentrations of 0.01 and 0.10 mg/kg (0.05 and 0.5 mg/kg in hops); DFA

was spiked at 0.02 and 0.20 mg/kg (0.10 and 1.0 mg/kg for hops), expressed in parent equivalents. Mean recoveries per fortification level for the primary method for both analytes and all matrices were in a range of 88-106%, with one exception at 62-65% (DFA, rape seed). Using the confirmatory conditions, mean values per fortification level were 70-108%, with one exception at 64% (DFA, rape seed, 0.02 mg/kg). In all three cases of lower recovery, the RSD was low (6.5-11.6%), so that these lower values were considered to be acceptable. The results are summarized below in tables 4.3-2 and 4.3-3.

Linearity

The correlation between the injected amount of substance and the detector response was linear for standards in matrix in the range from 0.125 to 500 µg/L, using at least 5 different concentration levels, for both compounds. The correlation coefficients of the 1/x weighted linear regression were > 0.99 in both cases. Linearity was proven for the confirmatory method as well, over the same concentration range and, again, with correlation coefficients of > 0.99.

Limit of Quantification

The limit of quantitation (LOQ) for BYI 02960, defined as the lowest validated fortification level, was 0.01 mg/kg in all matrices tested, except hop cones, in which in was 0.05 mg/kg. For DFA, the LOQ was 0.02 mg/kg in all matrices except hops, where it was 0.10 mg/kg. The calculated limit of detection (LOD) was estimated to be at least 3 times lower than the respective LOQ, based on the linearity response data and matrix interference observed in control samples.

Repeatability (precision)

As a measure of the precision, the intra-laboratory repeatability (n=5) is given as the relative standard deviation (% RSD) for different sample materials at fortification levels at the respective LOQ and, in general, 10×LOQ. For BYI 02960, these levels were 0.01 and 0.10 mg/kg for most matrices, or between 0.05 and 0.50 mg/kg in hop cones. Relative standard deviations were 1.1-8.8% for the primary method (confirmatory: 1.8-9.9%).

Fortification levels for DFA were 0.02 and 0.20 mg/kg, except for hop matrices, where they were 0.10 and 1.0 mg/kg. RSDs were 1.7-11.6% for the primary method, and 2.1-9.4% for the confirmatory.

The results are summarized below in tables 4.3-2 and 4.3-3.

Reproducibility (ILV)

An independent laboratory validation was conducted for method 01330 and reported as 2011/0134/01 (Konrad, 2012; KIIA 4.3/02). Samples of lettuce head, orange fruit, and wheat grain were fortified with BYI 02960 parent compound and DFA at the nominal fortification levels of 0.01 and 0.10 mg/kg, i.e. the LOQ and the 10-fold LOQ. Two replicate specimens per plant material were kept untreated, serving as blank controls.

Analysis of samples was performed according to method 01330 (Schulte & Bauer, 2012; KIIA 4.3/01). Two MRM transitions were measured for BYI 02960 (as described above under "specificity"), one for quantification and the second for confirmation. For all matrices, for both

fortification levels, and for both MRM transitions monitored, the mean recoveries were between 75% and 103%, with relative standard deviations of < 10%. Only minor interfering signals in the blank control specimens were detected, resulting in a limit of detection (LOD) of 0.003 mg/kg for all plant materials.

For DFA, as described above under "specificity", one MRM transition was determined using two different HPLC conditions. For all matrices, for both fortification levels, and for both the primary and the confirmatory HPLC procedure, the mean recoveries were between 73 and 100%, with the exception of wheat grain at the 10×LOQ, where the value was 68%. This value was considered to be acceptable nevertheless, as the RSD was 7.2% and the overall recovery for wheat grain over both spike levels was 77%. RSDs were < 10% in all cases. Only minor interfering signals in the blank control specimens were detected, resulting in a limit of detection (LOD) of 0.003 mg/kg for all plant materials when using the primary method. (For the confirmatory method, the S/N ratio > 3 was not achieved.)

Method 01330 was shown to fulfil the reproducibility requirements as defined in international guidelines and therefore to be suitable for the enforcement of residue levels of BYI 02960 and its metabolite DFA in plant matrices. A summary of the independent laboratory validation results is given in tables 4.3-4 and 4.3-5.

Extraction efficiency

The extraction efficiency of the residue method for the determination of the relevant residues of BYI 02960 in plant matrices, consisting of the parent compound and its metabolite DFA, was assured by choosing the same extraction procedures as used in the plant metabolism studies (cf. chapter 6.2 of this dossier). Nevertheless, an extraction efficiency study was conducted using method 01304 (KIIA 4.3/03 and /04, cf. below). As the extraction procedures for method 01330 are the same as for 01304, the results of the study prove satisfactory extraction efficiency with method 01330.

Stability of analytes

BYI 02960 and DFA were shown to be stable in standard solutions for at least 6 months as a part of the study RARVP013 (method 01304, cf. KIIA 4.3/03). During the course of the validations for this method 01330, stability was shown in all tested matrix extracts for at least 7-8 days, when stored in the dark in a refrigerator at 4°C ± 3°C.

Standard EU multi-residue methods, e.g. DFG S 19 or QuEChERS

The EU requires that a major multi-residue method, usually DFG S 19 and/or QuEChERS, be evaluated as to whether it can be used for monitoring purposes. When considering the proposed enforcement residue definition for BYI 02960, consisting of the sum of two components – parent compound and the metabolite DFA – it is evident that the small, polar molecule DFA cannot be sufficiently extracted via any of the recommended extraction systems in the two multimethods. Thus, a specific method is presented here for enforcement purposes.

Conclusion

As DFA cannot be determined by DFG multi-residue method S 19 or by the QuEChERS procedure, a specific method, such as 01330, is suitable to determine the proposed enforcement residue definition for BYI 02960.

Method 01330 meets all necessary performance requirements to determine residues of BYI 02960 and its metabolite DFA in plant materials, with an LOQ of 0.01 mg/kg for BYI 02960 (0.05 in hops), and of 0.02 mg/kg for DFA (0.10 in hops). Results of an ILV showed that method 01330 fulfills the reproducibility requirements and is, therefore, applicable as an enforcement method.

Table 4.3-2: Recovery results from the method validation of method 01330 (enforcement method) – Recoveries and relative standard deviations (RSDs) for **BYI 02960**

| Sample material | FL* [mg/kg] | Individual values [%] | | | | | Mean value [%] | RSD [%] | LOQ [mg/kg] |
|---|---|---|---|---|---|---|---|---|---|
| *QUANTIFICATION MRM (289/126)* | | | | | | | | | |
| lettuce / head | 0.01 | 102 | 84 | 94 | 105 | 102 | 97 | 8.8 | 0.01 |
| | 0.10 | 92 | 99 | 88 | 97 | 99 | 95 | 5.1 | |
| | | ***Overall recovery (n = 10)*** | | | | | ***96*** | ***6.9*** | |
| rape / seed | 0.01 | 99 | 98 | 97 | 92 | 91 | 95 | 3.8 | 0.01 |
| | 0.10 | 96 | 98 | 92 | 92 | 94 | 94 | 2.8 | |
| | | ***Overall recovery (n = 5)*** | | | | | ***95*** | ***3.2*** | |
| orange / fruit | 0.01 | 96 | 97 | 97 | 97 | 88 | 95 | 4.1 | 0.01 |
| | 0.10 | 100 | 106 | 103 | 99 | 104 | 102 | 2.8 | |
| | | ***Overall recovery (n = 10)*** | | | | | ***99*** | ***5.1*** | |
| wheat / grain | 0.01 | 100 | 104 | 106 | 104 | 109 | 105 | 3.1 | 0.01 |
| | 0.10 | 109 | 104 | 105 | 105 | 105 | 106 | 1.8 | |
| | | ***Overall recovery (n = 10)*** | | | | | ***105*** | ***2.5*** | |
| hop / cone | 0.01 | 86[1] | 89 | 94 | 90 | 91 | 91 | 2.4 | 0.05 |
| | 0.10 | 92 | 94 | 94 | 92 | 93 | 93 | 1.1 | |
| | | ***Overall recovery (n = 10)*** | | | | | ***92*** | ***2.0*** | |
| *CONFIRMATORY MRM (289/90)* | | | | | | | | | |
| lettuce / head | 0.01 | 103 | 85 | 95 | 107 | 104 | 99 | 9.0 | 0.01 |
| | 0.10 | 91 | 97 | 87 | 96 | 98 | 94 | 5.0 | |
| | | ***Overall recovery (n = 10)*** | | | | | ***96*** | ***7.5*** | |
| rape / seed | 0.01 | 100 | 99 | 97 | 93 | 91 | 96 | 4.0 | 0.01 |
| | 0.10 | 94 | 97 | 89 | 91 | 91 | 92 | 3.4 | |
| | | ***Overall recovery (n = 5)*** | | | | | ***94*** | ***4.1*** | |
| orange / fruit | 0.01 | 97 | 97 | 97 | 96 | 91 | 96 | 2.7 | 0.01 |
| | 0.10 | 98 | 105 | 102 | 98 | 104 | 101 | 3.2 | |
| | | ***Overall recovery (n = 10)*** | | | | | ***99*** | ***4.2*** | |
| wheat / grain | 0.01 | 100 | 101 | 107 | 104 | 108 | 104 | 3.4 | 0.01 |
| | 0.10 | 105 | 100 | 101 | 102 | 102 | 102 | 1.8 | |
| | | ***Overall recovery (n = 10)*** | | | | | ***103*** | ***2.8*** | |
| hop / cone | 0.01 | 84[1] | 88 | 94 | 88 | 88 | 90 | 3.4 | 0.05 |
| | 0.10 | 88 | 92 | 91 | 89 | 91 | 90 | 1.8 | |
| | | ***Overall recovery (n = 10)*** | | | | | ***90*** | ***2.5*** | |

* fortified compound BYI 02960 determined as BYI 02960 expressed as BYI 02960

*Footnotes:*

1: only 1 injection used for calculation

Table 4.3-3: Recovery results from the method validation of method 01330 (enforcement method) – Recoveries and relative standard deviations (RSDs) for **DFA**

| Sample material | FL* [mg/kg] | Individual values [%] | | | | | Mean value [%] | RSD [%] | LOQ [mg/kg] |
|---|---|---|---|---|---|---|---|---|---|
| *HILIC COLUMN (PRIMARY)* | | | | | | | | | |
| lettuce / head | 0.02 | 100 | 80 | 88 | 99 | 104 | 94 | 10.5 | 0.02 |
| | 0.20 | 91 | 100 | 88 | 99 | 101 | 96 | 6.1 | |
| | | ***Overall recovery (n = 10)*** | | | | | ***95*** | ***8.1*** | |
| rape / seed | 0.02 | 68 | 71 | 61 | 64 | 62 | 65[1] | 6.5 | 0.02 |
| | 0.20 | 63 | 74 | 61 | 56 | 57 | 62[1] | 11.6 | |
| | | ***Overall recovery (n = 5)*** | | | | | ***64***[1] | ***9.1*** | |
| orange / fruit | 0.02 | 101 | 103 | 104 | 94 | 84 | 97 | 8.6 | 0.02 |
| | 0.20 | 99 | 97 | 95 | 97 | 99 | 97 | 1.7 | |
| | | ***Overall recovery (n = 10)*** | | | | | ***97*** | ***5.8*** | |
| wheat / grain | 0.02 | 104 | 89 | 92 | 83 | 86 | 91 | 8.9 | 0.02 |
| | 0.20 | 96 | 87 | 88 | 82 | 87 | 88 | 5.7 | |
| | | ***Overall recovery (n = 10)*** | | | | | ***89*** | ***7.3*** | |
| hop / cone | 0.10 | 97 | 96 | 105 | 101 | 105 | 101 | 4.2 | 0.10 |
| | 1.0 | 105 | 102 | 105 | 97 | 102 | 102 | 3.2 | |
| | | ***Overall recovery (n = 10)*** | | | | | ***102*** | ***3.6*** | |
| *HYPERCARB COLUMN (CONFIRMATORY)* | | | | | | | | | |
| lettuce / head | 0.02 | 100 | 78 | 88 | 94 | 96 | 91 | 9.4 | 0.02 |
| | 0.20 | 94 | 101 | 90 | 100 | 100 | 97 | 4.9 | |
| | | ***Overall recovery (n = 10)*** | | | | | ***94*** | ***7.7*** | |
| rape / seed | 0.02 | 73 | 74 | 66 | 68 | 68 | 70 | 5.0 | 0.02 |
| | 0.20 | 67 | 71 | 60 | 60 | 61 | 64[1] | 7.8 | |
| | | ***Overall recovery (n = 5)*** | | | | | ***67***[1] | ***7.7*** | |
| orange / fruit | 0.02 | 101 | 99 | 101 | 97 | 89 | 97 | 5.1 | 0.02 |
| | 0.20 | 103 | 100 | 101 | 98 | 103 | 101 | 2.1 | |
| | | ***Overall recovery (n = 10)*** | | | | | ***99*** | ***4.1*** | |
| wheat / grain | 0.02 | 86 | 90 | 90 | 84 | 86 | 87 | 3.1 | 0.02 |
| | 0.20 | 92 | 92 | 93 | 87 | 92 | 91 | 2.6 | |
| | | ***Overall recovery (n = 10)*** | | | | | ***89*** | ***3.6*** | |
| hop / cone | 0.10 | 113 | 110 | 113 | 100 | 103 | 108 | 5.5 | 0.10 |
| | 1.0 | 97 | 94 | 96 | 101 | 102 | 98 | 3.5 | |
| | | ***Overall recovery (n = 10)*** | | | | | ***103*** | ***6.7*** | |

* fortified compound DFA determined as DFA expressed as BYI 02960

*Footnotes:*

1: mean recoveries were below 70%; this was accepted due to the RSD of < 20%

Table 4.3-4: Recovery results from **independent laboratory validation** of method 01330 – Recoveries and relative standard deviations (RSDs) for **BYI 02960**

| Sample material | FL [mg/kg] | Individual values [%] | | | | | Mean value [%] | RSD [%] | LOQ [mg/kg] |
|---|---|---|---|---|---|---|---|---|---|
| *QUANTIFICATION MRM (289/126)* | | | | | | | | | |
| lettuce / head | 0.01 | 89 | 91 | 90 | 92 | 89 | 90 | 1.4 | 0.01 |
| | 0.1 | 89 | 91 | 91 | 94 | 92 | 91 | 2.0 | |
| | | ***Overall recovery (n = 10)*** | | | | | ***91*** | ***1.8*** | |
| wheat / grain | 0.01 | 77 | 84 | 92 | 89 | 86 | 86 | 6.7 | 0.01 |
| | 0.1 | 80 | 92 | 95 | 95 | 92 | 91 | 6.8 | |
| | | ***Overall recovery (n = 10)*** | | | | | ***88*** | ***7.0*** | |
| orange / fruit | 0.01 | 89 | 92 | 96 | 93 | 92 | 92 | 3.0 | 0.01 |
| | 0.1 | 96 | 101 | 100 | 102 | 98 | 99 | 2.4 | |
| | | ***Overall recovery (n = 10)*** | | | | | ***96*** | ***4.5*** | |
| *CONFIRMATORY MRM (289/90)* | | | | | | | | | |
| lettuce / head | 0.01 | 94 | 88 | 93 | 93 | 90 | 92 | 2.5 | 0.01 |
| | 0.1 | 95 | 95 | 92 | 96 | 94 | 94 | 1.5 | |
| | | ***Overall recovery (n = 10)*** | | | | | ***93*** | ***2.5*** | |
| wheat / grain | 0.01 | 67 | 74 | 75 | 81 | 76 | 75 | 7.0 | 0.01 |
| | 0.1 | 84 | 95 | 98 | 98 | 93 | 93 | 6.3 | |
| | | ***Overall recovery (n = 10)*** | | | | | ***84*** | ***13.4*** | |
| orange / fruit | 0.01 | 94 | 92 | 103 | 100 | 101 | 98 | 4.5 | 0.01 |
| | 0.1 | 99 | 104 | 104 | 104 | 102 | 103 | 1.9 | |
| | | ***Overall recovery (n = 10)*** | | | | | ***100*** | ***4.0*** | |

* fortified compound BYI 02960 determined as BYI 02960 expressed as BYI 02960

Table 4.3-5: Recovery results from **independent laboratory validation** of method 01330 – Recoveries and relative standard deviations (RSDs) for **DFA**

| Sample material | FL [mg/kg] | Individual values [%] | | | | | Mean value [%] | RSD [%] | LOQ [mg/kg] |
|---|---|---|---|---|---|---|---|---|---|
| *HILIC COLUMN (PRIMARY)* | | | | | | | | | |
| lettuce / head | 0.01 | 95 | 92 | 93 | 97 | 93 | 94 | 2.1 | 0.01 |
| | 0.1 | 100 | 100 | 96 | 97 | 99 | 99 | 1.8 | |
| | | ***Overall recovery (n = 10)*** | | | | | ***96*** | ***3.2*** | |
| wheat / grain | 0.01 | 85 | 86 | 86 | 87 | 89 | 87 | 2.1 | 0.01 |
| | 0.1 | 71 | 70 | 72 | 65 | 60 | 68 | 7.2 | |
| | | ***Overall recovery (n = 10)*** | | | | | *77* | ***13.7*** | |
| orange / fruit | 0.01 | 87 | 88 | 106 | 91 | 94 | 93 | 8.3 | 0.01 |
| | 0.1 | 103 | 99 | 103 | 98 | 98 | 100 | 2.5 | |
| | | ***Overall recovery (n = 10)*** | | | | | ***97*** | ***6.8*** | |
| *HYPERCARB COLUMN (CONFIRMATORY)* | | | | | | | | | |
| lettuce / head | 0.01 | 79 | 78 | 81 | 72 | 80 | 78 | 4.3 | 0.01 |
| | 0.1 | 88 | 88 | 84 | 91 | 89 | 88 | 2.9 | |
| | | ***Overall recovery (n = 10)*** | | | | | ***83*** | ***7.1*** | |
| wheat / grain | 0.01 | 88 | 84 | 86 | 98 | 93 | 90 | 6.2 | 0.01 |
| | 0.1 | 74 | 72 | 71 | 76 | 71 | 73 | 2.7 | |
| | | ***Overall recovery (n = 10)*** | | | | | ***81*** | ***12.1*** | |
| orange / fruit | 0.01 | 89 | 96 | 81 | 92 | 87 | 89 | 6.4 | 0.01 |
| | 0.1 | 91 | 88 | 86 | 91 | 86 | 88 | 2.8 | |
| | | ***Overall recovery (n = 10)*** | | | | | ***89*** | ***4.7*** | |

* fortified compound DFA determined as DFA expressed as BYI 02960

| **Report:** | **KIIA 4.3/03, Li, Y., & Schoening, R.; 2012** |
|---|---|
| Title: | Amendment No. 1 - Validation of Bayer CropScience method RV-001-P10-02 - An analytical method for the determination of residues of BYI 02960, 6-chloronicotinic acid, difluoroacetic acid, and difluoroethyl-amino-furanone in plant matrices using LC/MS/MS |
| Report No. & Edition No. | RARVP013 (BCS EU method no. 01304, BCS US method no. RV-001-P10-02)<br>M-415504-02-1 |
| **Guidelines:** | – OPPTS 860.1340 – Crop Field Trials<br>– PMRA DACO 7.2.2 Residue Analytical Method<br>– EU Guidance document for residue analytical methods SANCO/825/00 rev. 7 |
| **GLP:** | yes (certified laboratory) |

| **Report:** | **KIIA 4.3/04, Justus, K.; 2011** |
|---|---|
| Title: | Extraction efficiency testing of the residue analytical method RV-001-P10-02 for the determination of BYI 02960, 6-chloronicotinic acid, difluoroacetic acid and difluoroethyl-amino-furanone in plant matrices using aged radioactive residues |
| Report No. & Edition No. | MEF-11/793<br>M-419323-01-1 |
| **Guidelines:** | – US EPA OPPTS 860.1340 |
| **GLP:** | yes (certified laboratory) |

Principle of the method

Residue analytical method 01304 (Li & Schoening, 2012; KIIA 4.3/03) was developed as a *data collection method* and a NAFTA enforcement method for the determination of the residues of BYI 02960 (parent compound), and its metabolites DFA, DFEAF, and 6-CNA in/on plant materials. The method validation is reported in study report RARVP013, which also contains the full method description, RV-001-P10-02. (As 6-CNA is not of relevance for this submission, further details relevant to this compound will not be discussed below. For more details on 6-CNA, please see the report.)

The residues were extracted twice from 5 g of plant material with acetonitrile/water (4/1, v/v) with 2.2 mL/L formic acid. The materials tested included bean (dry seed), wheat (grain & forage), orange (fruit), tomato (fruit), and soybean (seed), representing a wide variety of crops/crop types as requested by international guidance. (For further crops of relevance in the EU, additional materials were tested subsequent to this study; they and their source studies are also described below.) Aliquots of the extracts are purified through a C-18 solid-phase extraction column, then amended with a mixture of stable, isotopically labelled internal standards. The final solution was analyzed by HPLC-MS/MS.

Specificity

Although this method is used only for data collection in Europe, two MRM transitions for quantitation and confirmation were monitored for BYI 02960 (m/z 289/126 or 90) and DFEAF (m/z 162/94 or 98) in each matrix tested. An HPLC-MS/MS method is highly specific, but the confirmatory ions were tested, and, due to repeatability issues with BYI 02960 at the LOQ in some matrices, a second column system (Gemini C18, instead of HILIC as used in the primary method) was employed for confirmatory purposes with that compound.

For DFA, no second MRM transition is available. Thus, a Restek Allure Organic Acids HPLC column was employed as a different separation system (as opposed to HILIC for the primary

determination). The confirmatory methods were fully validated; hence the quantitation and confirmation methods can be used interchangeably if so desired.

Accuracy (recovery findings)

The accuracy of the method was assessed on the basis of the determined recovery rates. Samples were fortified with BYI 02960 at concentrations of 0.01 and 1.0 mg/kg, DFEAF at concentrations of 0.01, 0.013, and 1.3 mg/kg, and DFA at 0.05 and 1.0 mg/kg (for orange and tomato fruit samples, at 0.02 mg/kg as well). Metabolite levels were expressed in parent equivalents. Mean recoveries per fortification level for BYI 02960, DFEAF, and DFA for all matrices were in a range of 75-110%, using the primary conditions.

Confirmatory procedures for BYI 02960 and DFEAF called for the use of the same chromatographic system, but using a second MRM transition. For these two compounds, mean recoveries ranged from 81-107% – i.e. were in the acceptable range (70-110%) – for all matrices. However, as the repeatability was not within specification (relative standard deviation [RSDs] > 20%) for BYI 02960 in two matrices (orange fruit and wheat grain, at the LOQ), a second confirmatory method was developed, utilizing a different column system (Gemini C18, instead of HILIC as used in the primary method). Using this system, using both MRM transitions, mean recovery rates ranged from 85-104% in all matrices, always with acceptable RSD values.

For DFA, the confirmatory procedure is based on chromatography via an alternative column (Restek Allure Organic Acids, instead of HILIC as used in the primary method). Mean recovery values ranged from 77-105%, with a single exception: For tomato fruit, the mean recovery at the 50×LOQ was 111%. This value was considered to be acceptable, based on the fact that it was very close to 110%, the RSD was very low (1.0%), and the overall recovery over both fortification levels was in the acceptable range.

In addition to the matrices included in the report 01304 itself, for further crops of importance in the EU, additional materials were tested as required within the residue studies themselves; the ones of relevance to this dossier are described here. In those studies, limited validation sets were prepared for various matrices related to lettuce (head, washings), cereals (barley straw), hops (beer, brewer's yeast, draff), and sugar beet (root ["body"] and tops ["leaf"]). In addition, full validations were conducted for hop cones (both green and dried). Mean recovery values per fortification level in all matrices were 80-109%.

The results, including information on the source studies of the additional validations, are summarized below in tables 4.3-6 to 4.3-8.

Linearity

The correlation between the injected amount of substance and the detector response was linear for standards in solvent and in matrix and with/without ISTD in the range from 0.25 to 312.5 ng/L, using at least 5 different concentration levels, for all compounds. The correlation coefficients of the 1/x weighted linear regression were > 0.99 in all cases.

Limit of Quantification

The limit of quantitation (LOQ) for BYI 02960 and DFEAF, defined as the lowest validated fortification level, was 0.01 mg/kg in all matrices tested. All metabolite levels are expressed in parent equivalents. For DFA, the LOQ was 0.02 mg/kg in crop matrices high in acid and water content (e.g. oranges, tomatoes) or 0.05 mg/kg in dry/protein-rich matrices, fodder materials, and soybeans.

The calculated limit of detection (LOD) was calculated based on a statistical approach for each matrix and each compound MRM transition. Considering all sample materials and both compounds other than DFA, the LOD ranged from 0.0015-0.0065 mg/kg; for DFA, it was from 0.0048-0.0153 mg/kg.

Repeatability (precision)

As a measure of the precision, the intra-laboratory repeatability (n=5) is given as the relative standard deviation (% RSD) for different sample materials at fortification levels at the respective LOQ and at a higher level, 20×LOQ to 50×LOQ. For the primary determinations of BYI 02960, DFEAF, and DFA, RSDs were 0.8-16.5% for all matrices in the main method validation as presented in the method report itself. For the confirmatory procedures for DFEAF and DFA, the RSDs were 1.0-17.9%. As stated above (cf. "Accuracy"), based on some unacceptably high RSDs for the confirmatory validations with BYI 02960 (orange fruit, LOQ, 37.3%; wheat grain, LOQ, 34.9%), a second confirmatory procedure was developed. The repeatability of the new system was satisfactory, yielding RSDs of 1.8-16.9%.

For the additional crop matrices validated separately (as described above, cf. "Accuracy"), RSDs for all analytes at all fortification levels (generally LOQ and 10×LOQ) were 1.1-14.7%.

The results are summarized below in tables 4.3-6 to 4.3-8.

Reproducibility (ILV)

Since this method is a data collection method, no independent validation is required.

Extraction efficiency

The extraction efficiency of the residue method for the determination of the relevant residues of BYI 02960 in plant matrices, consisting of the parent compound and its metabolites DFEAF and DFA, was assured by choosing the same extraction procedures as used in the plant metabolism studies.

Nevertheless an extraction efficiency study was conducted (Justus, 2011; KIIA 4.3/04). Aged radioactive residues in samples from plant metabolism and confined rotational crop studies (cf. chapter 6 of this dossier) were analyzed using method 01304 (Li & Schoening, 2012; KIIA 4.3/03), and the recoveries of the extracted residues were then compared to those in the respective metabolism studies. The sample materials were tomato fruit, cotton seed, potato tuber, and wheat straw. The contribution of the metabolite DFA to the extraction efficiency was determined based on tomato fruit samples originating from the metabolism study with the [ethyl-1-$^{14}$C]-label – the only plant metabolism study conducted with this label – which metabolises to $^{14}$C-DFA.

Plant material from metabolism studies was extracted according to the residue analytical method. The total radioactivity in this extract was determined by Liquid Scintillation Counting and recalculated into mg parent equivalents using the specific radioactivity of the BYI 02960 originally used in the plant metabolism study. In addition, the same extract was analysed by HPLC using $^{14}C$ radiodetection, yielding the peak area of an analyte, relative to the total peak area of the whole chromatogram. This relative peak area was multiplied by the mg parent equivalents of the total extract to yield the mg parent equivalents represented by the respective peak. The identity of the peak was obtained by comparing the chromatograms with those of the plant metabolism study, which were obtained under the same chromatographic conditions. For confirmatory purposes, HPLC co-chromatography with a DFEAF reference standard was also conducted.

Relative amounts of the analytes relevant to the residue definition – BYI 02960, DFA, and DFEAF – were then compared between the original metabolism study extracts and those made using method 01304. The extraction efficiency values in tomato, cotton, potato, and wheat matrices were 100.7%, 160.2%, 104.0%, and 88.4%, respectively. The high value of 160% for cotton seeds may be due to losses of radioactivity observed during extraction and purification steps in the cotton metabolism study, but not observed in the extraction efficiency study.

In order to evaluate the storage stability in the samples used, comparison of the metabolic profiles recorded during this study with those from the original studies showed no significant changes, confirming that the results were not influenced by degradation of the representative components over the periods of sample storage subsequent to the original metabolism studies.

These results clearly demonstrate that residue method 01304 is suitable for the extraction and quantification of the total residues of BYI 02960, consisting of parent compound, DFA, and DFEAF, from plant matrices. This conclusion applies for other methods based on the same extraction procedures as well (i.e. all other methods presented under chapter point 4.3; cf. KIIA 4.3/01, /05, /06, and /08). (In addition to the components of the total residue, the method includes the determination of a fourth analyte, 6-CNA. This was also tested for extraction efficiency, but as it is not part of the residue definition in plants, it will not be discussed further here; please refer to the report.)

A summary of the extraction efficiency results is given in table 4.3-9.

Stability of analytes

In this validation study, the stability of BYI 02960, DFEAF, and DFA was tested in standard solutions (calibrations standards, spiking solutions, and internal standard solutions). All were shown to be stable in standard solutions for at least 6 months at temperatures of 4°C ± 3°C.

Also, during the course of the validations for this method 01304, the stability of BYI 02960, DFEAF, and DFA was tested in sample extracts, both before clean-up and in the final extracts for measurement. Stability was shown for all three compounds in all tested matrix extracts for approx. 20 days, when stored in the dark in a refrigerator at 4°C ± 3°C.

Conclusion

Method 01304 meets all necessary performance requirements to determine residues of BYI 02960 and its metabolites DFEAF and DFA in plant materials, with an LOQ of 0.01 mg/kg for BYI 02960 and DFEAF, and of 0.02 or 0.05 mg/kg for DFA, all expressed in parent compound equivalents. The method is valid as data collection method.

Table 4.3-6: Recovery results from method validation of method 01304 – Recoveries and relative standard deviations (RSDs) for **BYI 02960**
*(Recoveries as documented in the method report unless noted otherwise)*

| Sample material | FL [mg/kg] | Individual values [%] | Mean value [%] | SD* [%] | RSD [%] | LOQ [mg/kg] |
|---|---|---|---|---|---|---|
| *PRIMARY METHOD: HILIC COLUMN, MRM 289/126* | | | | | | |
| bean / dried bean | 0.01 | 92 88 94 85 91 108 | 93 | 8.1 | 8.6 | 0.01 |
| | 1.0 | 90 107 111 130 104 | 109 | 14.6 | 13.3 | |
| | | ***Overall recovery (n = 11)*** | ***100*** | | ***13.4*** | |
| wheat / forage | 0.01 | 102 104 99 92 86 | 97 | 7.5 | 7.7 | 0.01 |
| | 1.0 | 98 99 92 86 99 | 95 | 5.5 | 6.0 | |
| | | ***Overall recovery (n = 10)*** | ***96*** | | ***6.6*** | |
| orange / fruit | 0.01 | 104 105 89 109 88 | 99 | 9.9 | 9.9 | 0.01 |
| | 1.0 | 87 106 101 98 109 | 100 | 8.4 | 8.5 | |
| | | ***Overall recovery (n = 10)*** | ***100*** | | ***8.7*** | |
| soybean / seed | 0.01 | 83 92 105 75 83 | 87 | 11.3 | 13.1 | 0.01 |
| | 1.0 | 101 103 94 74 99 | 94 | 11.9 | 12.5 | |
| | | ***Overall recovery (n = 10)*** | ***91*** | | ***12.6*** | |
| tomato / fruit | 0.01 | 94 82 99 105 105 | 97 | 9.8 | 9.9 | 0.01 |
| | 0.1 | 88 86 102 103 108 | 97 | 9.8 | 10.0 | |
| | 1.0 | 87 91 81 85 96 | 88 | 5.8 | 6.5 | |
| | | ***Overall recovery (n = 15)*** | ***94*** | | ***9.7*** | |
| wheat /grain | 0.01 | 100 85 89 87 74 | 87 | 9.4 | 10.7 | 0.01 |
| | 1.0 | 93 92 98 81 73 | 88 | 10.0 | 11.6 | |
| | | ***Overall recovery (n = 10)*** | ***87*** | | ***10.5*** | |
| lettuce / head[1] | 0.01 | 92 97 107 | 99 | | 7.7 | 0.01 |
| | 0.1 | 90 92 93 | 92 | | 1.7 | |
| | | ***Overall recovery (n = 6)*** | ***95*** | | ***6.6*** | |
| lettuce / washings[2] | 0.01 | 99 102 106 109 117 | 107 | | 6.5 | 0.01 |
| | 0.5 | 95 97 111 | 101 | | 8.6 | |
| | | ***Overall recovery (n = 8)*** | ***105*** | | ***7.3*** | |
| barley / straw[3] | 0.01 | 104 106 106 108 111 | 107 | | 2.5 | 0.01 |
| | 0.1 | 105 107 111 | 108 | | 2.8 | |
| | | ***Overall recovery (n = 8)*** | ***107*** | | ***2.4*** | |
| hops / green cone[4] | 0.1 | 89 89 91 94 95 | 92 | | 3.0 | 0.1 |
| | 1.0 | 85 86 87 92 98 | 90 | | 6.0 | |
| | | ***Overall recovery (n = 10)*** | ***91*** | | ***4.6*** | |
| hops / kiln-dried cone[4] | 0.1 | 102 103 104 105 106 | 104 | | 1.5 | 0.1 |
| | 1.0 | 107 108 111 114 115 | 111 | | 3.2 | |
| | | ***Overall recovery (n = 10)*** | ***108*** | | ***4.2*** | |
| hops / beer[4] | 0.01 | 95 100 110 114 115 | 107 | | 8.3 | 0.01 |
| | 0.1 | 105 112 116 | 111 | | 5.0 | |
| | | ***Overall recovery (n = 8)*** | ***108*** | | ***7.1*** | |

fortified compound BYI 02960 determined as BYI 02960 expressed as BYI 02960
* as shown in report RARVP013

*Footnotes:*
1: this crop/matrix was validated as part of study 10-2213 (KIIA 6.3.1/03)
2: this crop/matrix was validated as part of study 10-3223 (KIIA 6.5.4/01)
3: this crop/matrix was validated as part of study 10-2237 (to be submitted later; data available on request)
4: this crop/matrix was validated as part of studies 10-2225 (KIIA 6.3.2/01) / 10-3407 (KIIA 6.5.4/02)

*Continued on next page…*

Table 4.3-6 (cont'd): Recovery results from method validation of method 01304 – Recoveries and relative standard deviations (RSDs) for **BYI 02960**
*(Recoveries as documented in the method report unless noted otherwise)*

| Sample material | FL [mg/kg] | Individual values [%] | Mean value [%] | SD* [%] | RSD [%] | LOQ [mg/kg] |
|---|---|---|---|---|---|---|
| *PRIMARY METHOD, HILIC COLUMN MRM 289/126 (cont'd)* | | | | | | |
| hops / brewer's yeast[4] | 0.1 | 98 109 111 111 113 | 108 | | 5.5 | 0.1 |
| | 1.0 | 77 99 102 | 93 | | 14.7 | |
| | | ***Overall recovery (n = 8)*** | ***103*** | | ***11.5*** | |
| hops / draff[4] | 0.1 | 84 94 96 105 108 | 97 | | 9.8 | 0.1 |
| | 1.0 | 101 102 105 | 103 | | 2.0 | |
| | | ***Overall recovery (n = 8)*** | ***99*** | | ***7.8*** | |
| sugar beet / body[5] | 0.01 | 89 97 98 99 100 | 97 | | 4.5 | 0.01 |
| | 0.5 | 92 94 95 | 94 | | 1.6 | |
| | | ***Overall recovery (n = 8)*** | ***96*** | | ***3.9*** | |
| sugar beet / leaf with root collar[5] | 0.01 | 90 94 94 | 93 | | 2.5 | 0.01 |
| | 0.5 | 93 97 97 | 96 | | 2.4 | |
| | | ***Overall recovery (n = 6)*** | ***94*** | | ***2.8*** | |
| *HILIC, CONFIRMATORY MRM 289/90* | | | | | | |
| bean / dried bean | 0.01 | 89 80 98 109 87 104 | 95 | 10.9 | 11.7 | 0.01 |
| | 1.0 | 102 103 110 137 112 | 113 | 14.2 | 12.6 | |
| | | ***Overall recovery (n = 11)*** | ***103*** | | ***14.8*** | |
| wheat / forage | 0.01 | 82 95 80 85 108 | 90 | 11.6 | 12.9 | 0.01 |
| | 1.0 | 96 108 100 97 96 | 99 | 4.9 | 5.1 | |
| | | ***Overall recovery (n = 10)*** | ***95*** | | ***10.3*** | |
| orange / fruit | 0.01 | 79 81 137 47 91 | 87 | 32.21 | 37.3 | 0.01 |
| | 1.0 | 83 99 90 95 103 | 94 | 7.6 | 8.3 | |
| | | ***Overall recovery (n = 10)*** | ***91*** | | ***24.9*** | |
| soybean / seed | 0.01 | 113 120 99 117 87 | 107 | 13.9 | 12.9 | 0.01 |
| | 1.0 | 104 111 95 103 90 | 101 | 8.1 | 8.2 | |
| | | ***Overall recovery (n = 10)*** | ***104*** | | ***10.9*** | |
| tomato / fruit | 0.01 | 81 92 73 119 103 | 93 | 18.2 | 19.4 | 0.01 |
| | 0.1 | 95 95 97 120 112 | 104 | 11.4 | 11.1 | |
| | 1.0 | 107 108 96 111 105 | 105 | 5.6 | 5.4 | |
| | | ***Overall recovery (n = 15)*** | ***101*** | | ***12.9*** | |
| wheat /grain | 0.01 | 65 116 157 89 84 | 102 | 35.6 | 34.9[6] | 0.01 |
| | 1.0 | 97 119 97 82 76 | 94 | 16.7 | 17.7 | |
| | | ***Overall recovery (n = 10)*** | ***98*** | | ***27.1***[6] | |
| *2ND CONFIRMATORY METHOD, GEMINI COLUMN, MRM 289/126* | | | | | | |
| bean / dried bean | 0.01 | 100 106 97 100 102 95 95 91 93 91 90 | 96 | 5.0 | 5.3 | 0.01 |
| | 1.0 | 92 99 114 113 97 | 103 | 9.7 | 9.6 | |
| | | ***Overall recovery (n = 16)*** | ***98*** | | ***7.5*** | |
| wheat / forage | 0.01 | 95 94 87 94 94 | 93 | 3.1 | 3.5 | 0.01 |
| | 1.0 | 86 95 86 85 81 | 87 | 5.3 | 5.9 | |
| | | ***Overall recovery (n = 10)*** | ***90*** | | ***5.8*** | |

fortified compound BYI 02960 determined as BYI 02960 expressed as BYI 02960
* as shown in report RARVP013
*Footnotes:*
4: this crop/matrix was validated as part of studies 10-2225 (KIIA 6.3.2/01) / 10-3407 (KIIA 6.5.4/02)
5: this crop/matrix was validated as part of study 10-2240 (to be submitted later; data available on request)
6: as the precision of this procedure was insufficient, an additional "2nd confirmatory procedure" was developed and validated

*Continued on next page…*

Table 4.3-6 (cont'd): Recovery results from method validation of method 01304 – Recoveries and relative standard deviations (RSDs) for **BYI 02960**
*(Recoveries as documented in the method report unless noted otherwise)*

| Sample material | FL [mg/kg] | Individual values [%] | Mean value [%] | SD* [%] | RSD [%] | LOQ [mg/kg] |
|---|---|---|---|---|---|---|
| *2ND CONFIRMATORY METHOD, GEMINI COLUMN, MRM 289/126 (cont'd)* | | | | | | |
| orange / fruit | 0.01 | 103 91 91 96 98 | 96 | 4.9 | 5.3 | 0.01 |
| | 1.0 | 97 98 96 91 95 | 96 | 2.6 | 2.8 | |
| | | ***Overall recovery (n = 10)*** | ***96*** | | ***4.0*** | |
| soybean / seed | 0.01 | 101 98 106 110 98 | 102 | 5.2 | 5.1 | 0.01 |
| | 1.0 | 90 92 96 96 90 | 93 | 2.8 | 3.3 | |
| | | ***Overall recovery (n = 10)*** | ***98*** | | ***6.7*** | |
| tomato / fruit | 0.01 | 97 96 88 114 79 | 95 | 13.0 | 13.7 | 0.01 |
| | 1.0 | 87 96 104 94 94 | 95 | 6.3 | 6.4 | |
| | | ***Overall recovery (n = 10)*** | ***95*** | | ***10.1*** | |
| wheat / grain | 0.01 | 86 91 101 87 94 | 92 | 5.9 | 6.6 | 0.01 |
| | 1.0 | 93 95 99 95 83 | 93 | 5.8 | 6.5 | |
| | | ***Overall recovery (n = 10)*** | ***92*** | | ***6.2*** | |
| *2ND CONFIRMATORY METHOD, GEMINI COLUMN, MRM 289/90* | | | | | | |
| bean / dried bean | 0.01 | 95 103 96 103 108<br>88 101 95 88 94<br>90 | 96 | 6.6 | 6.8 | 0.01 |
| | 1.0 | 93 98 115 114 97 | 104 | 10.4 | 10.0 | |
| | | ***Overall recovery (n = 16)*** | ***99*** | | ***8.4*** | |
| wheat / forage | 0.01 | 95 88 100 90 97 | 94 | 4.9 | 5.3 | 0.01 |
| | 1.0 | 83 93 85 86 80 | 85 | 4.9 | 5.7 | |
| | | ***Overall recovery (n = 10)*** | ***90*** | | ***7.2*** | |
| orange / fruit | 0.01 | 102 103 93 87 96 | 96 | 6.7 | 6.2 | 0.01 |
| | 1.0 | 92 98 94 93 95 | 95 | 2.4 | 2.4 | |
| | | ***Overall recovery (n = 10)*** | ***96*** | | ***4.6*** | |
| soybean / seed | 0.01 | 94 98 93 90 90 | 93 | 3.4 | 3.6 | 0.01 |
| | 1.0 | 92 90 91 94 90 | 92 | 1.8 | 1.8 | |
| | | ***Overall recovery (n = 10)*** | ***92*** | | ***2.8*** | |
| tomato / fruit | 0.01 | 100 92 82 117 77 | 93 | 15.7 | 16.9 | 0.01 |
| | 1.0 | 86 91 97 92 96 | 92 | 4.5 | 4.8 | |
| | | ***Overall recovery (n = 10)*** | ***93*** | | ***11.8*** | |
| wheat / grain | 0.01 | 105 90 102 82 101 | 96 | 9.6 | 10.1 | 0.01 |
| | 1.0 | 94 96 98 94 84 | 93 | 5.6 | 5.8 | |
| | | ***Overall recovery (n = 10)*** | ***95*** | | ***8.0*** | |

fortified compound BYI 02960 determined as BYI 02960 expressed as BYI 02960
* as shown in report RARVP013

Table 4.3.-7: Recovery results from method validation of method 01304 – Recoveries and relative standard deviations (RSDs) for **DFEAF**
*(Recoveries as documented in the method report unless noted otherwise)*

| Sample material | FL [mg/kg] | Individual values [%] | | | | | Mean value [%] | SD* [%] | RSD [%] | LOQ [mg/kg] |
|---|---|---|---|---|---|---|---|---|---|---|
| *PRIMARY ION TRANSITION, MRM 162/94* | | | | | | | | | | |
| bean / dried bean | 0.01 | 88 | 82 | 85 | 87 | 79 | 84 | 3.9 | 4.4 | 0.01 |
| | 0.013 | 110 | 96 | 88 | 102 | 94 | 96 | 8.8 | 9.1 | |
| | | 87 | | | | | | | | |
| | 1.3 | 90 | 95 | 135 | 118 | 110 | 109 | 18.1 | 16.5 | |
| | | ***Overall recovery (n = 16)*** | | | | | ***97*** | | ***15.5*** | |
| wheat / forage | 0.01 | 120 | 94 | 83 | 91 | 86 | 95 | 14.6 | 15.5 | 0.01 |
| | 0.013 | 93 | 103 | 91 | 99 | 94 | 96 | 4.7 | 5.1 | |
| | 1.3 | 82 | 95 | 91 | 84 | 100 | 90 | 7.8 | 8.3 | |
| | | ***Overall recovery (n = 15)*** | | | | | ***94*** | | ***10.2*** | |
| orange / fruit | 0.01 | 99 | 96 | 119 | 125 | 113 | 110 | 12.5 | 11.4 | 0.01 |
| | 0.013 | 89 | 99 | 82 | 98 | 95 | 93 | 7.3 | 7.7 | |
| | 1.3 | 93 | 104 | 103 | 94 | 106 | 100 | 6.0 | 6.0 | |
| | | ***Overall recovery (n = 15)*** | | | | | ***101*** | | ***11.2*** | |
| soybean / seed | 0.01 | 94 | 103 | 89 | 89 | 96 | 94 | 5.9 | 6.2 | 0.01 |
| | 0.013 | 93 | 81 | 106 | 112 | 105 | 99 | 12.5 | 12.5 | |
| | 1.3 | 94 | 98 | 91 | 103 | 94 | 96 | 4.8 | 4.8 | |
| | | ***Overall recovery (n = 15)*** | | | | | ***97*** | | ***8.3*** | |
| tomato / fruit | 0.01 | 96 | 136 | 100 | 94 | 104 | 106 | 17.4 | 16.2 | 0.01 |
| | 0.013 | 85 | 94 | 90 | 105 | 78 | 90 | 10.1 | 11.2 | |
| | 0.13 | 89 | 98 | 107 | 90 | 99 | 97 | 7.3 | 7.6 | |
| | 1.3 | 95 | 97 | 94 | 95 | 96 | 96 | 1.2 | 1.2 | |
| | | ***Overall recovery (n = 20)*** | | | | | ***97*** | | ***11.7*** | |
| wheat /grain | 0.01 | 97 | 79 | 106 | 97 | 116 | 99 | 13.4 | 13.8 | 0.01 |
| | 0.013 | 102 | 96 | 113 | 94 | 118 | 105 | 10.4 | 10.1 | |
| | 1.3 | 94 | 104 | 109 | 91 | 93 | 98 | 7.8 | 8.0 | |
| | | ***Overall recovery (n = 15)*** | | | | | ***101*** | | ***10.5*** | |
| lettuce / head[1] | 0.01 | 83 | 86 | 90 | | | 86 | | 4.1 | 0.01 |
| | 0.1 | 97 | 97 | 99 | | | 98 | | 1.2 | |
| | | ***Overall recovery (n = 6)*** | | | | | ***92*** | | ***7.2*** | |
| lettuce / washings[2] | 0.01 | 95 | 99 | 100 | 100 | 107 | 100 | | 4.3 | 0.01 |
| | 0.5 | 86 | 88 | 96 | | | 90 | | 5.9 | |
| | | ***Overall recovery (n = 8)*** | | | | | ***96*** | | ***7.1*** | |
| barley / straw[3] | 0.01 | 95 | 101 | 102 | 106 | 107 | 102 | | 4.7 | 0.01 |
| | 0.1 | 93 | 94 | 107 | | | 98 | | 8.0 | |
| | | ***Overall recovery (n = 8)*** | | | | | ***101*** | | ***5.9*** | |
| hops / green cone[4] | 0.1 | 68 | 73 | 79 | 85 | 96 | 80 | | 13.6 | 0.1 |
| | 1.0 | 76 | 77 | 78 | 84 | 91 | 81 | | 7.8 | |
| | | ***Overall recovery (n = 10)*** | | | | | ***81*** | | ***10.4*** | |
| hops / kiln-dried cone[4] | 0.1 | 89 | 106 | 107 | 107 | 108 | 103 | | 7.8 | 0.1 |
| | 1.0 | 108 | 109 | 110 | 112 | 114 | 111 | | 2.2 | |
| | | ***Overall recovery (n = 10)*** | | | | | ***107*** | | ***6.3*** | |
| hops / beer[4] | 0.01 | 92 | 94 | 102 | 111 | 115 | 103 | | 9.9 | 0.01 |
| | 0.1 | 102 | 107 | 112 | | | 107 | | 4.7 | |
| | | ***Overall recovery (n = 8)*** | | | | | ***104*** | | ***8.0*** | |

fortified compound DFEAF determined as DFEAF expressed as BYI 02960
* as shown in report RARVP013

*Continued on next page…*

*Footnotes:*
1: this crop/matrix was validated as part of study 10-2213 (KIIA 6.3.1/03)
2: this crop/matrix was validated as part of study 10-3223 (KIIA 6.5.4/01)
3: this crop/matrix was validated as part of study 10-2237 (to be submitted later; data available on request)
4: this crop/matrix was validated as part of studies 10-2225 (KIIA 6.3.2/01) / 10-3407 (KIIA 6.5.4/02)

Table 4.3-7 (cont'd): Recovery results from method validation of method 01304 – Recoveries and relative standard deviations (RSDs) for **DFEAF**
*(Recoveries as documented in the method report unless noted otherwise)*

| Sample material | FL [mg/kg] | Individual values [%] | Mean value [%] | SD* [%] | RSD [%] | LOQ [mg/kg] |
|---|---|---|---|---|---|---|
| *PRIMARY ION TRANSITION, MRM 162/94 (cont'd)* | | | | | | |
| hops / brewers yeast[4] | 0.1 | 97 107 107 110 113 | 106 | | 5.2 | 0.1 |
| | 1.0 | 85 99 109 | 98 | | 12.3 | |
| | | ***Overall recovery (n = 8)*** | ***103*** | | ***8.6*** | |
| hops / draff[4] | 0.1 | 103 105 105 105 109 | 105 | | 2.1 | 0.1 |
| | 1.0 | 100 104 107 | 104 | | 3.4 | |
| | | ***Overall recovery (n = 8)*** | ***105*** | | ***2.5*** | |
| sugar beet / body[5] | 0.01 | 91 92 93 94 96 | 93 | | 2.1 | 0.01 |
| | 0.50 | 95 101 110 | 102 | | 7.4 | |
| | | ***Overall recovery (n = 8)*** | ***97*** | | ***6.5*** | |
| sugar beet / leaf with root collar[5] | 0.01 | 83 89 94 | 89 | | 6.2 | 0.01 |
| | 0.5 | 91 108 111 | 103 | | 10.4 | |
| | | ***Overall recovery (n = 6)*** | ***96*** | | ***11.6*** | |
| *CONFIRMATORY ION TRANSITION, MRM 162/98* | | | | | | |
| bean / dried bean | 0.01 | 78 74 100 74 79 | 81 | 10.5 | 13.4 | 0.01 |
| | 0.013 | 119 107 88 93 104 96 | 101 | 11.1 | 11.1 | |
| | 1.3 | 101 97 109 108 92 | 101 | 7.3 | 7.1 | |
| | | ***Overall recovery (n = 16)*** | ***95*** | | ***14.2*** | |
| wheat / forage | 0.01 | 116 90 103 92 101 | 100 | 10.6 | 10.3 | 0.01 |
| | 0.013 | 86 95 82 87 92 | 88 | 5.1 | 5.8 | |
| | 1.3 | 89 92 92 90 110 | 94 | 8.7 | 9.2 | |
| | | ***Overall recovery (n = 15)*** | ***94*** | | ***9.8*** | |
| orange / fruit | 0.01 | 98 105 88 98 106 | 99 | 7.2 | 7.3 | 0.01 |
| | 0.013 | 94 83 90 92 91 | 90 | 4.4 | 4.6 | |
| | 1.3 | 91 99 98 97 94 | 96 | 3.1 | 3.4 | |
| | | ***Overall recovery (n = 15)*** | ***95*** | | ***6.5*** | |
| soybean / seed | 0.01 | 89 87 78 75 75 | 81 | 6.7 | 8.3 | 0.01 |
| | 0.013 | 92 97 89 96 94 | 94 | 3.5 | 3.4 | |
| | 1.3 | 90 93 87 86 91 | 89 | 3.0 | 3.2 | |
| | | ***Overall recovery (n = 15)*** | ***88*** | | ***7.9*** | |
| tomato / fruit | 0.01 | 97 104 74 114 94 | 96 | 14.9 | 15.3 | 0.01 |
| | 0.013 | 98 100 91 106 78 | 95 | 10.5 | 11.3 | |
| | 0.13 | 103 104 104 108 92 | 102 | 6.0 | 5.9 | |
| | 1.3 | 90 91 89 95 93 | 92 | 2.0 | 2.6 | |
| | | ***Overall recovery (n = 20)*** | ***96*** | | ***10.1*** | |
| wheat /grain | 0.01 | 101 86 95 93 84 | 92 | 7.1 | 7.5 | 0.01 |
| | 0.013 | 116 80 106 84 80 | 93 | 16.8 | 17.9 | |
| | 1.3 | 93 107 107 114 90 | 102 | 10.3 | 10.0 | |
| | | ***Overall recovery (n = 15)*** | ***96*** | | ***12.6*** | |

fortified compound DFEAF determined as DFEAF expressed as BYI 02960
* as shown in report RARVP013
*Footnotes:*
4: this crop/matrix was validated as part of studies 10-2225 (KIIA 6.3.2/01) / 10-3407 (KIIA 6.5.4/02)
5: this crop/matrix was validated as part of study 10-2240 (to be submitted later; data available on request)

Table 4.3-8: Recovery results from method validation of method 01304 – Recoveries and relative standard deviations (RSDs) for **DFA**
*(Recoveries as documented in the method report unless noted otherwise)*

| Sample material | FL [mg/kg] | Individual values [%] | Mean value [%] | SD* [%] | RSD [%] | LOQ [mg/kg] |
|---|---|---|---|---|---|---|
| *PRIMARY METHOD, HILIC COLUMN* | | | | | | |
| bean / dried bean | 0.05 | 73 73 73 76 80 73 | 75 | 2.9 | 3.9 | 0.05 |
| | 1.0 | 88 91 117 184** 91 | 97 | 13.6** | 12.6** | |
| | | ***Overall recovery (n = 10)*** | ***84*** | | ***16.8*** | |
| wheat / forage | 0.05 | 92 90 85 92 87 | 89 | 3.2 | 3.5 | 0.05 |
| | 1.0 | 96 100 99 97 103 | 99 | 2.6 | 2.8 | |
| | | ***Overall recovery (n = 10)*** | ***94*** | | ***6.2*** | |
| orange / fruit | 0.02 | 101 111 96 108 101 | 104 | 6.4 | 5.8 | 0.02 |
| | 0.05 | 94 100 90 97 87 | 93 | 5.2 | 5.6 | |
| | 1.0 | 99 101 100 100 101 | 100 | 1.0 | 0.8 | |
| | | ***Overall recovery (n = 15)*** | ***99*** | | ***6.1*** | |
| soybean / seed | 0.05 | 79 77 76 76 71 | 76 | 2.8 | 3.9 | 0.05 |
| | 1.0 | 78 73 75 76 77 | 76 | 2.1 | 2.5 | |
| | | ***Overall recovery (n = 10)*** | ***76*** | | ***3.1*** | |
| tomato / fruit | 0.02 | 98 100 108 107 97 | 102 | 4.9 | 5.0 | 0.02 |
| | 0.05 | 91 87 87 103 81 | 90 | 8.2 | 9.1 | |
| | 0.1 | 90 99 103 99 96 | 97 | 4.8 | 5.0 | |
| | 1.0 | 95 93 91 99 96 | 95 | 3.0 | 3.2 | |
| | | ***Overall recovery (n = 20)*** | ***96*** | | ***7.1*** | |
| wheat /grain | 0.05 | 86 86 89 87 96 | 89 | 4.4 | 4.7 | 0.05 |
| | 1.0 | 94 92 90 91 85 | 90 | 3.2 | 3.7 | |
| | | ***Overall recovery (n = 10)*** | ***90*** | | ***4.1*** | |
| lettuce / head[1] | 0.05 | 90 94 98 | 94 | | 4.3 | (0.02) |
| | 0.5 | 90 91 92 | 91 | | 1.1 | |
| | | ***Overall recovery (n = 6)*** | ***93*** | | ***3.3*** | |
| lettuce / head[2] | 0.02 | 90 93 94 95 97 112 112 116 86 89 93 95 | 98 | | 10.2 | 0.02 |
| | | ***Overall recovery (n = 12)*** | ***98*** | | ***10.2*** | |
| lettuce / washings[3] | 0.02 | 95 96 97 97 103 | 98 | | 3.2 | 0.02 |
| | 0.5 | 91 94 97 | 94 | | 3.2 | |
| | | ***Overall recovery (n = 8)*** | ***96*** | | ***3.5*** | |
| barley / straw[4] | 0.05 | 103 104 106 107 110 | 106 | | 2.6 | 0.05 |
| | 0.50 | 98 101 101 | 100 | | 1.7 | |
| | | ***Overall recovery (n = 8)*** | ***104*** | | ***3.7*** | |
| hops / green cone[5] | 0.2 | 91 92 95 99 100 | 95 | | 4.2 | 0.2 |
| | 1.0 | 76 79 83 84 94 | 83 | | 8.2 | |
| | | ***Overall recovery (n = 10)*** | ***89*** | | ***9.3*** | |

fortified compound DFA determined as DFA expressed as BYI 02960

* as shown in report RARVP013

** The sample was contaminated with DFA, is an outlier and is excluded. If the outlier is included, the average recovery is 114%, the SD is 40.8%, the RSD 35.7%, and the overall mean is 93%.

*Footnotes:*

1: this crop/matrix was validated as part of study 10-2213 (KIIA 6.3.1/03)
2: recoveries from studies 10-2212, 10-2213, 10-2223, 10-2503 (KIIA 6.3.1/05, /03, /01; and KIIA 6.6.3/01)
3: this crop/matrix was validated as part of study 10-3223 (KIIA 6.5.4/01)
4: this crop/matrix was validated as part of study 10-2237 (to be submitted later; data available on request)
5: this crop/matrix was validated as part of studies 10-2225 (KIIA 6.3.2/01) / 10-3407 (KIIA 6.5.4/02)

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Table 4.3-8 (cont'd): Recovery results from method validation of method 01304 – Recoveries and relative standard deviations (RSDs) for **DFA**
*(Recoveries as documented in the method report unless noted otherwise)*

| Sample material | FL [mg/kg] | Individual values [%] | | | | | Mean value [%] | SD* [%] | RSD [%] | LOQ [mg/kg] |
|---|---|---|---|---|---|---|---|---|---|---|
| *PRIMARY METHOD (cont'd)* | | | | | | | | | | |
| hops / kiln-dried cone[5] | 0.2 | 96 | 97 | 103 | 103 | 106 | 101 | | 4.3 | 0.2 |
| | 1.0 | 101 | 105 | 106 | 107 | 110 | 106 | | 3.1 | |
| | | ***Overall recovery (n = 10)*** | | | | | ***103*** | | ***4.3*** | |
| hops / beer[5] | 0.02 | 93 | 100 | 110 | 110 | 113 | 105 | | 8.0 | 0.02 |
| | 0.2 | 108 | 108 | 110 | | | 109 | | 1.1 | |
| | | ***Overall recovery (n = 8)*** | | | | | ***107*** | | ***6.2*** | |
| hops / brewers yeast[5] | 0.2 | 99 | 107 | 109 | 115 | 116 | 109 | | 6.3 | 0.2 |
| | 1.0 | 76 | 90 | 101 | | | 89 | | 14.1 | |
| | | ***Overall recovery (n = 8)*** | | | | | ***102*** | | ***13.2*** | |
| hops / draff[5] | 0.2 | 98 | 99 | 108 | 109 | 111 | 105 | | 5.8 | 0.2 |
| | 1.0 | 97 | 101 | 102 | | | 100 | | 2.6 | |
| | | ***Overall recovery (n = 8)*** | | | | | ***103*** | | ***5.3*** | |
| sugar beet / body[6] | 0.02 | 89 | 90 | 92 | 93 | 97 | 92 | | 3.4 | 0.02 |
| | 0.5 | 76 | 82 | 82 | | | 80 | | 4.3 | |
| | | ***Overall recovery (n = 8)*** | | | | | ***88*** | | ***8.0*** | |
| sugar beet / leaf with root collar[6] | 0.05 | 93 | 94 | 95 | | | 94 | | 1.1 | 0.05 |
| | 0.5 | 83 | 88 | 92 | | | 88 | | 5.1 | |
| | | ***Overall recovery (n = 6)*** | | | | | ***91*** | | ***5.0*** | |
| *CONFIRMATORY METHOD, RESTEK ALLURE ORGANIC ACIDS COLUMN* | | | | | | | | | | |
| bean / dried bean | 0.05 | 74 | 80 | 77 | 74 | 80 | 77 | 3.1 | 3.9 | 0.05 |
| | 1.0 | 79 | 77 | 80 | 84 | 75 | 79 | 3.7 | 4.3 | |
| | | ***Overall recovery (n = 10)*** | | | | | ***78*** | | ***4.1*** | |
| wheat / forage | 0.05 | 107 | 100 | 102 | 98 | 102 | 102 | 3.2 | 3.3 | 0.05 |
| | 1.0 | 106 | 106 | 105 | 101 | 105 | 105 | 2.0 | 2.0 | |
| | | ***Overall recovery (n = 10)*** | | | | | ***103*** | | ***2.9*** | |
| orange / fruit | 0.02 | 92 | 119 | 98 | 103 | 94 | 101 | 10.9 | 10.7 | 0.02 |
| | 1.0 | 105 | 101 | 101 | 100 | 100 | 101 | 1.9 | 2.0 | |
| | | ***Overall recovery (n = 10)*** | | | | | ***101*** | | ***7.2*** | |
| soybean / seed | 0.05 | 83 | 78 | 95 | 83 | 84 | 84 | 6.5 | 7.4 | 0.05 |
| | 1.0 | 85 | 88 | 81 | 80 | 88 | 84 | 3.6 | 4.5 | |
| | | ***Overall recovery (n = 10)*** | | | | | ***85*** | | ***5.8*** | |
| tomato / fruit | 0.02 | 102 | 96 | 100 | 98 | 118 | 103 | 8.6 | 8.5 | 0.02 |
| | 1.0 | 113 | 112 | 111 | 110 | 111 | 111 | 0.9 | 1.0 | |
| | | ***Overall recovery (n = 10)*** | | | | | ***107*** | | ***7.0*** | |
| wheat / grain | 0.05 | 91 | 96 | 78 | 84 | 89 | 87 | 6.7 | 7.9 | 0.05 |
| | 1.0 | 90 | 97 | 85 | 95 | 88 | 91 | 4.9 | 5.4 | |
| | | ***Overall recovery (n = 10)*** | | | | | ***89*** | | ***6.6*** | |

fortified compound DFA determined as DFA expressed as BYI 02960
* as shown in report RARVP013
*Footnotes:*

5: this crop/matrix was validated as part of studies 10-2225 (KIIA 6.3.2/01) / 10-3407 (KIIA 6.5.4/02)
6: this crop/matrix was validated as part of study 10-2240 (to be submitted later; data available on request)

Table 4.3-9: Recovery results of **extraction efficiency testing** of method 01304 – Recoveries from representative matrices taken from the plant metabolism studies

| Crop and matrix | Values determined in/with | | | | | | |
|---|---|---|---|---|---|---|---|
| | Metabolism studies | | | Method 01304 | | | |
| | TRR* [mg/kg] | Components of TTR** [mg/kg] | TTR/TRR [%] | TRR* [mg/kg] | Components of TTR** [mg/kg] | TTR/TRR [%] | Extraction efficiency† [%] |
| tomato fruit[1] | 0.201 | 0.198 | 98.8 | 0.191 | 0.190 | 99.5 | 100.7 |
| cotton seed[2] | 0.068 | 0.016 | 23.4 | 0.068 | 0.026 | 37.5 | 160.2 |
| potato tuber[3] | 0.171 | 0.102 | 59.8 | 0.191 | 0.119 | 62.2 | 104.0 |
| wheat grain[4] | 6.290 | 2.833 | 45.1 | 6.139 | 2.448 | 39.9 | 88.4 |

* TRR = total radioactive residues
** TTR = total toxic residue, i.e. the total residue of BYI 02960, comprising parent compound, DFA, and DFEAF
† Extr. efficiency = ratio of (TTR extracted with method 01304) ÷ (TTR extracted in metabolism study)
*Footnotes:*
1: metabolism study reported under no. MEF-11/498 (KIIA 6.2.1/03)
2: metabolism study reported under no. MEF-11/393 (KIIA 6.2.1/09)
3: metabolism study reported under no. MEF-10/769 (KIIA 6.2.1/04)
4: metabolism study reported under no. MEF-11/365 (KIIA 6.6.2/01)

| **Report:** | **KIIA 4.3/05, Rosati, D.; 2012** |
|---|---|
| Title: | Analytical method No. 01212 for the determination of residues of BYI 02960 and its metabolites BCS-AA56716 (DFA), AE F161089 (6CNA) and BCS-CC98193 (furanone) in/on plant materials by HPLC-MS/MS. |
| Report No. & Edition No. | Method no. 01212, report no. MR-10/174<br>M-428017-01-1 |
| **Guidelines:** | – EU Council Directive 91/414/EEC amended by Commission Directive 96/68/EC<br>– EU Guidance Document for residue analytical methods SANCO/3029/99<br>– OPPTS 860.1340 - Residue analytical methods |
| **GLP:** | yes (certified laboratory) |

Principle of the method

Residue analytical method 01212 (Rosati, 2012; KIIA 4.3/05) was developed as a *data collection method* for the determination of the residues of BYI 02960 (parent compound), and its metabolites DFA, DFEAF, and 6-CNA in/on plant materials. (As 6-CNA is not of relevance for this submission, further details relevant to this compound will not be discussed below. For more details on 6-CNA, please see the report.)

The residues were extracted twice from 5 g of plant material with acetonitrile/water (4/1, v/v) with 2.2 mL/L formic acid. The materials tested included bean (dry seed), barley (grain), grape (bunch), tomato (fruit), and rape (seed), representing a wide variety of crops/crop types as requested by international guidance. (For further crops of relevance in the EU, additional materials were tested subsequent to this study; they and their source studies are also described below.) Aliquots of the extracts are purified over a cationic resin (AG-50W-X8), then amended with a mixture of stable, isotopically labelled internal standards. The final solution was analyzed by HPLC-MS/MS.

Specificity

Apparent residues in control samples were below 30% of the LOQ, except for DFA in tomato fruit, for which a mean value of 32% was determined. Thus, recovery determinations conducted at the LOQ in this matrix were corrected for the interference.

As this is a data collection method, only one MRM transition was monitored for BYI 02960 (m/z 289/126), DFEAF (m/z 162/94), and DFA (m/z 95/51) in each matrix tested. Nevertheless, the HPLC-MS/MS method is highly specific and fully satisfactory for data collection.

Accuracy (recovery findings)

The accuracy of the method was assessed on the basis of the determined recovery rates. Samples were fortified with BYI 02960 and DFEAF at concentrations of 0.01 and 0.10 mg/kg; DFA was spiked at 0.02 and 0.20 mg/kg. (Metabolites were expressed in parent equivalents.) Mean recoveries per fortification level for BYI 02960, DFEAF, and DFA for all matrices were in a range of 70-110%, with three exceptions: In two cases, mean DFEAF recoveries were outside of this range (tomato fruit, LOQ, 111%; and barley grain, LOQ, 114%), and in another, mean DFA values were higher (tomato fruit, LOQ, 111%). These values were nevertheless considered to be acceptable, based on the fact that they were very close to 110% and, in all cases, the RSD was satisfactory (8.3-13.1%).

In addition to the matrices included in the report 01212 itself, for further crops of relevance in the EU, additional materials were tested as required within the residue studies themselves; the ones of relevance to this dossier are described here. In studies 11-2070 and 11-2958 (cf. KIIA 6.3.1/06 and KIIA 10.3.3/02), limited validation sets were prepared for the matrices lettuce head and barley green material, respectively. Mean recovery values in both matrices per fortification level were 82-108%, with one exception at 117% (DFA, green material, 0.02 mg/kg), which, however, was considered to be acceptable due to the very low RSD (3.3%).

The results are summarized below in tables 4.3-10 to 4.3-12.

Linearity

The correlation between the injected amount of substance and the detector response was linear for standards in solvent and in matrix and with/without ISTD in the range from 0.05 to 50 µg/L for BYI 02960 and DFEAF and from 0.15 to 0.50 µg/L for DFA, using more than 5 different concentration levels. The correlation coefficients of the 1/x weighted linear regression were > 0.99 in all cases.

Limit of Quantification

The limit of quantitation (LOQ) for BYI 02960 and DFEAF, defined as the lowest validated fortification level, was 0.01 mg/kg in all matrices tested. (All metabolite values are expressed in parent equivalents.) For DFA, the LOQ was 0.02 mg/kg in all matrices. The calculated limit of detection (LOD) was calculated based on a statistical approach for each matrix and each compound MRM transition. Considering all sample materials and all compounds except DFA, the LOD ranged from 0.0022-0.0010 mg/kg; for DFA, it was from 0.0043-0.0171 mg/kg.

Repeatability (precision)

As a measure of the precision, the intra-laboratory repeatability (n=5) is given as the relative standard deviation (% RSD) for different sample materials at fortification levels at the respective LOQ and, in general, 10×LOQ. For BYI 02960, DFEAF, and DFA, RSDs were 0.7-14.5% for all matrices in the main method validation, as presented in the method report itself. For the additional crop matrices validated separately (as described above, cf. "Accuracy"), RSDs for all analytes were 1.5-18.6%.

The results are summarized below in tables 4.3-10 to 4.3-12.

Reproducibility (ILV)

Since this method is a data collection method, no independent validation is required.

Extraction efficiency

The extraction efficiency of the residue method for the determination of the relevant residues of BYI 02960 in plant matrices, consisting of the parent compound and its metabolite DFA, was assured by choosing the same extraction procedures as used in the plant metabolism studies (cf. chapter 6.1 of this dossier). Nevertheless, an extraction efficiency study was conducted using method 01304 (KIIA 4.3/03 and /04, cf. above). As the extraction procedures for method 01212 are the same as for 01304, the results of the study prove satisfactory extraction efficiency with method 01212.

Stability of analytes

BYI 02960 and DFA were shown to be stable in standard solutions for at least 2 months. During the course of the validations for this method 01212, stability was not tested in matrix extracts. Thus, sample extracts analysed, using this method, were measured within 24 hours of their preparation.

Conclusion

Method 01212 meets all necessary performance requirements to determine residues of BYI 02960 and its metabolites DFEAF and DFA in plant materials, with an LOQ of 0.01 mg/kg for BYI 02960 and DFEAF, and of 0.02 mg/kg for DFA, all expressed in parent compound equivalents. The method is valid as data collection method.

Table 4.3-10: Recovery results from method validation of method 01212 – Recoveries and relative standard deviations (RSDs) for **BYI 02960**
*(Recoveries as documented in the method report unless noted otherwise)*

| Sample material | FL* [mg/kg] | Individual values [%] | | | | | Mean value [%] | RSD [%] | LOQ [mg/kg] |
|---|---|---|---|---|---|---|---|---|---|
| tomato / fruit | 0.01 | 94 | 105 | 95 | 102 | 125 | 104 | 12.0 | 0.01 |
| | 0.10 | 118 | 113 | 99 | 102 | 111 | 109 | 7.3 | |
| | | ***Overall recovery (n = 10)*** | | | | | ***106*** | ***9.5*** | |
| grape / bunch of grapes | 0.01 | 107 | 105 | 98 | 97 | 97 | 101 | 4.8 | 0.01 |
| | 0.10 | 110 | 101 | 107 | 113 | 100 | 106 | 5.3 | |
| | | ***Overall recovery (n = 5)*** | | | | | ***104*** | ***5.5*** | |
| kidney bean / dry seed | 0.01 | 104 | 117 | 110 | 96 | 98 | 105 | 8.2 | 0.01 |
| | 0.10 | 110 | 114 | 118 | 95 | 114 | 110 | 8.1 | |
| | | ***Overall recovery (n = 10)*** | | | | | ***108*** | ***8.1*** | |
| barley / grain | 0.01 | 94 | 85 | 109 | 96 | 121 | 101 | 13.9 | 0.01 |
| | 0.10 | 95 | 104 | 95 | 98 | 99 | 98 | 3.8 | |
| | | ***Overall recovery (n = 10)*** | | | | | ***100*** | ***9.9*** | |
| barley / green material[1] | 0.01 | 87 | 109 | 127 | | | 108 | 18.6 | 0.01 |
| | 0.10 | 96 | 105 | 113 | | | 105 | 8.1 | |
| | | ***Overall recovery (n = 6)*** | | | | | ***106*** | ***13.1*** | |
| summer rape / seed | 0.01 | 102 | 108 | 104 | 112 | 88 | 103 | 8.9 | 0.01 |
| | 0.10 | 107 | 97 | 98 | 97 | 104 | 101 | 4.6 | |
| | | ***Overall recovery (n = 10)*** | | | | | ***102*** | ***6.8*** | |
| lettuce / head[2] | 0.01 | 91 | 98 | 105 | | | 98 | 7.1 | 0.01 |
| | 0.10 | 98 | 103 | 107 | | | 103 | 4.4 | |
| | | ***Overall recovery (n = 6)*** | | | | | ***100*** | ***5.8*** | |

* fortified compound BYI 02960 determined as BYI 02960 expressed as BYI 02960

*Footnotes:*

1: this crop matrix was validated as part of study 11-2958 (KIIA 10.3.3/02)

2: this crop matrix was validated as part of study 11-2070 (KIIA 6.3.1/06)

Table 4.3-11: Recovery results from method validation of method 01212 – Recoveries and relative standard deviations (RSDs) for **DFA**
*(Recoveries as documented in the method report unless noted otherwise)*

| Sample material | FL* [mg/kg] | Individual values [%] | | | | | Mean value [%] | RSD [%] | LOQ [mg/kg] |
|---|---|---|---|---|---|---|---|---|---|
| tomato / fruit | 0.02[1] | 98 (130) | 127 (159) | 93 (125) | 119 (151) | 116 (148) | 111 | 13.1 | 0.02 |
| | 0.20 | 109 | 103 | 104 | 107 | 106 | 106 | 2.3 | |
| | | ***Overall recovery (n = 10)*** | | | | | ***108*** | ***9.3*** | |
| grape / bunch of grapes | 0.02 | 100 | 104 | 96 | 104 | 99 | 101 | 3.4 | 0.02 |
| | 0.20 | 104 | 99 | 97 | 102 | 101 | 101 | 2.7 | |
| | | ***Overall recovery (n = 5)*** | | | | | ***101*** | ***2.9*** | |
| kidney bean / dry seed | 0.02 | 88 | 83 | 103 | 96 | 88 | 92 | 8.6 | 0.02 |
| | 0.20 | 95 | 95 | 96 | 94 | 95 | 95 | 0.7 | |
| | | ***Overall recovery (n = 10)*** | | | | | ***93*** | ***6.0*** | |
| barley / grain | 0.02 | 98 | 92 | 100 | 90 | 110 | 98 | 8.0 | 0.02 |
| | 0.20 | 76 | 90 | 85 | 82 | 89 | 84 | 6.7 | |
| | | ***Overall recovery (n = 10)*** | | | | | ***91*** | ***10.6*** | |
| barley / green material[2] | 0.02 | 113 | 118 | 120 | | | 117 | 3.1 | 0.02 |
| | 0.20 | 100 | 102 | 103 | | | 102 | 1.5 | |
| | | ***Overall recovery (n = 6)*** | | | | | ***109*** | ***8.0*** | |
| summer rape / seed | 0.02 | 68 | 70 | 71 | 84 | 77 | 74 | 8.8 | 0.02 |
| | 0.20 | 71 | 68 | 69 | 71 | 69 | 70 | 1.9 | |
| | | ***Overall recovery (n = 10)*** | | | | | ***72*** | ***7.0*** | |
| lettuce / head[3] | 0.02 | 76 | 82 | 88 | | | 82 | 7.3 | 0.02 |
| | 0.20 | 90 | 91 | 101 | | | 94 | 6.5 | |
| | | ***Overall recovery (n = 6)*** | | | | | ***88*** | ***9.7*** | |

* fortified compound DFA determined as DFA expressed as BYI 02960

*Footnotes:*
1: values in ( ) are the uncorrected values (corrections done with mean control sample at 32% of the LOQ)
2: this crop matrix was validated as part of study 11-2958 (KIIA 10.3.3/02)
3: this crop matrix was validated as part of study 11-2070 (KIIA 6.3.1/06)

Table 4.3-12: Recovery results from method validation of method 01212 – Recoveries and relative standard deviations (RSDs) for **DFEAF**
*(Recoveries as documented in the method report unless noted otherwise)*

| Sample material | FL* [mg/kg] | Individual values [%] | | | | | Mean value [%] | RSD [%] | LOQ [mg/kg] |
|---|---|---|---|---|---|---|---|---|---|
| tomato / fruit | 0.01 | 109 | 120 | 119 | 111 | 97 | 111 | 8.3 | 0.01 |
| | 0.10 | 102 | 90 | 102 | 101 | 106 | 100 | 6.0 | |
| | | ***Overall recovery (n = 10)*** | | | | | ***106*** | ***8.9*** | |
| grape / bunch of grapes | 0.01 | 113 | 98 | 98 | 97 | 99 | 101 | 6.7 | 0.01 |
| | 0.10 | 96 | 105 | 113 | 105 | 103 | 104 | 5.8 | |
| | | ***Overall recovery (n = 5)*** | | | | | ***103*** | ***6.1*** | |
| kidney bean / dry seed | 0.01 | 89 | 105 | 88 | 100 | 111 | 99 | 10.2 | 0.01 |
| | 0.10 | 110 | 116 | 122 | 94 | 110 | 110 | 9.4 | |
| | | ***Overall recovery (n = 10)*** | | | | | ***105*** | ***11.0*** | |
| barley / grain | 0.01 | 104 | 109 | 106 | 116 | 133 | 114 | 10.4 | 0.01 |
| | 0.10 | 87 | 96 | 97 | 93 | 104 | 95 | 6.5 | |
| | | ***Overall recovery (n = 10)*** | | | | | ***105*** | ***12.5*** | |
| barley / green material[1] | 0.01 | 92 | 111 | 121 | | | 108 | 13.6 | 0.01 |
| | 0.10 | 101 | 106 | 118 | | | 108 | 8.1 | |
| | | ***Overall recovery (n = 6)*** | | | | | ***108*** | ***10.0*** | |
| summer rape / seed | 0.01 | 86 | 109 | 92 | 103 | 75 | 93 | 14.5 | 0.01 |
| | 0.10 | 109 | 112 | 99 | 108 | 103 | 106 | 4.9 | |
| | | ***Overall recovery (n = 10)*** | | | | | ***100*** | ***11.9*** | |
| lettuce / head[2] | 0.01 | **92** | **97** | **102** | | | **97** | **5.2** | 0.01 |
| | 0.10 | **94** | **95** | **98** | | | **96** | **2.2** | |
| | | ***Overall recovery (n = 6)*** | | | | | ***96*** | ***3.6*** | |

* fortified compound DFEAF determined as DFEAF expressed as BYI 02960

*Footnotes:*
1: this crop matrix was validated as part of study 11-2958 (KIIA 10.3.3/02)
2: this crop matrix was validated as part of study 11-2070 (KIIA 6.3.1/06)

*Animal matrices*

| **Report:** | **KIIA 4.3/06, Schulte, G., & Bauer, J.; 2012** |
|---|---|
| Title: | Analytical method 01214 for the determination of residues of BYI 02960 and its metabolite difluoroacetic acid in/on animal matrices by HPLC-MS/MS - Enforcement method animal |
| Report No. & Edition No. | Method no. 01214, report no.: MR-011/144<br>M-425837-01-1 |
| **Guidelines:** | – EU Council Directive 91/414/EEC amended by Commission Directive 96/68/EC<br>– EU Guidance document for residue analytical methods SANCO/825/00 rev. 7 |
| **GLP:** | yes (certified laboratory) |

| **Report:** | **KIIA 4.3/07, Konrad, S.; 2012** |
|---|---|
| Title: | Independent lab validation of BCS method 01214 for the determination of residues of BYI 02960 and its metabolite difluoroacetic acid in/on animal matrices by HPLC-MS/MS |
| Report No. & Edition No. | 2011/0164/01<br>M-427160-01-1 |
| **Guidelines:** | – EU Regulation (EC) No. 1107/2009<br>– EU Guidance Document for residue analytical methods SANCO/825/00 rev. 8.1<br>– EU Guidance Document for residue analytical methods SANCO/3029/99<br>– OECD Guidance Document on pesticide residue analytical methods ENV/JM/Mono (2007)<br>– US EPA Residue Chemistry Test Guideline OPPTS 860.1340, residue analytical methods |
| **GLP:** | yes (certified laboratory) |

Principle of the method

Residue analytical method 01214 (Schulte & Bauer, 2012; KIIA 4.3/06) was developed as an EU *enforcement method* for the determination of the residues of BYI 02960 (parent compound), and its metabolite DFA in animal matrices.

The residues were extracted twice from 5 g of animal-based material with acetonitrile/water (4/1, v/v), with the addition of n-heptane in the cases of fat and milk. The materials tested included bovine muscle, liver, kidney, and milk; and chicken fat and egg, representing the variety of matrix types requested by EU guidance. After the addition of formic acid and dilution, an aliquot of the raw extract was filtered for measurement. The solution was analyzed by HPLC-MS/MS; residues were quantified against matrix-matched standards.

Specificity

Apparent residues in control samples were below 30% of the LOQ. Two MRM transitions for quantitation and confirmation were monitored for BYI 02960 (m/z 289/126 or 90) in each matrix tested. Using this procedure, the HPLC-MS/MS method is highly specific, thus an additional confirmatory method based on another principle is not necessary. For DFA, no second MRM transition is available. Thus, a Hypercarb column was employed as a different separation system (as opposed to HILIC for the primary determination). The confirmatory methods were fully validated, hence the quantitation and confirmation methods can be used interchangeably if so desired.

Accuracy (recovery findings)

The accuracy of the method was assessed on the basis of the determined recovery rates. Samples were fortified with BYI 02960 at concentrations of 0.01 and 0.10 mg/kg; DFA was spiked at 0.02 and

0.20 mg/kg, expressed in parent equivalents. Mean recoveries per fortification level for the primary method for both analytes and all matrices were in a range of 90-108%, with one exception at 112% (BYI 02960, muscle, 0.01 mg/kg). Using the confirmatory conditions, mean values per fortification level were 91-108% in all matrices except for BYI 02960, muscle, at 0.01 mg/kg (112%). In both cases of higher recovery, the RSD was low (6.7 and 7.2%), so that these higher values were considered to be acceptable. The results are summarized below in tables 4.3-13 and 4.3-14.

Linearity

The correlation between the injected amount of substance and the detector response was linear for standards in matrix in the range from 0.125 to 100 µg/L, using at least 5 different concentration levels for both compounds. The correlation coefficients of the 1/x weighted linear regression were > 0.99 in both cases. Linearity was proven for the confirmatory method as well, over the same concentration range and, again, with correlation coefficients of > 0.99.

Limit of Quantification

The limit of quantitation (LOQ) for BYI 02960, defined as the lowest validated fortification level, was 0.01 mg/kg in all matrices tested. For DFA, the LOQ was 0.02 mg/kg. The calculated limit of detection (LOD) was estimated to be at least 3 times lower than the respective LOQ, based on the linearity response data and matrix interference observed in control samples.

Repeatability (precision)

As a measure of the precision, the intra-laboratory repeatability (n=5) is given as the relative standard deviation (% RSD) for different sample materials at fortification levels at the respective LOQ and, in general, 10×LOQ. For BYI 02960, these levels were 0.01 and 0.10 mg/kg. Relative standard deviations were 1.1-12.6% for the primary method (confirmatory: 1.5-13.6%).

Fortification levels for DFA were 0.02 and 0.20 mg/kg; RSDs were 0.9-13.3% for the primary method, and 1.5-13.6% for the confirmatory.

The results are summarized below in tables 4.3-13 and 4.3-14.

Reproducibility (ILV)

An independent laboratory validation was performed for method 01214 and reported as 2011/0164/01 (Konrad, 2012; KIIA 4.3/07). Samples of all matrices covered by the main method itself were fortified with BYI 02960 parent compound at the nominal fortification levels of 0.01 and 0.10 mg/kg, i.e. the LOQ and the 10-fold LOQ; and with DFA at 0.02 and 0.20 mg/kg. Two replicate specimens per animal material were kept untreated, serving as blank controls.

Analysis of samples was performed according to method 01214 (Schulte & Bauer, 2012; KIIA 4.3/06). Two MRM transitions were measured for BYI 02960 (as described above under "specificity"), one for quantification and the second for confirmation. For all matrices, for both fortification levels, and for both MRM transitions monitored, the mean recoveries were between 87% and 101%, with relative standard deviations of < 15%. Only minor interfering signals in the blank

control specimens were detected, resulting in a limit of detection (LOD) of 0.003 mg/kg for all animal materials.

For DFA, as described above under "specificity", one MRM transition was determined using two different HPLC conditions. For all matrices, for both fortification levels, and for both the primary and the confirmatory HPLC procedure, the mean recoveries were between 73 and 99%. RSDs were < 15% in all cases. Only minor interfering signals in the blank control specimens were detected, resulting in a limit of detection (LOD) of 0.006 mg/kg for all tested animal matrices.

Method 01214 was shown to fulfil the reproducibility requirements as defined in international guidelines and therefore to be suitable for the enforcement of residue levels of BYI 02960 and its metabolite DFA in animal matrices. A summary of the independent laboratory validation results is given in tables 4.3-15 and 4.3-16.

Extraction efficiency

The extraction efficiency of the residue method for the determination of the relevant residues of BYI 02960 in plant matrices, consisting of the parent compound and its metabolite DFA, was assured by choosing the same extraction procedures as used in the plant metabolism studies (cf. chapter 6.1 of this dossier).

Stability of analytes

BYI 02960 and DFA were shown to be stable in standard solutions for at least 6 months as a part of the study RARVP013 (method 01304, cf. reference IIA 4.3/03). During the course of the validations for this method 01214, stability was shown in all tested matrix extracts for at least 6-8 days, when stored in the dark in a refrigerator at 4°C ± 3°C.

Standard EU multi-residue methods, e.g. DFG S 19 or QuEChERS

The EU requires that a major multi-residue method, usually DFG S 19 and/or QuEChERS, be evaluated as to whether it can be used for monitoring purposes. When considering the proposed enforcement residue definition for BYI 02960, consisting of the sum of two components – parent compound and the metabolite DFA – it is evident that the small, polar molecule DFA cannot be sufficiently extracted via any of the recommended extraction systems in the two multimethods. Thus, a specific method is presented here for enforcement purposes.

Conclusion

As DFA cannot be determined by DFG multi-residue method S 19 or by the QuEChERS procedure, a specific method, such as 01214, is valid for the determination of the proposed enforcement residue definition for BYI 02960.

Method 01214 meets all necessary performance requirements to determine residues of BYI 02960 and its metabolite DFA in animal matrices, with LOQs of 0.01 mg/kg for BYI 02960 and 0.02 mg/kg for DFA. Results of an ILV showed that method 01214 fulfils the reproducibility requirements and is, therefore, applicable as an enforcement method.

Table 4.3-13: Recovery results from the method validation of method 01214 (enforcement method) – Recoveries and relative standard deviations (RSDs) for **BYI 02960**

| Sample material | FL* [mg/kg] | Individual values [%] | | | | | Mean value [%] | RSD [%] | LOQ [mg/kg] |
|---|---|---|---|---|---|---|---|---|---|
| *PRIMARY TRANSITION (289/126)* | | | | | | | | | |
| bovine muscle | 0.01 | 111 | 100 | 116 | 119 | 116 | 112[1] | 6.7 | 0.01 |
| | 0.10 | 105 | 105 | 110 | 106 | 112 | 108 | 3.0 | |
| | | ***Overall recovery (n = 10)*** | | | | | ***110*** | ***5.5*** | |
| bovine liver | 0.01 | 100 | 104 | 107 | 106 | 77 | 99 | 12.6 | 0.01 |
| | 0.10 | 99 | 100 | 101 | 100 | 96 | 99 | 1.9 | |
| | | ***Overall recovery (n = 5)*** | | | | | ***99*** | ***8.5*** | |
| bovine kidney | 0.01 | 100 | 105 | 104 | 104 | 105 | 104 | 2.0 | 0.01 |
| | 0.10 | 83 | 95 | 99 | 102 | 93 | 94 | 7.7 | |
| | | ***Overall recovery (n = 10)*** | | | | | ***99*** | ***7.1*** | |
| chicken fat | 0.01 | 101 | 106 | 113 | 110 | 106 | 107 | 4.2 | 0.01 |
| | 0.10 | 106 | 110 | 114 | 103 | 103 | 107 | 4.4 | |
| | | ***Overall recovery (n = 10)*** | | | | | ***107*** | ***4.1*** | |
| bovine milk | 0.01 | 103 | 109 | 103 | 103 | 104 | 104 | 2.5 | 0.01 |
| | 0.10 | 102 | 106 | 93 | 109 | 104 | 103 | 5.9 | |
| | | ***Overall recovery (n = 10)*** | | | | | ***104*** | ***4.3*** | |
| chicken egg | 0.01 | 97 | 96 | 97 | 97 | 99 | 97 | 1.1 | 0.01 |
| | 0.10 | 92 | 84 | 91 | 102 | 98 | 93 | 7.4 | |
| | | ***Overall recovery (n = 10)*** | | | | | ***95*** | ***5.3*** | |
| *CONFIRMATORY TRANSITION (289/90)* | | | | | | | | | |
| bovine muscle | 0.01 | 112 | 98 | 119 | 114 | 115 | 112[1] | 7.2 | 0.01 |
| | 0.10 | 106 | 105 | 106 | 105 | 112 | 107 | 2.8 | |
| | | ***Overall recovery (n = 10)*** | | | | | ***109*** | ***5.7*** | |
| bovine liver | 0.01 | 105 | 101 | 108 | 106 | 76 | 99 | 13.3 | 0.01 |
| | 0.10 | 101 | 100 | 104 | 97 | 96 | 100 | 3.2 | |
| | | ***Overall recovery (n = 5)*** | | | | | ***99*** | ***9.1*** | |
| bovine kidney | 0.01 | 104 | 104 | 105 | 106 | 104 | 105 | 0.9 | 0.01 |
| | 0.10 | 82 | 96 | 96 | 100 | 92 | 93 | 7.4 | |
| | | ***Overall recovery (n = 10)*** | | | | | ***99*** | ***7.7*** | |
| chicken fat | 0.01 | 104 | 105 | 109 | 103 | 105 | 105 | 2.2 | 0.01 |
| | 0.10 | 108 | 109 | 111 | 102 | 103 | 107 | 3.7 | |
| | | ***Overall recovery (n = 10)*** | | | | | ***106*** | ***2.9*** | |
| bovine milk | 0.01 | 102 | 106 | 109 | 103 | 99 | 104 | 3.7 | 0.01 |
| | 0.10 | 101 | 108 | 93 | 109 | 104 | 103 | 6.3 | |
| | | ***Overall recovery (n = 10)*** | | | | | ***103*** | ***4.9*** | |
| chicken egg | 0.01 | 96 | 93 | 95 | 93 | 104 | 96 | 4.7 | 0.01 |
| | 0.10 | 93 | 87 | 92 | 102 | 100 | 95 | 6.5 | |
| | | ***Overall recovery (n = 10)*** | | | | | ***96*** | ***5.4*** | |

* fortified compound BYI 02960 determined as BYI 02960 expressed as BYI 02960

*Footnotes:*

1: accepted as RSD was < 20%

Table 4.3-14: Recovery results from the method validation of method 01214 (enforcement method) – Recoveries and relative standard deviations (RSDs) for **DFA**

| Sample material | FL* [mg/kg] | Individual values [%] | | | | | Mean value [%] | RSD [%] | LOQ [mg/kg] |
|---|---|---|---|---|---|---|---|---|---|
| *PRIMARY METHOD (HILIC COLUMN)* | | | | | | | | | |
| bovine muscle | 0.02 | 106 | 106 | 105 | 107 | 104 | 106 | 1.1 | 0.02 |
| | 0.20 | 97 | 101 | 100 | 98 | 105 | 100 | 3.1 | |
| | | ***Overall recovery (n = 10)*** | | | | | ***103*** | ***3.5*** | |
| bovine liver | 0.02 | 97 | 99 | 105 | 102 | 72 | 95 | 13.9 | 0.02 |
| | 0.20 | 105 | 102 | 104 | 100 | 93 | 101 | 4.7 | |
| | | ***Overall recovery (n = 5)*** | | | | | ***98*** | ***10.1*** | |
| bovine kidney | 0.02 | 107 | 103 | 106 | 108 | 106 | 106 | 1.8 | 0.02 |
| | 0.20 | 94 | 96 | 100 | 101 | 96 | 97 | 3.0 | |
| | | ***Overall recovery (n = 10)*** | | | | | ***102*** | ***5.0*** | |
| chicken fat | 0.02 | 99 | 106 | 107 | 106 | 106 | 105 | 3.1 | 0.02 |
| | 0.20 | 103 | 104 | 111 | 101 | 103 | 104 | 3.7 | |
| | | ***Overall recovery (n = 10)*** | | | | | ***105*** | ***3.2*** | |
| bovine milk | 0.02 | 110 | 108 | 107 | 101 | 109 | 107 | 3.3 | 0.02 |
| | 0.20 | 103 | 108 | 92 | 105 | 102 | 102 | 5.9 | |
| | | ***Overall recovery (n = 10)*** | | | | | ***105*** | ***5.1*** | |
| chicken egg | 0.02 | 90 | 94 | 90 | 89 | 98 | 92 | 4.1 | 0.02 |
| | 0.20 | 90 | 81 | 84 | 94 | 99 | 90 | 8.1 | |
| | | ***Overall recovery (n = 10)*** | | | | | ***91*** | ***6.2*** | |
| *CONFIRMATORY METHOD (HYPERCARB COLUMN)* | | | | | | | | | |
| bovine muscle | 0.02 | 109 | 105 | 107 | 107 | 112 | 108 | 2.1 | 0.02 |
| | 0.20 | 103 | 105 | 105 | 103 | 107 | 105 | 1.6 | |
| | | ***Overall recovery (n = 10)*** | | | | | ***106*** | ***2.4*** | |
| bovine liver | 0.02 | 98 | 103 | 102 | 99 | 72 | 95 | 13.6 | 0.02 |
| | 0.20 | 92 | 90 | 94 | 91 | 88 | 91 | 2.5 | |
| | | ***Overall recovery (n = 5)*** | | | | | ***93*** | ***9.6*** | |
| bovine kidney | 0.02 | 100 | 102 | 97 | 104 | 96 | 100 | 3.4 | 0.02 |
| | 0.20 | 88 | 90 | 960 | 102 | 96 | 94 | 5.9 | |
| | | ***Overall recovery (n = 10)*** | | | | | ***97*** | ***5.3*** | |
| chicken fat | 0.02 | 105 | 105 | 109 | 107 | 106 | 106 | 1.6 | 0.02 |
| | 0.20 | 100 | 105 | 105 | 99 | 101 | 102 | 2.8 | |
| | | ***Overall recovery (n = 10)*** | | | | | ***104*** | ***3.1*** | |
| bovine milk | 0.02 | 104 | 105 | 103 | 101 | 104 | 103 | 1.5 | 0.02 |
| | 0.20 | 103 | 104 | 93 | 103 | 103 | 101 | 4.5 | |
| | | ***Overall recovery (n = 10)*** | | | | | ***102*** | ***3.4*** | |
| chicken egg | 0.02 | 100 | 101 | 102 | 98 | 100 | 100 | 1.5 | 0.02 |
| | 0.20 | 94 | 86 | 93 | 97 | 98 | 94 | 5.0 | |
| | | ***Overall recovery (n = 10)*** | | | | | ***97*** | ***4.9*** | |

* fortified compound DFA determined as DFA expressed as BYI 02960

Table 4.3-15: Recovery results from **independent laboratory validation** of method 01214 – Recoveries and relative standard deviations (RSDs) for **BYI 02960**

| Sample material | FL* [mg/kg] | Individual values [%] | | | | | Mean value [%] | RSD [%] | LOQ [mg/kg] |
|---|---|---|---|---|---|---|---|---|---|
| *PRIMARY TRANSITION (289/126)* | | | | | | | | | |
| chicken egg | 0.01 | 94 | 109 | 105 | 93 | 91 | 98 | 8.3 | 0.01 |
| | 0.1 | 90 | 84 | 84 | 88 | 87 | 87 | 3.1 | |
| | | ***Overall recovery (n = 10)*** | | | | | ***93*** | ***9.1*** | |
| bovine milk | 0.01 | 99 | 100 | 100 | 101 | 98 | 100 | 1.0 | 0.01 |
| | 0.1 | 97 | 91 | 94 | 96 | 95 | 95 | 2.2 | |
| | | ***Overall recovery (n = 10)*** | | | | | ***97*** | ***3.2*** | |
| bovine kidney | 0.01 | 92 | 94 | 94 | 93 | 93 | 93 | 0.8 | 0.01 |
| | 0.1 | 88 | 89 | 92 | 91 | 92 | 90 | 1.9 | |
| | | ***Overall recovery (n = 10)*** | | | | | ***92*** | ***2.2*** | |
| bovine fat | 0.01 | 98 | 90 | 96 | 100 | 96 | 96 | 4.2 | 0.01 |
| | 0.1 | 93 | 89 | 90 | 95 | 97 | 93 | 3.6 | |
| | | ***Overall recovery (n = 10)*** | | | | | ***94*** | ***4.2*** | |
| bovine muscle | 0.01 | 95 | 97 | 97 | 98 | 93 | 96 | 2.3 | 0.01 |
| | 0.1 | 90 | 96 | 94 | 96 | 95 | 94 | 2.5 | |
| | | ***Overall recovery (n = 10)*** | | | | | ***95*** | ***2.4*** | |
| bovine liver | 0.01 | 97 | 98 | 98 | 98 | 100 | 98 | 0.9 | 0.01 |
| | 0.1 | 96 | 97 | 99 | 101 | 98 | 98 | 1.8 | |
| | | ***Overall recovery (n = 10)*** | | | | | ***98*** | ***1.4*** | |
| *CONFIRMATORY TRANSITION (289/90)* | | | | | | | | | |
| chicken egg | 0.01 | 96 | 111 | 108 | 93 | 90 | 99 | 9.4 | 0.01 |
| | 0.1 | 92 | 89 | 89 | 89 | 91 | 90 | 1.7 | |
| | | ***Overall recovery (n = 10)*** | | | | | ***95*** | ***8.4*** | |
| bovine milk | 0.01 | 101 | 97 | 101 | 102 | 96 | 99 | 2.8 | 0.01 |
| | 0.1 | 100 | 94 | 95 | 97 | 97 | 97 | 2.3 | |
| | | ***Overall recovery (n = 10)*** | | | | | ***98*** | ***2.8*** | |
| bovine kidney | 0.01 | 93 | 94 | 95 | 96 | 98 | 95 | 2.2 | 0.01 |
| | 0.1 | 90 | 94 | 97 | 94 | 94 | 94 | 2.5 | |
| | | ***Overall recovery (n = 10)*** | | | | | ***94*** | ***2.4*** | |
| bovine fat | 0.01 | 100 | 93 | 98 | 98 | 95 | 97 | 2.6 | |
| | 0.1 | 97 | 92 | 92 | 97 | 99 | 95 | 3.5 | 0.01 |
| | | ***Overall recovery (n = 10)*** | | | | | ***96*** | ***3.0*** | |
| bovine muscle | 0.01 | 96 | 95 | 95 | 97 | 88 | 94 | 3.9 | |
| | 0.1 | 92 | 98 | 96 | 98 | 96 | 96 | 2.6 | 0.01 |
| | | ***Overall recovery (n = 10)*** | | | | | ***95*** | ***3.3*** | |
| bovine liver | 0.01 | 100 | 101 | 95 | 98 | 97 | 98 | 2.5 | |
| | 0.1 | 96 | 99 | 98 | 102 | 101 | 99 | 2.6 | 0.01 |
| | | ***Overall recovery (n = 10)*** | | | | | ***99*** | ***2.4*** | |

* fortified compound BYI 02960 determined as BYI 02960 expressed as BYI 02960

Table 4.3-16: Recovery results from **independent laboratory validation** of method 01214 – Recoveries and relative standard deviations (RSDs) for **difluoroacetic acid**

| Sample material | FL* [mg/kg] | Individual values [%] | | | | | Mean value [%] | RSD [%] | LOQ [mg/kg] |
|---|---|---|---|---|---|---|---|---|---|
| *PRIMARY METHOD (HILIC COLUMN)* | | | | | | | | | |
| chicken egg | 0.02 | 105 | 96 | 103 | 92 | 95 | 98 | 5.7 | 0.02 |
| | 0.2 | 90 | 102 | 93 | 91 | 93 | 94 | 5.0 | |
| | | ***Overall recovery (n = 10)*** | | | | | ***96*** | ***5.6*** | |
| bovine milk | 0.02 | 100 | 102 | 99 | 107 | 97 | 101 | 3.7 | 0.02 |
| | 0.2 | 97 | 96 | 94 | 98 | 96 | 96 | 1.6 | |
| | | ***Overall recovery (n = 10)*** | | | | | ***99*** | ***3.8*** | |
| bovine kidney | 0.02 | 93 | 102 | 98 | 106 | 102 | 100 | 4.9 | 0.02 |
| | 0.2 | 89 | 94 | 93 | 92 | 92 | 92 | 2.2 | |
| | | ***Overall recovery (n = 10)*** | | | | | ***96*** | ***5.8*** | |
| bovine fat | 0.02 | 100 | 100 | 90 | 100 | 95 | 97 | 4.4 | |
| | 0.2 | 91 | 86 | 96 | 92 | 90 | 91 | 4.1 | 0.02 |
| | | ***Overall recovery (n = 10)*** | | | | | ***94*** | ***5.2*** | |
| bovine muscle | 0.02 | 95 | 98 | 100 | 100 | 99 | 98 | 2.1 | |
| | 0.2 | 83 | 91 | 88 | 86 | 87 | 87 | 3.4 | 0.02 |
| | | ***Overall recovery (n = 10)*** | | | | | ***93*** | ***6.8*** | |
| bovine liver | 0.02 | 89 | 79 | 88 | 83 | 105 | 89 | 11.1 | |
| | 0.2 | 101 | 94 | 96 | 99 | 96 | 97 | 2.8 | 0.02 |
| | | ***Overall recovery (n = 10)*** | | | | | ***98*** | ***8.7*** | |
| *CONFIRMATORY METHOD (HYPERCARB COLUMN)* | | | | | | | | | |
| chicken egg | 0.02 | 80 | 80 | 83 | 78 | 78 | 80 | 2.3 | 0.02 |
| | 0.2 | 81 | 96 | 82 | 84 | 82 | 85 | 7.2 | |
| | | ***Overall recovery (n = 10)*** | | | | | ***82*** | ***6.2*** | |
| bovine milk | 0.02 | 76 | 79 | 77 | 85 | 75 | 78 | 5.0 | 0.02 |
| | 0.2 | 85 | 83 | 83 | 85 | 86 | 84 | 1.6 | |
| | | ***Overall recovery (n = 10)*** | | | | | ***81*** | ***5.2*** | |
| bovine kidney | 0.02 | 71 | 72 | 72 | 79 | 75 | 74 | 4.4 | 0.02 |
| | 0.2 | 76 | 78 | 78 | 78 | 76 | 77 | 1.4 | |
| | | ***Overall recovery (n = 10)*** | | | | | ***76*** | ***3.9*** | |
| bovine fat | 0.02 | 73 | 74 | 75 | 75 | 76 | 75 | 1.5 | |
| | 0.2 | 87 | 88 | 94 | 91 | 85 | 89 | 3.6 | 0.02 |
| | | ***Overall recovery (n = 10)*** | | | | | ***82*** | ***9.7*** | |
| bovine muscle | 0.02 | 73 | 72 | 74 | 72 | 73 | 73 | 1.3 | |
| | 0.2 | 79 | 83 | 84 | 88 | 85 | 84 | 3.8 | 0.02 |
| | | ***Overall recovery (n = 10)*** | | | | | ***78*** | ***8.0*** | |
| bovine liver | 0.02 | 78 | 72 | 73 | 77 | 96 | 79 | 12.3 | |
| | 0.2 | 89 | 86 | 92 | 92 | 90 | 90 | 2.8 | 0.02 |
| | | ***Overall recovery (n = 10)*** | | | | | ***84*** | ***10.3*** | |

* fortified compound DFA determined as DFA expressed as BYI 02960

| **Report:** | **KIIA 4.3/08, Moore, S.M., & Harbin, A.M.; 2012** |
|---|---|
| Title: | BYI 02960 – Magnitude of the residue in dairy cows |
| Report No. & Edition No. | RARVP050 (includes BCS US method no. RV-004-A11-04)<br>M-428416-01-1 |
| **Guidelines:** | – OPPTS 860.1480 – Meat/milk/poultry/eggs<br>– OECD Guideline 505<br>– APVMA Residue Guideline No. 23<br>– DACO 7.5 – Meat/milk/poultry/eggs |
| **GLP:** | yes (certified laboratory) |

| **Report:** | **KIIA 4.3/09, Wade, J.M., & Netzband, D.J.; 2012** |
|---|---|
| Title: | BYI 02960 – Magnitude of the residue in laying hens |
| Report No. & Edition No. | RARVP0041 (includes BCS US method no. RV-004-A11-04)<br>M-428933-01-1 |
| **Guidelines:** | – OPPTS 860.1480 – Meat/milk/poultry/eggs<br>– OECD Guideline 505<br>– APVMA Residue Guideline No. 23<br>– DACO 7.5 – Meat/milk/poultry/eggs<br>– OPPTS 860.1340 – Residue Analytical Method |
| **GLP:** | yes (certified laboratory) |

Principle of the method

A residue analytical method, RV-004-A11-04, was developed as a *data collection method* for the determination of the residues of BYI 02960 (parent compound), and its metabolites BYI 02960-acetyl-AMCP, and BYI 02960-OH in/on animal matrices The method validation is reported in feeding study reports RARVP050 (cattle; Moore & Harbin, 2012; KIIA 4.3/08) and RARVP041 (poultry; Wade & Netzband, 2012; KIIA 4.3/09), both of which also contain the full method description in a report appendix.

The residues were extracted twice from 2 g of animal material by diluting liquid matrices (milk, whey, cream, urine) or blending tissue matrices with acetonitrile/water (4/1, v/v) with 2.2 mL/L formic acid. The materials tested included all standard materials (muscle, liver, kidney, fat, eggs, milk), representing the variety of matrix types as requested by international guidance, plus bovine urine and poultry excreta. Aliquots of the extracts are purified through a C-18 solid-phase extraction column, then amended with a mixture of stable, isotopically labelled internal standards. The final solution was analyzed by HPLC-MS/MS.

Specificity

Apparent residues in control samples were generally below 30% of the LOQ, except in a few isolated cases. Recovery determinations conducted with the affected control samples were corrected for the interference.

As this is a data collection method, only one MRM transition was required. Nevertheless, two were monitored for BYI 02960 (m/z 289/126 or 90), BYI 02960-acetyl-AMCP (m/z 185/107 or 143), and BYI 02960-OH (m/z 305/126 or 90) in each matrix tested. Generally, the HPLC-MS/MS method is highly specific and fully satisfactory for data collection.

Nevertheless, additional confirmatory procedures were developed for parent BYI 02960 and BYI 02960-OH, as pre-testing showed that the confirmatory ion's sensitivity might be too low in some matrices. Thus, a Gemini C-18 column was employed (as opposed to HILIC in the primary method). However, as all validation experiments were successful using HILIC, these alternative confirmatory procedures were not validated further.

For DFA, only one MRM transition was available (m/z 95/51), so an alternative chromatographic system was chosen for confirmation (Restek Organic Acids column as opposed to HILIC).

Accuracy (recovery findings)

The accuracy of the method was assessed on the basis of the determined recovery rates. Samples were fortified with BYI 02960, BYI 02960-acetyl-AMCP, and BYI 02960-OH at concentrations of 0.01 mg/kg in all matrices as well as 4.0 mg/kg in poultry matrices and 0.10 mg/kg and various other levels in bovine matrices. Metabolite levels were expressed in parent equivalents.

Mean recoveries per fortification level for BYI 02960 parent ranged from 89-110% for all matrices. For DFA, the values were in the range from 71-119%. All were in acceptable ranges. For the remaining two compounds, most mean recoveries were also within the range of 70-110%. Values of 88-109% were determined for BYI 02960-acetyl-AMCP, except for in 4 cases: in poultry muscle at 0.01 mg/kg, bovine cream at 0.10 and 1.0 mg/kg, and in bovine uring at 0.01 mg/kg, mean recoveries were 111, 112, 113, and 111%, respectively. However, in all of these cases, the relative standard deviations were acceptable (1.5-11.7%), the overall recoveries were as well, and the deviations from the "norm" were only minimal, so these were considered to be acceptable values as well.

For BYI 02960-OH, mean recoveries per fortification level ranged from 88-110% in all matrices except five: poultry fat at 4.0 mg/kg; bovine cream at 0.01, 0.10, and 1.0 mg/kg; and bovine urine at 0.10 mg/kg, for which mean recoveries of 116, 114, 111, 113, and 112% were determined, respectively. Again, relative standard deviations were very low (1.0-4.9%) and the deviations from the "norm" were less than 10%, so these values were considered to be acceptable.

The results discussed above are for the primary methods and are summarized below in tables 4.3-17a to 4.3-20a. (Further details of the confirmatory methods can be taken from tables 4.3-17b to 4.3-20b.)

Linearity

The correlation between the injected amount of substance and the detector response was linear in the range from 0.5 to 250 ng/mL, using at least 5 different concentration levels, for all compounds. The correlation coefficients of the 1/x weighted linear regression were > 0.99 in all cases.

Limit of Quantification

The limit of quantitation (LOQ) for BYI 02960, BYI 02960-acetyl-AMCP, and BYI 02960-OH, defined as the lowest validated fortification level, was 0.01 mg/kg in all matrices tested. For DFA, the LOQ was 0.01 mg/kg in all poultry matrices and 0.02 mg/kg in bovine matrices, except whey, where it was 0.05 mg/kg.

The calculated limit of detection (LOD) was calculated based on a statistical approach for each matrix and each compound (primary method). Considering all sample materials and all compounds except DFA, the LOD ranged from 0.0005-0.005 mg/kg; for DFA, it was from 0.002-0.006 mg/kg.

Repeatability (precision)

As a measure of the precision, the intra-laboratory repeatability (n=5) is given as the relative standard deviation (% RSD) for different sample materials at fortification levels at the respective LOQ and at a higher level, in general, 10×LOQ. During the validation of the methods for animal matrices, the similarity of many matrices (e.g. poultry and bovine fat; poultry and bovine muscle; liver and kidney; etc.) was considered when preparing the validation sets. Thus, although validation sets included 5-17 repetitions at the respective LOQ (0.01 mg/kg for BYI 02960, BYI 02960-acetyl-AMCP, and BYI 02960-OH, and for DFA in poultry matrices, and 0.02 mg/kg for DFA in bovine matrices), multiple higher fortification levels were used, but generally only as limited validation sets, i.e. n=3.

For all analytes and all matrices, over all validation sets, relative standard deviations (RSDs) ranged between 0-15.9%. All values were in the acceptable range.

The results discussed above are for the primary methods and are summarized below in tables 4.3-17a to 4.3-20a. (Further details of the confirmatory methods can be taken from tables 4.3-17b to 4.3-20b.)

Reproducibility (ILV)

Since this method is a data collection method, no independent validation is required.

Extraction efficiency

The extraction efficiency of the residue method for the determination of the relevant residues of BYI 02960 in animal matrices, consisting of the parent compound and its metabolites DFA, BYI 02960-acetyl-AMCP, and BYI 02960-OH, was assured by choosing the same extraction procedures as used in the plant metabolism studies, except that formic acid (2.2 mL/L) was added to the extraction solution.

Nevertheless an extraction efficiency examination was conducted as part of the poultry (Wade & Netzband, 2012; KIIA 4.3/09) and cattle (Moore & Harbin, 2012; IIA 4.3/08) feeding studies. Aged residues in respective samples were analyzed using procedures described in method RV-004-A11-04 and, in parallel, using the procedures described in the metabolism studies (cf. chapter 6.1 of this dossier). The sample materials were eggs, fat, liver, and muscle in the poultry report, and kidney and milk in the ruminant report.

Following addition of appropriate isotopically labelled internal standards and clean-up on a C-18 Bond Elut column, the samples were analyzed by HPLC-MS/MS. The animal residue method RV-004-A11-04 was able to extract and measure relevant aged residues from all tested matrices, with extraction efficiency ranging from 81-91% for the poultry matrices and 90 and 105% for cattle kidney and milk, and thus, within the respective guideline values (70-120%).

Stability of analytes

BYI 02960, DFA, BYI 02960-acetyl-AMCP and BYI 02960-OH were shown to be stable in standard solutions for at least 163 days as a part of the study RARVP050 (Moore & Harbin, 2012; KIIA 4.3/08). During the course of the validations in that study, stability was shown in all tested matrix extracts (milk, liver, kidney, muscle, fat, urine, cream, whey) for at least 4-8 days, when stored in the dark in a refrigerator at 4°C ± 3°C. In the case of the chicken study (Wade & Netzband, 2012; KIIA 4.3/09), all extracts were analyzed within 4 days of sample extraction; acceptable concurrent recoveries were generated with each set of samples, thus ensuring sample stability over the given period of storage.

Conclusion

Method RV-004-A11-04 meets all necessary performance requirements to determine residues of BYI 02960 and its metabolites DFA, BYI 02960-acetyl-AMCP, and BYI 02960-OH in animal materials, with an LOQ of 0.01 mg/kg for all analytes in all matrices except for 0.02 mg/kg for DFA in bovine matrices (0.05 mg/kg for whey).

Table 4.3-17a: Recovery results from method validation of method RARVP040 – Recoveries and relative standard deviations (RSDs) for **BYI 02960** **– primary** transition (289/126)
*(Recoveries documented in the feeding study reports RARVP050 and RARVP041)*

| Sample material | FL* [mg/kg] | Individual values [%] | Mean value [%] | RSD [%] | LOQ [mg/kg] |
|---|---|---|---|---|---|
| poultry egg | 0.01 | 113 115 118 103 109<br>94 98 87 84 77<br>93 76 115 94 84<br>94 84 | 96 | 14.2 | 0.01 |
| | 4.0 | 100 81 95 | 92 | 10.7 | |
| | | ***Overall recovery (n = 20)*** | ***96*** | ***13.7*** | |
| poultry fat | 0.01 | 87 88 77 85 95<br>103 96 94 88 93<br>75 105 | 91 | 10.1 | 0.01 |
| | 4.0 | 110 104 106 | 107 | 2.9 | |
| | | ***Overall recovery (n = 15)*** | ***94*** | ***11.3*** | |
| poultry liver | 0.01 | 93 104 121 114 102<br>114 90 94 110 95<br>84 92 | 101 | 11.4 | 0.01 |
| | 4.0 | 112 102 100 | 104 | 6.1 | |
| | | ***Overall recovery (n = 15*** | ***102*** | ***10.4*** | |
| poultry muscle | 0.01 | 117 106 115 119 96<br>119 82 81 116 94<br>115 98 | 105 | 13.5 | 0.01 |
| | 4.0 | 108 112 110 | 109 | 2.1 | |
| | | ***Overall recovery (n = 15)*** | ***106*** | ***12.1*** | |
| poultry excreta | 0.01 | 87 110 104 91 112<br>109 88 111 | 102 | 10.8 | 0.01 |
| | 14.0 | 98 96 93 | 95 | 2.6 | |
| | | ***Overall recovery (n = 11)*** | ***100*** | ***9.6*** | |
| bovine milk | 0.01 | 109 109 108 110 108<br>107 102 | 108 | 2.5 | 0.01 |
| | 0.025 | 107 | | | |
| | 0.05 | 100 102 97 105 105 | 102 | 3.4 | |
| | 0.10 | 103 109 104 | 105 | | |
| | 0.25 | 103 | | | |
| | 2.0 | 100 102 103 | 102 | 1.5 | |
| | | ***Overall recovery (n = 20)*** | ***105*** | ***3.5*** | |
| bovine cream | 0.01 | 109 112 107 118 105<br>110 106 | 110 | 4.0 | 0.01 |
| | 0.10 | 109 107 113 | 110 | 2.8 | |
| | 1.0 | 111 109 111 | 110 | 1.0 | |
| | | ***Overall recovery (n = 13)*** | ***110*** | ***3.1*** | |
| bovine whey | 0.01 | 98 99 100 98 105<br>101 104 | 101 | 2.8 | 0.01 |
| | 0.025 | 104 106 102 102 101<br>102 106 | 103 | 2.0 | |
| | 0.10 | 101 102 102 | 102 | 0.6 | |
| | 0.25 | 105 103 104 | 104 | 1.0 | |
| | 1.0 | 102 100 104 | 102 | 2.0 | |
| | | ***Overall recovery (n = 23)*** | ***102*** | ***2.3*** | |

* fortified compound BYI 02960 determined as BYI 02960 expressed as BYI 02960

*Continued on next page…*

Table 4.3-17a (cont'd): Recovery results from method validation of method RARVP040 – Recoveries and relative standard deviations (RSDs) for **BYI 02960 – primary** transition (289/126)
*(Recoveries documented in the feeding study reports RARVP050 and RARVP041)*

| Sample material | FL* [mg/kg] | Individual values [%] | Mean value [%] | RSD [%] | LOQ [mg/kg] |
|---|---|---|---|---|---|
| bovine fat | 0.01 | 104 101 105 96 107 106 103 | 103 | 3.6 | 0.01 |
| | 0.05 | 93 100 | 97 | | |
| | 0.1 | 97 100 101 | 99 | 2.1 | |
| | 1.5 | 92 95 99 | 95 | 3.7 | |
| | | ***Overall recovery (n = 15)*** | ***100*** | ***4.6*** | |
| bovine kidney | 0.01 | 98 99 95 91 94 98 95 | 96 | 2.9 | 0.01 |
| | 0.05 | 93 87 98 | 93 | 5.9 | |
| | 0.10 | 96 96 94 | 95 | 1.2 | |
| | 6.0 | 90 92 97 | 93 | 3.9 | |
| | | ***Overall recovery (n = 16)*** | ***95*** | ***3.5*** | |
| bovine liver | 0.01 | 98 90 89 93 91 85 84 | 90 | 5.3 | 0.01 |
| | 0.05 | 96 98 96 | 97 | 1.2 | |
| | 0.10 | 87 88 91 | 89 | 2.3 | |
| | 4.0 | 90 93 91 | 91 | 1.7 | |
| | | ***Overall recovery (n = 16)*** | ***91*** | ***4.7*** | |
| bovine muscle | 0.01 | 92 97 92 100 96 98 100 | 96 | 3.5 | 0.01 |
| | 0.05 | 95 99 | 97 | | |
| | 0.10 | 93 98 93 | 95 | 3.0 | |
| | 2.0 | 92 90 89 | 90 | 1.7 | |
| | | ***Overall recovery (n = 15)*** | ***95*** | ***3.8*** | |
| bovine urine | 0.01 | 102 104 103 103 104 103 101 | 103 | 1.0 | 0.01 |
| | 0.10 | 102 102 102 | 102 | 0.0 | |
| | 40.0 | 109 103 104 | 105 | 3.1 | |
| | | ***Overall recovery (n = 13)*** | ***103*** | ***1.9*** | |

* fortified compound BYI 02960 determined as BYI 02960 expressed as BYI 02960

Table 4.3-17b: Recovery results from method validation of method RARVP040 – Recoveries and relative standard deviations (RSDs) for **BYI 02960 – confirmatory** transition (289/90)
*(Recoveries documented in the feeding study reports RARVP050 and RARVP041)*

| Sample material | FL* [mg/kg] | Individual values [%] | Mean value [%] | RSD [%] | LOQ [mg/kg] |
|---|---|---|---|---|---|
| bovine milk | 0.01 | 100 110 106 107 109 112 99 | 106 | 4.7 | 0.01 |
| | 0.10 | 105 108 104 | 106 | 2.0 | |
| | | ***Overall recovery (n = 10)*** | ***106*** | ***3.9*** | |
| bovine cream | 0.01 | 106 117 104 123 105 112 113 | 111[1] | 6.3 | 0.01 |
| | 0.10 | 104 111 112 | 109 | 4.0 | |
| | 1.0 | 115 108 111 | 111[1] | 3.2 | |
| | | ***Overall recovery (n = 13)*** | ***111***[1] | ***5.0*** | |
| bovine whey | 0.01 | 112 112 102 104 111 107 106 | 108 | 3.7 | 0.01 |
| | 0.10 | 103 99 100 | 101 | 2.1 | |
| | | ***Overall recovery (n = 10)*** | ***106*** | ***4.6*** | |
| bovine fat | 0.01 | 99 115 109 108 101 113 101 | 107 | 5.9 | 0.01 |
| | 0.10 | 96 100 103 | 100 | 3.5 | |
| | | ***Overall recovery (n = 10)*** | ***105*** | ***6.1*** | |
| bovine kidney | 0.01 | 98 93 93 103 92 94 98 | 96 | 4.1 | 0.01 |
| | 0.10 | 96 92 95 | 94 | 2.2 | |
| | | ***Overall recovery (n = 10)*** | ***95*** | ***3.6*** | |
| bovine liver | 0.01 | 91 98 93 98 85 89 87 | 92 | 5.6 | 0.01 |
| | 0.10 | 90 88 90 | 89 | 1.3 | |
| | | ***Overall recovery (n = 10)*** | ***91*** | ***4.8*** | |
| bovine muscle | 0.01 | 104 105 105 100 112 101 105 | 105 | 3.7 | 0.01 |
| | 0.10 | 92 97 91 | 93 | 3.4 | |
| | | ***Overall recovery (n = 10)*** | ***101*** | ***6.4*** | |
| poultry egg | 0.01 | 96 102 80 87 94 85 80 | 89 | 9.4 | 0.01 |
| | 4.0 | 95 73 92 | 87 | 13.8 | |
| | | ***Overall recovery (n = 10)*** | ***88*** | ***10.1*** | |
| poultry fat | 0.01 | 74 75 99 94 130 73 128 | 96 | 25.6[2] | 0.01 |
| | 4.0 | 106 103 103 | 104 | 1.7 | |
| | | ***Overall recovery (n = 10)*** | ***99*** | ***20.8***[2] | |
| poultry liver | 0.01 | 107 117 74 95 120 109 111 | 105 | 15.0 | 0.01 |
| | 4.0 | 108 96 99 | 101 | 6.2 | |
| | | ***Overall recovery (n = 10)*** | ***104*** | ***12.8*** | |

* fortified compound BYI 02960 determined as BYI 02960 expressed as BYI 02960

*Footnotes:*
1: accepted as RSD was < 20%
2: accepted, as this is a data collection method and only the primary method was used in the study

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Table 4.3-17b (cont'd): Recovery results from method validation of method RARVP040 – Recoveries and relative standard deviations (RSDs) for **BYI 02960 – confirmatory** transition (289/90)
*(Recoveries documented in the feeding study reports RARVP050 and RARVP041)*

| Sample material | FL* [mg/kg] | Individual values [%] | Mean value [%] | RSD [%] | LOQ [mg/kg] |
|---|---|---|---|---|---|
| poultry muscle | 0.01 | 117 123 113 81 98 106 104 | 106 | 13.1 | 0.01 |
| | 4.0 | 107 102 104 | 104 | 2.4 | |
| | | ***Overall recovery (n = 10)*** | ***106*** | ***10.8*** | |
| poultry excreta | 0.01 | 119 110 102 112 98 104 109 | 108 | 6.5 | 0.01 |
| | 14.0 | 96 94 89 | 93 | 3.9 | |
| | | ***Overall recovery (n = 10)*** | ***103*** | ***9.0*** | |

* fortified compound BYI 02960 determined as BYI 02960 expressed as BYI 02960

Table 4.3-18a: Recovery results from method validation of method RARVP040 – Recoveries and relative standard deviations (RSDs) for **BYI 02960-acetyl-AMCP – primary** transition (185/107)
*(Recoveries documented in the feeding study reports RARVP050 and RARVP041)*

| Sample material | FL* [mg/kg] | Individual values [%] | Mean value [%] | RSD [%] | LOQ [mg/kg] |
|---|---|---|---|---|---|
| poultry egg | 0.01 | 84 97 89 120 92 103 100 76 86 100 70 89 99 104 91 90 | 93 | 12.7 | 0.01 |
| | 4.0 | 93 81 90 | 88 | 7.1 | |
| | | ***Overall recovery (n = 19)*** | ***92*** | ***12.1*** | |
| poultry fat | 0.01 | 119 81 96 85 99 77 100 92 95 107 95 87 | 94 | 12.2 | 0.01 |
| | 4.0 | 109 111 108 | 109 | 1.4 | |
| | | ***Overall recovery (n = 15)*** | ***97*** | ***12.2*** | |
| poultry liver | 0.01 | 108 112 116 117 96 116 96 114 115 89 96 94 | 106 | 10.0 | 0.01 |
| | 4.0 | 115 100 99 | 105 | 8.6 | |
| | | ***Overall recovery (n = 15)*** | ***106*** | ***9.5*** | |
| poultry muscle | 0.01 | 114 116 117 135 103 119 118 116 110 101 92 88 | 111[1] | 11.7 | 0.01 |
| | 4.0 | 108 107 101 | 105 | 3.6 | |
| | | ***Overall recovery (n = 15)*** | ***110*** | ***10.7*** | |
| poultry excreta | 0.01 | 112 106 98 101 99 108 109 109 | 105 | 5.0 | 0.01 |
| | 14.0 | 98 91 91 | 93 | 4.3 | |
| | | ***Overall recovery (n = 11)*** | ***102*** | ***7.2*** | |
| bovine milk | 0.01 | 103 105 104 104 109 106 101 | 105 | 2.4 | 0.01 |
| | 0.025 | 112 | | | |
| | 0.05 | 103 102 103 109 107 | 105 | 2.9 | |
| | 0.10 | 108 112 107 | 109 | 2.4 | |
| | 0.25 | 103 | | | |
| | 2.0 | 98 99 99 | 99 | 0.6 | |
| | | ***Overall recovery (n = 20)*** | ***105*** | ***3.9*** | |
| bovine cream | 0.01 | 106 108 102 113 93 98 93 | 102 | 7.5 | 0.01 |
| | 0.10 | 111 111 114 | 112[1] | 1.5 | |
| | 1.0 | 114 109 115 | 113[1] | 2.9 | |
| | | ***Overall recovery (n = 13)*** | ***107*** | ***7.3*** | |
| bovine whey | 0.01 | 99 100 102 103 104 103 106 | 102 | 2.3 | 0.01 |
| | 0.10 | 102 102 103 | 102 | 0.6 | |
| | | ***Overall recovery (n = 10)*** | ***102*** | ***1.9*** | |

* fortified compound BYI 02960-acetyl-AMCP determined as BYI 02960-acetyl-AMCP expressed as BYI 02960

*Footnotes:*
1: accepted as RSD was < 20%

*Continued on next page…*

Table 4.3-18a (cont'd): Recovery results from method validation of method RARVP040 – Recoveries and relative standard deviations (RSDs) for **BYI 02960-acetyl-AMCP – primary** transition (185/107)
*(Recoveries documented in the feeding study reports RARVP050 and RARVP041)*

| Sample material | FL* [mg/kg] | Individual values [%] | Mean value [%] | RSD [%] | LOQ [mg/kg] |
|---|---|---|---|---|---|
| bovine fat | 0.01 | 94 97 100 98 99 97 96 | 97 | 2.0 | 0.01 |
| | 0.05 | 97 98 | 98 | | |
| | 0.1 | 96 98 101 | 98 | 2.6 | |
| | 1.5 | 101 102 106 | 103 | 2.6 | |
| | | ***Overall recovery (n = 15)*** | ***99*** | ***3.0*** | |
| bovine kidney | 0.01 | 102 103 105 99 99 99 96 | 100 | 3.0 | 0.01 |
| | 0.05 | 100 94 90 | 95 | 5.3 | |
| | 0.10 | 98 99 97 | 98 | 1.0 | |
| | 6.0 | 91 93 95 | 93 | 2.2 | |
| | | ***Overall recovery (n = 16)*** | ***98*** | ***4.3*** | |
| bovine liver | 0.01 | 99 85 104 93 98 86 80 | 92 | 9.5 | 0.01 |
| | 0.05 | 103 109 95 | 102 | 6.9 | |
| | 0.10 | 93 92 98 | 94 | 3.4 | |
| | 4.0 | 93 94 94 | 94 | 0.6 | |
| | | ***Overall recovery (n = 16)*** | ***95*** | ***7.7*** | |
| bovine muscle | 0.01 | 89 101 92 98 98 101 99 | 97 | 4.7 | 0.01 |
| | 0.05 | 95 103 | 99 | | |
| | 0.10 | 93 103 91 | 96 | 6.7 | |
| | 2.0 | 96 96 95 | 96 | 0.6 | |
| | | ***Overall recovery (n = 15)*** | ***97*** | ***4.5*** | |
| bovine urine | 0.01 | 112 119 106 109 113 111 110 | 111[1] | 3.6 | 0.01 |
| | 0.10 | 108 109 107 | 108 | 0.9 | |
| | 40.0 | 109 105 107 | 107 | 1.9 | |
| | | ***Overall recovery (n = 13)*** | ***110*** | ***3.3*** | |

* fortified compound BYI 02960-acetyl-AMCP determined as BYI 02960-acetyl-AMCP expressed as BYI 02960

*Footnotes:*
1: accepted as RSD was < 20%

Table 4.3-18b: Recovery results from method validation of method RARVP040 – Recoveries and relative standard deviations (RSDs) for **BYI 02960-acetyl-AMCP – confirmatory** transition (185/143)
*(Recoveries documented in the feeding study reports RARVP050 and RARVP041)*

| Sample material | FL* [mg/kg] | Individual values [%] | Mean value [%] | RSD [%] | LOQ [mg/kg] |
|---|---|---|---|---|---|
| bovine milk | 0.01 | 106 107 103 102 105 105 106 | 105 | 1.7 | 0.01 |
| | 0.10 | 110 111 110 | 110 | 0.5 | |
| | | ***Overall recovery (n = 10)*** | ***107*** | ***2.8*** | |
| bovine cream | 0.01 | 106 112 101 114 110 112 99 | 108 | 5.4 | 0.01 |
| | 0.10 | 112 110 113 | 112[1] | 1.4 | |
| | 1.0 | 112 108 112 | 111[1] | 2.1 | |
| | | ***Overall recovery (n = 13)*** | ***109*** | ***4.3*** | |
| bovine whey | 0.01 | 102 103 105 99 101 100 101 | 102 | 2.0 | 0.01 |
| | 0.10 | 102 102 102 | 102 | 0.0 | |
| | | ***Overall recovery (n = 10)*** | ***102*** | ***1.6*** | |
| bovine fat | 0.01 | 93 94 98 93 100 100 95 | 96 | 3.3 | 0.01 |
| | 0.10 | 98 99 99 | 99 | 0.6 | |
| | | ***Overall recovery (n = 10)*** | ***97*** | ***2.9*** | |
| bovine kidney | 0.01 | 103 106 97 94 99 104 92 | 99 | 5.3 | 0.01 |
| | 0.10 | 95 97 96 | 96 | 1.0 | |
| | | ***Overall recovery (n = 10)*** | ***98*** | ***4.7*** | |
| bovine liver | 0.01 | 101 96 93 92 95 85 82 | 92 | 7.1 | 0.01 |
| | 0.10 | 95 96 93 | 95 | 1.6 | |
| | | ***Overall recovery (n = 10)*** | ***93*** | ***6.0*** | |
| bovine muscle | 0.01 | 90 88 94 96 91 103 102 | 95 | 6.2 | 0.01 |
| | 0.10 | 91 102 92 | 95 | 6.4 | |
| | | ***Overall recovery (n = 10)*** | ***95*** | ***5.9*** | |
| poultry egg | 0.01 | 90 97 93 99 92 75 75 | 89 | 11.1 | 0.01 |
| | 4.0 | 97 85 86 | 89 | 7.5 | |
| | | ***Overall recovery (n = 10)*** | ***89*** | ***9.7*** | |
| poultry fat | 0.01 | 105 82 91 86 114 84 108 | 96 | 13.6 | 0.01 |
| | 4.0 | 111 109 106 | 109 | 2.3 | |
| | | ***Overall recovery (n = 10)*** | ***100*** | ***12.4*** | |
| poultry liver | 0.01 | 91 120 96 119 115 120 113 | 111[1] | 10.9 | 0.01 |
| | 4.0 | 91 85 82 | 86 | 5.3 | |
| | | ***Overall recovery (n = 10)*** | ***103*** | ***15.1*** | |

* fortified compound BYI 02960-acetyl-AMCP determined as BYI 02960-acetyl-AMCP expressed as BYI 02960

*Footnotes:*
1: accepted as RSD was < 20%

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Table 4.3-18b (cont'd): Recovery results from method validation of method RARVP040 – Recoveries and relative standard deviations (RSDs) for **BYI 02960-acetyl-AMCP – confirmatory** transition (185/143)
*(Recoveries documented in the feeding study reports RARVP050 and RARVP041)*

| Sample material | FL* [mg/kg] | Individual values [%] | Mean value [%] | RSD [%] | LOQ [mg/kg] |
|---|---|---|---|---|---|
| poultry muscle | 0.01 | 113 111 104 115 98 114 119 | 111[1] | 6.5 | 0.01 |
| | 4.0 | 102 110 100 | 104 | 5.1 | |
| | | ***Overall recovery (n = 10)*** | ***109*** | ***6.6*** | |
| poultry excreta | 0.01 | 90 97 96 75 92 104 107 | 94 | 11.1 | 0.01 |
| | 14.0 | 96 91 94 | 94 | 2.7 | |
| | | ***Overall recovery (n = 10)*** | ***94*** | ***9.2*** | |

* fortified compound BYI 02960-acetyl-AMCP determined as BYI 02960-acetyl-AMCP expressed as BYI 02960

*Footnotes:*
1: accepted as RSD was < 20%

Table 4.3-19a: Recovery results from method validation of method RARVP040 – Recoveries and relative standard deviations (RSDs) for **DFA**
**– primary** method (HILIC column)
*(Recoveries documented in the feeding study reports RARVP050 and RARVP041)*

| Sample material | FL* [mg/kg] | Individual values [%] | Mean value [%] | RSD [%] | LOQ [mg/kg] |
|---|---|---|---|---|---|
| poultry egg | 0.01 | 100 112 109 78 87<br>74 81 76 78 68<br>77 70 83 73 79<br>76 71 | 82 | 15.9 | 0.01 |
| | 4.0 | 75 70 71 | 72 | 3.7 | |
| | | ***Overall recovery (n = 20)*** | ***80*** | ***15.6*** | |
| poultry fat | 0.01 | 97 91 94 97 84<br>80 88 96 83 85<br>75 74 | 87 | 9.4 | 0.01 |
| | 4.0 | 100 96 92 | 96 | 4.2 | |
| | | ***Overall recovery (n = 15)*** | ***89*** | ***9.3*** | |
| poultry liver | 0.01 | 75 90 83 97 89<br>93 81 103 84 77<br>78 82 | 86 | 9.9 | 0.01 |
| | 4.0 | 91 85 82 | 86 | 5.3 | |
| | | ***Overall recovery (n = 15)*** | ***86*** | ***9.0*** | |
| poultry muscle | 0.01 | 91 95 101 85 82<br>92 88 118 98 87<br>86 104 | 95 | 10.8 | 0.01 |
| | 4.0 | 86 83 80 | 83 | 3.6 | |
| | | ***Overall recovery (n = 15)*** | ***92*** | ***11.0*** | |
| poultry excreta | 0.01 | 118 110 103 114 112<br>121 99 92 | 109 | 9.1 | 0.01 |
| | 14.0 | 86 86 88 | 87 | 1.3 | |
| | | ***Overall recovery (n = 11)*** | ***103*** | ***12.8*** | |
| bovine milk | 0.02 | 85 88 80 88 92<br>76 84 | 85 | 6.3 | 0.02 |
| | 0.05 | 81 77 78 89 91<br>93 93 | 86 | 8.5 | |
| | 0.20 | 91 92 94 | 92 | 1.7 | |
| | 0.40 | 85 85 93 | 88 | 5.3 | |
| | | ***Overall recovery (n = 19)*** | ***87*** | ***6.7*** | |
| bovine cream | 0.02 | 103 95 89 93 85<br>103 96 | 95 | 7.1 | 0.02 |
| | 0.20 | 101 102 106 | 103 | 2.6 | |
| | | ***Overall recovery (n = 10)*** | ***97*** | ***7.0*** | |
| bovine whey | 0.05 | 96 94 95 95 87<br>96 89 | 93 | 3.9 | 0.02 |
| | 0.50 | 102 94 99 | 98 | 4.1 | |
| | | ***Overall recovery (n = 10)*** | ***95*** | ***4.6*** | |

* fortified compound DFA determined as DFA expressed as BYI 02960

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Table 4.3-19a (cont'd): Recovery results from method validation of method RARVP040 – Recoveries and relative standard deviations (RSDs) for **DFA** **– primary** method (HILIC column)
*(Recoveries documented in the feeding study reports RARVP050 and RARVP041)*

| Sample material | FL* [mg/kg] | Individual values [%] | Mean value [%] | RSD [%] | LOQ [mg/kg] |
|---|---|---|---|---|---|
| bovine fat | 0.02 | 81 96 88 95 92 86 87 | 89 | 6.0 | 0.02 |
| | 0.05 | 89 93 | 91 | | |
| | 0.60 | 88 89 90 | 89 | 1.1 | |
| | | ***Overall recovery (n = 12)*** | ***90*** | ***4.6*** | |
| bovine kidney | 0.02 | 74 73 74 79 66 69 66 | 72 | 6.7 | 0.02 |
| | 0.05 | 69 72 | 71 | | |
| | 0.80 | 84 79 84 | 82 | 3.5 | |
| | | ***Overall recovery (n = 12)*** | ***74*** | ***8.5*** | |
| bovine liver | 0.02 | 87 75 97 68 73 73 82 | 79 | 12.7 | 0.02 |
| | 0.05 | 64 72 | 68[1] | | |
| | 0.60 | 79 80 89 | 83 | 6.7 | |
| | | ***Overall recovery (n = 12)*** | ***78*** | ***12.0*** | |
| bovine muscle | 0.02 | 66 71 76 76 77 78 73 | 74 | 5.7 | 0.02 |
| | 0.05 | 71 78 70 | 73 | 6.0 | |
| | 0.50 | 67 65 73 | 68[1] | 6.1 | |
| | | ***Overall recovery (n = 13)*** | ***72*** | ***6.2*** | |
| bovine urine | 0.02 | 78 85 67 73 79 70 76 | 75 | 8.0 | 0.02 |
| | 0.20 | 92 91 92 | 92 | 0.6 | |
| | 5.0 | 106 102 101 | 103 | 2.6 | |
| | | ***Overall recovery (n = 13)*** | ***86*** | ***15.0*** | |

* fortified compound DFA determined as DFA expressed as BYI 02960

*Footnotes:*
1: accepted as RSD was < 20%

Table 4.3-19b: Recovery results from method validation of method RARVP040 – Recoveries and relative standard deviations (RSDs) for **DFA** **– confirmatory** method (RESTEK organic acids column)
*(Recoveries documented in the feeding study reports RARVP050 and RARVP041)*

| Sample material | FL* [mg/kg] | Individual values [%] | Mean value [%] | RSD [%] | LOQ [mg/kg] |
|---|---|---|---|---|---|
| bovine milk | 0.02 | 84 88 78 94 94 95 94 | 90 | 7.3 | 0.02 |
| | 0.20 | 88 88 91 | 89 | 1.9 | |
| | | ***Overall recovery (n = 10)*** | ***89*** | ***6.0*** | |
| bovine cream | 0.02 | 90 92 83 87 78 84 90 | 86 | 5.7 | 0.02 |
| | 0.20 | 98 97 98 | 98 | 0.6 | |
| | | ***Overall recovery (n = 10)*** | ***90*** | ***7.6*** | |
| bovine whey | 0.05 | 93 94 85 92 97 93 89 | 92 | 4.2 | 0.02 |
| | 0.50 | 93 97 99 | 96 | 3.2 | |
| | | ***Overall recovery (n = 10)*** | ***93*** | ***4.4*** | |
| bovine fat | 0.02 | 87 89 96 95 100 89 89 | 92 | 5.3 | 0.02 |
| | 0.60 | 85 90 87 | 87 | 2.5 | |
| | | ***Overall recovery (n = 10)*** | ***89*** | ***6.9*** | |
| bovine kidney | 0.02 | 89 86 83 78 76 79 84 | 82 | 5.7 | 0.02 |
| | 0.80 | 82 85 81 | 83 | 2.5 | |
| | | ***Overall recovery (n = 10)*** | ***82*** | ***4.8*** | |
| bovine liver | 0.02 | 76 72 75 70 70 76 77 | 74 | 4.0 | 0.02 |
| | 0.60 | 79 76 86 | 80 | 6.4 | |
| | | ***Overall recovery (n = 10)*** | ***76*** | ***6.2*** | |
| bovine muscle | 0.02 | 63 61 66 62 69 73 65 | 66[1] | 6.5 | 0.02 |
| | 0.50 | 71 67 73 | 70 | 4.3 | |
| | | ***Overall recovery (n = 10)*** | ***67*** | ***6.6*** | |
| poultry egg | 0.01 | 101 90 88 82 98 83 74 | 88 | 10.7 | 0.01 |
| | 4.0 | 77 67 70 | 71 | 7.2 | |
| | | ***Overall recovery (n = 10)*** | ***83*** | ***13.7*** | |
| poultry fat | 0.01 | 72 72 84 86 83 82 90 | 81 | 8.4 | 0.01 |
| | 4.0 | 95 91 94 | 93 | 2.2 | |
| | | ***Overall recovery (n = 10)*** | ***85*** | ***9.6*** | |
| poultry liver | 0.01 | 83 102 105 93 94 94 89 | 94 | 7.9 | 0.01 |
| | 4.0 | 95 90 84 | 90 | 6.1 | |
| | | ***Overall recovery (n = 10)*** | ***93*** | ***7.5*** | |

* fortified compound DFA determined as DFA expressed as BYI 02960

*Footnotes:*
1: accepted as RSD was < 20%

*Continued on next page…*

Table 4.3-19b (cont'd): Recovery results from method validation of method RARVP040 – Recoveries and relative standard deviations (RSDs) for **DFA**
**– confirmatory** method (RESTEK organic acids column)
*(Recoveries documented in the feeding study reports RARVP050 and RARVP041)*

| Sample material | FL* [mg/kg] | Individual values [%] | Mean value [%] | RSD [%] | LOQ [mg/kg] |
|---|---|---|---|---|---|
| poultry muscle | 0.01 | 104 117 100 90 112 79 86 | 98 | 14.2 | 0.01 |
| | 4.0 | 80 81 81 | 81 | 0.7 | |
| | | ***Overall recovery (n = 10)*** | ***93*** | ***15.3*** | |
| poultry excreta | 0.01 | 118 110 103 114 112 121 99 | 111[1] | 7.1 | 0.01 |
| | 14.0 | 89 89 88 | 89 | 0.7 | |
| | | ***Overall recovery (n = 10)*** | ***104*** | ***12.0*** | |

* fortified compound DFA determined as DFA expressed as BYI 02960

*Footnotes:*
1: accepted as RSD was < 20%

Table 4.3-20a: Recovery results from method validation of method RARVP040 – Recoveries and relative standard deviations (RSDs) for **BYI 02960-OH – primary** transition (305/126)
*(Recoveries documented in the feeding study reports RARVP050 and RARVP041)*

| Sample material | FL* [mg/kg] | Individual values [%] | Mean value [%] | RSD [%] | LOQ [mg/kg] |
|---|---|---|---|---|---|
| poultry egg | 0.01 | 102 109 101 89 99<br>90 96 83 90 72<br>95 89 113 107 73<br>96 99 | 94 | 12.0 | 0.01 |
| | 4.0 | 102 89 96 | 96 | 6.8 | |
| | | ***Overall recovery (n = 20)*** | ***95*** | ***11.2*** | |
| poultry fat | 0.01 | 95 106 100 87 100<br>88 90 93 87 86<br>90 102 | 94 | 7.3 | 0.01 |
| | 4.0 | 117 113 118 | 116[1] | 2.3 | |
| | | ***Overall recovery (n = 15)*** | ***98*** | ***11.3*** | |
| poultry liver | 0.01 | 84 85 120 83 74<br>88 89 97 101 72<br>91 71 | 88 | 15.6 | 0.01 |
| | 4.0 | 108 106 107 | 107 | 0.9 | |
| | | ***Overall recovery (n = 15)*** | ***92*** | ***15.8*** | |
| poultry muscle | 0.01 | 100 112 114 97 105<br>93 120 119 111 107<br>107 81 | 106 | 10.7 | 0.01 |
| | 4.0 | 108 112 110 | 110 | 1.8 | |
| | | ***Overall recovery (n = 15)*** | ***106*** | ***9.6*** | |
| poultry excreta | 0.01 | 102 104 100 110 102<br>105 101 91 | 102 | 5.3 | 0.01 |
| | 14.0 | 97 99 91 | 95 | 4.4 | |
| | | ***Overall recovery (n = 11)*** | ***100*** | ***5.7*** | |
| bovine milk | 0.01 | 115 110 111 105 113<br>110 108 | 110 | 2.9 | 0.01 |
| | 0.025 | 106 | | | |
| | 0.05 | 101 104 100 106 106 | 103 | 2.7 | |
| | 0.10 | 110 108 105 | 108 | 2.3 | |
| | 0.25 | 105 | | | |
| | 2.0 | 101 105 101 | 102 | 2.3 | |
| | | ***Overall recovery (n = 20)*** | ***102*** | ***3.9*** | |
| bovine cream | 0.01 | 116 115 113 124 107<br>109 111 | 114[1] | 4.9 | 0.01 |
| | 0.10 | 110 111 113 | 111[1] | 1.4 | |
| | 1.0 | 114 111 114 | 113[1] | 1.5 | |
| | | ***Overall recovery (n = 13)*** | ***112***[1] | ***3.7*** | |
| bovine whey | 0.01 | 104 104 104 104 102<br>100 103 | 103 | 1.5 | 0.01 |
| | 0.025 | 107 112 104 109 107<br>108 107 | 108 | 2.3 | |
| | 0.10 | 101 100 102 | 101 | 1.0 | |
| | 0.25 | 106 112 107 | 108 | 3.0 | |
| | | ***Overall recovery (n = 20)*** | ***105*** | ***3.3*** | |

* fortified compound BYI 02960-OH determined as BYI 02960-OH expressed as BYI 02960

*Footnotes:*
1: accepted as RSD was < 20%

*Continued on next page…*

Table 4.3-20a (cont'd): Recovery results from method validation of method RARVP040 – Recoveries and relative standard deviations (RSDs) for **BYI 02960-OH – primary** transition (305/126)
*(Recoveries documented in the feeding study reports RARVP050 and RARVP041)*

| Sample material | FL* [mg/kg] | Individual values [%] | Mean value [%] | RSD [%] | LOQ [mg/kg] |
|---|---|---|---|---|---|
| bovine fat | 0.01 | 106 107 109 101 108 107 105 | 106 | 2.5 | 0.01 |
| | 0.05 | 98 105 | 102 | | |
| | 0.1 | 97 101 106 | 101 | 4.4 | |
| | 1.5 | 94 97 98 | 96 | 2.2 | |
| | | ***Overall recovery (n = 15)*** | ***103*** | ***4.7*** | |
| bovine kidney | 0.01 | 101 90 99 94 90 105 98 | 97 | 5.8 | 0.01 |
| | 0.05 | 97 93 95 | 95 | 2.1 | |
| | 0.10 | 98 98 98 | 98 | 0.0 | |
| | 6.0 | 95 94 100 | 96 | 3.3 | |
| | | ***Overall recovery (n = 16)*** | ***97*** | ***4.1*** | |
| bovine liver | 0.01 | 93 88 88 90 95 90 87 | 90 | 3.2 | 0.01 |
| | 0.05 | 98 100 93 | 97 | 3.7 | |
| | 0.10 | 91 91 92 | 91 | 0.6 | |
| | 4.0 | 97 98 95 | 97 | 1.6 | |
| | | ***Overall recovery (n = 16)*** | ***93*** | ***4.3*** | |
| bovine muscle | 0.01 | 93 100 106 95 101 101 99 | 99 | 4.3 | 0.01 |
| | 0.05 | 117 96 | 107 | | |
| | 0.10 | 96 101 94 | 97 | 3.7 | |
| | 2.0 | 92 92 92 | 92 | 0.0 | |
| | | ***Overall recovery (n = 15)*** | ***98*** | ***6.8*** | |
| bovine urine | 0.01 | 109 116 110 108 113 113 112 | 112[1] | 2.5 | 0.01 |
| | 0.10 | 107 105 105 | 106 | 1.1 | |
| | 40.0 | 110 106 105 | 107 | 2.5 | |
| | | ***Overall recovery (n = 13)*** | ***109*** | ***3.3*** | |

* fortified compound BYI 02960-OH determined as BYI 02960-OH expressed as BYI 02960

*Footnotes:*
1: accepted as RSD was < 20%

Table 4.3-20b: Recovery results from method validation of method RARVP040 – Recoveries and relative standard deviations (RSDs) for **BYI 02960-OH – confirmatory** transition (305/90)
*(Recoveries documented in the feeding study reports RARVP050 and RARVP041)*

| Sample material | FL* [mg/kg] | Individual values [%] | Mean value [%] | RSD [%] | LOQ [mg/kg] |
|---|---|---|---|---|---|
| bovine milk | 0.01 | 108 106 102 108 110 112 96 | 106 | 5.1 | 0.01 |
| | 0.10 | 111 108 102 | 107 | 4.3 | |
| | | ***Overall recovery (n = 10)*** | ***106*** | ***4.7*** | |
| bovine cream | 0.01 | 121 111 111 120 108 119 110 | 114[1] | 4.8 | 0.01 |
| | 0.10 | 110 110 114 | 111[1] | 2.1 | |
| | 1.0 | 114 107 110 | 110 | 3.2 | |
| | | ***Overall recovery (n = 13)*** | ***113***[1] | ***4.1*** | |
| bovine whey | 0.01 | 117 103 119 95 125 133 123 | 116[1] | 11.3 | 0.01 |
| | 0.10 | 118 111 116 | 115[1] | 3.1 | |
| | | ***Overall recovery (n = 10)*** | ***116***[1] | ***9.4*** | |
| bovine fat | 0.01 | 111 96 101 102 94 106 99 | 101 | 5.7 | 0.01 |
| | 0.10 | 97 103 103 | 101 | 3.4 | |
| | | ***Overall recovery (n = 10)*** | ***101*** | ***5.0*** | |
| bovine kidney | 0.01 | 93 92 93 97 92 83 92 | 92 | 4.6 | 0.01 |
| | 0.10 | 101 100 100 | 100 | 0.6 | |
| | | ***Overall recovery (n = 10)*** | ***94*** | ***5.7*** | |
| bovine liver | 0.01 | 91 92 92 97 93 85 93 | 92 | 3.9 | 0.01 |
| | 0.10 | 88 94 93 | 92 | 3.5 | |
| | | ***Overall recovery (n = 10)*** | ***92*** | ***3.6*** | |
| bovine muscle | 0.01 | 78 81 94 104 89 89 102 | 91 | 10.8 | 0.01 |
| | 0.10 | 95 107 92 | 98 | 8.1 | |
| | | ***Overall recovery (n = 10)*** | ***93*** | ***10.2*** | |
| poultry egg | 0.01 | 94 101 96 98 77 80 83 | 90 | 10.7 | 0.01 |
| | 4.0 | 95 80 84 | 86 | 9.0 | |
| | | ***Overall recovery (n = 10)*** | ***89*** | ***9.9*** | |
| poultry fat | 0.01 | 97 113 100 91 70 97 111 | 97 | 14.7 | 0.01 |
| | 4.0 | 109 106 105 | 107 | 2.0 | |
| | | ***Overall recovery (n = 10)*** | ***100*** | ***12.6*** | |
| poultry liver | 0.01 | 72 89 115 93 111 91 119 | 99 | 17.2 | 0.01 |
| | 4.0 | 100 102 97 | 100 | 2.5 | |
| | | ***Overall recovery (n = 10)*** | ***99*** | ***14.1*** | |

* fortified compound BYI 02960-OH determined as BYI 02960-OH expressed as BYI 02960

*Footnotes:*
1: accepted as RSD was < 20%

*Continued on next page…*

Table 4.3-20b (cont'd): Recovery results from method validation of method RARVP040 – Recoveries and relative standard deviations (RSDs) for **BYI 02960-OH – confirmatory** transition (305/90)
*(Recoveries documented in the feeding study reports RARVP050 and RARVP041)*

| Sample material | FL* [mg/kg] | Individual values [%] | Mean value [%] | RSD [%] | LOQ [mg/kg] |
|---|---|---|---|---|---|
| poultry muscle | 0.01 | 72 105 104 79 89 92 97 | 91 | 13.5 | 0.01 |
| | 4.0 | 104 109 103 | 105 | 3.1 | |
| | | ***Overall recovery (n = 10)*** | ***95*** | ***12.9*** | |
| poultry excreta | 0.01 | 111 96 99 109 74 76 90 | 94 | 15.6 | 0.01 |
| | 14.0 | 84 92 89 | 88 | 4.6 | |
| | | ***Overall recovery (n = 10)*** | ***92*** | ***13.4*** | |

* fortified compound BYI 02960-OH determined as BYI 02960-OH expressed as BYI 02960

## IIA 4.4 Description of methods for analysis of soil (parent and metabolites)

| | |
|---|---|
| **Report:** | **KIIA 4.4/01, Brumhard, B., Reineke, A.; 2009** |
| Title: | Analytical Method 01074 for the Determination of BYI 02960 in Soil using LC/MS/MS |
| Report No & Document No: | MR-07/337<br>M-337752-01-1 |
| **Guidelines:** | **EC Guidance Document on Residue Analytical Methods, SANCO/825/00 rev.7 of March 17, 2004; OPPTS 860.1340** |
| **GLP:** | **GLP** |

Report MR-07/337 provides a method description and validation data for the method 01074 that was developed for determination of BYI 02960 in soils by HPLC-MS/MS using two MRM transitions.
The method is the recommended enforcement method for monitoring of BYI08330 residues in soil.

**Principle of the Method:**
Soil samples of 20 g are extracted in a microwave extractor with 40 mL of a mixture of acetonitrile (1:4; v/v). Then a subsample is centrifuged to remove fine particles of the soil. An aliquot of the sample solution was injected into the high performance liquid chromatograph and subjected to reversed phase chromatography coupled with tandem mass spectrometry (MS/MS) with electrospray ionisation. The MS/MS instrument was operated in the Multiple Reaction Monitoring mode (MRM). The pseudomolecular ions of the analytes ([M+H]+) were selected by the first quadrupole. These precursor ions were impulsed with nitrogen in the collision cell (second quadrupole) and the resulting fragment ions (product ions) were separated according to their m/z ratio in the third quadrupole. Two of these product ions per analyte were selected: one product ion (MRM-transition) serving for quantitation and the second for confirmation. The first MRM transition of BYI 02960 is the quantification ion with the mass 126 [m/z 126] and the second MRM transition is the confirmatory ion with the mass 99 [m/z 99].
The method was validated using a silt soil (Höfchen) and a sandy loam soil (Laacher Hof). Two different soils were used in order to assess a possible influence of different soil characteristics.

Variations in equipment or sample characteristics and/or deterioration of system performance may require slight modifications in the chromatographic or detector conditions listed in order to obtain adequate chromatographic peak shapes or sensitivity. Instrument parameters and mobile phase may be adjusted to improve separation from unexpected interfering peaks. Therefore, the given LC/MS/MS parameters listed (see following table) may require adaptation.

The detection by MS/MS was performed on a triple-quadrupole tandem mass spectrometer, equipped with a Turbo IonSpray (ESI) interface operated in positive ion mode and multiple reaction monitoring (MRM). Optimal collisionally-activated dissociation (CAD) conditions for fragmentation of the pseudomolecular ions of the analyte was applied with nitrogen as the collision gas.

For calculation of the concentrations, calibration curves were used. These curves were calculated automatically after each sequence run with the Applied Biosystem quantitation software Analyst (Version 1.4.1) using linear regression. Further calculations were performed using the software EXCEL 2003 (Office 2003®).

| | |
|---|---|
| HPLC System | API 3000 or equivalent |
| Injector | PAL, CTC Analytics or equivalent |
| HPLC Column | Luna 2.5 µm, C18 (2), length 50 mm, i.d. 2 mm, Phenomenex or equivalent |
| Injection Volume | 10 µL or as needed for the sensitivity |
| HPLC Method | Bin Pump A: Water (0.9 L), methanol (0.1 L), formic acid (0.12 mL), ammonium formiate (10 mMol)<br>Bin Pump B: Water (0.1L) / methanol (0.9 L) / formic acid (0.12 mL) / ammonium formiate (10 mMol)<br>Iso Pump C: Methanol (1 L) /ammonium formiate (10 mMol)<br>Oven Temperature: 60°C<br>Flow rate (column): 0.4 mL/min<br>Flow rate (into MS): 0.4 mL/min<br>Gradient: |

| Time [min] | A [%, v/v] | B [%, v/v] | Into MS | Into Waste |
|---|---|---|---|---|
| 0.00 | 80 | 20 | Iso pump | Bin pump |
| 2.00 | 40 | 60 | Bin pump | Iso pump |
| 2.10 | 5.0 | 95 | Bin pump | Iso pump |
| 4.00 | 5.0 | 95 | Bin pump | Iso pump |
| 4.10 | 80 | 20 | Bin pump | Iso pump |
| 7.50 | 80 | 20 | Iso pump | Bin pump |
| 7.50 | Stop time | | | |

| | | | | | | |
|---|---|---|---|---|---|---|
| Detector | Triple Quadrupole Tandem Mass Spectrometer, Mass Spectrometer, API 3000 with Turbo-IonSpray (ESI), gas temperature 350 °C or as needed for the sensitivity; mass selective detector (MS/MS), Windows XP, Analyst 1.4.1 software versions or any equivalent HPLC-MS/MS System | | | | | |
| MS/MS operating parameters | | Precursor Ion Q1 Mass (amu) | Product Ion Q3 Mass (amu) | Dwell Time (msec) | Collision Energy (eV) | Polarity |
| | BYI 02960 Quantification | 289 | 126 | 250 | 35 | positive |
| | BYI 02960 Confirmatory | 289 | 99 | 250 | 71 | positive |
| Retention time | BYI 02960: approx. 2.0 min | | | | | |

Note: Different MS/MS-instruments may result in different MRM transitions or signal intensity.

**Selectivity**: The high selectivity of the method resulted from the HPLC separation in combination with MS/MS detection. Two MRM transitions were monitored for BYI 02960 (m/z 289 → 126 for quantitation and m/z 289 → 99 for confirmation). No signals / peaks interfering with the detection of the analyte were observed in extracts of untreated blank control specimens.

**Linearity**: For both mass transitions (m/z 289 → 126 and m/z 289 → 99) of BYI 02960 the correlation between the injected amount of substance and the detector response was linear for standards in matrix (both soil matrices: Höfchen and Laacher Hof) (1.0 µg/L to 100 µg/L) corresponding to a concentration in soil of 2 to 200 µg/kg. The correlation coefficients of the 1/x weighted linear regression ranged from 0.9960 to 0.9973.

**Untreated Control Samples**: Apparent residues in control samples were below 0.3 × LOQ (1.5 µg/kg) for BYI 02960.

**Recovery Rates (Accuracy) and Precision (Repeatability)**: Recovery rates were determined at fortification levels of 5 µg/kg (= LOQ level), and 50 µg/kg. The lowest fortification level experimentally providing a mean recovery between 70 and 110% with a relative standard deviation of ≤ 20% per definition is the Limit of Quantitation (LOQ), provided that the blank values were below 30% of this level. Results are presented in the following tables.
As a measure for the precision of the method, the intra-laboratory repeatability (n = 5) is given as relative standard deviation (% RSD) for all sample materials at fortification levels of 5 and 50 µg/kg. The RSD of the repeatability tests at each recovery set ranged from 1.3 to 3.8 for the quantifier mass transition and from 1.5 to 3.3 % for the confirmatory mass transition.

**Recoveries for BYI 02960 Quantifier Mass Transition (m/z 126) RSD: Relative Standard Deviation**

| Fortification [µg/kg] | Soil | Single values [%] | | | | | Mean [%] | RSD [%] |
|---|---|---|---|---|---|---|---|---|
| 5.0 | Höfchen | 94 | 92 | 92 | 92 | 90 | 92 | 1.3 |
| 5.0 | Laacher Hof | 102 | 100 | 99 | 97 | 96 | 99 | 2.3 |
| | | | | | | Mean of all 5.0 µg/kg single values | 95 | 4.1 |
| 50.0 | Höfchen | 90 | 88 | 85 | 85 | 85 | 86 | 2.5 |
| 50.0 | Laacher Hof | 100 | 94 | 93 | 94 | 91 | 94 | 3.8 |
| | | | | | | Mean of all 50.0 µg/kg single values | 90 | 5.6 |
| | | | | | | Mean of all Höfchen samples | 89 | 3.8 |
| | | | | | | Mean of all Laacher Hof samples | 97 | 3.7 |
| | | | | | | Overall mean | 93 | 5.5 |

**Recoveries for BYI 02960 Confirmatory Mass Transition (m/z 99) RSD: Relative Standard Deviation**

| Fortification [µg/kg] | Soil | Single values [%] | | | | | Mean [%] | RSD [%] |
|---|---|---|---|---|---|---|---|---|
| 5.0 | Höfchen | 93 | 93 | 91 | 94 | 91 | 92 | 1.5 |
| 5.0 | Laacher Hof | 100 | 99 | 98 | 101 | 94 | 98 | 2.8 |
| | | | | | | Mean of all 5.0 µg/kg single values | 95 | 4.0 |
| 50.0 | Höfchen | 91 | 90 | 88 | 87 | 87 | 88 | 1.9 |
| 50.0 | Laacher Hof | 98 | 95 | 92 | 94 | 90 | 94 | 3.3 |
| | | | | | | Mean of all 50.0 µg/kg single values | 91 | 4.0 |
| | | | | | | Mean of all Höfchen samples | 90 | 2.7 |
| | | | | | | Mean of all Laacher Hof samples | 96 | 3.9 |
| | | | | | | Overall mean | 93 | 4.5 |

**Limit of quantification (LOQ)**: The target limit of quantitation of the method is 5 µg/kg for BYI 02960. The target limit of detection of the method is 1.5 µg/kg for BYI 02960.

Remarks: The target LOQ is defined as the lowest fortification level experimentally providing a mean recovery between 70 and 110% with a relative standard deviation of ≤ 20%, provided that the blank values were below 30% of this level. The target LOD is defined as the lowest value of a compound showing a signal, which significantly differs from the blank values. In this particular case, the LOD was deduced from the measurements/chromatograms of the standard solution 0.75 µg/L corresponding to 1.5 µg/kg of BYI 02960. It could be shown that the chromatographic peaks of a solution representing 1.5 µg/kg of BYI 02960, were clearly detectable.

**Conclusions**:
The method 01074 (report MR-07/337) was developed for determination of BYI 02960 in soil with target limit of quantitation of 5 µg/kg for BYI 02960. All method validation data are in compliance with the guideline requirements for residue data generation and enforcement. Thus, the method is the recommended enforcement method for monitoring of BYI 02960 residues in soil

## IIA 4.5 Description of methods of analysis of water (parent and metabolites)

| | |
|---|---|
| **Report:** | **KIIA 4.5/01, Fargeix, G., Rosati, D.; 2012** |
| Title: | Analytical Method N°01213 for the Determination of Residues of BYI 02960 in Drinking and Surface Water by HPLC-MS/MS |
| Report No & Document No: | MR-12/022<br>M-428019-01-1 |
| **Guidelines:** | **EC Guidance Document on Residue Analytical Methods, SANCO/825/00 rev. 8 of June 30, 2010**<br>**US EPA Residue Chemistry Test Guideline OPPTS 860.1340** |
| **GLP:** | **GLP** |

Report MR-12/022 provides a method description and validation data for the method N 01213 that was developed for determination of BYI 02960 in drinking and surface water by HPLC-MS/MS using two MRM transitions.
The method is the recommended enforcement method for monitoring of BYI08330 residues in drinking and surface water.

**Principle of the Method:**
BYI 02960 is determined by direct injection of filtered aliquot of sample into the high performance liquid chromatograph and subjected to reversed phase chromatography coupled with tandem mass spectrometry (MS/MS) with electrospray ionization, using positive ion mode without further clean-up. Concentrations are quantified using external matrix-matched standard solutions or standards in solvent: it is advised to quantify against matrix matched standards.
The MS/MS instrument is operated in the Multiple Reaction Monitoring mode (MRM). The pseudomolecular ions of the analytes ($[M+H]^+$ or any adducts) were selected by the first quadrupole. These precursor ions were impulsed with nitrogen in the collision cell (second quadrupole), and the resulting fragment (product ions) are separated according to their m/z ratio in the third quadrupole. Two of these product ions per analyte were selected: one product ion (MRM-transition) for quantitation and the second for confirmation. The first MRM transition of BYI 02960 is the quantification ion with the mass 126 [m/z 126] and the second MRM transition is the confirmatory ion with the mass 90 [m/z 90].

Variations in equipment or sample characteristics and/or deterioration of system performance may require slight modifications in the chromatographic or detector conditions listed in order to obtain adequate chromatographic peak shapes or sensitivity. Instrument parameters and mobile phase may be adjusted to improve separation from unexpected interfering peaks. Therefore, the given LC/MS/MS parameters listed (see following table) may require adaptation. Before running a batch of analyses, it is necessary to sufficiently equilibrate the LC column for the analysis of this compound. It is advised to inject 5 calibration points for equilibrium time.

<table>
<tr><td>HPLC System</td><td colspan="6">Agilent 1100 or equivalent</td></tr>
<tr><td>Injector</td><td colspan="6">HTC PAL, CTC Analytics or equivalent</td></tr>
<tr><td>HPLC Column</td><td colspan="6">Ascentis Express C18 : 2.7 µm (supelco), ,50 x 2.1 mm or equivalent<br>Phenomenex C18 ODS 4 x 2.0mm pre-column or equivalent</td></tr>
<tr><td>Injection Volume</td><td colspan="6">100 µL or as needed for the sensitivity</td></tr>
<tr><td>HPLC Method</td><td colspan="6">Binary Pump 2 - Channel A : $H_2O$/MeOH/HCOOH (900/100/0.12 v/v/v) +10 mM ammonium formiate<br>Binary Pump 2 - Channel B : $H_2O$/MeOH/HCOOH (100/900/0.12 v/v/v) +10 mM ammonium formiate<br>Oven Temperature: at room temperature<br><br>
<table>
<tr><td>Gradient:</td><td>Time [min]</td><td>Flow [µL/min]</td><td>Channel A [%, v/v]</td><td>Channel B [%, v/v]</td></tr>
<tr><td></td><td>0.00</td><td>500</td><td>95</td><td>5</td></tr>
<tr><td></td><td>1.00</td><td>500</td><td>95</td><td>5</td></tr>
<tr><td></td><td>3.00</td><td>500</td><td>5</td><td>95</td></tr>
<tr><td></td><td>4.00</td><td>500</td><td>5</td><td>95</td></tr>
<tr><td></td><td>4.10</td><td>500</td><td>95</td><td>5</td></tr>
<tr><td></td><td>5.50</td><td>500</td><td>95</td><td>5</td></tr>
</table></td></tr>
<tr><td>Detector</td><td colspan="6">Triple Quadrupole Tandem Mass Spectrometer, AB Sciex API 4000, Windows XP, Analyst 1.4.1 software versions or any equivalent HPLC-MS/MS System</td></tr>
<tr><td>Interface</td><td colspan="6">Turbo IonSpray (ESI) – positive mode<br>Gas Temperature: 500 °C or as needed for the sensitivity</td></tr>
<tr><td>Scan Type</td><td colspan="6">MRM (Multiple Reaction Monitoring)</td></tr>
<tr><td>MS/MS operating parameters</td><td colspan="6">
<table>
<tr><td></td><td>Precursor Ion Q1 Mass (amu)</td><td>Product Ion Q3 Mass (amu)</td><td>Dwell Time (msec)</td><td>Collision Energy (eV)</td><td>Cell Exit Potential CXP (V)</td></tr>
<tr><td>BYI 02960 Quantification</td><td>289.1</td><td>126.0</td><td>150</td><td>30</td><td>6</td></tr>
<tr><td>BYI 02960 Confirmatory</td><td>289.1</td><td>90.0.</td><td>150</td><td>60</td><td>6</td></tr>
</table></td></tr>
<tr><td>Retention time</td><td colspan="6">BYI 02960: approx. 3.5 min</td></tr>
</table>

Note: Different MS/MS-instruments may result in different MRM transitions or signal intensity.

The analytical method was validated for surface water. A validation for drinking water was not necessary because the limit of quantitation for surface water is below the drinking water limit (i.e. < 0.1 µg/L). For method validation surface water from the river Rhine sampled at Leverkusen-Hitdorf was used. Characteristics of the test system are listed in following table.

**Characteristics of Surface Water from River Rhine, Sampled on 2007-07-29 at LEV-Hitdorf (GER)**

| Parameter | Value |
|---|---|
| Total organic carbon (TOC) | 2 mg/L |
| Dissolved organic carbon (DOC) | 2 mg/L |
| Conductivity | 448 µS/cm |
| pH | 7.3 |
| Water hardness | 9.9 °dH |
| Dry residue after filtration | 290 mg/L |

**Selectivity/Specificity**: The high selectivity of the method resulted from the HPLC separation in combination with MS/MS detection. Two MRM transitions were monitored for BYI 02960 (m/z 289 → 126 for quantitation and m/z 289 → 90 for confirmation). Therefore, the HPLC-MS/MS method is highly specific and an additional confirmatory method is not necessary. No signals / peaks interfering with the detection of the analyte were observed in extracts of untreated control samples. Apparent concentrations in control samples were below 0.3 × LOQ.

**Linearity**: The correlation between the injected amount of substance and the detector response was linear (1/x weighted) for aqueous standard solutions ranging from 0.01 to 20 µg/L. The correlation coefficients were > 0.9998 for both MRM transitions.

**Limit of Quantification (LOQ) and Limit of Detection (LOD)**: The LOQ for BYI 02960 is 0.05 µg/L (rounded value) in surface water, and the LOD is 0.02 µg/L.

**Repeatability (Precision)**: As a measure for the precision of the method, the intra-laboratory repeatability is given as relative standard deviation (% RSD) for surface water samples at fortification levels of 0.05 µg/L and 0.5 µg/L. The relative standard deviation for the peak area of the quantification MRM was 5.2% (0.05 µg/L) and 2.5% (0.5 µg/L).

**Recovery Rates (Accuracy)**: Because of the direct measurement of fortified samples without separate extraction and clean-up steps it is not possible to determine recovery rates in a classical way and therefore, an estimate of the accuracy of the analytical technique was made by an assessment of the linearity of matrix calibration and by determination of the repeatability of sample analysis.

However, for additional demonstration of the reliability of the method, the validation samples were evaluated like recovery rates. Mean recoveries for each fortification level and the overall mean recoveries were within the 70 - 110% range for both MRM transitions. The results are shown in the following tables. All method validation data are in compliance with the guideline requirements for enforcement methods

**Recoveries for BYI 02960 Quantifier Mass Transition (m/z 126); RSD = Relative Standard Deviation**

| Fortification [µg/L] | Matrix (measured by) | Single values [%] | | | | | Mean [%] | RSD [%] |
|---|---|---|---|---|---|---|---|---|
| 0.05 | Surface Water (matrix matched standards) | 82 | 88 | 87 | 80 | 79 | 83 | 4.7 |
| 0.5 | | 84 | 88 | 86 | 84 | 89 | 86 | 5.7 |
| Mean | | | | | Mean single values | | 85 | 4.1 |
| 0.05 | Surface Water (solvent standards) | 76 | 82 | 81 | 75 | 74 | 78 | 4.7 |
| 0.5 | | 81 | 84 | 83 | 73 | 77 | 80 | 5.7 |
| Mean | | | | | Mean single values | | 79 | 5.1 |

**Recoveries for BYI 02960 Confirmatory Mass Transition (m/z 90); RSD = Relative Standard Deviation**

| Fortification [µg/L] | Matrix (measured by) | Single values [%] | | | | | Mean [%] | RSD [%] |
|---|---|---|---|---|---|---|---|---|
| 0.05 | Surface Water (matrix matched standards) | 94 | 94 | 94 | 90 | 90 | 92 | 2.4 |
| 0.5 | | 81 | 82 | 79 | 84 | 84 | 82 | 2.6 |
| Mean | | | | | Mean single values | | 87 | 6.7 |
| 0.05 | Surface Water (solvent standards) | 78 | 78 | 78 | 74 | 74 | 76 | 2.9 |
| 0.5 | | 77 | 78 | 74 | 72 | 72 | 75 | 3.7 |
| Mean | | | | | Mean single values | | 76 | 3.4 |

**Matrix Effects**: The MS/MS detection of BYI 02960 was slightly affected by the matrix. Even if the results meet all guideline criteria on method validation, the method users are advice to use matrix matched standards because the recovery results are slightly closer to 100%, then.

**Storage Stability of the Analyte**: The analyte was stable in aqueous solution when stored in a freezer at T ≤ -18 °C for a period of 3 days.

**Conclusions**: All method validation data are in compliance with the guideline requirements for residue data generation and enforcement. Thus, the method is the recommended enforcement method for monitoring of BYI 02960 residues in drinking and surface water at 0.05 µg/L.

## IIA 4.6 Method for determining pesticides in sediment

Methods for analysis of sediments are not a specific data requirement according to European Regulation 1107/2009. Hence data/documents do not need to be submitted. However, in case residues of BYI 02960 must be determined in sediments, it is suggested to use and if necessary adapt the method developed for the determination of residues in soil (see report **KIIA 4.4/01**).

## IIA 4.7 Methods for analysis of air (parent and metabolites)

| | |
|---|---|
| **Report:** | **KIIA 4.7/01, Heinz, N.; 2011** |
| Title: | BYI 02960: Analytical Method for Determination in Air |
| Report No & Document No: | P 2419 G<br>M-420657-01-1 |
| **Guidelines:** | **EC Guidance document on residue analytical methods, SANCO/825/00 rev. 8.1, 16/11/2010** |
| **GLP:** | **GLP** |

Report P 2419 G provides a method description and validation data for the method that was developed for determination of BYI 02960 in air by HPLC-MS/MS using two parent-daughter ion transitions. The method achieves a limit of quantification (LOQ) of 7 µg/m$^3$. Method validation for the determination of BYI 02960 in warm (approx. 35 °C), humid air (relative humidity approx. 92 %) was performed at the LOQ and at 10-fold LOQ at approx. 70 µg/m$^3$.

**Principle of the Method:**
Air sampling uses adsorption tubes, e.g. flame sealed glass tubes (OD: 8 mm, L: 100 mm) filled with two layers (front layer A: 100 mg, mesh size 20 and rear layer B: 50 mg, mesh size 40) of porous polymer (XAD: ORBO$^{TM}$-44, Supelco Cat. No. 20260-U), held in place by three glass wool plugs. Particles and aerosols are trapped by filtration or impact onto the adsorbent material. Both ends of sealed cartridges were opened and the fortification solution (10 µL) was dosed directly onto the front layer XAD adsorbent material (layer A, 100 mg adsorption material). The solvent was allowed to evaporate.
The sampling cartridges (including two blank control cartridges) were placed on a SPE station with the fortified layer A at the upper end. A laboratory air pump was attached to the pressure gauge of the station with a tube providing an air flow of 0.83 L/min. The SPE station was placed in a chamber where the air could be heated with a hot air fan. For air sampling under warm and humid conditions, warm air (heated by the fan in the partially closed cabinet) was passed over warm water before diverting the humidified air flow over the sampling cartridges. The water load of the air was calculated from the water loss in the round bottomed flask. The total relative humidity of the air passing through the sampling cartridges was obtained as the sum of the original humidity measured with a humidity sensor at the air inlet, plus the humidity added by evaporation.
After sampling of air (6 hours, i.e. a total air sampling volume of approx. 0.3 m$^3$), the cartridges were sealed with the provided plastic plugs. If not analyzed immediately, the cartridges may be stored at freezer temperature for up to 5 days. Storage stability was demonstrated as part of this study by storing two fortified cartridges (at LOQ level) for 5 days in a freezer at approx. -20°C. All solutions were stored in a refrigerator (at approximately ≤ 8 °C) when not in use.
The adsorbent is extracted three times with approx. 3 mL of acetonitrile. The extracts are combined and the volume is adjusted to 10 mL. Then a 100-µL or 50-µL aliquot of the raw extract is further diluted into water with 0.1% formic acid resulting in a final volume of 1.0 mL.

The analyte is determined by liquid chromatography with tandem mass spectrometric detection (LC-MS/MS), monitoring two parent-daughter ion transitions. The pseudomolecular ions of the analytes ([M+H]$^+$) were selected by the first quadrupole. These precursor ions were impulsed with nitrogen in the collision cell (second quadrupole) and the resulting fragment ions (product ions) were separated according to their m/z ratio in the third quadrupole. Two of these product ions per analyte were

selected: one product ion (MRM-transition) serving for quantitation and the second for confirmation. The first MRM transition of BYI 02960 is the quantification ion with the mass 126 [m/z 126] and the second MRM transition is the confirmatory ion with the mass 99 [m/z 99]. These parameters are given only as guidance and were established for the specimens of the present study, using the mentioned apparatus and analytical column.

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| HPLC System/ Detector | Agilent 1100 Series HPLC system (vacuum solvent degasser, binary HPLC pump, column oven), and CTC Analytics HTC-Pal autosampler.<br>Applied Biosystems MDS Sciex API 3000 triple quadrupole LC-MS/MS system with TurboIonspray ESI source. Analyst 1.4.2 Instrument control and data acquisition software, or equivalent | | | | | | | |
| HPLC Column | Supelco Ascentis Express C18, 50 mm length x 2.1 mm i.d.; 2.7 micron particle size with pre-column Phenomenex C18, 4 mm x 3 mm, or equivalent | | | | | | | |
| Injection Volume | 50 µL | | | | | | | |
| Mobile Phase | A –0.1 % formic acid in water with 10 mmol ammonium formiate<br>B –0.1 % formic acid in methanol with 10 mmol ammonium formiate | | | | | | | |
| HPLC Method | Oven Temperature: 60°C<br>Flow rate (column): 0.6 mL/min<br>Gradient: | | | | | | | |
| | Time [min] | A [%, v/v] | B [%, v/v] | | | | | |
| | 0.0 | 90 | 10 | | | | | |
| | 1.0 | 90 | 10 | | | | | |
| | 2.0 | 10 | 90 | | | | | |
| | 3.0 | 10 | 90 | | | | | |
| | 3.1 | 90 | 10 | | | | | |
| | 5.0 | 90 | 10 | | | | | |
| Interface | TurboIonSpray | | | | | | | |
| Polarity | Positive | | | | | | | |
| Scan Type | MRM | | | | | | | |
| Resolution | Q1 – unit, Q3 – unit | | | | | | | |
| Curtain gas (CUR) | 9 | | | | | | | |
| Collision gas (CAD) | 2.0 | | | | | | | |
| Temperature (TEM) | 480 °C | | | | | | | |
| Nebulizer Gas (NEB): | 12 | | | | | | | |
| IonSpray Voltage (IS): | 4500 V | | | | | | | |
| MS/MS operating parameters | Compound | Q1 Mass (amu) | Q2 Mass (amu) | Dwell Time (msec) | DP, V | FP, V | CE, V | CXP, V |
| | BYI 02960 Quantification | 290 | 126 | 500 | 61 | 270 | 29 | 8 |
| | BYI 02960 Confirmatory | 290 | 99 | 500 | 61 | 270 | 67 | 18 |
| Retention time | BYI 02960 approx. 3.2 min | | | | | | | |

Note: Different MS/MS-instruments may result in different MRM transitions or signal intensity.

Repeatability of HPLC-MS/MS determination was demonstrated by duplicate injection of selected fortified specimen extracts.

Linear LC-MS/MS calibration functions were established by injecting standard solutions (50 µL) and using both, the 126 m/z and the 99 m/z daughter ion peak areas for separate quantification/ confirmation. Calibration levels ranged from, e.g. 2.0 ng to 200 ng/mL. The concentration of BYI 02960 in the extracts from the control and recovery specimens was evaluated by external calibration, employing the LC/MS software.

Extraction efficiency was examined by fortifying the analyte (duplicates fortified at 2 µg) onto adsorbent portions of sampling cartridges. Subsequently the analyte was extracted as described above and analysed by LC-MS/MS. After sampling (approximately 6 hours) the XAD layer A was analysed for recovery. For all cartridges fortified at the higher level the rear layer B was analysed for breakthrough determination.

Storage stability of adsorbed BYI 02960 was examined by fortifying the analyte onto adsorbent layers of sampling cartridges (duplicates fortified at 2 µg). The solvent was allowed to evaporate. The cartridges were sealed and stored for a 5-day period at freezer temperature (approximately -19 to -23 °C). After storage the adsorbent layers A and B were extracted combined as described above and analysed by LC-MS/MS.

The analytical method was validated for the determination of BYI 02960 in warm (approx. 35 °C), humid air (relative humidity approx. 92%). For method validation, adsorption tubes were fortified with BYI 02960 using a syringe. Exactly 10 µL of the 0.20 µg/µL fortification solution (2.0 µg fortified at LOQ) or 10 µL of the 2.0 µg/µL stock solution (20 µg fortified at 10xLOQ) were dosed onto the adsorption material layer A. Five replicates were analysed per fortification level.

**Selectivity**: HPLC-MS/MS, monitoring two parent to daughter ion transitions, for BYI 02960 (m/z 289 → 126 for quantitation and m/z 289 → 99 for confirmation), is considered to be highly selective, thus not requiring further confirmation of detected residues. It does not result in mass spectra when applying full scan mass spectrometry, but in daughter fragment ion chromatograms which are highly selective, and, with a chromatographic peak present at the correct HPLC retention time, which is considered highly selective for results ≥ LOQ.

**Linearity and Sensitivity**: For both mass transitions (m/z 289 → 126 and m/z 289 → 99) of BYI 02960 the correlation between the injected amount of substance and the detector response was linear for standards in matrix (levels of calibration ranged from 2.0 to 200 ng/mL). The correlation coefficients of the 1/x weighted linear regression were > 0.999.
Based on the detectability of 2.0 ng/mL BYI 02960 in the final extracts, assuming a theoretical final volume of 100 mL and an air sample volume of approx. 0.3 $m^3$, the limit of detection (LOD) is estimated to be <0.7 µg/$m^3$.

**Untreated Control Samples**: The chromatograms of the control specimens showed no signals (<0.7 µg/$m^3$) at the retention time of BYI 02960.

**Retention Efficiency (Recovery) and Precision (Repeatability)**: The results of five replicates per fortification level showed that the average recoveries for the analyte fortification levels and MS/MS transitions after air sampling ranged between 107 to 109%, the relative standard deviations were always ≤ 2%. No breakthrough into the back layer of the adsorption tubes was observed (resp. values were < 1%). The results of validation are summarised below. Extraction efficiency was demonstrated with average recoveries of 103 and 105%, the recoveries from storage stability tests were in the range of 101 to 105%.

**Recovery Results: Extraction Efficiency and Storage Stability**

| Specimen Type | Fortified BYI 02960 µg | 290 m/z --> 126 m/z | | 290 m/z --> 99 m/z | |
|---|---|---|---|---|---|
| | | Recovery | Mean * | Recovery | Mean * |
| Extraction Efficiency | 2.0 | 101%<br>109% | 105% | 99%<br>108% | 103% |
| Stability of Extracts Refrigerated for 6 days at < 8 °C | 2.0 | 105%<br>105% | 105% | 105%<br>103% | 104% |
| Storage Stability of Adsorbed BYI 02960 for 5 days at Freezer Temperature: -19 to -23 °C | 2.0 | 100%<br>103% | 101% | 100%<br>102% | 101 |

*: each two specimens included in calculation

**Recovery Results: Retention Recoveries**

| Specimen Type | Fortified BYI 02960 µg | Average $C_{Air}$ µg / $m^3$ | 290 m/z --> 126 m/z | | 290 m/z --> 99 m/z | |
|---|---|---|---|---|---|---|
| | | | Average Recovery | Mean* (RSD) | Average Recovery | Mean* (RSD) |
| Warm Humid Air (temp. 35 °C, relative humidity 92%) | 2.0 | 6.7 (at LOQ) | 109%<br>109%<br>110%<br>110%<br>110% | 109% (0.3%) | 106%<br>107%<br>109%<br>109%<br>109% | 108% (1%) |
| | 20 | 67 (at 10xLOQ) | 107%<br>105%<br>110%<br>107%<br>104% | 107% (2%) | 108%<br>105%<br>109%<br>106%<br>106% | 107% (1%) |

*: each five specimens included in calculation
Average $C_{Air}$: Average fortified concentration of BYI 02960 in air.

**Limit of quantification (LOQ)**: The method achieves a limit of quantification (LOQ) of 7 µg BYI 02960/$m^3$ air. Based on the detectability of 2.0 ng BYI 02960/mL and assuming a theoretical final volume of 100 mL and an air sample volume of approx. 0.3 $m^3$, the limit of detection (LOD) in the final extracts is estimated to be <0.7 µg/$m^3$.

**Conclusions**:
The LC-MS/MS based analytical method (report no. P 2419 G) was developed for determination of BYI 02960 in air with target limit of quantitation of 7 µg BYI 02960/$m^3$. All method validation data were in compliance with the guideline requirements for residue data generation and enforcement. Thus, the method is the recommended enforcement method for monitoring of BYI 02960 residues in air.

## IIA 4.8 Methods for analysis of body fluid/tissues (parent and metabolites)

A method of analysis for body fluids and tissues is not required, since the active substance is of low acute toxicity (GHS category 4). Please refer to KIIA, sect. 3, point 5.2.

## IIA 4.9 Other/special studies

No other or special studies have been conducted.