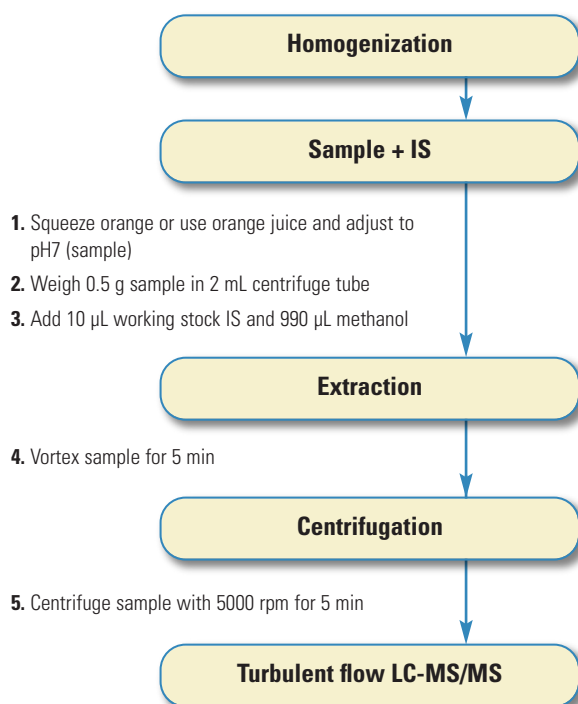# Determination of Carbendazim and Benomyl Residues in Oranges and Orange Juice by Automated Online Sample Preparation Using TLX-LC-MS/MS

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1. Schematic of Method

Homogenization

Sample + IS

1. Squeeze orange or use orange juice and adjust to pH7 (sample)

2. Weigh 0.5 g sample in 2 mL centrifuge tube

3. Add 10 μL working stock IS and 990 μL methanol

Extraction

4. Vortex sample for 5 min

Centrifugation

5. Centrifuge sample with 5000 rpm for 5 min

Turbulent flow LC-MS/MS

2. Introduction

Methyl 2-benzimidazole carbamate, most commonly known as carbendazim, is a widely used broad-spectrum benzimidazole fungicide and a decomposition product of benomyl. Carbendazim is used to control plant diseases in cereals and fruit, including citrus, bananas, strawberries, pineapples, and pome fruits. Although not permitted for use to treat citrus fruit in the USA and Australia, it is permitted in the EU and European Regulation 559/2011 sets a limit

for carbendazim and benomyl (sum of carbendazim and benomyl expressed as carbendazim) of 0.2 mg/kg in oranges. Incidences of MRL exceedance have been common in the EU, with 23 Rapid Alert Notifications in 2011 for levels of carbendazim as high at 4 mg/kg in fruit, vegetables and herbs from Africa, S. America and Asia.[1] The most common occurrence was in yams and no instances of carbendazim in oranges or orange juice were reported. Orange juice from Brazil imported into the USA has been found to contain carbendazim and an action limit of 0.01 m/kg has been applied by the FDA.[2]

Many methods in widespread use for monitoring carbendazim have been developed for multi-residue determination of fungicides and employ a variety of sample preparation and cleanup techniques. In recent years the QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) method has become widely adopted for handling

Thermo Scientific Transcend TLX system coupled with the TSQ Quantum Access MAX triple quadrupole mass spectrometer

Thermo

S C I E N T I F I C

fruit such as oranges. However, despite its undoubted advantages, it requires many manual sample manipulation steps, making it labor-intensive, especially when large numbers of samples have to be analyzed. Therefore, it is beneficial to consider options for automation of multi-residue methods, which can be both cost-effective as well as offer a high degree of reliability in recovery and repeatability. While the preliminary stages of homogenization and solvent extraction of food matrices inevitably require manual intervention, once a crude extract has been obtained there is scope for a fully automated procedure thereafter. The method described in this document is an adaptation of an existing online, multi-residue pesticide method (Thermo Scientific Method 52213[3]) proven and verified specially for the actual carbendazim contamination issue of orange juices in the US.

3. Scope

This method can be applied to oranges and orange juice at a limit of quantification (LOQ) below 0.01 mg/kg, the action limit used by the FDA for monitoring purposes. The method has been validated for carbendazim and the sum of benomyl + carbendazim in oranges and orange juice, but can be readily extended to a larger number of residues.

4. Principle

This method is the adaptation of carbendazim and extension for benomyl of an online sample preparation technique based on an existing in-house validated method (Thermo Scientific Method 52213[3]) for the determination of 50 pesticides in grape, baby food and wheat flour matrices. The method uses TurboFlow technology as a possible alternative to the QuEChERS method since TurboFlow is more suitable for high-throughput fungicide analysis. Sample pre-concentration, cleanup and analytical separation is carried out in a single run, using an online coupled TurboFlow method (Thermo Scientific Transcend TLX). TurboFlow technology serves as a novel sample preparation technique due to its special flow profile, size exclusion, reversed phase column chemistry and very effective separation of matrix and target compounds, resulting in relatively clean sample extracts. Macromolecules such as sugars, fats and proteins are removed from the sample extract with high efficiency, while target analytes are retained on the column based on different chemical interactions. After application of a wash step, the trapped compounds are transferred onto the analytical LC column and separated conventionally. The complete method involves internal standardization, solvent extraction of the homogenized orange juice, solvent extraction, centrifugation and injection into an automated cleanup system. Cleanup using Transcend TLX system has been optimized for max-imum recovery of carbendazim or benomyl and minimal injection of co-extractives into the MS/MS. Identification of carbendazim and benomyl is based on retention time, ion-ratios using selected reaction monitoring (SRM) of characteristic transition ions, and quantification using matrix-matched standards of one of the selected SRM ions.

5. Reagent List

		Part Number
5.1	Acetone, HPLC Grade	A/0606/17
5.2	Acetonitrile, LC-MS Grade	A/0638/17
5.3	Ammonium formate, for HPLC	A/5080/53
5.4	Methanol, Optima LC/MS grade	A456-212
5.5	Formic acid, extra pure for HPLC	F/1850/PB08
5.6	Isopropanol, HPLC grade	P/7507/17
5.7	Water, LC-MS grade	W/0112/17
5.8	Ammonia (35% solution)	10508610

6. Calibration Standards

6.1 Standards

6.1.1 Carbendazim (analytical standard) from Sigma-Aldrich®

6.1.2 Benomyl (analytical standard) from Sigma-Aldrich

6.2 Internal standards:

6.2.1 Imidaclorprid-4,4,5,5-d$_4$ (analytical standard) from Sigma-Aldrich

7. Standards and Reagent Preparation

7.1 **Stock solution:** Weigh 10.00 mg of the compounds (recalculate the amount regarding actual purity of the standard) into a volumetric flask, dissolve in methanol and dilute to 100 mL. The final concentration of the two fungicides is 100 µg/mL. The solution of carbendazim can be used for 3 months when stored refrigerated, however benomyl stock solution remains stable only for 0.5 days.

7.2 **Individual working mixture:** Transfer 50 µL of stock solution of either carbendazim or benomyl (100 µg/mL), respectively, to a 50 mL volumetric flasks and dilute to the mark with methanol. The solution should be prepared fresh every time before using. Final concentration of each standard is 0.1 µg/mL.

7.3 **Stock standard solution of internal standard:** Weigh 10.00 mg of Imidacloprid-d$_4$ (recalculate the amount regarding actual purity of the standard) into volumetric flask, dissolve in methanol and dilute to 100 mL. The solution can be stored at 4 °C for at least 3 months. Final concentration is 100 µg/mL.

7.4 **Working standard solution of internal standard:** Transfer 100 µL of stock solution of imidacloprid-d4 (100 µg/mL) to a 10 mL volumetric flask and dilute to marked volume with methanol. The solu-tion should be prepared fresh every time before using. The final concentration of imidacloprid-d$_4$ is 1 µg/mL.

7.5 **5 M Ammonia solution:** Weigh 24.3 g of ammonia (35% solution) to 100 mL volumetric flask and dilute to marked volume with deionized water.

8. Apparatus

		Part Number
8.1	Sartorius analytical balance	ME235S
8.2	Thermo Scientific Barnstead EASYpure II water	3125753
8.3	Vortex shaker	3205025
8.4	Vortex universal cap	3205029
8.5	Accu-Jet pipettor	3140246
8.6	Orion™ 2 Star, pH meter	10539752
8.7	Thermo Scientific Heraeus Fresco 17 micro centrifuge	208590
8.8	Transcend TLX-1 system with TSQ Quantum Access MAX MS/MS	40500

9. Consumables

		Part Number
9.1	LC vials	3205111
9.2	LC caps	3151266
9.3	Thermo Scientific Pipette Finnpipette 100–1000 µL	321453
9.4	Pipette Finnpipette™ 10–100 µL	3166472
9.5	Pipette Finnpipette 500–5000 µL	3166473
9.6	Pipette holder	3651211
9.7	Pipette tips 0.5–250 µL, 500/box	3270399
9.8	Pipette tips 1–5 mL, 75/box	3270420
9.9	Pipette tips 100–1000 µL, 200/box	3270410
9.10	Spatula, 18/10 steel	3458179
9.11	Spatula, nylon	3047217
9.12	Tube holder	3204844
9.13	Wash bottle, PTFE	3149330
9.14	Vial rack (2 mL)	12211001
9.15	Centrifuge plastic tube (2 mL)	3150968
9.16	TurboFlow Cyclone MCX-2 (50 × 0.5 mm) column	CH-953457
9.17	Thermo Scientific Hypersil GOLD 150 × 4.6 mm, 5 µm column	25005-154630
9.18	UNIGUARD holder	850-00
9.19	Hypersil GOLD™ 10 × 4 mm, 5 µm guard column	25005-014001

10. Glassware

		Part Number
10.1	Volumetric flask, 10 mL	FB50143
10.2	Volumetric flask, 25 mL	FB50147
10.3	1 mL glass pipette	FB50211
10.4	1 L bottle	9653650
10.5	500 mL bottle	9653640
10.6	100 mL volumetric flask	FB50151

11. Procedure

11.1 Sample Preparation

11.1.1 **Orange samples:** Prepare orange samples prior to injection into TLX-MS/MS system: Collect at least 10 representative oranges (min 1 kg) and cut into two halves.[4] Squeeze them on a kitchen squeezer and collect the pressed juice. Adjust the pH of the juice to 7 by adding 5 M ammonia solution.

11.1.2 **Orange juice samples:** Orange juice can be used directly after vigorous shaking and adjusting the pH to 7 with 5 M ammonia solution.

11.2 Sample Extraction

11.2.1 Weigh 0.5 g sample on an analytical balance into a 2 mL centrifuge tube

11.2.2 Add 990 µL methanol and 10 µL working IS solution

11.2.3 Vortex the sample for 5 min

11.2.4 Centrifuge in the centrifuge at 5000 rpm for 5 min

11.2.5 Transfer the supernatant into the LC vial for TLX-LC-MS/MS clean up and determination

		TurboFlow								Analytical					
Step	Duration [s]	Flow mL/min	Grad	A%	B%	C%	D%	Tee	Loop	Flow mL/min	Grad	A%	B%	C%	D%
1	60	1.50	step		100			–	out	0.50	step		100		
2	60	1.50	step		95		5	–	out	0.50	step		100		
3	80	0.16	step		100			Tee	in	1.44	step		100		
4	60	1.00	step			100		–	in	1.60	ramp		55		45
5	60	1.00	step	10			90	–	in	1.60	ramp		40		60
6	220	0.20	step		100			–	out	1.60	ramp				100
7	60	0.20	step		100			–	out	1.60	step				100
8	180	0.20	step		100			–	out	1.00	step		100		

Mobile phases for the TurboFlow:
  A: water pH=3
  B: water
  C: 40% acetonitrile 40% isopropanol and 20% acetone
  D: 5 mM ammonium-formiate in methanol
    + 0.1% formic acid

Solvent channels for analytical:
  A: not in use
  B: 5 mM ammonium-formiate in water + 0.1% formic acid
  C: not in use
  D: 5 mM ammonium-formiate in methanol + 0.1%
    formic acid

Table 1: Gradient program table for Thermo Scientific Aria control software

12. Analysis

12.1 LC Operating Conditions

The TLX system was optimized for both TurboFlow methods and analytical separation.

12.1.1 LC conditions for TurboFlow and analytical columns

Operation was carried out in focus mode setup (Figure 1) with 1:0.75 splitting before MS/MS entrance using a divert valve connection. A TurboFlow Cyclone MCX-2 column was installed (9.17) and a Hypersil Gold column equipped with guard column was used (9.18–9.20). Installed loop volume was 200 µL.

Table 1 gives details of the method program. Sample load (Step 1) was applied with 1.5 mL/min flow rate in turbulent flow, whereby matrix components were eluted in the waste and target fungicides were trapped on the TurboFlow column. After washing the TurboFlow column with a 5% organic/aqueous mixture (Step 2), the trapped fungicides were eluted and transferred (Step 3) after 2 minutes from the TurboFlow to the analytical column with simultaneous dilution of the eluate enabling pre-concentration of fungicides at the beginning of the analytical column. The analytical column was equilibrated and conditioned during loading and washing steps. After transfer of the fungicides, the analytical separation started with gradient elution (Step 4–7), while the TurboFlow column was washed and conditioned and the loop was filled with the TurboFlow eluent. After the gradient run, analytical column was washed in acetonitrile and conditioned for the next run. The total run time of the method with TurboFlow sample preparation and analytical separation, with preparation for the next run, is 13 minutes to keep method capable for multi-fungicide residue analysis. In order to minimize sample carry-over and cross-contamination, the injection needle and valve were washed with both strong and weak wash solvents 4 times (conditions in 12.1.2).
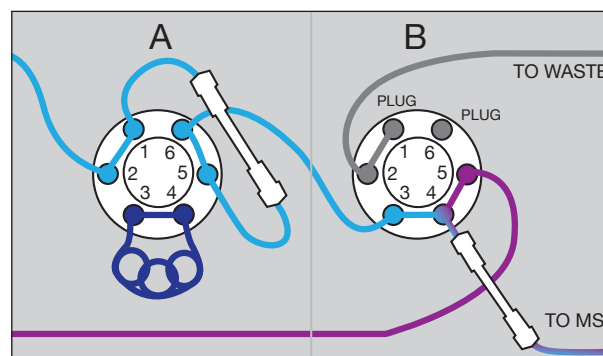

Figure 1: Focus mode system set up and method setting in Aria control software on the Transcend TLX system

12.1.2 Injector set up

Injector: Thermo Scientific Pal injector with 100 µL
    injection syringe volume

Sample holder temperature: 10 °C

Cleaning solvents: Solvent channel 1 – 80:20
                    methanol/acetone
                    Solvent channel 2 – acetonitrile

Injector settings:
- Pre clean with solvent 1 [steps]: 2
- Pre clean with solvent 2 [steps]: 2
- Pre clean with sample [steps]: 1
- Filling speed [µL/s]: 50
- Filling strokes [steps]: 2
- Injection port: LC Vlv1 (TX channel)
- Pre inject delay [ms]: 500
- Post inject delay [ms]: 500
- Post clean with solvent 1 [steps]: 4
- Post clean with solvent 2 [steps]: 4
- Valve clean with solvent 1 [steps]: 4
- Valve clean with solvent 2 [steps]: 4
- Injection volume: 20 µL

12.2 Mass Spectrometric Conditions

Mass spectrometric detection was carried out using a TSQ Quantum Access MAX triple quadrupole mass spectrometer in SRM mode. All SRM traces were individually tuned for the target fungicides (Table 2). MS software programming was set in Thermo Scientific Xcalibur Eazy mode set up.

MS settings:

- Scan type: SRM (details in Table 2)
- Cycle time [s]: 0.3
- Peak width: 0.7 Da FWHM
- Collision gas pressure [mTorr]: 1.5
- Capillary temperature [°C]: 290 °C
- Vaporizer temperature [°C]: 290 °C
- Sheath gas pressure [arb]: 40
- Aux gas pressure [arb]: 10
- Ion sweep pressure [arb]: 0
- Spray voltage [V]: 3200
- Skimmer offset [V]: 2
- Polarity: positive for all compounds
- Trigger: 1.00e5

13. Calculation of Results

Calibration by internal standardization is applied for the determination of carbendazim and benomyl. This quantification method requires determination of response factors R_f defined by the equation below. Calculation of final result is performed using the following equations.

Calculation of the response factor:

$$R_f = \frac{A_{St} \times c_{[IS]}}{A_{[IS]} \times c_{St}}$$

R_f – the response factor

A_{St} – the area of the fungicide peak in the calibration standard

$A_{[IS]}$ – the area of the internal standard peak of the calibration standard

c_{St} – fungicide concentration of the calibration standard solution

$c_{[IS]}$ – the internal standard concentration of the calibration standard solution

Calculations for each sample of the absolute amount of fungicide that was extracted from the sample:

$$X_{analyte} = \frac{A_{analyte} \times X_{[IS]}}{A_{[IS]} \times R_f}$$

$X_{analyte}$ – the absolute amount of fungicide that was extracted from the sample

$A_{analyte}$ – the area of fungicide peak in the sample

$A_{[IS]}$ – the area of the internal standard peak in the sample

$X_{[IS]}$ – the absolute amount of internal standard added to the sample

The concentration of fungicide in the sample [ng/g]:

$$c = \frac{X_{analyte}}{m}$$

m – the weight of sample [g]
$X_{analyte}$ – absolute analyte amount [ng]

14. Method Performance Characteristics

In-house validation of the method was carried out according to IUPAC and AOAC guidelines for single laboratory validation and it was also demonstrated that method performance characteristics fulfilled the legislative criteria set for pesticide residue methods.[5-8]

Samples used for the determination of method performance characteristic parameters were prepared by spiking of appropriate amount of working standard solution and work solution of internal standard into the 0.5 g sample and total volume was adjusted to 1 mL with methanol (equivalent total volume according to 10.2.).

With reference to the low stability and fast transformation of benomyl into carbendazim, the validation study was carried out with samples spiked only with carbendazim to establish the method performance parameters.[6] After establishing validation parameters, samples were run additionally with spiked carbendazim and benomyl, in order to check degradation and contribution of benomyl to the carbendazim peak area (Figure 2). In order to keep control on benomyl degradation, all these samples were analyzed within 2 hours after preparation.

14.1 Selectivity

Method (SRM) selectivity was confirmed based on presence of specific ion transitions at the corresponding retention time (Table 2), as well as the observed ion ratio values corresponding to those of the standards. Acceptance criteria for retention time and ion ratios were set according to Reference 4.

14.2 Linearity, Response Factor

The linearity of calibration curves was assessed by internal standardization over the range from 0–0.1 mg/kg. The matrix-matched calibration curves were created at seven levels (and blank) and injected in duplicate. Calibration levels were 0, 0.005, 0.010, 0.015, 0.025, 0.035, 0.050 and 0.100 mg/kg. R_f values for internal standardization were determined from the calibration curves by calculating cumulative average response factor over the whole calibration range and resulted $R_f = 3.2$, which was used for quantitative analysis. The details on calibration are shown in Table 3.

14.3 Accuracy

Method accuracy and precision was assessed by recovery studies using blank matrices spiked at three concentration levels injected in six individually prepared replicates. Samples were spiked at 0.005, 0.010 and 0.050 mg/kg concentration levels. Found concentrations, recovery and relative standard deviation (% RSD) were calculated (Table 3). Recovery values were in the range 96–115% and were deemed to be acceptable (criteria 70–120%).

14.4 Repeatability and Intermediate Precision

Method within-day (repeatability) and between-day precision (intermediate precision) values ranged from 6.8–9.8% (Table 4) and were deemed acceptable (below 20%).

14.5 Limits of Detection (LODs) and Quantification (LOQs)

Limits of detection and quantification were estimated following the IUPAC approach which consisted of analyzing the blank sample to establish noise levels and then testing experimentally estimated LODs and LOQs for signal/noise, 3 and 10 respectively. The method LOD and LOQ values resulted as 0.00015 mg/kg and 0.0005 mg/kg (Figures 3 and 4). The expectation of the method was to meet the US rejection limit for orange juices set by the FDA at 0.010 mg/kg as well as the European MRL value (0.2 mg/kg) at LOQ level. Method LOQ fulfilled both legislation criteria.

14.6 Matrix Effect

Matrix effect was investigated by comparison of calibration results in solvent and in matrix. Youden plot of both calibration series was applied. Slope of fitted linear resulted $y=0.8497\times$ which represents less than 20 % deviation from the idealistic $y=\times$ value indicating no matrix effect for the investigated matrix (Figure 5).

14.7 Survey Samples

The method was applied to 6 different orange juice samples (n=3) and oranges (n=3) purchased from local stores. Survey samples were of organic origin from Spain and Germany. No carbendazim was found above 0.01 mg/kg in any of survey samples (Table 5).

15. Conclusion

This method enables convenient, fast and cost-effective automated determination of carbendazim and benomyl in oranges and orange juice. Based on the short total run time and a simple online sample preparation technique, 100 samples per day can be analyzed at a level of 0.01 mg/kg, with faster and more precise analysis compared to the QuEChERS technique. Method performance characteristics were established by in-house validation for oranges and orange juice. Based on its method performance parameters, the developed TLX system is suitable for routine use for regulatory purposes and possesses potential as alternative to the widely used QuEChERS method. The TLX system can readily be extended to a larger and wider range of fungicide residues, and has previously been demonstrated as being applicable to other matrices such as cereals, grapes and baby food.[1]

16. References

1. https://webgate.ec.europa.eu/rasff-window/portal/index.cfm?event=searchResultList

2. http://www.fda.gov/Food/FoodSafety/Product-SpecificInformation/FruitsVegetablesJuices/ucm287783.htm

3. https://www.thermo.com/eThermo/CMA/PDFs/Product/productPDF_58039.PDF

4. Comission Directive 2002/63/EC, L187/30 establishing Community methods of sampling for the official control of pesticides residues in and on products of plant and animal origin and repealing directive 79/700/EEC.

5. http://www.aoac.org/Official_Methods/slv_guidelines.pdf

6. http://www.scribd.com/doc/4922271/Harmonized-Guidelines-for-Single-Laboratory-Validation-of-Methods-Of

7. http://ec.europa.eu/sanco_pesticides/public/index.cfm

8. E. Mallat, D. Barcelo, R.Tauler, Chromatographia 46 (1997) 342-350

17. Annex

17.1 Tables, Chromatograms and Matrix Study

Analyte	Retention time [min]	Precursor Ion	Product Ion (Ecoll)	Product Ion2 (Ecoll)	Ion Ratio	Tube Lens
Carbendazim	6.01	191.8	160.1 (18)	132.1 (29)	0.25	100
d4-Imidacloprid	6.21	259.9	213.1 (17)	179.1 (20)	0.82	97
Benomyl	9.03	291.1	192.1 (12)	160.1 (27)	0.85	101

Table 2: Ion transitions of target compounds for SRM setting

Compound	Linearity			Recovery [%] (RSD%)		
	Slope	Intercept	R^2	0.005 mg/kg	0.010 mg/kg	0.050 mg/kg
Carbendazim	0.1501	0.1787	0.9981	99 (5.5)	101 (6.8)	108 (4.0)
Carbendazim + Benomyl	0.3377	0.1840	0.9891	115 (14.6)	96 (9.4)	104 (3.6)

Table 3: Linearity (n=2) and recovery (n=6) of target compounds

Compound	Precision [%]			
	Identification (tr)		Quantification (Peak Area)	
	Repeatability	Intermediate Precision	Repeatability	Intermediate Precision
Carbendazim	0.1	0.1	6.8	9.5
Carbendazim + Benomyl	–	–	7.5	9.8

Table 4: Repeatability and intermediate precision of target compounds

Sample #	Type of Sample	Carbendazim [mg/kg]
1	juice	0.001
2	juice	0.002
3	juice	0.005
4	orange	0.001
5	orange	<LOD
6	orange	<LOD

Table 5: Survey sample results
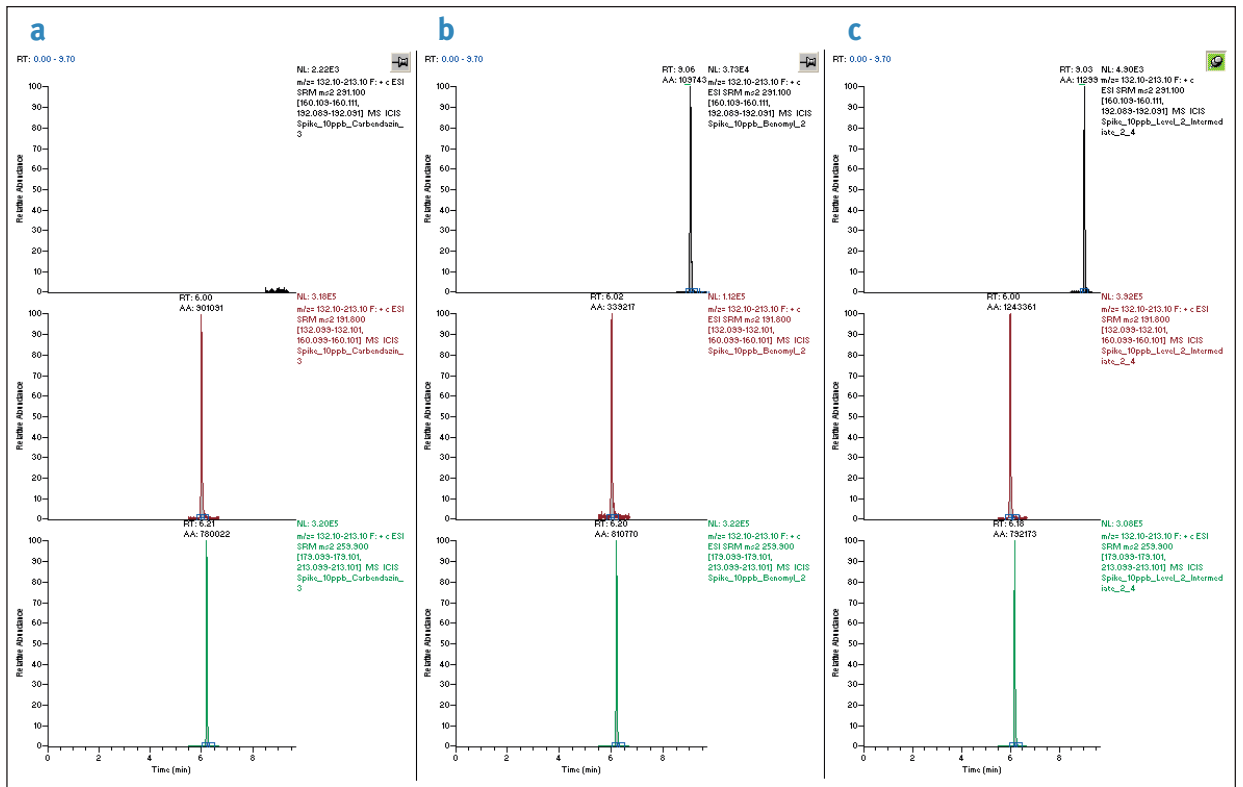
Figure 2: Demonstration of transformation of benomyl into carbendazim. Traces from top: benomyl, carbendazim and d4-imidacloprid (IS). Chromatograms showing a) 10 ng/mL carbendazim solution, b) 10 ng/mL benomyl solution after 2 hrs of preparation, c) chromatogram of solution containing 10 ng/mL carbendazim and benomyl after 2 hrs of preparation. Significant amount of benomyl transforms into carbendazim.
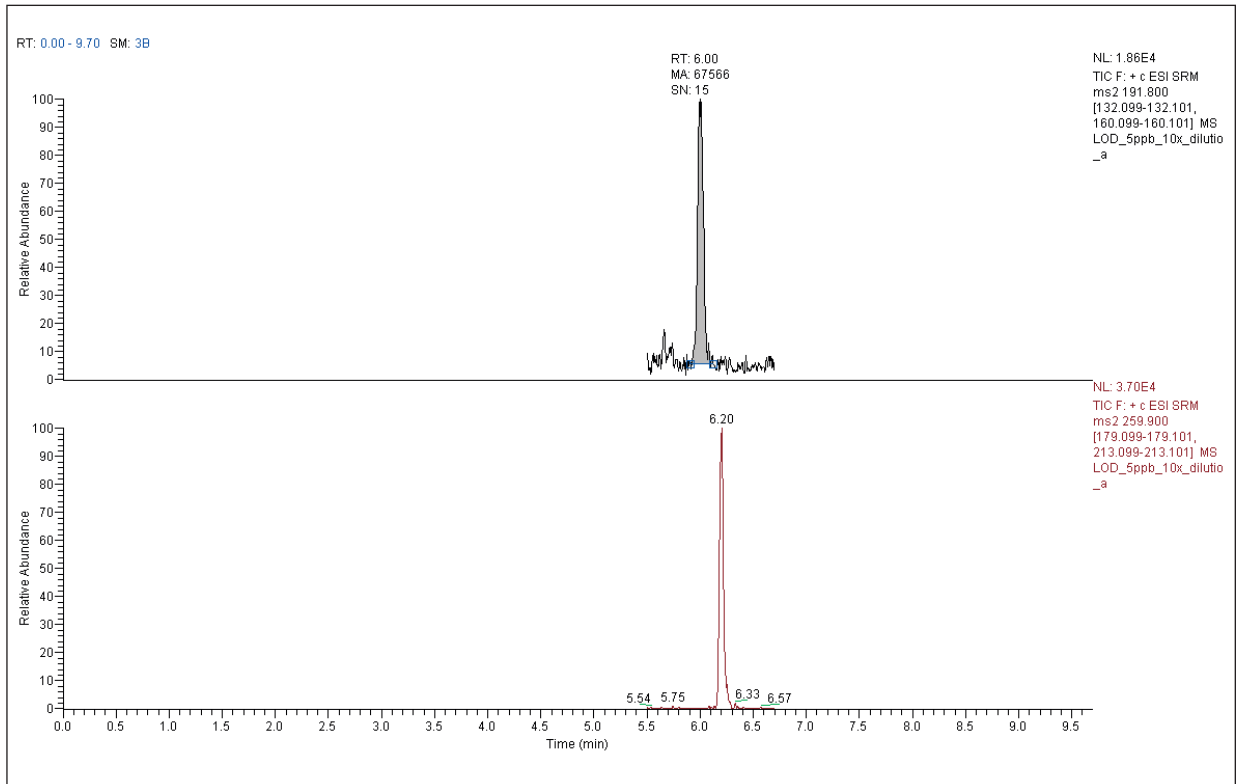
Figure 3: Chromatogram of 0.0005 mg/kg carbendazim in orange juice representing signal intensity at LOQ level.
On top: carbendazim, below: d4-imidacloprid (IS).
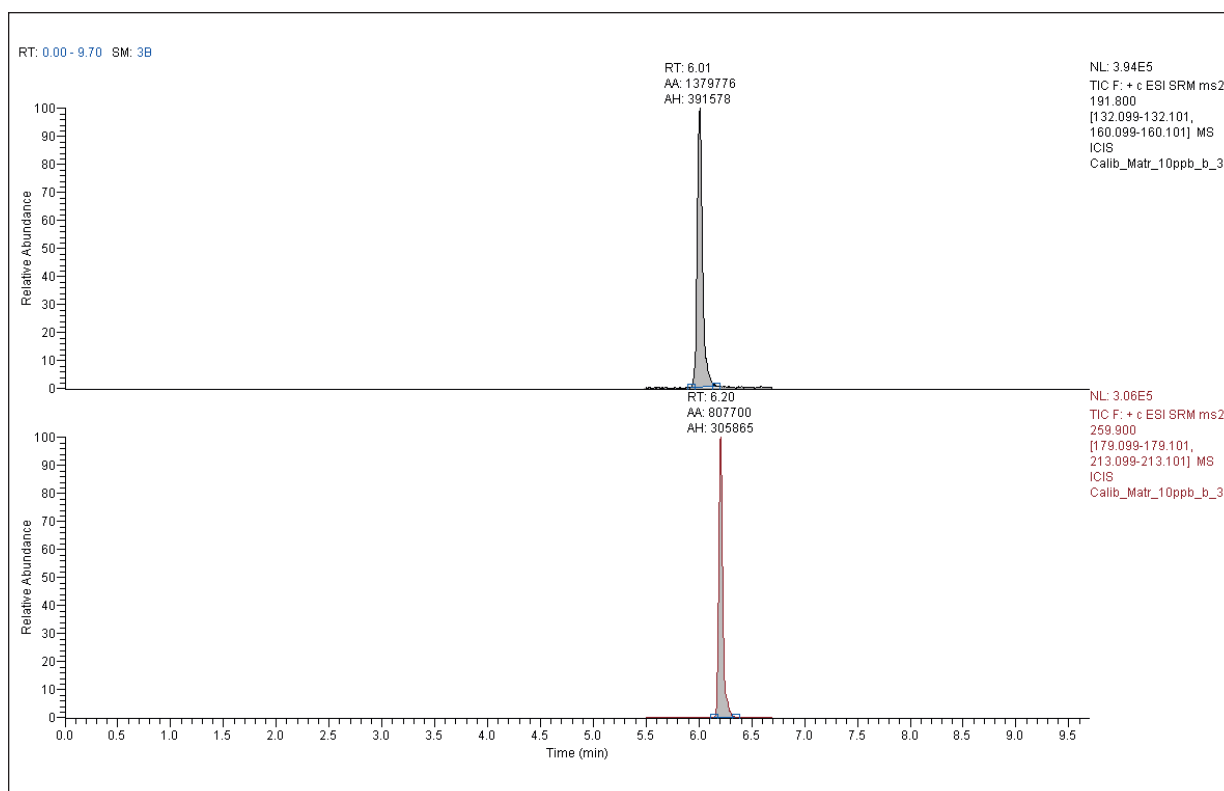
Figure 4: Chromatogram of 0.01 mg/kg carbendazim matrix (orange juice) matched calibration standard representing peak intensity at current US (FDA) rejection level. On top: carbendazim, below: d4-imidacloprid (IS).
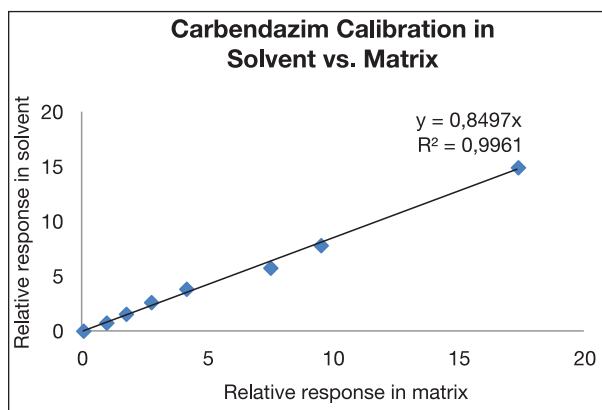
Figure 5: Matrix effect study. Plot of relative responses of calibration levels in solvent vs in orange juice.

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