

Analysis of 510 Pesticides in Black Pepper

Using Captiva EMR sequential passthrough cleanup and LC/MS/MS

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Abstract

This application note describes an advanced sample preparation strategy optimized for the reliable quantitation of 510 pesticides in black pepper by LC/MS/MS. The workflow was demonstrated on an Agilent 1290 Infinity II LC system coupled to an Agilent 6470B triple quadrupole LC/MS using a previously developed dynamic multiple reaction monitoring (dMRM) method.

The black pepper sample preparation strategy used the Agilent Bond Elut QuEChERS EN extraction kit, followed by Agilent Captiva Enhanced Matrix Removal–General Pigment Dry (EMR–GPD) and Captiva Enhanced Matrix Removal–General Pigment Fresh (EMR–GPF) cartridges for sequential passthrough cleanup. The workflow performance was evaluated and verified according to SANTE/11312/2021 based on method sensitivity, calibration curve linearity, prespiked QC recovery, and precision using matrix matched calibration standards from 0.25 to 100 µg/L. Matrix effect (ME) was assessed in terms of ion suppression and enhancement of MS response of 510 targets. Over 85% of analytes demonstrated linearity with $R^2 \geq 0.99$ for the calibration range of limit of detection (LOD) to 50 or 100 µg/L, while 75% of targets achieved 40 to 120% recovery with $RSD \leq 20\%$. The newly developed sample preparation method significantly reduced matrix effect and improved the extraction efficiency, as well as simplifying the overall workflow.

Introduction

Vietnam, Indonesia, India, Malaysia, Sri Lanka, and Brazil are the major producers and exporters of black pepper. To achieve the harvest and quality of black pepper, pesticides have been applied widely for pest and disease control during black pepper planting, storage, and production. These widely used pesticides raise concerns for food safety and environmental impact. Therefore, regulatory agencies worldwide, such as the European Union (EU) and Codex Alimentarius Commission (CAC), have established maximum residue limits (MRLs) for pesticides in black pepper.

Black pepper, which is considered as a difficult or unique commodity under SANTE guidelines, is a well-known, challenging matrix.¹ The sample matrix interferences are extremely difficult to clean through traditional sample preparation approaches. Current sample preparation methods either apply high dilution factor to reduce the ME, or use extensive sample cleanup to achieve a cleaner extract. These traditional approaches often achieve the desired outcome at the expense of reduced detection sensitivity.

In this work, an improved sample preparation method was developed using QuEChERS EN extraction followed by Captiva EMR–GPD and EMR–GPF passthrough cleanup. Both Captiva EMR–GPD and EMR–GPF cartridges are expanded Captiva EMR products, containing optimized blended sorbents, including the newly developed Agilent Carbon S sorbent. Carbon S sorbent is an advanced hybrid carbon material with optimized carbon content and pore structure. It provides selective and

efficient matrix passthrough cleanup to plant-origin sample matrices, including fresh and dry matrices. The Captiva EMR–GPD is designed for general pigmented dry matrix, while Captiva EMR–GPF is designed for general pigmented fresh matrix. After the traditional QuEChERS extraction, the two Captiva EMR cartridges were used sequentially for black pepper matrix cleanup. Two type of Captiva EMR cartridges were used to deliver the enhanced matrix cleaning of the complex black pepper matrix.

The sample preparation procedure, the LC/MS/MS detection method, targets acquisition, and data analysis steps are available from Agilent.²

Experimental

Reagents and chemicals

Agilent LC/MS grade acetonitrile (ACN) (part number 5191-4496) and methanol (MeOH) (part number 5191-4497), as well as ultrapure Milli-Q water were used. LC/MS grade formic acid, acetic acid, and ammonium formate were purchased from Sigma-Aldrich.

Standards and solutions

Ten custom premixed pesticide standards used for the study were from Agilent, and the standard details are listed in Table 1.³

An intermediate standard mix comprised of 510 targets at a concentration of 1,000 µg/L was prepared in ACN from stock standard solutions and used for the rest of experiment.

Neat standard solutions were prepared in ACN for ME assessment by comparing the responses in the corresponding matrix matched calibration standards.¹

The intermediate standard solution 1,000 µg/L was diluted appropriately to make the nine calibration concentration levels of 0.25, 0.5, 1, 2, 5, 10, 25, 50, and 100 µg/L in ACN.

Note: calibration standard solutions must be freshly prepared and stored in the refrigerator at 4 °C if not immediately used.

Sample preparation

The ground, dry black pepper powder was purchased from a local grocery store and used for the experimental work without further treatment.

Table 1. Agilent premixed pesticide standards.

Part Number	Part Description	Analyte Concentration	Matrix	No. of Vials	Total Analytes
5190-0551	LC/MS pesticide comprehensive test mix	100 µg/mL	Acetonitrile	8	254
CUS-00000635	Custom pesticide test mix 1	100 µg/mL	Acetonitrile	1	27
CUS-00000636	Custom pesticide test mix 2	100 µg/mL	Acetonitrile	1	26
CUS-00000637	Custom pesticide test mix 3	100 µg/mL	Acetonitrile	1	27
CUS-00000638	Custom pesticide test mix 4	100 µg/mL	Acetonitrile	1	28
CUS-00000639	Custom pesticide test mix 5	100 µg/mL	Acetonitrile	1	25
CUS-00000641	Custom pesticide test mix 6	100 µg/mL	Acetonitrile	1	26
CUS-00000640	Custom pesticide test mix 7	100 µg/mL	Acetonitrile	1	28
CUS-00000642	Custom pesticide test mix 8	100 µg/mL	Acetonitrile	1	29
CUS-00000643	Custom pesticide test mix 9	100 µg/mL	Acetonitrile	1	30
CUS-00004663	Custom pesticide test mix 10	100 µg/mL	Acetonitrile	1	25

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 Captiva Enhanced Matrix Removal–General Pigmented Fresh (EMR-GPF) cartridge (part number 5610-2090)
- Geno/Grinder (SPEX, Metuchen, NJ, USA)
- Centrifuge 5804 R (Eppendorf, Hamburg, Germany)
- Vortexer and multitube vortexer (VWR, Plainfield, NJ, USA)
- Agilent positive pressure manifold 48 processor (PPM-48) (part number 5191-4101)

A 0.5 ± 0.01 g portion of ground black pepper was weighed into a 50 mL centrifuge tube. An appropriate volume of intermediate pesticide standard solution (1,000 $\mu\text{g/L}$) was spiked into the black pepper matrix to generate the fortified quality control (QC) samples (prespiked QC). Matrix blank (MB) was prepared using unfortified black pepper samples. Prespiked QC samples were then capped tightly, vortexed, and equilibrated for 15 to 20 minutes to allow the analytes to infiltrate the sample matrix. A 10 mL aliquot of water was added into all samples. Samples were vortexed for 10 to 15 minutes to improve the extraction efficiency of low moisture commodities (dried samples).¹ An aliquot of 10 mL ACN with 1% acetic acid was added to the tubes, followed by the addition of QuEChERS EN extraction salts and the ceramic homogenizer. Tubes were capped and vigorously shaken using the Geno/grinder at 1,500 rpm for 5 minutes.

All the samples were then centrifuged at 4,000 rpm for 5 minutes. A 2.5 mL aliquot of supernatant was transferred to the 15 mL tube, and mixed with 250 μL of water with 1% formic acid. The entire mixture was homogenized well and then loaded into a Captiva EMR–GPD cartridge and placed on the Agilent PPM-48 processor with labelled 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 of sample was passing through the cartridge with no visible liquid in the cartridge, high pressure (~ 10 psi) was applied to dry the EMR–GPD cartridge completely for 2 minutes. Next, the entire eluent was transferred to a Captiva EMR–GPF cartridge for the second step cleanup with gravity elution. Upon no more dripping by gravity, the cartridge was dried completely with 10 psi pressure. The eluent was mixed well and ready for LC/MS/MS analysis. The entire sample preparation workflow introduced 22-times dilution factor. Figure 1 shows the comprehensive sample preparation workflow.

Matrix matched calibration standards preparation

Matrix matched calibration standards were prepared by postspiking the intermediate standard solution into MB. Preparation of matrix matched calibration levels was identical to solvent standards preparation, except using MB instead of ACN solvent blank.

Instrumentation

Chromatographic separation was performed using an Agilent ZORBAX Rapid Resolution High Definition Eclipse Plus C18 column (part number 959759-902) on the Agilent 1290 Infinity II LC, and the detection was performed using an Agilent 6470B triple quadrupole LC/MS equipped with an Agilent Jet Stream (AJS) electrospray ion source, operated in dMRM mode. The LC/TQ autotune was performed in unit and wide modes. All data was acquired by Agilent LC/MS Data Acquisition software (version 10.1 or higher) and processing were performed using Agilent Quantitative Analysis for QQQ software (version 10.2 or higher).

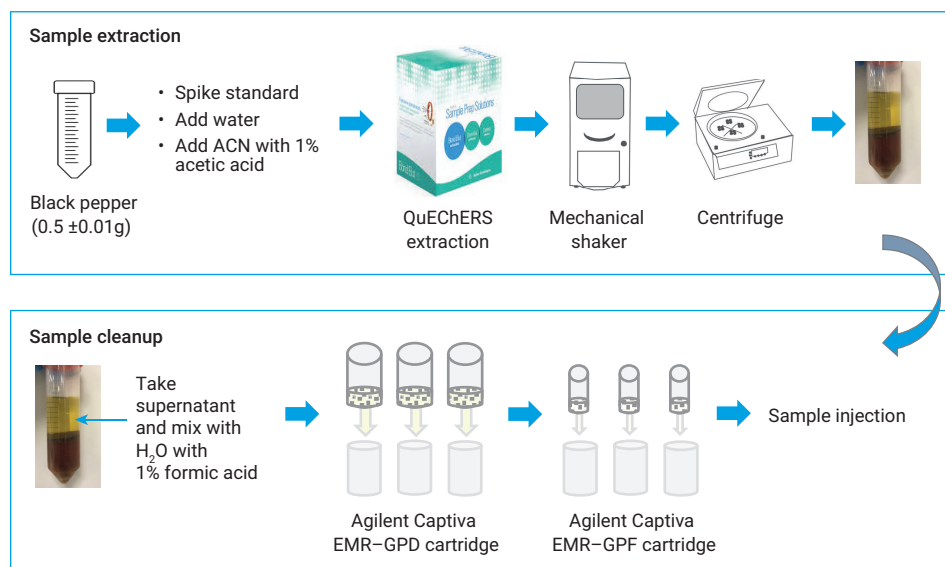


Figure 1. Black pepper sample preparation workflow using Bond Elut QuEChERS EN extraction kit followed with Captiva EMR–GPD plus Captiva EMR–GPF sequential passthrough cleanup.

The LC system conditions and TQ parameters used in the workflow are available in previous studies.^{4,5}

Result and discussion

Black pepper sample preparation and matrix cleanup efficiency

Captiva EMR passthrough cleanup methodology offers high selectivity and efficiency at matrix removal without significant impact on targets recoveries, making it a convenient, rapid, and reliable sample cleanup technique. It is especially suitable for multiclass, multiresidue analysis, providing satisfactory overall targets pass rate and decent matrix cleanliness. Compared to traditional dSPE cleanup, the passthrough cleanup provides simplified workflow steps, such as the elimination of uncapping and capping the dSPE tubes, vortexing, and centrifuging.

The new Captiva EMR cartridges adopted the advanced Carbon S sorbent blended with other sorbent using the optimized formula, and thus enables selective and efficient matrix passthrough cleanup to plant-origin sample matrices, including fresh and dry matrices.^{6,7}

Given the significant matrix complexity and challenges to matrix cleanup for black pepper, tremendous investigation work was done to develop a simple and robust sample preparation strategy that can provide the improved matrix cleanup, and acceptable targets quantitation results. The method performance evaluation was based on black pepper matrix removal and target recovery and reproducibility. The matrix dried residue weight was mostly used for a quick comparison of cleanup efficiency.⁸ A smaller representative targets list, including about 100 pesticides, was used during method development. The sequential passthrough cleanup using Captiva EMR-GPD and EMR-GPF

cartridges demonstrated the best matrix removal efficiency with overall the best target recoveries and lowest failure rate.

Figure 2 shows the black pepper matrix removal evaluation by (A) dried

residue visualization and weight, and (B) a GC/MS full-scan background test; and Figure 3 shows the total ion chromatogram (TIC) by LC/TQ for the demonstration of black pepper matrix cleanliness comparison. The comparison

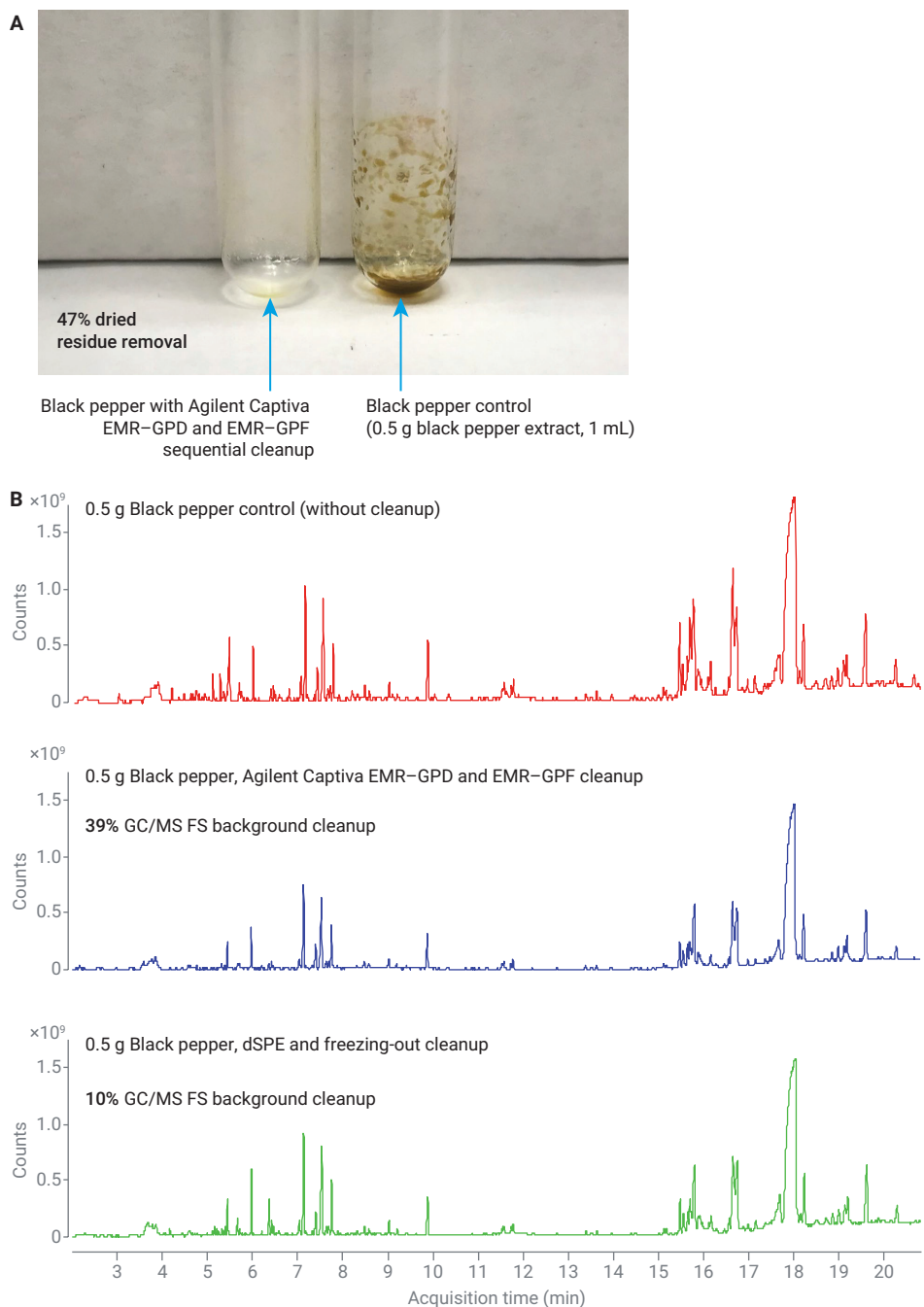


Figure 2. Black pepper matrix cleanup evaluation with Agilent Captiva EMR-GPD and Captiva EMR-GPF sequential cleanup. (A) Matrix dried residue weight test demonstrated removal of 47% of dried co-extractive residue. (B) A GC/MS full scan background test showed that 39% of sample background interferences or noise were cleaned.

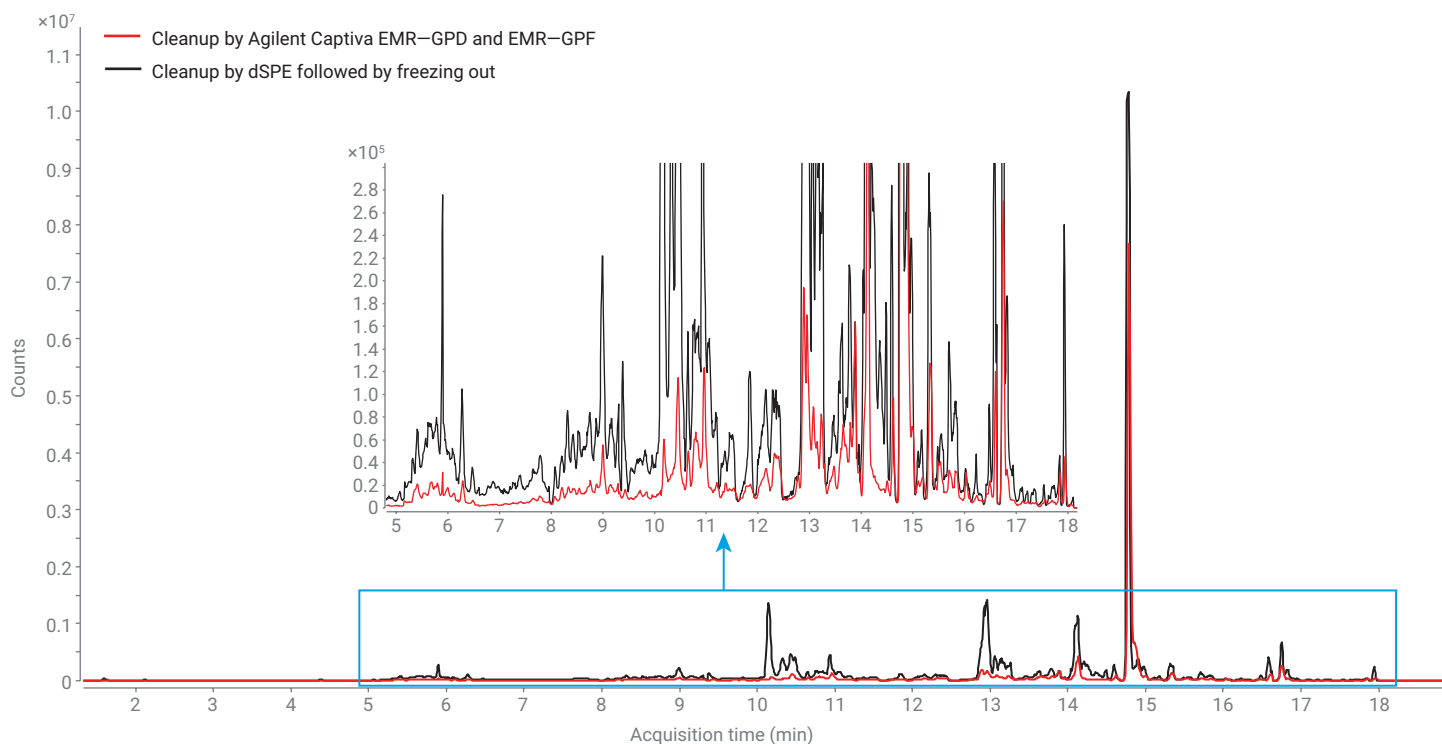


Figure 3. TIC chromatograms of black pepper matrix blank by LC/TQ using the sequential Agilent Captiva EMR-GPD and EMR-GPF passthrough cleanup, compared to dSPE followed by freezing-out cleanup.

included the sample prepared by the sequential Captiva EMR passthrough cleanup (EMR-GPD and EMR-GPF), sample prepared by the traditional dSPE plus freezing-out cleanup⁹, and black pepper crude extract without any cleanup. All these matrix cleanliness evaluation results demonstrate that the sequential Captiva EMR passthrough cleanup produced the cleanest matrix extract with lower background for the black pepper matrix.

Matrix effect (ME) evaluation

In this study, ME was evaluated for 510 pesticide residues in black pepper cleaned by two different methods: a) the dSPE with freezing-out cleanup⁹, and b) the new Captiva EMR sequential passthrough cleanup. ME evaluation following SANTE protocols was obtained by the ratio of target response in black pepper matrix matched standards to that in corresponding solvent standards.¹

Table 2 shows the ME data using two different preparation methods. Overall, 85% of 510 compounds showed ME within 40 to 120% in black pepper after the sequential Captiva EMR passthrough cleanup. This demonstrates the outstanding cleanup efficiency of the new Captiva EMR cartridges, and their quantitation can be compensated effectively by matrix matched calibration.

Table 2. ME data using the sequential cleanup with the Agilent Captiva EMR-GPD and EMR-GPF cartridges, compared to dSPE with freezing-out cleanup.

Matrix	Extraction Method	Total 510 Targets	
		No. of Targets with Insignificant ME 80 to 120%	No. of Targets with ME 40 to 120%
Black Pepper	A) QuEChERS extraction + dSPE with freezing-out cleanup	16 (3%)	136 (26%)
	B) QuEChERS extraction + Captiva EMR-GPD followed by EMR-GPF	120 (24%)	435 (85%)

Figure 4 shows a representative MRM chromatogram for all targets at 10 µg/L in black pepper extract prepared by QuEChERS EN extraction followed with Captiva EMR sequential passthrough cleanup. The symmetric sharp peaks and sensitive response demonstrated the efficient matrix cleanup and improved ME for over 500 targets using the new Captiva EMR cartridges.

Verification of the entire workflow performance

The workflow performance was verified based on calibration curve linearity, method sensitivity, recovery, and precision according to SANTE guidelines.¹ Intra- and inter-batch analyses were carried out to assess method repeatability and reproducibility.

Each batch included solvent blank, matrix matched calibration standards, matrix blank, and prespiked and postspiked QCs. Three technical replicates of prespiked QCs were prepared per batch.

1) Calibration curve linearity

The calibration curve linearity for all targets was evaluated using matrix matched standards from the dynamic range of 0.25 to 100 µg/L. Overall, 85% of 510 targets met the calibration curve linearity requirement of $R^2 \geq 0.99$, from LOD to 50 or 100 µg/L. The accuracy of linearity was also assessed based on the deviation of back calculated concentration accuracy, compared to the spiking concentration at all levels. As an example, more than 90% of 510 compounds at calibration level 6

(10 µg/L) showed a back calculated accuracy within the acceptance window of $100 \pm 20\%$ in black pepper extract.

2) Method sensitivity

Method sensitivity was evaluated based on LOD and limit of quantitation (LOQ). In this study, method LOD was established using the lowest calibration standard, with consideration of signal-to-noise ratio (S/N) of ≥ 3 , and accuracy within 70 to 130%.¹ For 97% of 510 targets in black pepper extract, the method LOD was obtained with ≤ 10 µg/L. Furthermore, 67% and 81% of 510 targets showed LOD ≤ 0.25 and ≤ 1 µg/L, respectively. The LOD results in black pepper extract demonstrated high sensitivity of the 6470B LC/MS/MS, and excellent matrix cleanliness provided by the

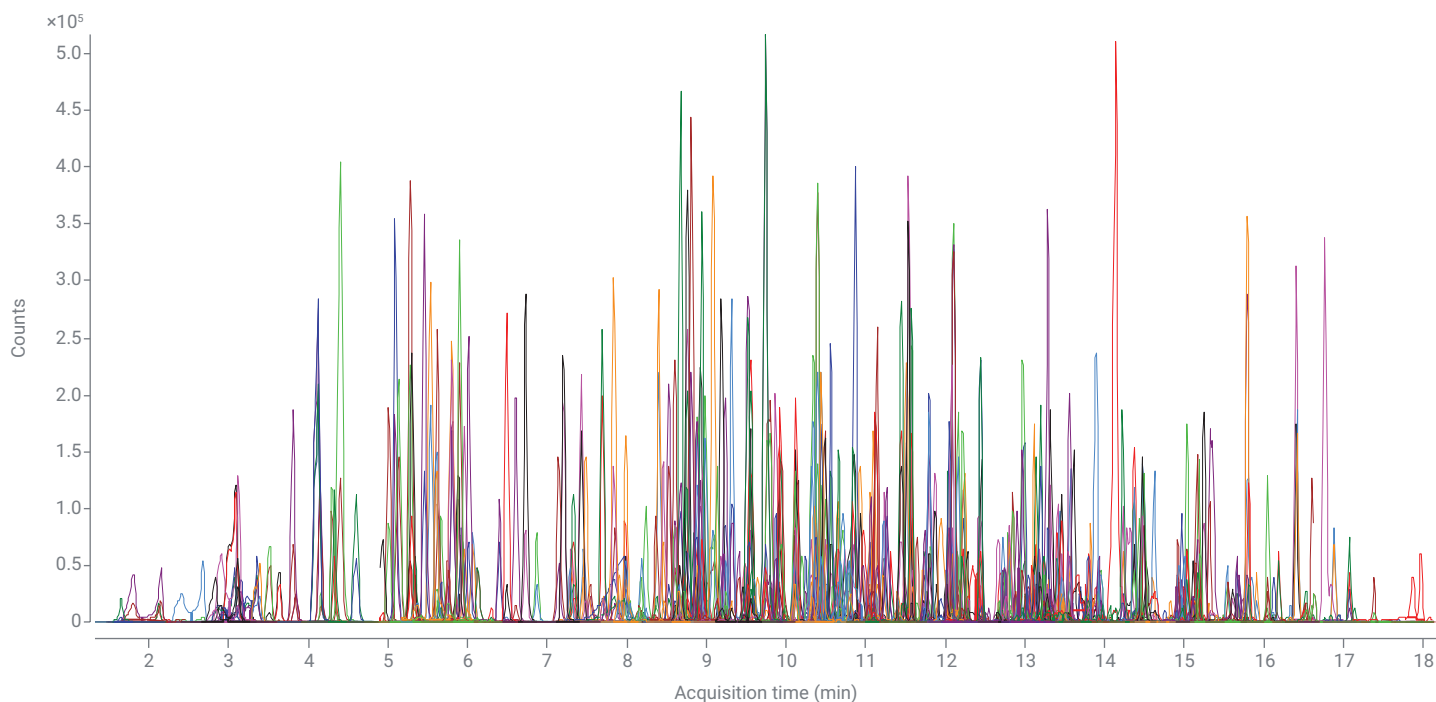


Figure 4. Representative MRM chromatogram of 510 pesticides at 10 µg/L in black pepper matrix extract.

newly developed sample preparation method. Carbendazim, carbofuran, and triazophos are identified as typical planar, alkaline, or acidic compounds, where loss could happen easily during sample cleanup. Therefore, these three challenging compounds were selected to demonstrate the method performance in black pepper matrix. Figure 5 shows the LC/MS/MS MRM chromatograms

overlay of carbendazim, carbofuran, and triazophos from 0.25 to 100 µg/L in black pepper, illustrating the excellent linearity and sensitivity of the workflow.

According to SANTE guidelines, LOQ was obtained from the prespiked samples at the lowest level providing S/N of 10 and up, with additional consideration of target identification and method

performance criteria for analyte recovery and precision in black pepper matrix. Table 3 compares MRLs established by the EU for black pepper matrix with LOQs measured in this study.¹⁰ The results here demonstrate that the analytical workflow performance provided acceptable method sensitivity to meet the regulatory requirements.

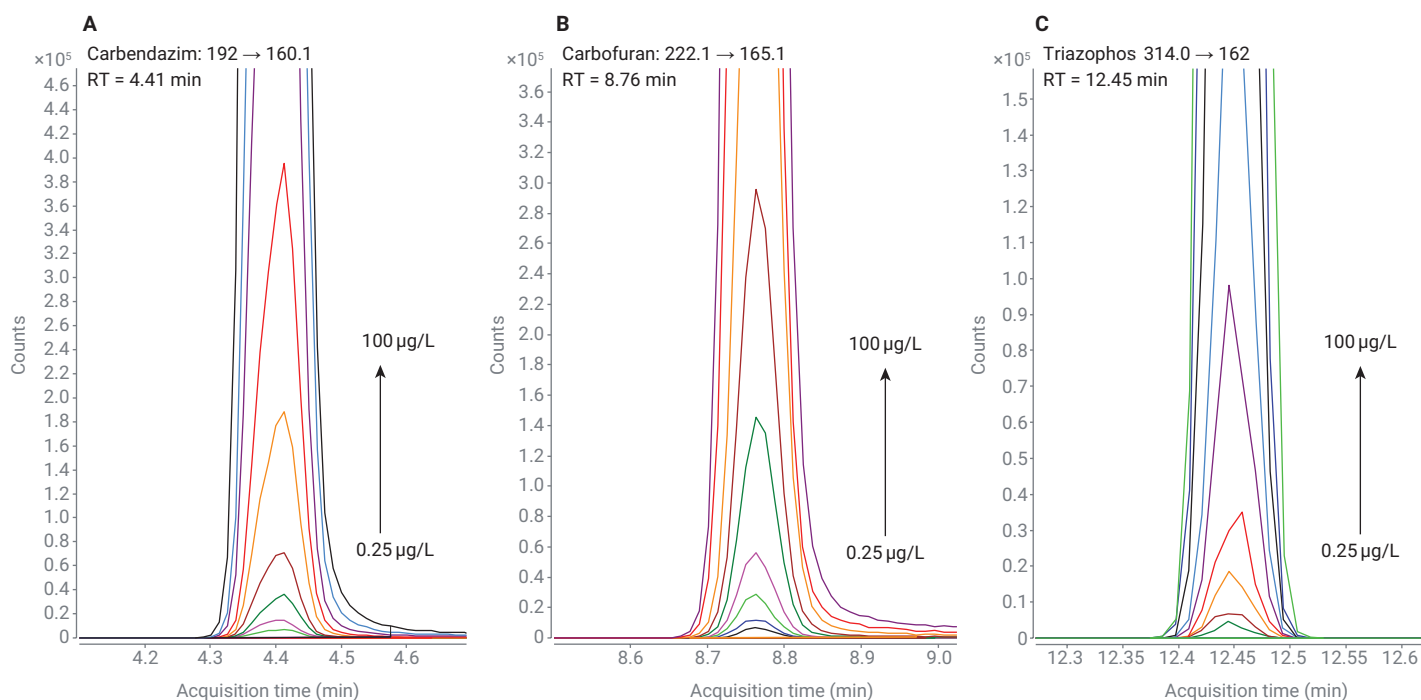


Figure 5. MRM chromatograms overlay of (A) carbendazim, (B) carbofuran, and (C) triazophos from 0.25 to 100 µg/L in black pepper extract.

Table 3. Comparison between MRLs established by EU for black pepper matrix and corresponding LOQs measured following the workflow described in this application note.

Pesticide	MRLs in EU (µg/kg)	LOQs in Study (µg/kg)
Acetamiprid	100	10
Atrazine	100	10
Buprofezin	50	10
Carbendazim	100	10
Carbofuran	50	10
Clothianidin	50	10
Cyprodinil	100	10
Difenoconazole	300	10
Epoxiconazole	100	25*
Florasulam	50	10

Pesticide	MRLs in EU (µg/kg)	LOQs in Study (µg/kg)
Flutriafol	50	10
Hexaconazole	50	10
Imidacloprid	50	10
Isoproturon	50	10
Metalaxyl	50	10
Methomyl	50	10
Paclobutrazol	50	10
Pirimicarb	50	10
Prochloraz	150	10
Pyridaben	50	10

Pesticide	MRLs in EU (µg/kg)	LOQs in Study (µg/kg)
Pyrimethanil	50	10
Tebuconazole	50	10
Thiabendazole	50	10
Thiamethoxam	50	10
Triazophos	70	10
Tricyclazole	50	10

* Raised LOQ due to positive occurrence in black pepper matrix.

Figure 6 shows the MRM chromatograms overlay of carbendazim, carbofuran, and triazophos for three technical replicates at prespiked QC 10 µg/kg (LOQ level). Considering the 22x dilution factor introduced during sample preparation, this corresponds to ~0.45 µg/L in black pepper extract. As shown in Figure 6, high response with $RSD_r < 3\%$ was obtained for these three challenging compounds at LOQ level, demonstrating the excellent method sensitivity and reproducibility.

3) Method recovery

Target recovery is a key indicator of the sample preparation method performance and trueness of measurement for pesticide residues in food matrices. Considering 50 µg/kg is the lowest MRL established for pesticides in pepper matrix, prespiked QC at 10 µg/kg was used to evaluate target recovery in this study. The recovery was obtained based on the ratio of calculated concentration of analyte from the matrix matched

calibration curve to the expected concentration in prespiked QCs ($n = 3$). According to the method performance acceptability criteria in SANTE guidelines, the average recovery must not be lower than 30% or above 140% if they are consistent ($RSD_r \leq 20\%$). In this study, the acceptable recovery range was even more stringently defined from 40 to 120% with $RSD_r \leq 20\%$. Figure 7 shows the recovery distribution of 510 targets in black pepper matrix. Overall, 382 of 510 compounds (75%) met the above criteria with the developed workflow. Furthermore, 358 of 510 targets (70%) showed 70 to 120% recovery at 10 µg/kg in black pepper. The recovery results confirmed that the developed sample preparation method provided acceptable analyte recoveries for most targeted pesticides in black pepper. The results demonstrate that the newly developed sample preparation method is suitable for analysis of a large panel of pesticides in a complex matrix.

The unacceptable recoveries of the failed pesticides were mainly related to the positive occurrence of the targets and matrix interferences in the black pepper. Positive occurrence of pesticide residues is quite common in black pepper matrices; and it is difficult to find a completely clean matrix blank. As a result, the positive occurrence for certain pesticides significantly impacted the accuracy of the matrix matched calibration curve for target quantitation. These compounds included diuron, DEET, epoxiconazole, fluometuron, quinalphos, and so on. In addition, matrix interferences caused by unidentified matrix background also contributed to the difficulty of accurate peak integration.¹ Targets including anilazine, diniconazole, mepronil, nicotine, pencycuron, terbutryn, and so on were shown to have significant matrix interferences, resulting in difficult consistent targets quantitation.

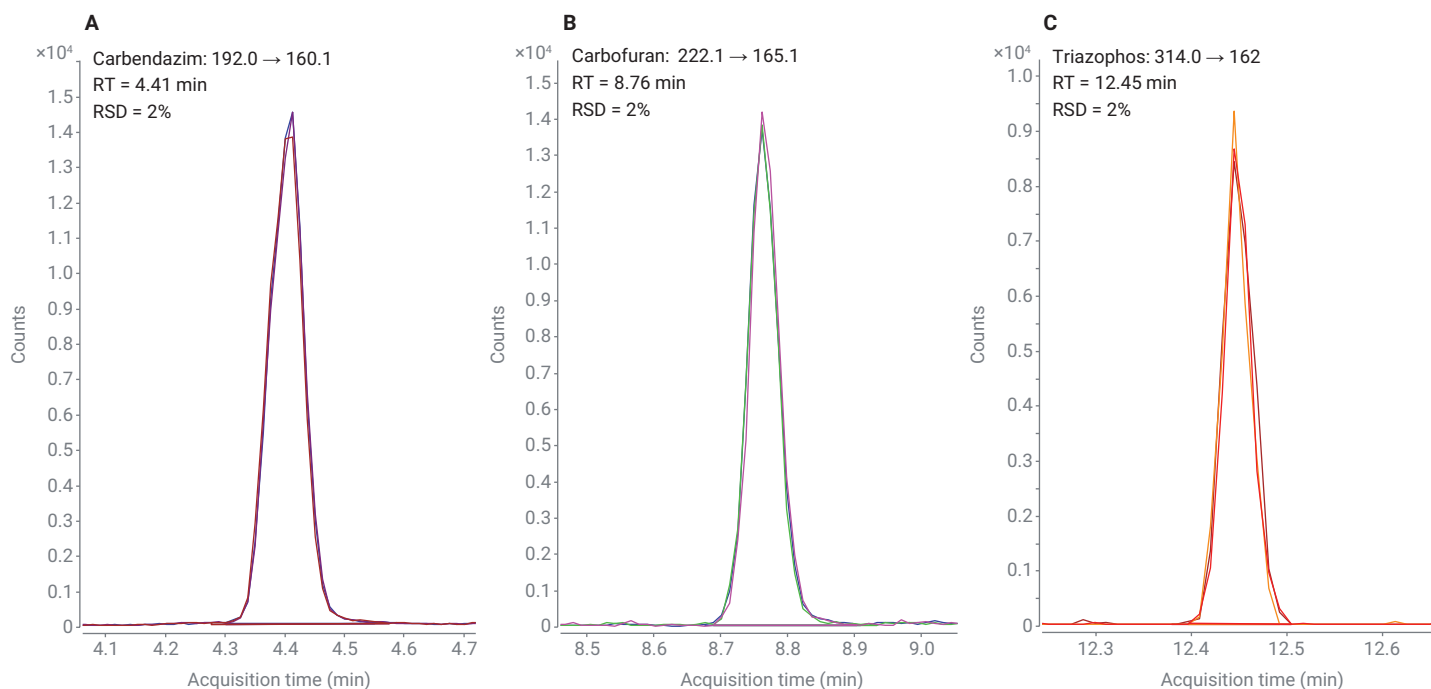


Figure 6. MRM chromatograms overlay of (A) carbendazim, (B) carbofuran, and (C) triazophos for three technical replicates at prespiked QC 10 µg/kg in black pepper matrix within a batch.

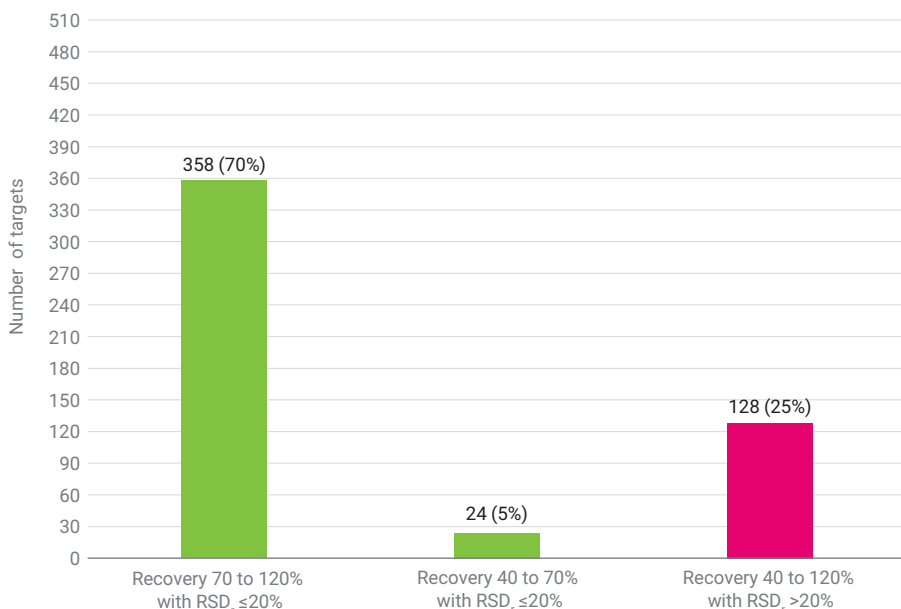


Figure 7. Prespiked QC recovery distribution of all 510 targets in black pepper.

4) Method precision

Method precision was evaluated using the intrabatch recovery repeatability and inter-batch recovery reproducibility, based on the technical replicates of prespiked QCs at 10 µg/kg. RSD_r % was calculated based on the recoveries of three technical replicates of prespiked QCs within a batch. Overall, 80% of targets in black pepper provided

RSD_r ≤20%, which demonstrates the consistent performance of the sample preparation method.

RSD_{ir} % was calculated based on the recoveries of six replicates of prespiked QCs across two batches, prepared by two lab scientists using different lots of sample matrix on different days. Of 510 targets, 78% gave out RSD_{ir} ≤20% in black pepper. Figure 8 shows

inter-batch recovery values of three representative targets (carbendazim, carbofuran, and triazophos) for six technical replicates at prespiked QC 10 µg/kg in black pepper. The results showed that the developed analytical workflow provided consistent quantitation results for day-to-day routine analyses.

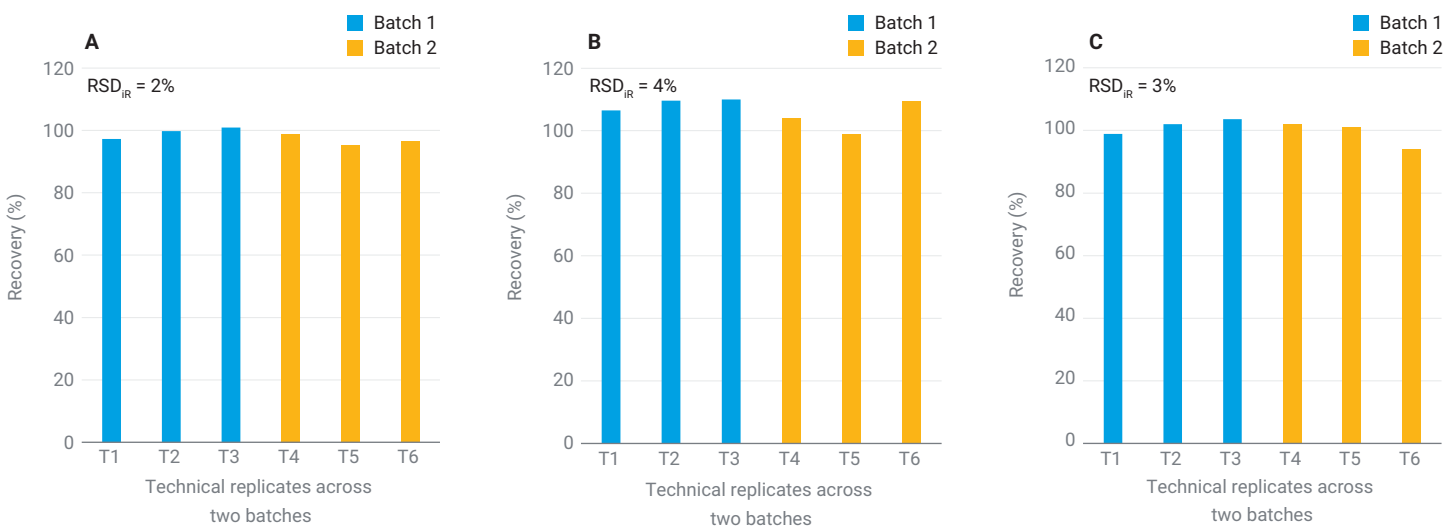


Figure 8. Recovery values of (A) carbendazim, (B) carbofuran, and (C) triazophos for six technical replicates at prespiked QC 10 µg/kg across two batches.

Conclusion

The total workflow described here, including sample preparation, chromatographic separation, and MS detection, provided a selective and sensitive quantitative analysis for 510 pesticide residues in the black pepper matrix.

The simplified sample preparation protocol using the Agilent Bond Elut QuEChERS EN extraction kit followed by sequential passthrough cleanup using the Agilent Captiva EMR–GPD and EMR–GPF cartridges provided efficient black pepper matrix removal, reduced the matrix effect, and cleaned more matrix interferences in black pepper. The method delivered acceptable quantitation results for 75% of pesticides. These results are shown to exceed traditional sample preparation approaches. Cleaner extracted samples also prevent LC column and MS source contamination and carryover, thus reducing maintenance frequency and improving the long term overall workflow robustness.

Method LOD was achieved at a sub-1 and 10 ng/mL for 81 and 97% of targets in black pepper, respectively. The LOQs met MRL requirements for compounds in pepper matrix established in the EU pesticides database, delivering a prominent result to laboratories that run pesticide residue analyses routinely. The workflow reproducibility was evaluated

from intra- and inter-batch analysis, demonstrating the highly reproducible and reliable analytical performance for the quantification of more than 500 pesticide residues in black pepper. It also demonstrates a possibility for extension to other complex dry food matrices containing high pigment and fat content.

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Printed in the USA, April 28, 2022

5994-4768EN