Application Note

Food

# Quantitation of Pesticide Residues in Milk Using the Agilent 6470 Triple Quadrupole LC/MS

## Analysis of pesticide residues in milk with reporting of lipophilic pesticides based on milk fat percentage

**Figure 1.** Agilent 1290 Infinity II LC coupled to an Agilent 6470 triple quadrupole LC/MS.


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## Abstract

Pesticides are used to support the growth of food crops, control vector-borne diseases, or to control animal parasites. Such pesticides may transfer to livestock animals primarily through feed and fodder. After ingestion, some pesticides are metabolized and deposited throughout the fat and muscle tissue of the animal's body. Milk from these animals contains a considerable amount of fat. Many regulatory agencies specify the maximum residue limits (MRLs) for pesticides based on fat (%) in milk and milk-based products. To detect the MRLs required in milk and milk-based products, the instrument must deliver good sensitivity for analytes in matrix. This application note provides a method for the analysis of pesticide residue based on fat percentage in milk samples. Additionally, this application note describes a detailed methodology for the quantitation of pesticide residues carbofuran, carbendazim, benomyl, edifenphos, and 2,4-D in milk matrix using LC/MS/MS. Quantitation of pesticides in milk is based on QuEChERS extraction, followed by instrument analysis without adopting extensive cleanup procedures.

# Introduction

Pesticides used in agriculture may enter an animal's body through feed and drinking water. Some of these pesticides are fat soluble and deposit into the animal's fat and muscle tissue. Milk contains a considerable amount of fat; therefore, regulatory agencies mention a maximum residue limit (MRL) for lipophilic pesticides, based on milk fat – for safe consumption.

QuEChERS methodology has been a widely accepted method to extract pesticide residues from various food commodities such as fruits and vegetables. For milk, a QuEChERS-based extraction of pesticide residues was found to be a straightforward sample preparation to adopt. For detection and quantitation, a highly selective multiple reaction monitoring (MRM)-based LC/MS/MS method was developed using the Agilent 6470 triple quadrupole LC/MS (LC/TQ). The sensitivity of the 6470 LC/TQ can provide analysis of milk samples at the required limits of detection, and report the results based on milk fat percentage.

# Experimental

## Chemicals and reagents

LC/MS pesticide standards were purchased from Sigma-Aldrich. These standards were used for method development and analysis of milk samples. Agilent Bond Elut QuEChERS extraction kit (part number 5982-5650) was used for sample preparation. LC/MS-grade solvents such as acetonitrile and water were purchased from Honeywell (Charlotte, NC, USA). Acetic acid of MS grade was purchased from Fluka (now of Honeywell).

## Instrument configuration

- Agilent 1290 Infinity II high-speed pump (G7120A)
- Agilent 1290 Infinity II multisampler (G7167B)
- Agilent 1290 Infinity II multicolumn thermostat (G7116B)
- Agilent 6470 triple quadrupole LC/MS (G6470B)

## Data acquisition and data analysis

All samples were acquired using the Agilent MassHunter Data Acquisition software version 10.1. Chromatograms were reviewed through MassHunter qualitative analysis software version 10.0. Quantitation of each batch was carried out using MassHunter quantitative analysis software version 10.1.

## Sample preparation

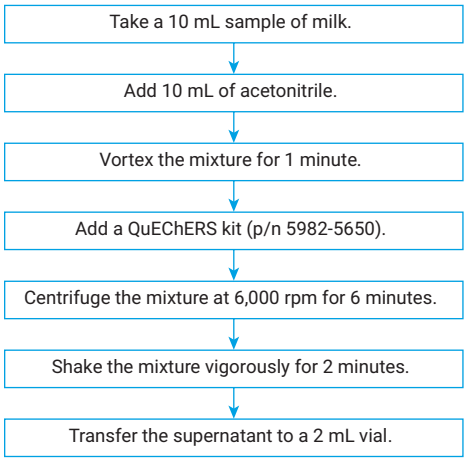


**Figure 2.** Flowchart for sample preparation.

**Table 1.** Chromatography conditions.

| Parameter | Value |
|---|---|
| Mobile Phase A | 0.1% acetic acid in water |
| Mobile Phase B | Acetonitrile |
| Flow Rate | 0.3 mL/min |
| Injection Volume | 5 µL |
| Column Temperature | 40 °C |
| Sample Diluent | Acetonitrile/water (60/40) |
| Needle Wash | MeOH/acetonitrile/water (25/50/25) |
| Column | Agilent XDB C18, 3.0 × 150 mm, 3.5 µm (p/n 963954-302) |

**Table 2.** Gradient.

| Time (min) | %A | %B |
|---|---|---|
| 0 | 95 | 5 |
| 1.5 | 95 | 5 |
| 4 | 50 | 50 |
| 9 | 0 | 80 |
| 12 | 0 | 80 |
| Post Run | 2 minutes | |

**Table 3.** MS source parameters.

| Ionization Source | AJS ESI |
|---|---|
| Ionization Mode | ESI Positive |
| Gas Temperature | 200 °C |
| Gas Flow | 12 L/min |
| Nebulizer | 35 psi |
| Sheath Gas | 390 °C |
| Sheath Gas Flow | 11.5 L/min |
| Capillary Voltage | 2,000 V |
| Nozzle Voltage | 2,000 V |

**Table 4.** MRM parameters.

| Compound ID | Precursor Ion (*m/z*) | Product Ion (*m/z*) | Frag. | CE | CAV | Ionization |
|---|---|---|---|---|---|---|
| Edifenphos | 311 | 111 | 88 | 24 | 4 | ESI Positive |
| | 311 | 109 | 88 | 40 | 4 | ESI Positive |
| Carbofuran | 222 | 165.1 | 84 | 12 | 4 | ESI Positive |
| | 222 | 123 | 84 | 24 | 4 | ESI Positive |
| Carbendazim | 192 | 160 | 78 | 20 | 4 | ESI Positive |
| | 192 | 132 | 78 | 36 | 4 | ESI Positive |
| 2,4-D | 221 | 163 | 56 | 12 | 4 | ESI Negative |
| | 219 | 161 | 56 | 12 | 4 | ESI Negative |

**Table 5.** Dilution chart for prespike calibration curve.

| Working Standard Concentration | Volume Taken (µL) | Volume of Milk (mL) | Obtained Concentration (ng/mL) |
|---|---|---|---|
| 100 ppb | 40 | 10 | 0.4 |
| 100 ppb | 100 | 10 | 1 |
| 100 ppb | 200 | 10 | 2 |
| 1 ppm | 50 | 10 | 5 |
| 1 ppm | 100 | 10 | 10 |
| 1 ppm | 200 | 10 | 20 |
| 10 ppm | 50 | 10 | 50 |
| 10 ppm | 75 | 10 | 75 |
| 10 ppm | 100 | 10 | 100 |
| 10 ppm | 200 | 10 | 200 |

## Results and discussion

Individual stock solutions were made and mixed to appropriate volumes of stock solutions to make working standards. Working standards prepared were of concentrations 1 ppm, 10 ppm, and 100 ppb. Individual volumes of working standards were spiked in 10 mL of milk samples, then followed the sample preparation flowchart to generate matrix-based calibration (prespike) points.

Matrix-based calibration curves (Figure 4) were found to be linear for carbofuran and edifenphos from 0.4 ng/mL to 100 ng/mL with both linear regression and 1/x. A linear regression line was plotted for carbendazim and 2,4-D with $1/x^2$ weighing. Regression coefficient values were above 0.995 for all four compounds. Benomyl was found to be very unstable and converted to carbendazim, therefore, no calibration curve or measurement was made. In this case, benomyl was reported as carbendazim.

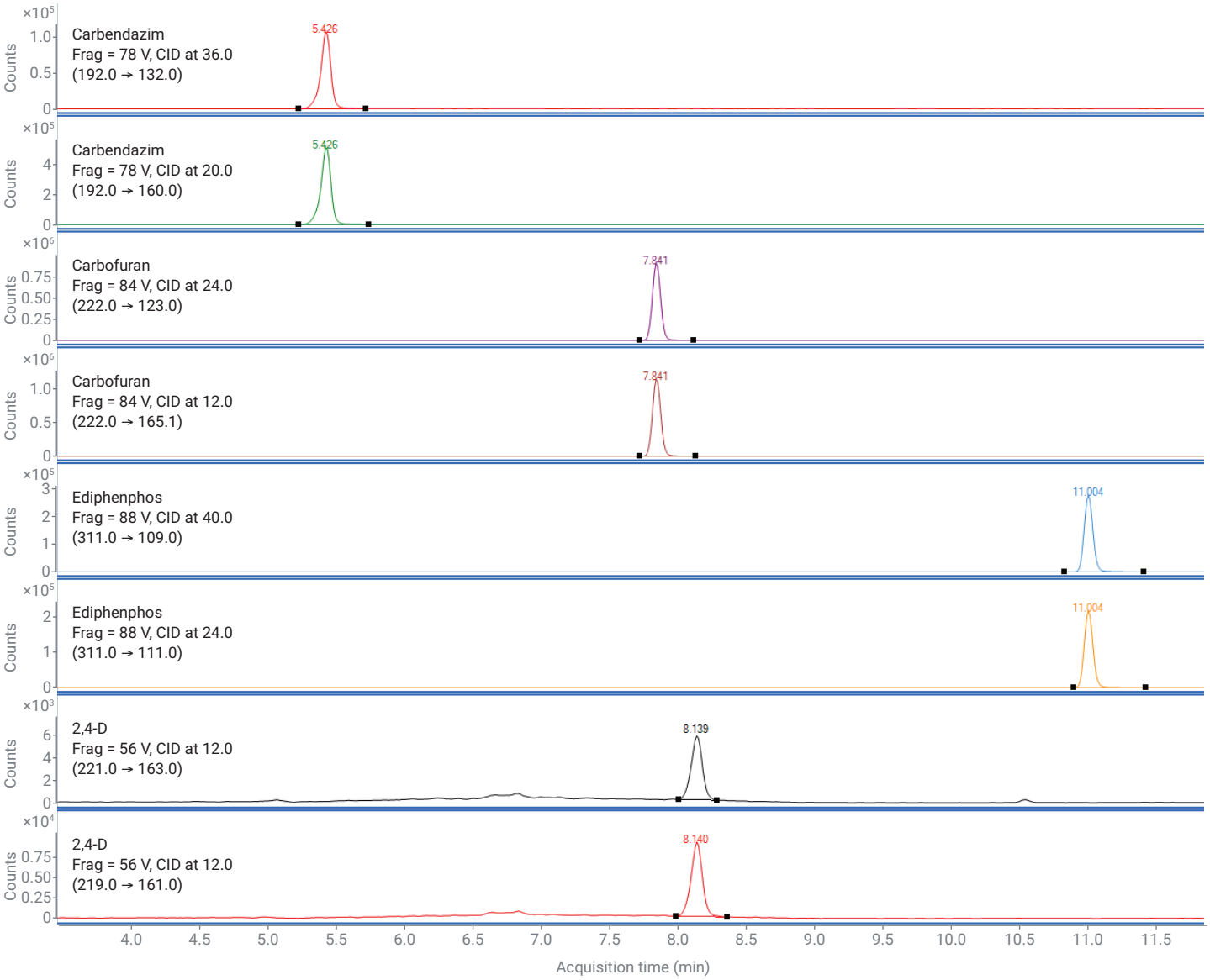


**Figure 3.** Extracted ion chromatogram (EIC) at 10 ng/mL prespiked in milk matrix.

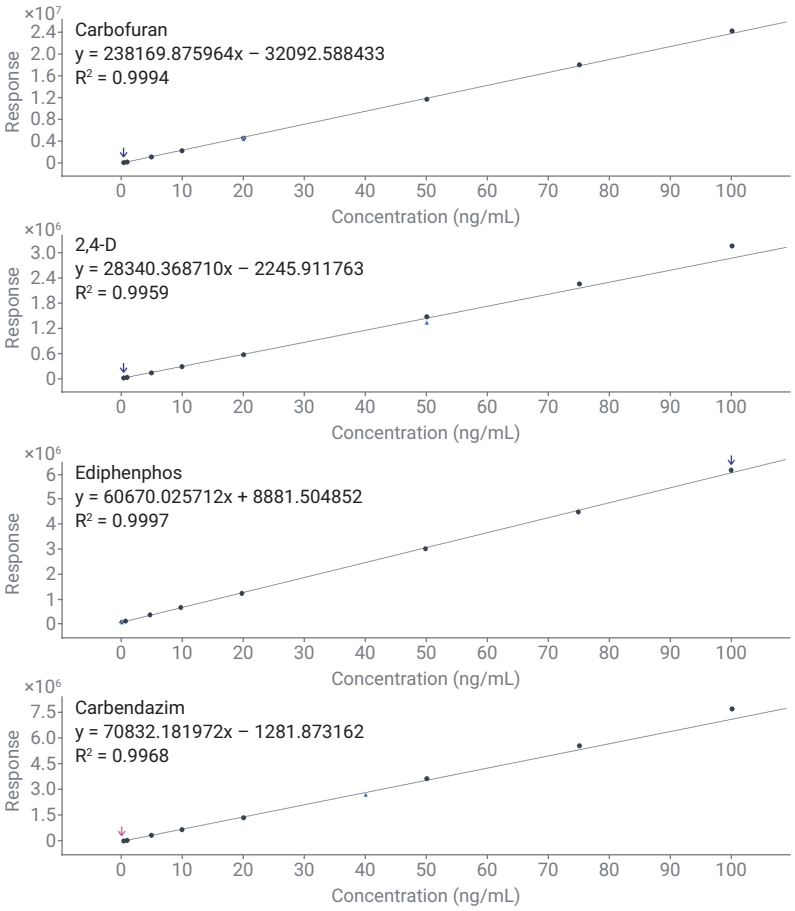


**Figure 4.** Matrix-based calibration curves from 0.4 ng/mL to 100 ng/mL.

Spiking was made per the maximum residue limits mentioned in BIS (Bureau of Indian Standards) regulation documents. Recovery percentage was calculated based on this experiment.

Spiking was made for edifenphos using the maximum residue limit of 10 ppb fat

- This analysis considered milk fat percentage to be 4%.
- Fat level in milk: 4 g in 100 mL
- Sample volume: 10 mL
- Fat (g) in 10 mL: 0.4 g
- To get a spike level of 10 ppb (fat basis), a spike concentration of 4 ng in 10 mL milk sample is needed.
- The calculation is as follows: 4 ng/10 mL = 4 ng/0.4 g fat = 10 ng/g fat = 10 ppb fat.
- To spike 4 ng in 10 mL, 40 µL of 100 ppb in 10 mL milk must be spiked.
- A spike sample at the 0.4 ppb level showed a recovery of more than 84%.

Batch Table

Sample: MB 100 PPB  Sample Type: <All>  Compound: Ediphenphos  ISTD:

| Sample | | | | | | Ediphenp... | Ediphenphos Results | | | | | Qualifi... |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Name | Data File | Type | Level | Exp. Conc. | RT | Resp. | MI | Final Conc. | Accuracy | Ratio |
| | | MB BLANK | MB BLANK-r003... | Sample | | | 10.992 | 1896 | | 0.0000 | | 14.2 |
| | | MB 0.4 PPB | MB 0.4 PPB.d | Cal | 1 | 0.4000 | 10.981 | 35672 | | 0.4416 | 110.4 | 62.1 |
| | | MB 1 PPB | MB 1.0 PPB.d | Cal | 2 | 1.0000 | 10.969 | 71705 | | 1.0355 | 103.5 | 63.4 |
| | | MB 5 PPB | MB 5.0 PPB.d | Cal | 3 | 5.0000 | 10.957 | 319565 | | 5.1209 | 102.4 | 74.2 |
| | | MB 10 PPB | MB 10.0 PPB.d | Cal | 4 | 10.0000 | 10.957 | 635246 | | 10.3241 | 103.2 | 75.1 |
| | | MB 20 PPB | MB 20.0 PPB.d | Cal | 5 | 20.0000 | 10.957 | 1201605 | | 19.6592 | 98.3 | 77.6 |
| | | MB 50 PPB | MB 50.0 PPB.d | Cal | 6 | 50.0000 | 10.957 | 2998108 | | 49.2702 | 98.5 | 77.9 |
| | | MB 75 PPB | MB 75.0 PPB.d | Cal | 7 | 75.0000 | 10.969 | 4489234 | | 73.8479 | 98.5 | 78.6 |
| | | MB 100 PPB | MB 100.0 PPB.d | Cal | 8 | 100.0000 | 10.969 | 6183108 | | 101.7673 | 101.8 | 78.2 |
| | | MB SPIKE | MB SPIKE.d | QC | 9 | 0.4000 | 10.969 | 29451 | | 0.3390 | 84.8 | 63.3 |

**Figure 5.** Edifenphos results using the maximum residue limit of 10 ppb fat.

Spiking was made for carbofuran at the maximum residue limit of 50 ppb fat

- To get a spike level of 50 ppb (fat basis), a spike concentration of 20 ng in 10 mL milk sample is needed.
- The calculation is as follows: 20 ng/10 mL = 20 ng/0.4 g fat = 50 ng/g fat = 50 ppb fat.
- To spike 20 ng in 10 mL, 200 µL of 100 ppb in 10 mL milk must be spiked.
- A spike sample at the 20 ppb level showed a recovery of more than 92%.

Spiking was made for carbendazim at the maximum residue limit of 100 ppb fat

- To get a spike level of 100 ppb (fat basis), a spike concentration of 40 ng in 10 mL milk sample is needed.
- The calculation is as follows: 40 ng/10 mL = 40 ng/0.4 g fat = 100 ng/g fat = 100 ppb fat.
- To spike 40 ng in 10 mL, 40 µL of 1 ppm in 10 mL milk must be spiked.
- A spike sample at the 40 ppb level showed a recovery of more than 95%.

Spiking was made for 2,4-D at the maximum residue limit of 50 ppb

- To get a spike level of 50 ppb, a spike concentration of 50 ng in 1 mL milk sample is needed.
- The calculation is as follows: 500 ng/10 mL = 50 ppb.
- To spike 500 ng in 10 mL, 50 µL of 10 ppm in 10 mL milk must be spiked.
- A spike sample at the 50 ppb level showed a recovery of more than 92%.

Batch Table

Sample: MB 100 PPB  Sample Type: <All>  Compound: Carbofuran  ISTD:

| Sample | | | | | Carbofur... | Carbofuran Results | | | | Qualifi... |
|---|---|---|---|---|---|---|---|---|---|---|
| Name | Data File | Type | Level | Exp. Conc. | RT | Resp. | MI | Final Conc. | Accuracy | Ratio |
| MB BLANK | MB_BLANK-r003... | Sample | | | 7.806 | 229 | | 0.1357 | | |
| MB 0.4 PPB | MB 0.4 PPB.d | Cal | 1 | 0.4000 | 7.818 | 77814 | | 0.4615 | 115.4 | 75.2 |
| MB 1 PPB | MB 1.0 PPB.d | Cal | 2 | 1.0000 | 7.818 | 205061 | | 0.9957 | 99.6 | 77.8 |
| MB 5 PPB | MB 5.0 PPB.d | Cal | 3 | 5.0000 | 7.818 | 1088528 | | 4.7051 | 94.1 | 76.8 |
| MB 10 PPB | MB 10.0 PPB.d | Cal | 4 | 10.0000 | 7.818 | 2213712 | | 9.4294 | 94.3 | 78.5 |
| MB 20 PPB | MB 20.0 PPB.d | Cal | 5 | 20.0000 | 7.818 | 4522421 | | 19.1230 | 95.6 | 78.7 |
| MB 50 PPB | MB 50.0 PPB.d | Cal | 6 | 50.0000 | 7.818 | 11654697 | | 49.0691 | 98.1 | 79.5 |
| MB 75 PPB | MB 75.0 PPB.d | Cal | 7 | 75.0000 | 7.818 | 18041329 | | 75.8846 | 101.2 | 78.2 |
| MB 100 PPB | MB 100.0 PPB.d | Cal | 8 | 100.0000 | 7.818 | 24197302 | | 101.7316 | 101.7 | 80.3 |
| MB SPIKE | MB SPIKE.d | QC | 9 | 20.0000 | 7.818 | 4387832 | | 18.5579 | 92.8 | 80.0 |

**Figure 6.** Carbofuran results using the maximum residue limit of 50 ppb fat.

Batch Table

Sample: MB 100 PPB  Sample Type: <All>  Compound: Carbendazim  ISTD:

| Sample | | | | Carbenda... | Carbendazim Results | | | | Qualifi... |
|---|---|---|---|---|---|---|---|---|---|
| Name | Data File | Type | Level | Exp. Conc. | RT | Resp. | MI | Final Conc. | Accuracy | Ratio |
| MB BLANK | MB_BLANK-r003... | Sample | | | 5.414 | 4606 | | 0.0831 | | 24.9 |
| MB 0.4 PPB | MB 0.4 PPB.d | Cal | 1 | 0.4000 | 5.414 | 27874 | | 0.4116 | 102.9 | 23.1 |
| MB 1 PPB | MB 1.0 PPB.d | Cal | 2 | 1.0000 | 5.414 | 65455 | | 0.9422 | 94.2 | 22.1 |
| MB 5 PPB | MB 5.0 PPB.d | Cal | 3 | 5.0000 | 5.414 | 332331 | | 4.7099 | 94.2 | 19.7 |
| MB 10 PPB | MB 10.0 PPB.d | Cal | 4 | 10.0000 | 5.414 | 680043 | | 9.6189 | 96.2 | 19.8 |
| MB 20 PPB | MB 20.0 PPB.d | Cal | 5 | 20.0000 | 5.414 | 1379767 | | 19.4975 | 97.5 | 20.3 |
| MB 50 PPB | MB 50.0 PPB.d | Cal | 6 | 50.0000 | 5.402 | 3629817 | | 51.2634 | 102.5 | 20.8 |
| MB 75 PPB | MB 75.0 PPB.d | Cal | 7 | 75.0000 | 5.414 | 5540561 | | 78.2391 | 104.3 | 21.1 |
| MB 100 PPB | MB 100.0 PPB.d | Cal | 8 | 100.0000 | 5.414 | 7659812 | | 108.1584 | 108.2 | 21.0 |
| MB SPIKE | MB SPIKE.d | QC | 9 | 40.0000 | 5.414 | 2718107 | | 38.3920 | 96.0 | 20.8 |

**Figure 7.** Carbendazim results using the maximum residue limit of 100 ppb fat.

Batch Table

Sample: MB 100 PPB  Sample Type: <All>  Compound: 2,4-D  ISTD:

| Sample | | | | 2,4-D Met... | 2,4-D Results | | | | Qualifi... |
|---|---|---|---|---|---|---|---|---|---|
| Name | Data File | Type | Level | Exp. Conc. | RT | Resp. | MI | Final Conc. | Accuracy | Ratio |
| MB BLANK | MB_BLANK-r003... | Sample | | | 8.140 | 54 | | 0.0811 | | |
| MB 0.4 PPB | MB 0.4 PPB.d | Cal | 1 | 0.4000 | 8.187 | 9467 | | 0.4133 | 103.3 | 65.0 |
| MB 1 PPB | MB 1.0 PPB.d | Cal | 2 | 1.0000 | 8.187 | 24200 | | 0.9331 | 93.3 | 60.8 |
| MB 5 PPB | MB 5.0 PPB.d | Cal | 3 | 5.0000 | 8.187 | 130273 | | 4.6760 | 93.5 | 62.4 |
| MB 10 PPB | MB 10.0 PPB.d | Cal | 4 | 10.0000 | 8.175 | 269695 | | 9.5955 | 96.0 | 61.1 |
| MB 20 PPB | MB 20.0 PPB.d | Cal | 5 | 20.0000 | 8.175 | 549477 | | 19.4677 | 97.3 | 62.1 |
| MB 50 PPB | MB 50.0 PPB.d | Cal | 6 | 50.0000 | 8.175 | 1447667 | | 51.1607 | 102.3 | 62.5 |
| MB 75 PPB | MB 75.0 PPB.d | Cal | 7 | 75.0000 | 8.175 | 2216920 | | 78.3041 | 104.4 | 62.7 |
| MB 100 PPB | MB 100.0 PPB.d | Cal | 8 | 100.0000 | 8.175 | 3110061 | | 109.8188 | 109.8 | 62.2 |
| MB SPIKE | MB SPIKE.d | QC | 9 | 50.0000 | 8.175 | 1316350 | | 46.5271 | 93.1 | 63.1 |

**Figure 8.** 2,4-D results using the maximum residue limit of 50 ppb.

A matrix-based standard, prepared by prespiking the pesticide standards at the 10 ppb level in milk matrix, was injected with six replicates. Consistency of the chromatogram with respect to the response and retention time demonstrates the repeatability of the instrument result.

## Conclusion

A highly sensitive and robust polarity-switching method based on Multiple Reaction Monitoring was developed to quantify pesticide residues in milk matrix.

Method performance was evaluated for sensitivity, specificity, linearity, reproducibility, and recovery. This note describes the reporting of pesticides based on the percentage of milk fat. This method can be adopted for routine pesticide quantitation in milk samples.

## References


1. Comprehensive LC/MS/MS Workflow of Pesticide Residues in Food Using the Agilent 6470 Triple Quadrupole LC/MS System. *Agilent Technologies application note*, publication number 5994-2370EN, **2020**.
2. Bedi, J. S. *et al.* Pesticide residues in milk and their relationship with pesticide contamination of feedstuffs supplied to dairy cattle in Punjab (India). *Journal of Animal and Feed Sciences* **2018**, *27*, 18–25.


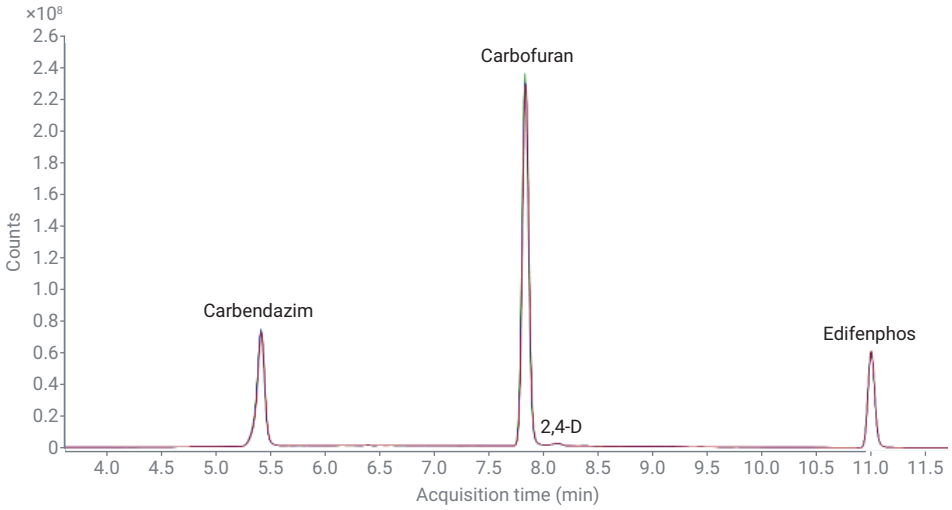


**Figure 9.** Overlaid chromatograms of six replicate injections at 10 ppb concentration spiked in milk matrix.

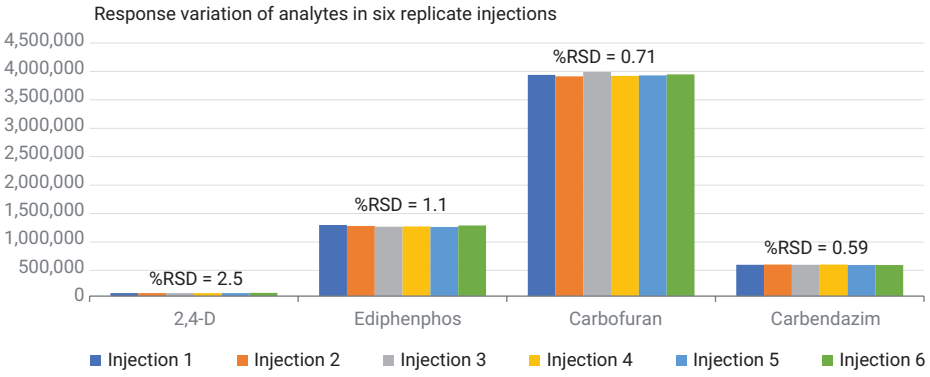


**Figure 10.** Repeatability in area response for six replicate injections at 10 ppb in milk matrix.

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