



**Evaluation of the three main multiresidue
methods for the validation of new substances of
SANCO/12745/2013 and those with low
analytical coverage**



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1. Aim and scope

This document describes the validation data for 15 pesticides included in the Working document SANCO/12745/2013 rev.16(3) using QuEChERS extraction method by LC-QqQ-MS/MS and GC-QqQ-MS/MS in tomato, orange and avocado.

2. Short description

Homogenous samples were extracted using QuEChERS extraction. Validation was carried out at two concentration levels: 0.005 and 0.100 mg/Kg. Recoveries, repeatability, linearity and matrix effects were evaluated in the validation of the method, in matrices covering the main commodity groups in fruits and vegetables: high water content (tomato), high acid and high water content (orange) and high oil content and intermediate water content (avocado).

3. Experimental

3.1. Citrate-buffered QuEChERS extraction procedure

1. Weigh 10 g \pm 0.1 g of sample in 50 mL PTFE centrifuge tube.
2. Add 10 mL of acetonitrile and 10 μ L of 10 mg/L carbendazim-d3, dichlorvos-d6 and malathion-d10 (procedure internal standards).
3. Shake the sample using an automatic axial shaker for 4 min.
4. Add 4 g of anhydrous magnesium sulfate, 1 g of sodium chloride, 1 g of trisodium citrate dihydrate and 0.5 g of disodium hydrogenocitrate sesquihydrate.
5. Shake the samples again in the automatic shaker for 4 min.
6. Centrifuge the tubes at 3700 rpm for 5 min.
7. Transfer 5 mL of the supernatant to a 15 mL PTFE tube containing:
 - a. 750 mg anhydrous magnesium sulfate and 125 mg PSA for matrices with high water content.
 - b. 750 mg anhydrous magnesium sulfate and 125 mg Z-Sep for matrices with high fat content.
8. Vortex the tube for 30 sec.
9. Centrifuge the tubes at 3700 rpm for 5 min.
10. Add 40 μ L (10 μ L/mL) of formic acid 5% in acetonitrile.
11. Injection vials preparation:
 - a. for LC analysis, dilute 100 μ L extract with 400 μ L of water containing dimethoate-d6 at 0.0625 mg/L (Injection Internal Standard) and add 50 μ L of acetonitrile.
 - b. for GC analysis, dilute 50 μ L of extract with 50 μ L of orange extract in ethyl acetate. Additionally, 4 μ L of lindane-d6 (Injection Internal Standard) were added to the final vials.

3.2. Instrumentation and analytical conditions for the LC- MS/MS system

All samples were analyzed by a 1200 UHPLC Instrument coupled to a 6490A LC/MS Triple Quad (Agilent Technologies). The analytical parameters are detailed below.

3.2.1. 1290 UHPLC (Agilent)

- Column: Zorbax Eclipse Plus C8 2.1 x 100 mm and 1.8 μ m particle size (Agilent).
- Mobile phase A: Water (0.1% formic acid, 5mM ammonium formate, 2% MeOH).



- Mobile phase B: Methanol (0.1% formic acid, 5mM ammonium formate, 2% H₂O).
- Column temperature: 35°C
- Flow rate: 0.3 mL/min
- Injection volume: 5 µL.
- Mobile phase gradient:

Time (min)	Mobile phase A (%)	Mobile phase B (%)
0	100	0
2	80	20
15	0	100
18	0	100

- Re-equilibration with initial mobile phase: 2.5 minutes.

3.3.2. 6490 triple quadrupole system (Agilent)

- Ionization mode: Positive and negative mode.
- Capillary (positive and negative): 3000 V.
- Nebulizer and collision gas: Nitrogen.
- Nebulizer: 45 psi.
- Nozzle: 400 V.
- Drying gas flow: 15 L/min.
- Drying gas temperature: 120°C.
- Sheath gas flow: 10 L/min.
- Sheath gas temperature: 375°C.
- High Pressure RF (positive): 150 V.
- High Pressure RF (negative): 110 V.
- Low Pressure RF (positive): 60 V.
- Low Pressure RF (negative): 60 V.

3.3. Instrumentation and analytical conditions for the GC- MS/MS system

Samples were analyzed by an Intuvo 9000 GC Instrument coupled to a 7410 GC/MS Triple Quad (Agilent Technologies). The analytical parameters are detailed below. The mass transitions used are presented in **Appendix I (Table 1.1)** for LC-MS/MS and **Table 1.2** for GC-MS/MS parameters).

3.3.1. Intuvo 9000 GC system (Agilent)

- Column: 2 Planar columns HP-5MS UI (15 m long × 0.25 mm i.d. × 0.25 µm film thickness) (Agilent).
- Injection mode: splitless, 1 µL sample and 0.5 µL matrix blank in ethyl acetate (in this case orange matrix blank).
- Ultra-inert inlet liner with glass wool frit (Agilent).
- Injector temperature: 280 °C (hold time: 0.1 min)
- Carrier gas: Helium (purity 99.999%) at constant flow = 1.611 mL/min.



- Carrier gas purity: 99.999%.
- Oven temperature: 60 °C for 0.5 min, up to 170 °C (80 °C/min) and up to 310 °C (20 °C/min).

3.3.2. 7010B triple quadrupole mass spectrometer (Agilent)

- Ionization mode: electron impact ionization.
- Temperature of the transfer line: 280 °C.
- Temperature of ion source: 280 °C.
- Collision gas: Nitrogen.
- Collision gas purity: 99.999%.
- Solvent delay: 2.6 minutes.

4. Results and discussion

The performance of the QuEChERS method for pesticide residues analysis was evaluated in tomato, orange and avocado at two concentration levels: 0.005 and 0.100 mg/kg.

4.1. Linearity

Linearity of the MS/MS system was evaluated by assessing the signal responses of the target analytes from matrix-matched calibration solutions prepared by spiking blank extracts at seven concentration levels, from 0.002 to 0.200 mg/L. In most cases, coefficient of determination (R^2) was higher than 0.99 and back-calculated concentrations were within $\pm 20\%$.

Table 1.1 and **Table 1.2** of **Appendix I** show the detection and chromatographic parameters for the selected compounds analyzed by LC-MS/MS and GC-MS/MS, respectively.

Linearity ranges for all pesticides are summarized in **Table 2.1** of **Appendix II**.

4.2. Matrix effects

Matrix effects were assessed by comparison of the slopes of seven-point matrix-matched calibration curves with the slopes of the calibration curves in solvent (LC) or in tomato (GC). For values (in absolute terms) between 0% and 20%, matrix effect was considered low; a moderate matrix effect would have values between 20% and 50%, and for compounds with a value above 50 %, matrix effect was considered strong.

Values of matrix effects are summarized in **Table 2.1** of **Appendix II**.

4.3. Ion ratio and peak shape

The ion ratio was calculated between the qualitative peak area and the quantitative peak area. In all cases, the obtained values of ion ratio were within $\pm 30\%$ of the average of calibration standards ion ratios from the same sequence.

All the studied pesticides showed acceptable peak shape.

4.4. Recoveries and repeatability

The mean recovery ($n=5$) and repeatability in terms of relative standard deviation (RSDr) of 15 pesticides were evaluated at two spiking levels (0.005 and 0.100 mg/kg) in the three matrices



included in the present study (tomato, orange and avocado). The results are summarized in **Table 2.2** of **Appendix II**.

All recovery results were within the range of 70-120% (RSD \leq 20%), except for fluazinam, for which the clean-up step is critical and affects its recoveries. Therefore, fluazinam was re-analysed using the QuEChERS method without clean-up.

5. References

Analytical quality control and method validation procedures for pesticide residues analysis in food and feed. Document SANTE/11312/2021 (V2).

Working document on pesticides to be considered for inclusion in the national control programmes to ensure compliance with maximum residue levels of pesticides residues in and on food of plant and animal origin. Document SANCO/12745/2013 rev. 16(3).

APPENDIX I: MASS TRANSITIONS

Table 1.1 Detection and chromatographic parameters for the selected compounds analyzed by LC-MS/MS.

	Name	t _R (min)	Precursor ion 1 (m/z)	Product ion 1 (m/z)	CE 1 (V)	Precursor ion 2 (m/z)	Product ion 2 (m/z)	CE 2 (V)	Polarity
1	4-Bromophenyl-urea	7.72	215.2	93.1	35	215.2	171.9	10	Positive
2	Azadirachtin	9.39	703.3	585.2	15	703.3	567.2	10	Positive
3	Florpyrauxifen-benzyl	12.59	439.2	91.1	40	441.2	90.9	55	Positive
4	Fluazinam	13.66	462.9	416.0	15	462.9	398.0	35	Negative
5	Metamitron	5.47	203.2	174.9	15	203.2	104.1	15	Positive
6	Metazachlor	9.79	278.0	210.1	5	278.0	134.2	30	Positive
7	Metobromuron	9.40	259.0	170.0	15	259.0	148.0	10	Positive
8	Milbemectin A3	14.58	511.3	493.0	10	511.3	457.1	10	Positive
9	Milbemectin A4	14.88	525.3	109.1	20	525.3	489.1	10	Positive
10	Phendimethan	10.20	301.1	167.8	5	301.1	136.1	25	Positive
11	Thiencarbazone-methyl	7.5	391.0	229.9	10	391.0	129.7	10	Positive

Table 1.2. Detection and chromatographic parameters for the selected compounds analyzed by GC-MS/MS.

	Name	t _R (min)	Precursor ion 1 (m/z)	Product ion 1 (m/z)	CE 1 (V)	Precursor ion 2 (m/z)	Product ion 2 (m/z)	CE 2 (V)
1	1,4-Dimethylnaphthalene	4.13	156	141	15	156	115	30
2	Chlorpyrifos-methyl	6.05	286	271	15	288	93	25
3	Cyhalofop-butyl	8.93	256	120	10	357	229.2	10
4	Fluensulfone	3.37	119	92	10	226	206	20



APPENDIX II: VALIDATION RESULTS

Table 2.1. Linearity ranges, coefficients of determination and matrix effects for selected matrices studied by using citrate-buffered QuEChERS method. Negative values of matrix effects mean suppression of the signal, and positives values, enhancement.

No.	Compound	Linear Range (mg/kg)			R ²			Matrix effect (%)		
		Tomato	Orange	Avocado	Tomato	Orange	Avocado	Tomato	Orange	Avocado
1	4-Bromophenyl-urea	0.002-0.200	0.002-0.200	0.002-0.100	> 0.99	> 0.99	> 0.99	-11	-45	-9
2	Azadirachtin	0.002-0.200	0.002-0.200	0.002-0.100	> 0.99	> 0.99	> 0.99	-1	19	4
3	Florpyrauxifen-benzyl	0.002-0.100	0.002-0.100	0.002-0.100	> 0.99	> 0.99	> 0.99	-17	-30	18
4	Fluazinam	0.002-0.100	0.005-0.100	0.002-0.100	> 0.99	> 0.99	> 0.99	-3	-63	63
5	Metamitron	0.002-0.200	0.002-0.200	0.002-0.100	> 0.99	> 0.99	> 0.99	-8	-41	3
6	Metazachlor	0.005-0.200	0.005-0.200	0.005-0.200	> 0.99	> 0.99	> 0.99	-3	-24	-1
7	Metobromuron	0.005-0.200	0.005-0.200	0.005-0.200	> 0.99	> 0.99	> 0.99	1	-18	9
8	Milbemectin A3	0.002-0.200	0.005-0.200	0.005-0.100	> 0.99	> 0.99	> 0.99	-30	-44	-33
9	Milbemectin A4	0.002-0.200	0.005-0.100	0.005-0.200	> 0.99	> 0.99	> 0.99	-25	-54	-58
10	Phendimethan	0.005-0.200	0.005-0.200	0.002-0.100	> 0.99	> 0.99	> 0.99	2	-36	8
11	Thiencarbazone-methyl	0.002-0.200	0.002-0.200	0.002-0.200	> 0.99	> 0.99	> 0.99	-4	50	17
12	<i>1,4-Dimethylnaphthalene</i>	0.002-0.200	0.002-0.200	0.002-0.200	> 0.99	> 0.99	> 0.99	0	8	8
13	<i>Chlorpyrifos-methyl</i>	0.002-0.200	0.002-0.200	0.002-0.200	> 0.99	> 0.99	> 0.99	0	2	-3
14	<i>Cyhalofop-butyl</i>	0.002-0.200	0.002-0.200	0.002-0.200	> 0.99	> 0.99	> 0.99	0	9	10
15	<i>Fluensulfone</i>	0.002-0.200	0.002-0.200	0.002-0.200	> 0.99	> 0.99	> 0.99	0	14	15

In bold, pesticides analysed by LC-MS/MS

In italic, pesticides analysed by GC-MS/MS



Table 2.2. Accuracy data (as % recovery) and precision data (as repeatability RSDr, n=5) at 0.005 and 0.100 mg/kg for tomato, orange and avocado by using citrate-buffered QuEChERS method with clean-up.

No.	Compound	Tomato				Orange				Avocado			
		0.005 mg/kg		0.100 mg/kg		0.005 mg/kg		0.100 mg/kg		0.005 mg/kg		0.100 mg/kg	
		Recov (%)	RSD (%)	Recov (%)	RSD (%)	Recov (%)	RSD (%)	Recov (%)	RSD (%)	Recov (%)	RSD (%)	Recov (%)	RSD (%)
1	4-Bromophenyl-urea	98	5	99	2	109	5	108	5	85	14	84	12
2	Azadirachtin	84	11	99	4	90	13	82	4	110	16	100	10
3	Florpyrauxifen-benzyl	84	3	105	11	75	6	72	18	106	8	99	8
4	Fluazinam	123*	8*	113*	6*	93*	2*	76*	9*	109	7	98	11
5	Metamitron	97	8	94	4	104	3	108	3	101	7	91	10
6	Metazachlor	94	8	95	5	103	5	97	3	108	3	103	9
7	Metobromuron	102	8	98	2	99	3	94	4	110	6	97	10
8	Milbemectin A3	97	16	100	10	95	9	89	3	102	14	81	15
9	Milbemectin A4	96	9	99	5	85	15	76	10	100	14	84	2
10	Phendimethan	116	4	93	4	113	4	98	1	116	2	97	7
11	Thiencarbazon-methyl	83	13	81	6	86	6	78	6	101	11	87	12
12	<i>1,4-Dimethylnaphthalene</i>	105	5	99	5	106	11	95	3	89	5	71	1
13	<i>Chlorpyrifos-methyl</i>	94	6	95	3	104	3	86	3	100	4	84	3
14	<i>Cyhalofop-butyl</i>	90	6	99	4	105	2	92	3	109	2	97	3
15	<i>Fluensulfone</i>	95	4	103	4	100	8	96	3	108	3	100	2

*QuEChERS without clean-up

In bold, pesticides analysed by LC-MS/MS

In italic, pesticides analysed by GC-MS/MS