Application Note

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A Newly Modified QuEChERS Method for Multiresidue Pesticide Determination in Black Pepper Using LC/TQ

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Abstract

This application note presents several investigations of different sample treatment methods for the simultaneous analysis of 400 pesticides on black pepper samples. The work used an Agilent 1290 Infinity II liquid chromatography (LC) system coupled to an Agilent 6495C triple quadrupole mass spectrometer (LC/TQ) using a dynamic multiple reaction monitoring (dMRM) method.

Based on the QuEChERS method, different cleanup techniques such as solid phase extraction (SPE), dispersive solid phase extraction (dSPE), and passthrough cleanup combined with the dilution technique were evaluated. The data were evaluated based on SANTE criteria such as matrix effect, method sensitivity, calibration curve linearity, recovery, and precision. A simple, efficient, new sample processing was introduced with results of more than 84% of 400 targets meeting the SANTE requirements 11312/2021, demonstrating the performance and sensitivity of the 6495C LC/TQ system. The application note also suggests a sample preparation to suit existing conditions in different labs.

Introduction

In addition to the nutritional and medical values that black pepper brings to people, the pepper agriculture industry also provides economic value to many countries such as Brazil, India, Indonesia, and Vietnam. However, one of the challenges for the pepper industry are the problems of diseases, pests, and mold, etc., which are harmful during cultivation and storage. Especially in the cultivation and preservation of pepper, the humid tropical monsoon climate is favorable for the development of pests and molds. For this reason, pesticides are used to prevent the growth of pests and molds. Therefore, to meet the requirements of import-export and consumer health, it is necessary to meet the requirements and standards of countries and organizations around the world. Countries such as the United Kingdom, United States, Russia, and others, have regulations for the maximum allowable residue limit of pesticides.

However, black pepper is one of the most challenging samples for pesticide analysis. A bottleneck for cleanup methods is to ensure that pesticides are not lost during the cleaning process but still effectively remove the matrix interference, such as volatile oils, peptides, carbohydrates, fibers, lipids, and pigments. Traditionally, the QuEChERS technique for the complex matrix octadecyl (C18), primary secondary amine (PSA), and graphitized carbon black (GCB) are used. These kits are used in combination to increase the efficiency of the removal of coextracts, limit the influence of the matrix, and help to obtain accurate quantitative results and be more precise. However, PSA and GCB both have limitations. PSA can mostly adsorb acid-based analytes, and GCB may have an affinity for compounds with planar structures; thus, there is a risk of losing analytes with this type of structure.[1] Therefore, mass, material structure, and dilution factor must be optimized to achieve the allowable recovery and effectively remove the matrix. In this study, the dilution technique combined with the QuEChERS method with cleanup techniques such as dSPE, SPE, and passthrough cleanup was used. These different materials were investigated to analyze 400 pesticides in black pepper samples using a 6495C LC/TQ system.

Experimental

Reagents and chemicals

Glacial acetic acid (AA), HPLC-grade acetonitrile (ACN), LC/MS-grade methanol (MeOH), and LC/MS-grade water were obtained from Merck (Darmstadt, Germany)

LC/MS-grade formic acid and ammonium formate were purchased from Sigma-Aldrich, St. Louis, MO, USA.

Standards solution and standards preparation

The pesticide standard solution kits for 204 pesticides were obtained from Restek (part number 31971) in Bellefonte, PA, USA, and all other compounds were obtained from Sigma-Aldrich, LGC, and Chem Service (see Table A1 in the Appendix).

Standard mix solutions of 400 targets at concentrations of 1 mg/L were prepared from the stock solutions to optimize and validate each method.

For the matrix effect assessment experiment, solvent calibration curves were prepared from mixed standards at 0.05, 0.1, 0.2, 0.5, 1, 2, 5, and 10 µg/L in MeOH:H_2O (1:1) solution and matrix calibration curves with the same concentration level were prepared from blank matrix solution for each method.

Sample preparation

Black pepper was obtained from a local market store in Vietnam. This sample was homogenized into a powder using a blender and passed through a 2 mm sieve as a blank sample for experimental work.

The following products and equipment were used for sample preparation:

- Agilent Bond Elut QuEChERS EN extraction kit (part number 5982-5650CH)
- Agilent Captiva Enhanced Matrix Removal–General Pigmented Dry (EMR-GPD) cartridge (part number 5610-2091)
- Agilent Bond Elut QuEChERS Dispersive Universal kit (part number 5982-0028)
- Agilent Bond Elut SPE Plexa (part number 12109603)
- Agilent Bond Elut BE Carbon (part number 12102042C250)
- Geno/Grinder (SPEX, Metuchen, NJ, USA)
- Centrifuge (Eppendorf, Centrifuge 5804R and 5430R)
- Vortexer and multitube vortexer (VWR, Plainfield, NJ, USA)
- Agilent positive pressure manifold 48 processor (PPM-48) (part number 5191-4101)

To optimize recovery and matrix removal, two extraction solvents (ACN and ACN acidified with 1% acetic acid) and four different cleanup procedures were used. The cleanup procedures included dSPE with Bond Elut QuEChERS Dispersive Universal kit C18, PSA, and GCB (part number 5982-0028). Also used was Captiva EMR–GPD cartridge passthrough cleanup, mixed-mode SPE using mixed-mode BE/PSA in the cartridge format, and SPE through Bond Elut Plexa and dSPE with C18, PSA, and GCB, were evaluated.

Captiva EMR–GPD products contain optimized mixed sorbents, including the newly developed Agilent Carbon S sorbent. Carbon S absorbent material is an advanced hybrid carbon material with optimized carbon content and pore structure. It provides efficient and selective matrix pass cleanup for plant-derived sample matrices.[2] Bond Elut Plexa is a new generation of polymeric SPE products designed for simple method development, ease of use, and improved analytical performance. The Plexa surface consists of a hydroxylated ligand, which is highly polar and entirely amide free, with advanced polymeric architecture and selectivity.[3] Optimized polymeric design, in combination with a narrow particle size distribution, ensures consistent flow rates and reproducible, high analyte recoveries. The advanced polymeric design and particle surface modification minimize the common matrix interferences that are the primary source of ion suppression, thus improving analytical sensitivity and data quality.

Procedure 1. QuEChERS-d-SPE

QuEChERS extraction: Black pepper samples (2.0 g) were weighed into 50 mL centrifuge tubes and fortified with an appropriate volume of pesticides mix standard as a prespike QC sample. Next, 10.0 mL of water was added and capped tightly and vigorously vortexed for 2 minutes. The samples were equilibrated for 10 to 15 minutes, then 10.0 mL of acetonitrile/1% acetic acid was added. After the extraction, EN extraction salt and one ceramic homogenizer (part number 5982-5650CH) were added into a centrifuge, capped tightly, and shaken immediately for 30 seconds, followed by centrifugation at 3,800 rpm (2,260 rcf) for 5 minutes.

Disperse-SPE cleanup: the supernatant extract (1.0 mL) was transferred into a 2 mL dSPE tube containing a sorbent mixture of 150 mg of anhydrous $MgSO_4$ + 50 mg of PSA + 50 mg of C18 + 7.5 mg of GCB. The tubes were vortexed for 1 minute, then centrifuged at 3,800 rpm (2,260 rcf) for 3 minutes. Then, the supernatants were diluted ten-fold with MeOH:H_2O (1:1) and filtered through a 0.22 µm PTFE membrane into an autosampler vial for LC/TQ analysis.

Procedure 2. QuEChERS-MeOH dSPE

QuEChERS extraction was the same as procedure 1.

Disperse-SPE cleanup: the supernatant extract was diluted two-fold with MeOH, and 1.0 mL was transferred into a 2 mL dSPE tube containing a sorbent mixture of 150 mg of anhydrous $MgSO_4$ + 50 mg of PSA + 50 mg of C18 + 7.5 mg of GCB. The tubes were vortexed for 1 minute, then centrifuged at 3,800 rpm (2,260 rcf) for 3 minutes. Then, the supernatants were diluted fivefold with MeOH:H_2O (1:1) and filtered through a 0.22-µm PTFE membrane into an autosampler vial for LC/TQ analysis.

Procedure 3. Captiva EMR–GPD cartridge passthrough cleanup

QuEChERS extraction: Black pepper samples were weighed (0.5 g) then extracted following the QuEChERS extraction in procedure 1.

Passthrough cleanup: A 2.5 mL aliquot of the supernatant was transferred to the 15 mL tube and mixed with 250 µL of water with 1% formic acid. The mixture was well homogenized, loaded into a Captiva EMR–GPD cartridge, and placed on the PPM48 processor with a labeled collection tube beneath. Low-level pressure (1 to 3 psi) was applied to control the flow rate at 3 to 5 seconds per drop. When all samples passed through the cartridge with no visible liquid, high pressure (~10 psi) was applied to dry the EMR–GPD cartridge for 2 minutes. The eluent was then ready for LC/TQ analysis.

Procedure 4. Mixed-mode SPE

QuEChERS extraction: Black pepper samples were weighed (0.5 g) then extracted following the QuEChERS extraction in procedure 1.

SPE cleanup: The GCB/PSA SPE cartridges were preconditioned with 6 mL of ACN:toluene (3:1). Then, 2 mL of ACN extract obtained was introduced to the cartridge, and 20 mL of ACN:toluene (3:1, v/v) was used as the eluting solvent. All of the extract solutions and eluent solvents were collected. Then, 10 mL of extract was dried under N_2 gas and the residue was redissolved with 0.5 mL of MeOH and vortexed for 1 minute. Next, this solution was diluted two-fold with H_2O and filtered through a 0.22 µm PTFE membrane into an autosampler vial for LC/TQ analysis.

Procedure 5. SPE through Bond Elut Plexa and dSPE

QuEChERS extraction was the same as procedure 1.

SPE cleanup: The Bond Elute Plexa SPE cartridges were preconditioned with 1 mL of MeOH and 1 mL of DI water. Then, 1 mL of ACN extract was introduced to the cartridge, the eluent was collected, and the column was washed with 1 mL of methanol:water (5:95, v/v). The solution was eluted with 1 mL of MeOH and combined with the eluent at the loading step.

Disperse-SPE cleanup: The entire mixture was homogenized well and 1 mL was transferred into a 2 mL dSPE tube containing a sorbent mixture of 150 mg of anhydrous $MgSO_4$ + 50 mg of PSA + 50 mg of C18 + 7.5 mg of GCB. The tubes were vortexed for 1 minute, then centrifuged at 3,800 rpm (2,260 rcf) for 3 minutes. Then, the supernatants were diluted five-fold with MeOH:H_2O (1:1) and filtered through a 0.22 µm PTFE membrane into an autosampler vial for LC/TQ analysis.

Instrumentation

A 1290 Infinity II HPLC and a 6495C triple quadrupole LC/MS was used. LC and MS configuration and operating parameters appear in Tables 1 and 2. All data were acquired by Agilent LC/MS Data Acquisition software (version 10.1 or higher), and processing was performed using Agilent Quantitative Analysis for triple quadrupole software (version 10.2 or higher).

Result and discussion

Development of LC/TQ method

Dynamic-multiple reaction monitoring (dMRM) mode was used for data acquisition. The acquisition windows and dwell times were adjusted to optimize acquisition frequency of at least 10 data points for each peak. The MRM transitions were referenced from the Pesticides Triggered MRM (tMRM) Database for triple quadrupole LC/MS (G1733CA) and were optimized using the MassHunter Optimizer software. At least two MRM transitions were selected per compound (except for chlorpropham and procymidone because only one transition was stable enough to be monitored) to satisfy the SANTE requirements for the identification and confirmation by LC/TQ.[4]

Table 1. LC configuration and operating parameters.

Parameter	Value
Instruments	Agilent 1290 Infinity II High Speed Pump (G7120A) Agilent 1290 Infinity II Multisampler with multiwash capability (G7167B) Agilent 1290 Infinity II Multicolumn Thermostat Column Compartment (G7116B)
Needle Wash	Standard wash (MeOH:H_2O 1:9)
Thermostat Temperature	4 °C
Injection Volume	3 µL
Analytical Column	Agilent ZORBAX RRHD Eclipse Plus C18, 2.1 × 150 mm, 1.8 µm (p/n 959759-902)
Column Temperature	40 °C
Mobile Phase A	5 mM ammonium formate and 0.1% formic acid in water
Mobile Phase B	5 mM ammonium formate and 0.1% formic acid in methanol
Flow Rate Gradient	0.4 mL/min
Gradient	Time (min) A (%) B (%) Flow (mL/min) 0.5 95 5 0.4 3.5 50 50 0.4 21 0 100 0.4 24 0 100 0.4 24.1 95 5 0.4 26 95 5
Stop Time	At 26 min

Table 2. Ion source parameters used for the 6495C LC/TQ.

Parameter	Value
MS Acquisition	Agilent Jet Stream Electrospray ionization
Gas Temperature	200 °C
Gas Flow	11 L/min
Nebulizer	35 psi
Sheath Gas Heater	400 °C
Sheath Gas Flow	12 L/min
Capillary	2,500 (+V) and 3,000 (–V)
Nozzle Voltage	500

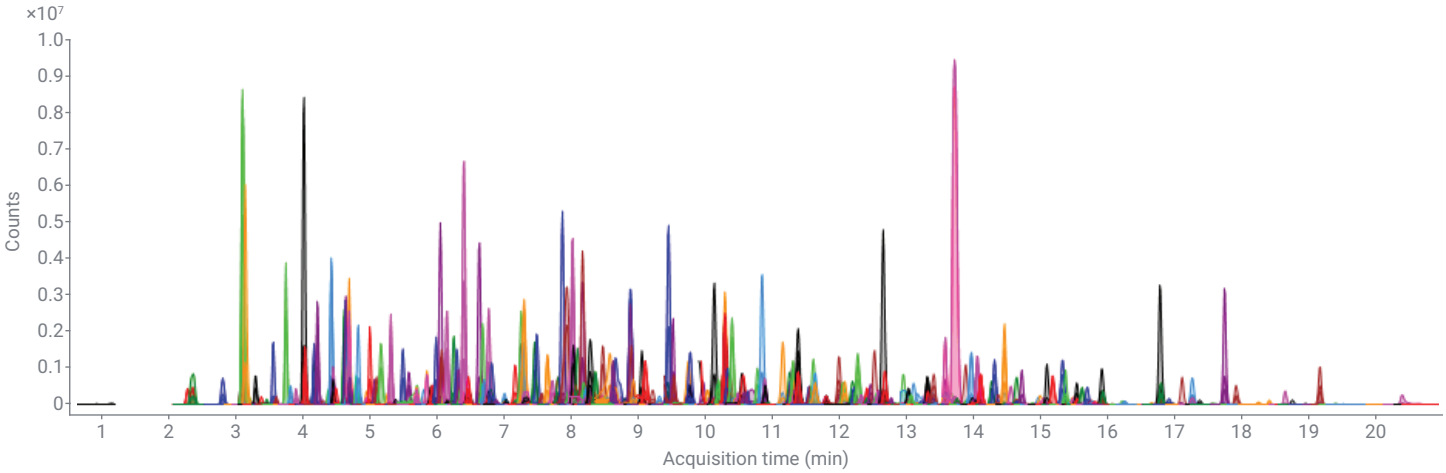


Figure 1. LC/TQ MRM chromatogram of 400 pesticides analyzed at 10 µg/L in black pepper.

The narrow, symmetrical peak shape, and targets were eluted and spread throughout the chromatography, demonstrating the efficiency of the chromatographic separation.

Several factors, such as analyte behavior in the LC column, solvent effect, and matrix effect, lead to some problems with the peak shape of some compounds. Similar to the previous study[2], some compounds with isotopes still showed split peaks due to unresolved isomers such as sulfoxaflor, difenoconazole, and benalaxyl. The unsymmetric sharp peaks were a problem with some chloroacetamide pesticides (metazachlor, dimethachlor), oxadixyl, and clethodim in black pepper. The standard solution was prepared in a weak solvent system as MeOH:H_2O (1:1) to avoid the influence of the solvent effect on peak shape. However, cypromazine still showed split peaks at high concentrations. The rest of the early eluted analytes had good peak shape. However, integration was consistent between standard and sample since it insignificantly affected the quantitative result.

Sample preparation

Extraction solvents were screened for efficiency. With simultaneous analysis of 400 different acidic, basic, polar, and nonpolar pesticides, the choice of extraction solvent significantly affects the extraction efficiency. To support efficient extraction of acidic analytes, and at the same time protect the base-sensitive pesticides, the extraction of pesticides was investigated. These extractions were done with and without acidified ACN (1% acetic acid) using method EN 15662 partitioning salts. In both cases, dSPE cleanup (150 mg anhydrous $MgSO_4$ + 50 mg PSA + 50 mg C18 + 7.5 mg GCB) was conducted. The recovery experiments were conducted at a spiking level of 0.05 mg/kg (n = 6) in all cases.

With the standard QuEChERS method (EN 15662), although recovery rates were satisfactory (>80% number of compounds) in black pepper, recovery rates met SANTE 11312/2021. However, recoveries were still lower than with the acidified ACN method (340 and 353 compounds) and the average recovery rates were significantly lower; for certain compounds, recovery dropped below 40%. In the case of the florasulam group (florasulam, flumetsulam, and metosulam) and the pyrimdinylsulfonylurea group (oxasulfuron, triasulfuron, triasulfuron methyl, nicosulfuron, metsulfon-methyl, furamsulfuron, chlorsulfuron, mesosulfuron-methyl, and flazasulfuron), bromoxynil and warfarin recovery was <40%; but, with the acidified ACN method, recovery was improved within the allowed range of SANTE 11312/2021[4] (Table 3).

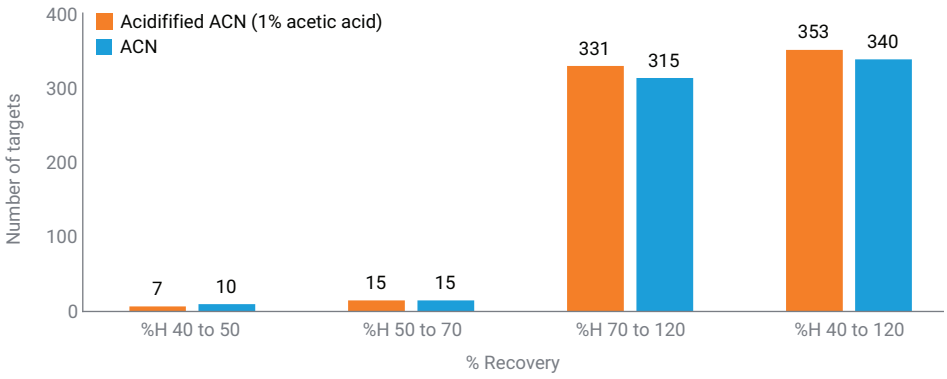


Figure 2. Comparison of the recoveries obtained by analysis of pesticide residues using different solvent extraction (with and without acidified acetonitrile).

The extraction at lower pH probably prevented or minimized the interaction between the analytes and the matrix through their charged functional groups. In addition, the presence of acetic acid in the extract helped to limit the loss of the acidic analyte absorbed dependent on the PSA stationary phase during the cleanup phase, thus improving recovery. However, acetic acid may reduce the matrix cleaning efficiency of the PSA stationary phase.[1]

Dilution factor

The dilution technique will be used after the cleanup step for complex matrices to minimize the matrix effect. However, this technique requires highly sensitive equipment to meet the analyte maximum residue limit (MRL) requirements. The 6945C LC/TQ system is a high-sensitivity instrument and delivers confidence into the ppt range and beyond so that it can meet this requirement. To evaluate the effect of the dilution factor on the matrix effect (ME), evaluation was obtained by the ratio of target response in black pepper extract. The evaluation was done after the cleanup step following procedure 1 (QuEChERS Acidified ACN + dSPE) with three samples with dilution factors (1, 5, 10) compared to that in corresponding solvent standards (Figure 3).

Despite a 10-fold increase in the dilution factor, the number of pesticides that were not significantly affected by the matrix increased from 3.5 to 39.5%. However, a percentage between –20% and 20% was considered no matrix effect. The number of pesticides severely affected by the matrix is still more than 60%. These substances will probably need to be diluted more; however, this will increase minimum detection limit (MDL). Therefore, considering the instrument sensitivity and the compound MRL value, a dilution factor of 10 is considered appropriate to meet the quantitative validity requirements of the method.

Table 3. Recoveries obtained by analysis of pesticide residues using different solvent extractions.

Compound	Extraction Method	Recovery% (0.05 mg/kg)	RSD% (n = 6)
Flumetsulam	ACN	21.45	2.66
	ACN + 1% acetic acid	73.03	3.11
Florasulam	ACN	40.95	1.89
	ACN + 1% acetic acid	76.18	4.75
Oxasulfuron	ACN	31.25	1.22
	ACN + 1% acetic acid	89.17	1.74
Triasulfuron (Logran)	ACN	26.38	6
	ACN + 1% acetic acid	87.78	2.66
Thifensulfuron-methyl	ACN	32.23	5.7
	ACN + 1% acetic acid	58.7	2.06
Nicosulfuron	ACN	16.66	6.1
	ACN + 1% acetic acid	40.58	1.7
Metsulfuron-methyl	ACN	31.81	3.42
	ACN + 1% acetic acid	62.2	1.41
Metosulam	ACN	18.23	5.78
	ACN + 1% acetic acid	82.87	2.72
Chlorsulfuron	ACN	31.86	4.84
	ACN + 1% acetic acid	53.1	4.76
Rimsulfuron	ACN	26.43	8.23
	ACN + 1% acetic acid	51.72	8.36
Foramsulfuron	ACN	16.48	5.95
	ACN + 1% acetic acid	59.5	6.39
Bromoxynil	ACN	32.95	7.15
	ACN + 1% acetic acid	46.67	8.89
Mesosulfuron-methyl	ACN	27.46	2.77
	ACN + 1% acetic acid	82.74	2.88

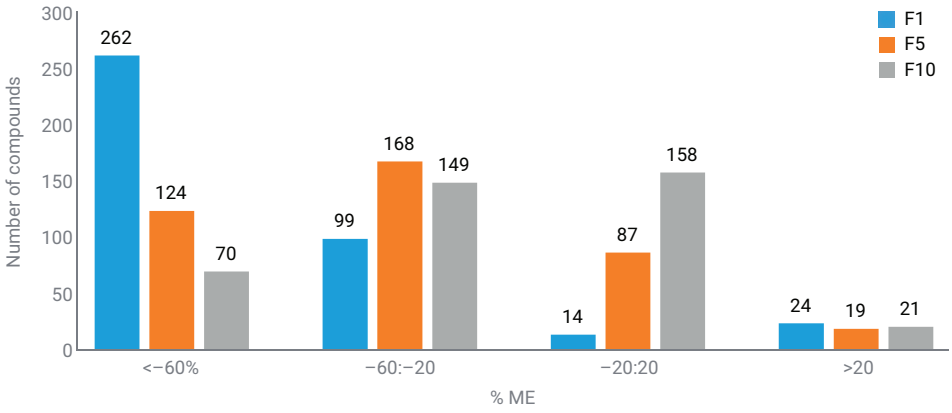


Figure 3. Numbers of pesticides in each ME range obtained by LC/TQ of black pepper extract after the cleanup step with dilution factors (F) of 1, 5, and 10.

Comparison of black pepper sample preparation method and matrix cleanup efficiency

The method performance was evaluated through three parameters: matrix removal, matrix effect, and recovery.

Matrix removal and matrix effect

The total ion chromatogram (TIC) of black pepper extract without cleanup and five extracts following five procedures by LC/TQ for the demonstration of black pepper matrix cleanliness comparison is shown in Figure 4.

The matrix cleanliness of all methods combining dilution and cleanup has a significantly lower background than the TIC of the extract without cleanup (trace black), indicating that these methods effectively remove the matrix. In particular, the SPE mixed-mode GCB/PSA method (Figure 4, violet trace) shows the best background removal efficiency with the cleanest matrix extract and the lowest background. However, this technique is complicated and time-consuming and needs to optimize the SPE. Also, the loss of pesticides during the cleanup process leads to poor recovery performance.

For the rest of the methods, the matrix cleanliness of the sample blank showed insignificant background difference between methods; however, due to the conversion factor of procedure 3 (Captiva EMR–GPD cartridge passthrough cleanup, Figure 4, yellow trace) was 2.2 times smaller than the other methods. So, the matrix cleanliness has a higher background. Once again, the results demonstrate the importance of instrument sensitivity and the effectiveness of the dilution technique in removing the sample matrix.

Matrix effect

The matrix effects were evaluated by comparing slopes of matrix-matched calibration curves with slopes of solvent calibration curves. Calibration curves (eight points from 0.05 to 10 µg/L) were prepared in the solvent ($MeOH/H_2O$) and matrix (black pepper extract obtained from the five procedures). The matrix effects (%) are summarized in Figure 5.

Similar to the results in Figure 5, procedure 4 (mixed-mode SPE GCB/PSA) was least affected by the matrix effect. In procedure 4, 256 compounds (64%) were insignificantly affected by the matrix and 338 (84.5%) compounds showed ME within 40 to 120%. Procedure 3 (Captiva EMR–GPD cartridge passthrough cleanup) had a higher dilution factor, so this procedure matrix effect was more severe, with 98 (24%) compounds insignificantly affected by the matrix and 276 (69%) compounds showing ME within 40 to 120%. The cleanup step for the other three methods was based on the dSPE technique ($MgSO_4$/PSA/C18/GCB). However, procedure 5 (SPE through Bond Elut Plexa and dSPE) used the SPE Plexa column before the dSPE phase, and the results were insignificantly different. Compounds insignificantly affected by the matrix were 42%, 39%, and 40%. These compounds showed ME% within 40 to 120%, 76.5%, 77%, and 78% for procedure 1, procedure 5, and procedure 2, respectively. The results showed that the cleanup phase with the SPE Plexa column was not effective for black pepper. For procedure 2 (MeOH-dSPE), the presence of MeOH in the extract helps to limit the loss of the acidic analyte absorbed in the PSA stationary phase during the cleanup phase. Nevertheless, it may reduce the matrix cleaning efficiency of the PSA stationary phase. Experiments showed insignificant differences in matrix effect compared with the cleanup phase of

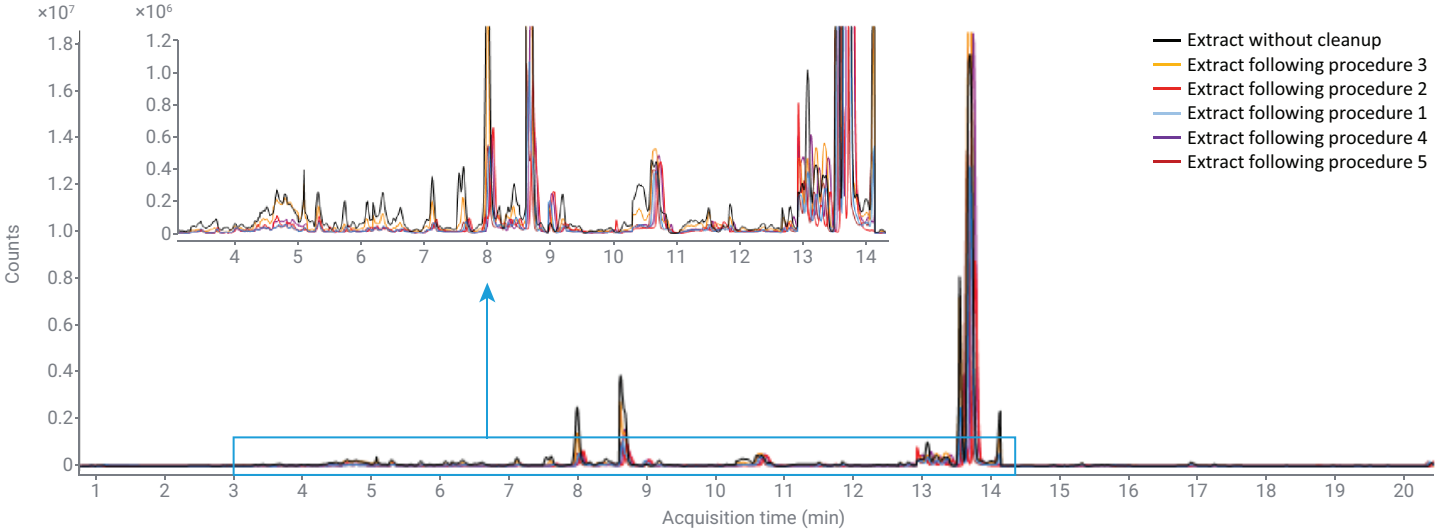


Figure 4. The total ion chromatogram (TIC) of black pepper extract without cleanup and five extracts following five procedures by LC/TQ.

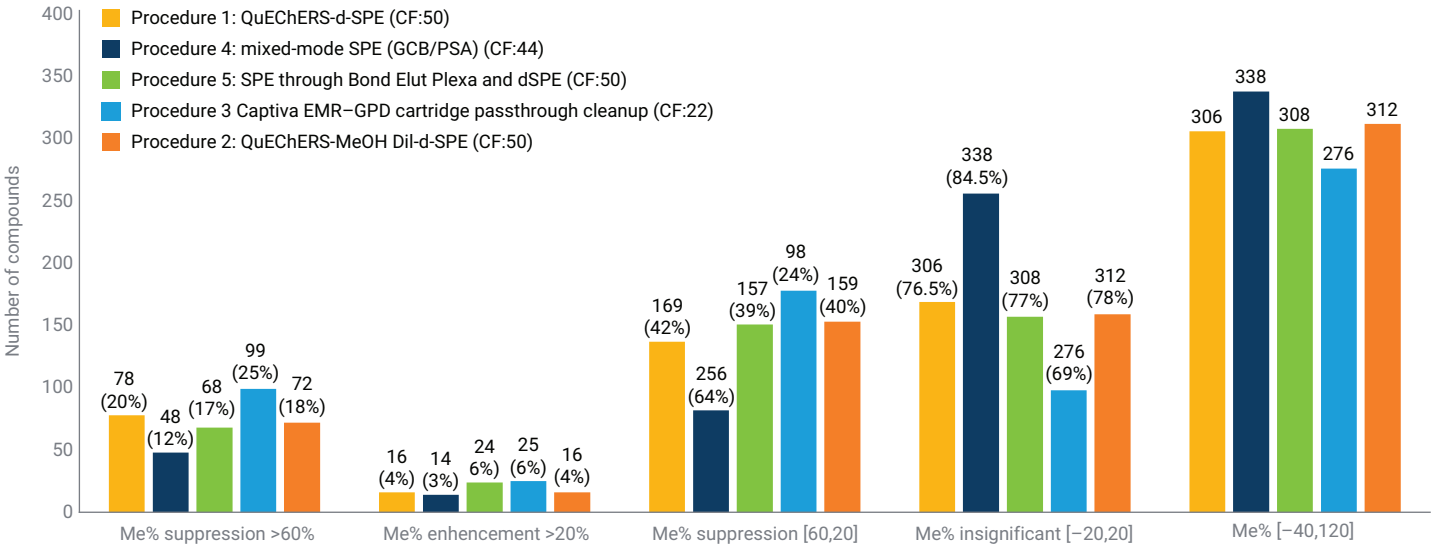


Figure 5. Numbers of pesticides in each ME range obtained by LC/TQ of black pepper extract with a different procedure.

dSPE in the absence of MeOH in the extract. Based on the experimental results for all procedures, the use of matrix calibration curves is highly recommended to compensate for the matrix effect and to achieve more reliable and consistent quantitation results. Although procedure 4 (mixed-mode SPE GCB/PSA) has a good matrix removal efficiency compared to other methods, removing only the matrix without loss of analyte is a mandatory criterion (Figure 6).

Method recovery

The recovery efficiency of the methods was evaluated based on the recovery results. These results were calculated from the matrix calibration curves for each method of the prespiked black pepper sample at 50 µg/kg (n = 6).

Although the matrix removal efficiency of the GCB/PSA mixed-mode SPE technique was the best compared with other methods, only 261 (65%) compounds had a recovery efficiency in the range of 40 to 120%. Of these samples, 187 (47%) had a recovery rate from 70 to 120%, caused by loss of acidic pesticide in the cleaning step (2,4-D, 2,4,5-T, imidazolinone

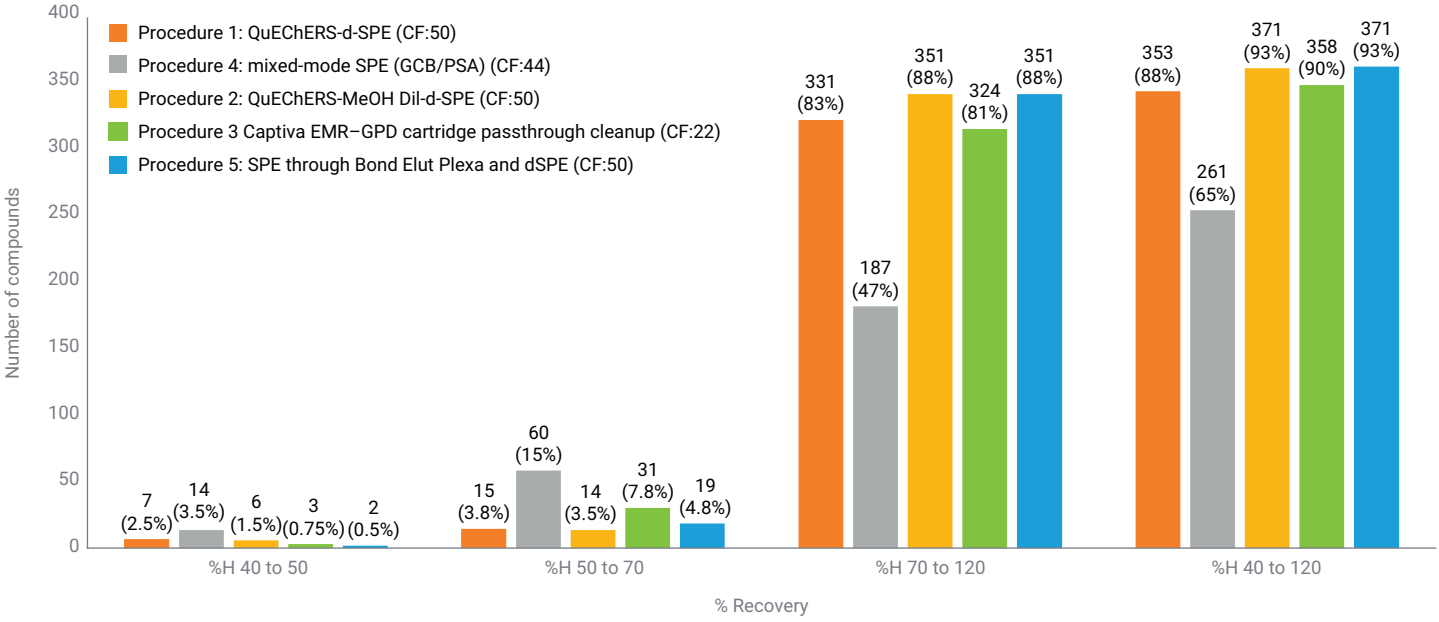


Figure 6. Numbers of pesticides in each recovery range were obtained by LC/TQ of black pepper extract with a different procedure.

group, pyrimdinylsulfonylurea group, quinolinecarboxylic acid, etc.) due to PSA, loss of planar compounds (hydramethylnon, cypromazine, pymetrozine, etc.) due to GCB and decomposition, and solvent evaporation (clethodim, hydramethylnon, aspon, ethion, etc.). Procedure 2 (MeOH-dSPE) and procedure 5 (SPE through Bond Elut Plexa and dSPE) had a higher number of pesticides with better recovery than the other methods, with 371 (93%) compounds having a recovery in the range of 40 to 120%, of which 351 (88%) had recovery rates from 70 to 120%. Procedures 1 and 3 had fewer than 353 (88%) and 358 (90%) pesticides in the 40 to 120% range, respectively. This difference is mainly due to the improved loss of pesticides by PSA (2,4-D, 2,4,5-T, 2,4,5-TP, quimerac, pyridate, imazapyr, fluroxypyr, bispyribac, imazapic, imazamox, MCPA, MCPD, dodine, etc.) and improved loss of planar pesticides by GCB.

In general, the cleaning technique combined with the dilution method has shown a particular effect on black pepper. Specifically, in procedure 3, although the dilution factor is insignificant, 358 (90%) compounds with recovery in the range of 40 to 120% are suitable for instruments with limited sensitivity. Procedure 2 and procedure 5 have a better recovery than the remaining methods. To simplify the sample processing method and take advantage of the high sensitivity of 6495C LC/TQ, procedure 2 was selected for validation in this study.

Table 4. The recoveries of some pesticides sensitive to PSA were obtained by analysis of pesticide residues using a different procedure.

Compound	Validation Parameter	Procedure 1, Agilent QuEChERS-d-SPE	Procedure 2, Agilent QuEChERS-MeOH-d-SPE	Procedure 3, Agilent Captiva EMR–GPD Cartridge Passthrough Cleanup	Procedure 4, Mixed-Mode SPE	Procedure 5, SPE Through Agilent Bond Elut Plexa and dSPE
2,4-D	Recovery% (0.05 mg/kg)	N/D	92.18	59.61	N/D	76.67
	RSD%(n = 6)	–	4.09	10.74	–	6.70
Imazapyr	Recovery% (0.05 mg/kg)	22.35	80.26	8.30	N/D	77.62
	RSD%(n = 6)	13.47	1.20	16.88	–	1.88
Imazapic	Recovery% (0.05 mg/kg)	33.63	87.81	27.02	4.60	79.56
	RSD%(n = 6)	10.43	1.15	5.07	10.54	1.54
MCPA	Recovery% (0.05 mg/kg)	21.48	80.13	59.85	N/D	72.35
	RSD%(n = 6)	6.31	2.75	6.91	–	12.00
Pyridate	Recovery% (0.05 mg/kg)	23.95	40.73	8.33	5.24	63.90
	RSD%(n = 6)	2.57	1.37	18.39	2.17	3.25
Quinmerac	Recovery% (0.05 mg/kg)	N/D	80.20	N/D	N/D	70.58
	RSD%(n = 6)	–	2.38	–	–	2.54
Dodine	Recovery% (0.05 mg/kg)	43.07	69.60	N/D	13.58	83.80
	RSD% (n = 6)	3.05	2.85	–	17.88	2.84

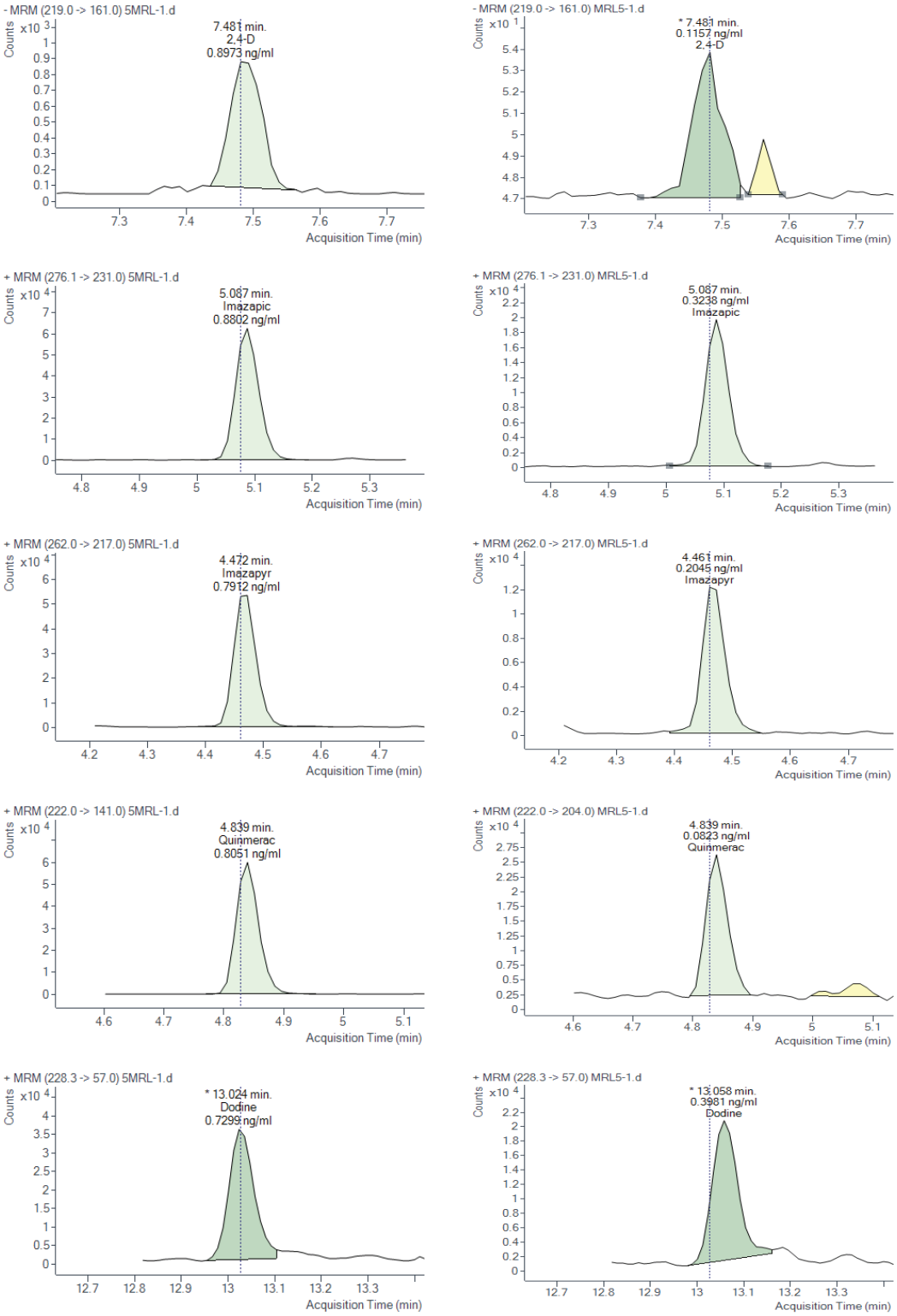


Figure 7. MRM chromatograms of some pesticides sensitive to PSA of prespike QC 50 µg/kg in black pepper with procedure 1, Agilent QuEChERS-d-SPE (right) and procedure 2, QuEChERS-MeOH/d-SPE (left).

Verification of the entire workflow performance

Calibration curve linearity: A linear least-squares regression weighted by the inverse concentration (1/x) was applied to all target compounds using external calibration. The matrix calibration curves were prepared from 0.05 to 10 µg/L (eight points). Overall, 95% of 400 targets met the calibration curves linearity requirement of R^2 ≥0.99, from LOQ to 10 µg/L. For all other standards, the calculated concentration must be within 80 to 120% of the actual concentration. As an example, the bias % of more than 95% of 400 targets was in the acceptable range of 80 to 120% of the actual concentration at calibration level 3 (0.2 µg/L).

Method recovery and precision: Recovery and precision were determined based on prespiked samples at 10 and 50 µg/kg (six samples for each level over two days). The relative standard deviation for reproducibility (RSDr) % was calculated based on the recoveries of six technical replicates of prespiked QC samples within a batch. The RSDr % was calculated based on the recoveries of 12 replicates of prespiked sample across two batches. The results are presented in detail in Table 5.

Table 5. Evaluating recovery based on prespiked samples at 10 and 50 µg/kg.

Validation Parameter	Prespike 10 µg/kg (n = 6)	Prespike 50 µg/kg (n = 6)
Repeatability		
H: 70–120% and RSDr % ≤20%	336 (84%)	351 (88%)
H: 40–120% and RSDr % ≤20%	358 (90%)	371 (93%)
Reproducibility		
H: 70–120% and RSDiR% ≤20%	336 (84%)	351 (88%)
H: 40–120% and RSDiR% ≤20%	349 (87%)	363 (91%)

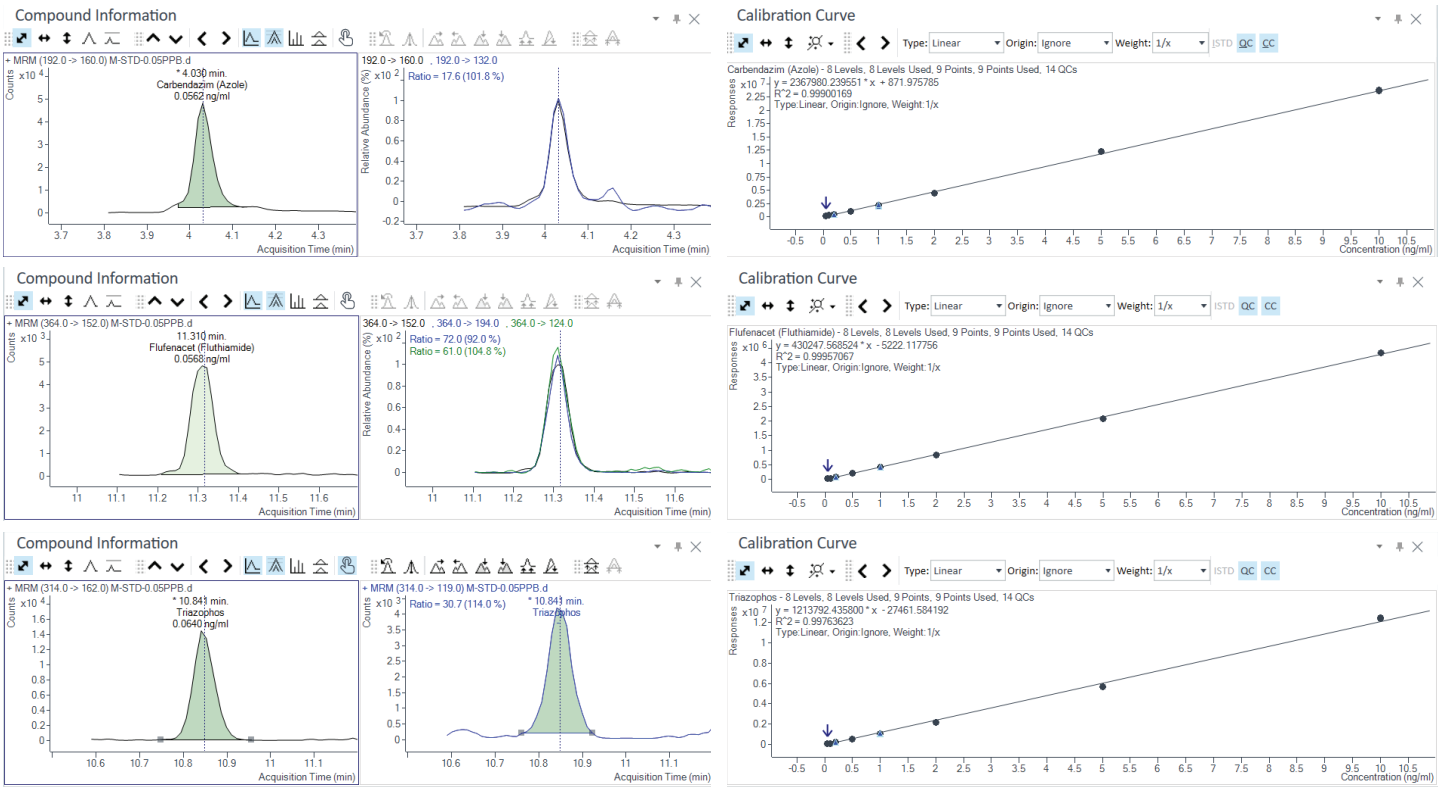


Figure 8. MRM chromatograms and black pepper matrix calibration curves of some pesticides at the lowest level (0.05 µg/L).

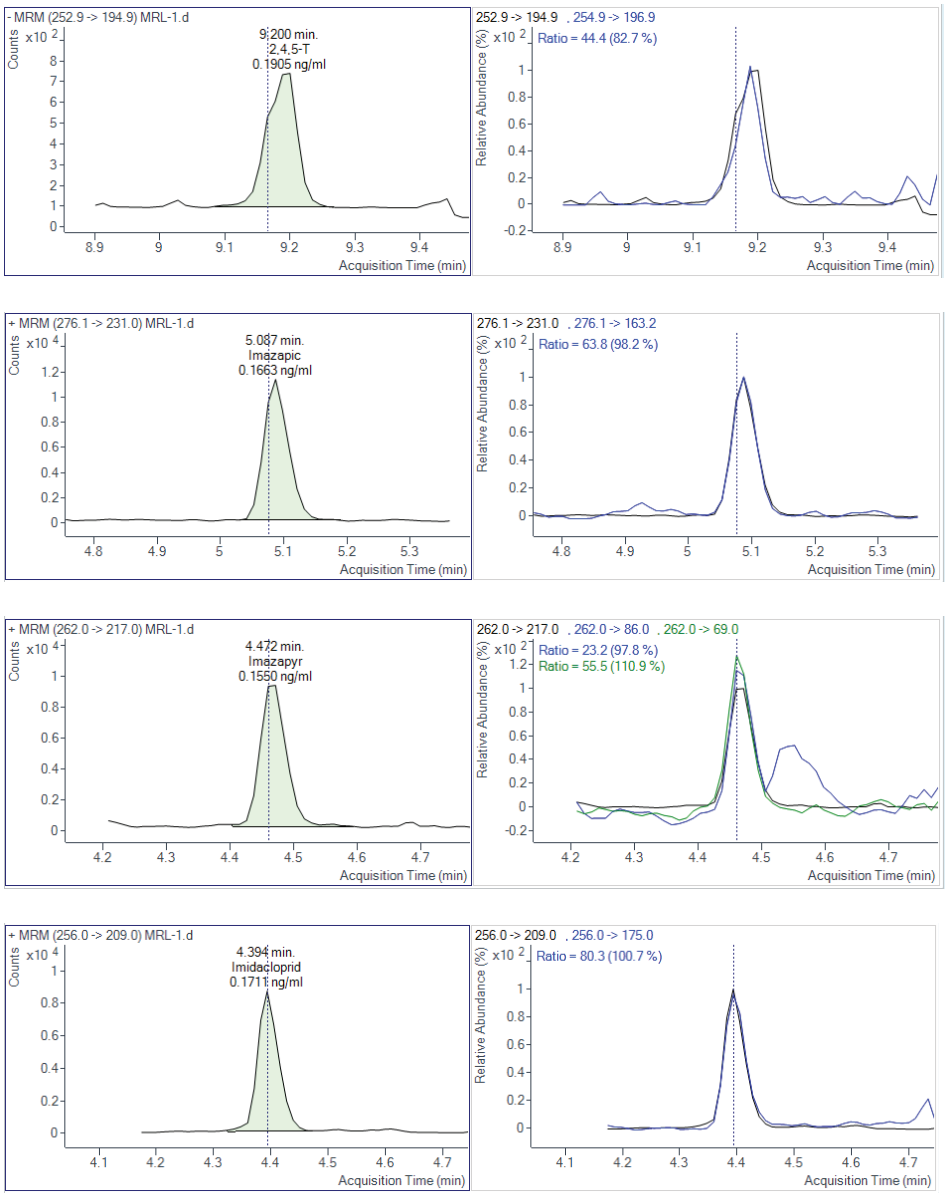
For recovery, according to the method performance acceptability criteria in SANTE guidelines, the average recovery must be within 30 to 140% and RSDr ≤20%. This study's acceptable recovery range was even more rigorously defined, from 40 to 120% with an RSDr ≤20%. Overall, the results showed that at 10 µg/kg (low concentration), and 50 µg/kg (high concentration), most of the pesticides had the recovery, repeatability, and reproducibility to meet the criteria in Table 5 with the developed workflow. These results also met the requirements of SANTE 11312/2021.[4] The results demonstrate that this method is suitable for analyzing a large group of pesticides in a complex matrix and provides consistent quantitative results for routine everyday analyses.

Compounds that do not meet the requirements of SANTE 11312/2021[4] were mainly related to the positive occurrence of the targets and matrix interferences. Although calibration curves are used to overcome the matrix effect, some substances are severely affected by the loss of sensitivity or noise, contributing to difficulties in peak integration. Some compounds had substantial ion suppression including Amitrole, Prohexadione, Halosulfuron-methyl, Propiconazole, etc. Some compounds related to the positive occurrence of the targets include Isoxathion, Dioxabenzofos, Tebuconazole, etc.

Method limit of quantitation (MLOQ)

According to SANTE/11312/2021[4], MLOQ is defined as the lowest concentration spike for the sample in which the repeatability (RSDr) and reproducibility (RSDir) are less than 20%, and recovery efficiency from 70 to 120%. The method is meaningful when the MLOQ is equal to or less than the compound MRL. Considering 50 µg/kg is the lowest MRL established for most pesticides in pepper matrix, prespiked QC at 10 µg/kg (six samples each day over two days) is sufficient for evaluating MLOQ.

The results in Figure 9 demonstrate that the analytical workflow performance provided acceptable method sensitivity with more than 336 (84%) of 400 targets. These results meet the regulatory requirements of 10 µg/kg and 15 compounds with MLOQ of 50 µg/kg because low sensitivity, matrix interferences, and positive occurrence in black pepper matrix include 2,4-D, dicamba, fenpropimorph, fluroxypyr, fluquinconazole, tepraloxydim, haloxyfop, tolclofos-methyl, bitertanol, cyanophos, benzoximate, disulfoton, chlorpyriphos-methyl, allethrin, and acrinathrin.



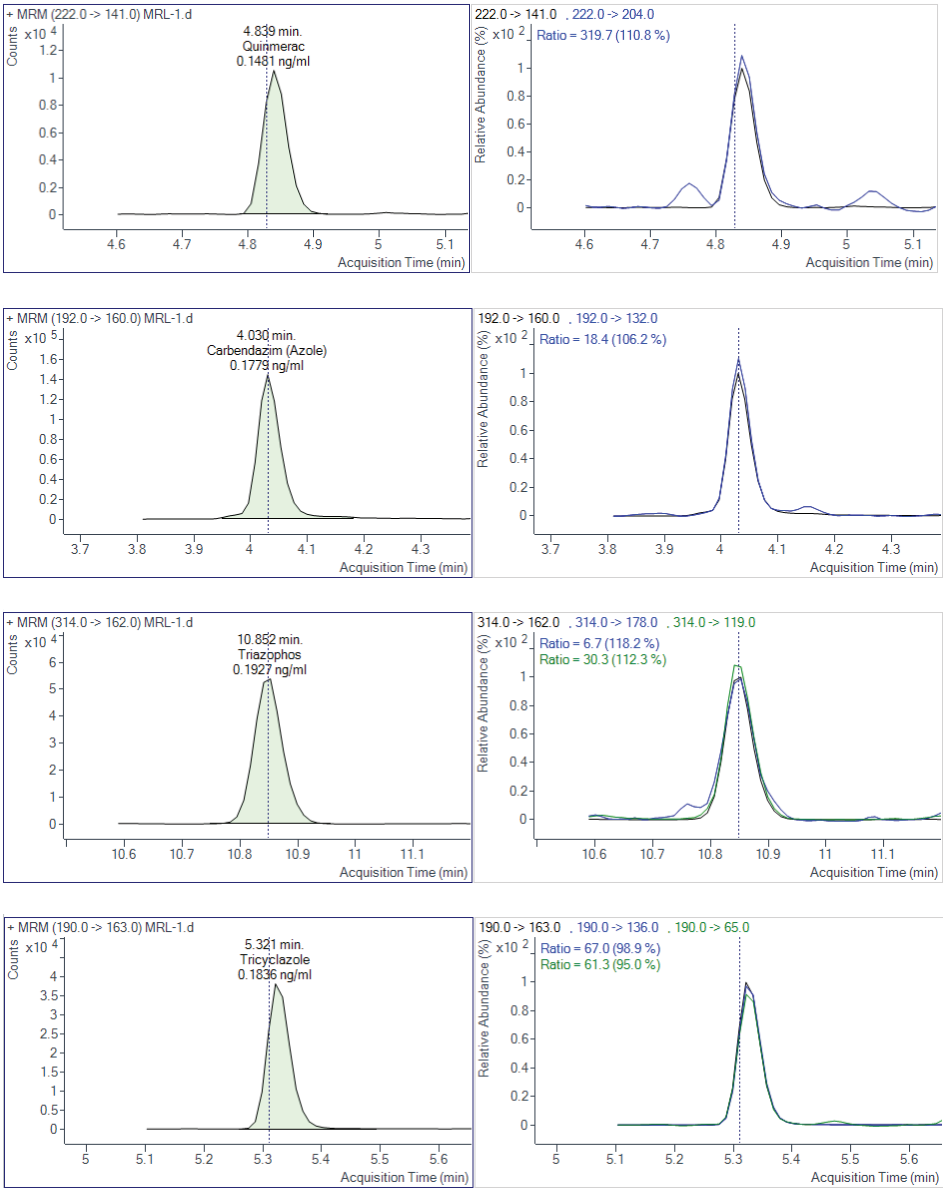


Figure 9. MRM chromatograms of some pesticides at prespike QC 10 µg/kg (MLOQ level).

Robustness assessment: Instrument method robustness is crucial for reliable analysis as part of routine, day-to-day laboratory testing. To investigate the method robustness, an 18-fold repeated intercalation analysis of the QC sample at 10 µg/L was performed (Figure 10). The analysis was done on a batch with more than 140 injections of black pepper extract over 2 days.

The results demonstrate excellent instrument method robustness for sustainable and reliable, day-to-day, routine analyses, with 312 (78%) of 400 targets having area RSDir <5% and 100% of compounds having area RSDir <20%.

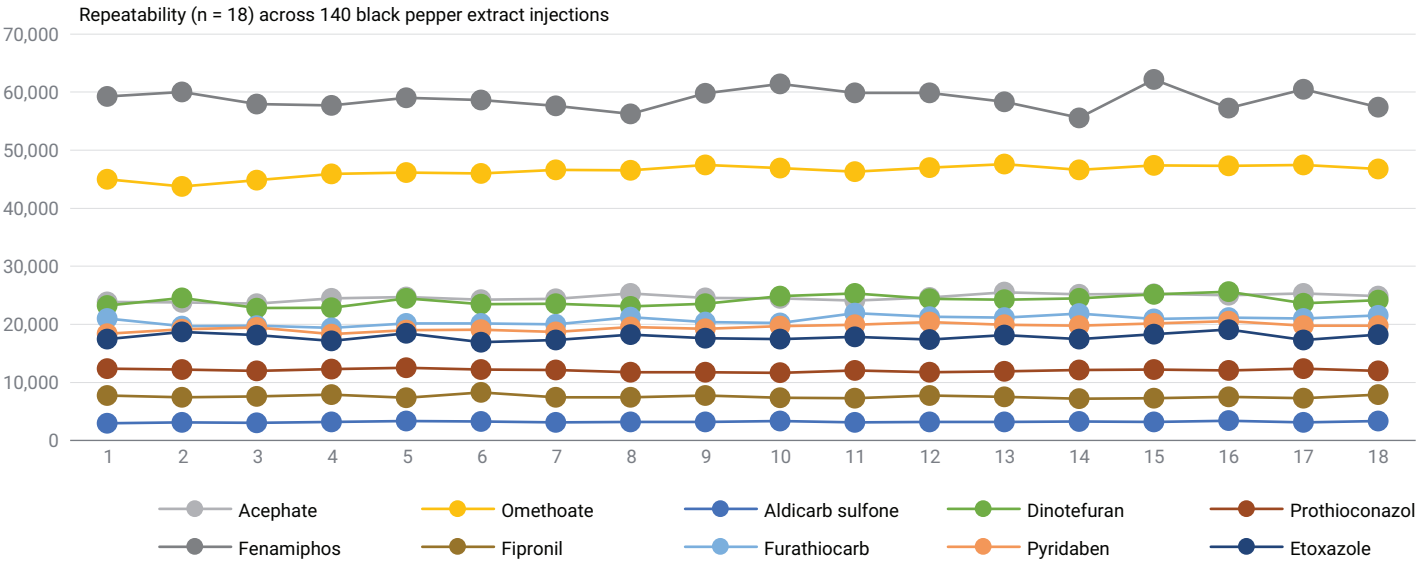


Figure 10. Distribution of some pesticides (n = 18) across 140 black pepper extract injections.

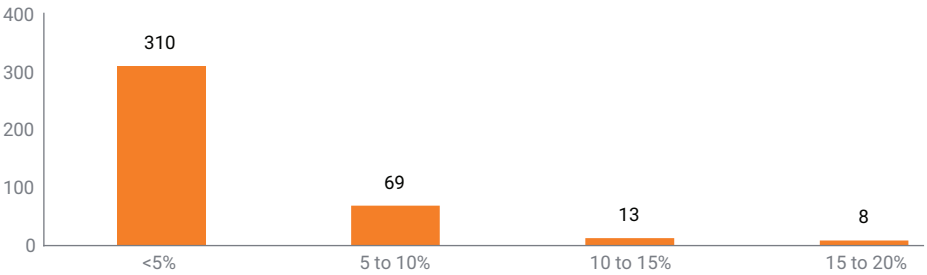


Figure 11. Distribution area RSD% of some pesticides (n = 18) across 140 black pepper extract injections.

Conclusion

This application note presents several workflows for pesticide analysis, including sample preparation, chromatographic separation, and MS detection. Each method has its advantages and disadvantages. For an instrument with excellent sensitivity, such as the Agilent 6495C LC/TQ, procedure 2 showed these results to exceed traditional sample preparation approaches in simplicity, cost savings in sample processing, reliability, and high performance in results analysis. Cleaner extract for injection also prevents contamination and carryover for the LC column and MS source, thus reducing maintenance frequency and improving the long-term overall workflow robustness. The robustness and effectiveness of the method was evaluated following the requirements of SANTE 11312/2021. The method demonstrated reliable and highly reproducible analytical performance for quantifying 400 pesticide residues in black pepper with an MLOQ achieved at 10 and 50 µg/kg for 84 and 87% of targets in black pepper, respectively, meeting the requirements of SANTE 11312/2021.

If the lab has an instrument that is not demanding in terms of sensitivity, the simplified sample preparation protocol using an Agilent Bond Elut QuEChERS EN extraction kit and passthrough cleanup using Agilent Captiva EMR–GPD is also a suitable protocol. The method does not have significant dilution factors, provides efficient black pepper matrix removal, reduces matrix effect, and cleans more matrix interferences in black pepper. More than 80% of 400 targets met SANTE 11312/2021[4] in recovery requirements.

References

1. Casado, N.; Morante-Zarcero, S.; Sierra, I. Application of the QuEChERS Strategy as a Useful Sample Preparation Tool for the Multiresidue Determination of Pyrrolizidine Alkaloids in Food and Feed Samples: A Critical Overview. *Appl. Sci.* **2022**, *12*, 4325. https://doi.org/10.3390/app12094325
2. Zou, A. *et al.* Analysis of 510 Pesticides in Black Pepper, *Agilent Technologies application note*, publication number 5994-4768EN, **2022**.
3. Boonjob, W. *et al.* Retention and Selectivity of Basic Drugs on Solid-Phase Extraction Sorbents: Application to Direct Determination of B-Blockers in Urine. *Anal. Bioanal. Chem.* **2014** Jul, *406(17)*, 4207–15. doi: 10.1007/s00216-014-7753-4. Epub 2014 May 2. PMID: 24788887.
4. SANTE/11312/2021: Analytical Quality Control and Method Validation Procedures for Pesticide Residues Analysis in Food and Feed.

Appendix

Table A1. List of pesticides analyzed in this study.

- Amitrole
- Cyromazine
- Methamidophos
- Acephate
- Omethoate
- Pymetrozine
- Oxamyl oxime
- Aminocarb
- Propamocarb
- Aldicarb sulfoxide
- Dinotefuran
- Butoxycarboxim
- Aldicarb-sulfone
- (Aldoxycarb)
- Oxamyl
- Dazomet
- Nitenpyram
- Oxydemeton-methyl
- Methomyl
- Amitraz
- Thiamethoxam
- Flucarbazone
- Imazapic
- Mevinphos (Phosdrin)
- Mexacarbate
- Dicamba
- Florasulam
- Tricyclazole
- Butocarboxim
- Prohexadione
- Aldicarb
- Metoxuron
- Ethirimol
- Aminopyralid
- Oxadixyl
- Metolcarb
- Phosphamidon (mix of isomers)
- Bentazone
- Cyanazine (Fortrol)
- Quinoclamine
- Oxasulfuron
- Amidosulfuron
- Fenthion-sulfone
- Rimsulfuron
- Foramsulfuron
- Ethiofencarb
- Cyantraniliprole
- Bromoxynil (Brominal)
- Thiodicarb
- Fluometuron
- Imazalil (Enilconazole)
- Secbumeton
- Prometon
- Fosthiazate
- Procymidone
- Thiofanox
- Disulfoton-sulfoxide
- 2,4-D
- Chlorotoluron
- Metobromuron
- Dimethachlor
- Ethoxyquin
- Desmedipham
- Pyrimethanil

- Azinphos-methyl (Guthion)
- Fenpiclonil
- Flazasulfuron
- Phenmedipham
- Clomazone
- Phosmet
- Chlorantraniliprole
- Flumioxazin
- Demeton-S
- Fenpropimorph (Ro 14-3169)
- Fluroxypyr
- Triclopyr
- 2,4,5-T
- Fenobucarb
- Saflufenacil
- Linuron
- Triazamate
- Azinphos-ethyl
- Fenarimol
- Fluoxastrobin
- Mecarbam
- Bupirimate
- Butafenacil
- Flufenacet (Fluthiamide)
- Tetraconazole
- Triticonazole
- Spirotetramat
- Pethoxamid
- Napropamide
- Oryzalin
- Epoxiconazole (BAS 480F)
- Cyazofamid
- Cyprodinil
- Fipronil-desulfinyl
- Metolachlor
- Fenbuconazole
- Carbendazim (Azole)
- Monocrotophos (Azodrin)
- Atrazin-2-hydroxy
- Dicrotophos (Bidrin)
- Atrazine-desisopropyl
- Imidacloprid
- Thiabendazole
- Clothianidin
- Ethidimuron
- Imazapyr
- Flumetsulam
- Fuberidazole
- Fenuron (N,N-Dimethyl-N-phenylurea)
- Vamidothion
- Flupyradifurone
- 3-Hydroxy Carbofuran
- Acetamiprid
- Dimethoate
- Flonicamid
- Tifatol (Cymiazole)
- Metamitron
- Trichlorfon
- Dioxacarb
- Sulfoxaflor
- Chloridazon (Pyrazon)
- Azamethiphos
- Pirimicarb
- Triasulfuron (Logran)
- Thifensulfuron-methyl
- Mephosfolan
- Dichlorvos
- Nicosulfuron
- Thiophanate-methyl
- Propoxur
- Bendiocarb
- Thidiazuron
- Metsulfuron-methyl
- Metribuzin
- Simazine
- Carbofuran
- Fenamiphos-sulfoxide
- Malaoxon
- Metosulam
- Pyracarbolid
- Fenthion sulfoxide (Mesulfenfos)
- Tebuthiuron
- Fenamiphos-sulfone
- Sulfentrazone
- Chlorpropham
- Desmetryn
- Thiometon
- Propham
- Paraoxon
- Isoprocarb
- Mesosulfuron-methyl
- Flutriafol
- Atrazine
- Triamiphos
- Tribenuron-methyl
- Metazachlor
- Thionazin
- MCPA
- Lenacil
- DEET
- Isoproturon
- Fensulfothion
- 2,3,5-Trimethacarb
- Isoxaflutole
- Metalaxyl
- Diuron
- Forchlorfenuron
- Heptenophos
- Azaconazole
- Dodemorph
- Trinexapac-ethyl
- Acibenzolar-s-methyl
- Propanil
- Atrazine-desethyl
- Ethofumesate
- Diethofencarb
- Furalaxyl
- Azoxystrobin
- Spiroxamine
- Methiocarb
- Chlorbromuron
- Halofenozide
- Dimethenamid-P
- Fludioxonil
- Fenamidone
- Propazine
- Terbuthylazine
- Promecarb
- Acifluorfen
- Picloram
- Boscalid (Nicobifen)
- Warfarin
- Bispyribac
- Terbutryn
- Prometryn
- Paraoxon-methyl
- Zoxamide
- Fipronil-sulfone
- Propiconazole
- Benalaxyl
- Diazinon
- Coumaphos
- Dodine
- Prothioconazole
- Prochloraz
- Tebuconazole
- Chlorfenvinphos
- Famoxadone
- Hexaconazole
- Phoxim
- Metconazole
- Pirimifos-methyl
- Clofentezin
- Isoxathion
- Metaldehyde
- Pyraclostrobin
- Hexaflumuron
- Cycloxydim
- Spinetoram J
- Prosulfocarb
- Clethodim
- Quinmerac
- Cymoxanil
- Imazamox
- Thiacloprid
- Mesotrione
- Isoprothiolane
- Fluopicolid
- Isoxaben
- Barban
- Fluxapyroxad
- Triforine
- Dimethomorph(E)
- Mepronil
- Cyproconazole
- Molinate
- Triadimefon
- Methoxyfenozide
- Myclobutanil
- Pyrifenox
- Propetamphos
- Flamprop-methyl
- Sedaxane
- Chloroxuron
- Triazophos
- Bifenazate (D 2341)
- Mepanipyrim

- 2,4,5-TP (Silvex)
- Paclobutrazol
- Halosulfuron-methyl
- Aminocyclopyrachlor
- Carbaryl
- Carboxin
- Chlorsulfuron
- Diflufenzopyr
- Diflubenzuron
- Uniconazole-P
- Flusilazol
- Fenamiphos
- Fipronil
- Rotenone
- Fenoxycarb
- Dioxabenzofos
- Bromuconazole
- Aclonifen
- Tebufenozide
- Silthiofam
- Quinalphos
- Bixafen
- Dimoxystrobin
- Haloxyfop
- Bensulide
- Phenthoate (Fenthoate)
- Fipronil-sulfide
- Kresoxim methyl
- Dinoseb
- Penconazole
- Beflubutamid
- Isofenphos methyl
- Cycluron
- Naled
- Methoprotryne
- Methidathion
- Methacrifos
- Tolclofos-methyl
- Triflumuron
- Phosalone
- Bitertanol
- Cyanophos
- Thiobencarb
- Cyflufenamid
- Spinosad A
- Benzoximate
- Pinoxaden
- Isofenphos
- Diniconazole
- Disulfoton (Thiodemeton)
- Metrafenone
- Chlorpyrifos-methyl
- Pencycuron
- Dialifos
- Ametoctradin
- Cycloate
- Cadusafos
- Difenoconazole (mix of isomers)
- Isopyrazam
- Hydramethylnon
- Triflumizol
- Pronamide
- Sebuthylazine
- Mandipropamid
- Malathion
- Isocarbophos
- Propaquizafop
- Tebupirimfos
- Tolfenpyrad
- Tralkoxydim
- Oxadiazon
- Temephos
- Fluazinam (Shirlan (VAN))
- Metaflumizone
- Allethrin
- Pyriproxyfen
- Quinoxyfen
- Ethion
- Emamectin benzoate
- Lufenuron
- Chlorpyriphos
- Pendimethalin (Penoxalin)
- Hexythiazox
- Carbophenothion
- Spiromesifen
- Flufenoxuron
- Etoxazole
- Propargite
- Butralin
- Pyrethrin
- Novaluron
- Profenofos
- Fenoxaprop-P-ethyl
- Quizalofop-ethyl
- Cyflumetofen
- Fenpicoxamid
- Benfuracarb
- Diclofop-methyl
- Buprofezin
- Terbufos
- Furathiocarb
- Spinetoram L
- Tebufenpyrad
- Teflubenzuron
- Picolinafen
- Meptyldinocap
- Fenazaquin
- Pyridaben
- Esfenvalerate
- Acrinathrin
- Brodifacoum
- Pyridate
- Carbosulfan
- Phenothrin
- Avermectin B1a (Abamectin B1a)
- Flumethrin
- Etofenprox
- Bifenox
- Bifenthrin
- Fluopyram
- Fluquinconazole
- Triadimenol
- Trietazine
- Tepraloxydim
- Iprovalicarb (mix of isomers)
- Tolylfluanide
- Etrimfos
- Penthiopyrad
- Flubendiamide
- Alanycarb
- Benzovindiflupyr
- 2-Pivaloyl-1,3-indandione
- Diflufenican
- Indoxacarb
- Trifloxystrobin
- Spinosin D
- Ipconazole
- Aspon
- Fenpyroximate
- Flumetrian
- Diafenthiuron
- Proquinazid
- Spirodiclofen
- Moxidectin
- Ivermectin B1a
- Silafluofen
- Azocyclotin
- Dinobuton
- Cyhexatin

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