



Optimizing Recoveries of Planar Pesticides in Spinach Using Toluene and Agilent Bond Elut QuEChERS AOAC Kits with Graphitized Carbon

Application Note

Food Safety

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Abstract

This application note describes the impact of toluene addition in the dispersive solid phase extraction (SPE) step on the analysis of pesticides in spinach using Agilent Bond Elut QuEChERS AOAC kits for highly pigmented fruits and vegetables. Graphitized carbon black (GCB) is required in the dispersive SPE kits in order to remove high levels of pigments from the matrix. However, it also retains pesticides with planar structures resulting in poor recovery and precision. The eight problematic pesticides found in the original AOAC method, by either LC/MS/MS or GC/MS, generated poor results with about 20% to 60% recovery with >15% relative standard deviation (RSD). In the modified AOAC method, an aliquot of toluene was added to the dispersive SPE clean-up tube, in a ratio of 8:3 (acetonitrile (ACN) extracts/toluene). It significantly improved the extraction efficiency of the problematic planar pesticides. With the modified AOAC method, the eight problematic pesticides generated substantially improved recoveries, 50% to 100%, and < 10% RSD. However, the addition of toluene also introduced more matrix impurities into the final sample, and caused problems for some pesticides which gave good results originally. Therefore, the modified AOAC method cannot be considered a "drop in" replacement for the original AOAC method; but it can be a very useful alternative for the problematic pesticides affected by GCB in the pesticides analysis of highly pigmented matrix.



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Introduction

The AOAC quick, easy, cheap, effective, rugged, safe (QuEChERS) method has been widely applied in the analysis of pesticides in food since it was introduced by USDA scientists. [1-3] In general, it contains two major steps: extraction and dispersive SPE clean-up. In the extraction step, the method uses a single-step buffered acetonitrile (1% HAc) extraction while simultaneously salting out water from the sample using anhydrous magnesium sulfate (MgSO_4) to induce liquid-liquid partitioning. For cleanup, a dispersive solid phase extraction (dispersive SPE) step is employed using a combination of primary secondary amine (PSA) to remove polar organic acids as well as other components, and anhydrous MgSO_4 to reduce the remaining water in the extract. After mixing and centrifugation, the upper layer is ready for analysis.

Various food matrices require modifications to the dispersive SPE clean-up step. For general fruits and vegetables, 50 mg PSA and 150 mg MgSO_4 , per mL of ACN extracts are used for clean-up to remove polar organic acids, some sugars and lipids, and excess water. Pigmented fruits and vegetables kits, besides PSA and MgSO_4 , include 50 mg GCB per mL of ACN extracts to remove pigments like chlorophyll and carotinoides. For fruits and vegetables with fats and waxes, 50 mg C18 per mL of ACN extracts is added with PSA and MgSO_4 removing lipids and sterols. Therefore, according to the food matrix, analysts need to select a suitable dispersive SPE kit in order to analyze pesticides of interest.

Previously, we demonstrated the excellent performance of Bond Elut QuEChERS AOAC buffered AOAC extraction kits and dispersive SPE kits for general fruits and vegetables on a representative group of pesticides in apple by LC/MS/MS and GC/MS. [4, 5] For the Bond Elut QuEChERS AOAC kits for pigmented fruits and vegetables, spinach was selected as the matrix in order to evaluate the extraction and performance of the dispersive kit. GCB was added to the dispersive SPE kit to remove the high level of pigments, such as chlorophyll and carotinoides, which can cause more matrix effect and introduce more interferences. Conversely, GCB can cause a significant loss of planar pesticides, for example, thiabendazole, chlorothalonil, coumaphos, cyprodinil. [3, 6] Therefore, the use of GCB is recommended when planar pesticides are not being analyzed; greatly limiting the usefulness of GCB to the clean-up of pigmented matrix. In previous GCB SPE column extractions [7], solvent mixtures containing toluene were commonly used to elute pesticides through GCB columns. ACN/toluene (3:1) mixtures have been used for the multiclass multiresidue method (MRM) elution of pesticides through

tandem GCB- NH_2 [8], GCB-PSA [9], and GCB SAX-PSA. [10] In this study, toluene was added into the ACN extracts in the second step of QuEChERS, the dispersive SPE clean-up. We determined that the ratio of 8:3 (ACN extract toluene) generated higher recoveries (50% to 300% higher), and substantially better precision ($< 10\%$ RSD) for the eight GCB retained pesticides. However, it was noted that the addition of toluene caused adverse affects, such as additional matrix impurities in the final extracted samples, lower recovery and higher imprecision for certain pesticides which originally produced good results without the addition of toluene.

Experimental

Reagents and Chemicals

All reagents and solvents were HPLC or analytical grade. Methanol (MeOH), and toluene were from Honeywell (Muskegon, MI, USA). Acetonitrile (ACN), dimethyl sulfoxide (DMSO) and acetic acid, glacial (HAc) were from Sigma-Aldrich (St Louis, MO, USA). Ammonium acetate (NH_4OAc) was from Fisher Chemicals (Fair Lawn, NJ, USA). Formic acid (FA) was from Fluka (Sleinheim, Germany). The pesticide standards and internal standard triphenyl phosphate, (TPP) were purchased from Sigma-Aldrich (St Louis, MO, USA), ChemService (West Chester, PA, USA), Ultra (North Kingstown, RI, USA), or AlfaAesar (Ward Hill, MA, USA).

Solutions and Standards

The 1 M ammonium acetate pH 5 stock solution was made by dissolving 19.27 g NH_4OAc powder in 250 mL Milli-Q water, and the pH adjusted to 5 with glacial acetic acid. The solution was stored at 4 °C. Methanol/ H_2O (20:80) containing 5 mM ammonium acetate pH 5 was made by combining 200 mL MeOH and 800 mL Milli-Q water, adding 5 mL of 1M ammonium acetate pH 5 stock solution and mixing well. A 5 mM ammonium acetate in ACN solution was prepared by adding 5 mL of 1 M ammonium acetate pH 5 stock solution to 1 L ACN, mixing well and sonicating 5 min. 1% HAc in ACN was prepared by adding 10 mL of glacial acetic acid to 1 L of ACN, and mixing well.

Standard and internal standard (IS) stock solutions (2.0 mg/mL for all, except 0.5 mg/mL for carbendazim) were made in MeOH, 0.1% FA in ACN, or DMSO, respectively, and stored at -20 °C. Three QC spiking solutions of 1.5, 7.5, and 30 $\mu\text{g}/\text{mL}$ were made fresh daily in 1:1 ACN/ H_2O containing 0.1% FA. A 10 $\mu\text{g}/\text{mL}$ standard spiking solution in 1:1 ACN/ H_2O containing 0.1% FA was made for preparation of LC/MS/MS calibration curves in the matrix blank extract by appropriate dilution.

A 2.5 µg/mL standard solution in ACN containing 0.1% FA was used to prepare the GC/MS calibration curves in the matrix blank extract by appropriate dilution. A 15 µg/mL IS spiking standard of TPP in 1:1 ACN/H₂O containing 0.1% FA was made.

Equipment and material

Agilent 1200 Series HPLC with Diode Array Detector (Agilent Technologies Inc., CA, USA).

Agilent 6410 triple quadrupole MS/MS system with Electrospray Ionization (Agilent Technologies Inc., CA, USA).

Agilent Gas Chromatograph (Agilent Technologies Inc., Santa Clara, CA, USA).

Agilent 5975C Mass Spectrometer (Agilent Technologies Inc., Santa Clara, CA, USA).

Agilent Bond Elut QuEChERS AOAC Extraction kits, p/n 5982-5755, and Bond Elut QuEChERS AOAC dispersive SPE kits for Pigmented Fruits and Vegetables, p/n 5982-5222 and 5982-5258 (Agilent Technologies Inc., DE, USA).

CentraCL3R Centrifuge (Thermo IEC, MA, USA)

Bottle top dispenser (VWR, South Plainfield, NJ, USA)

Eppendorf microcentrifuge (Brinkmann Instruments, Westbury, NY, USA)

Instrument Conditions

HPLC conditions

Column: Agilent ZORBAX Solvent Saver Plus Eclipse Plus Phenyl-Hexyl, 3.0 × 150 mm, 3.5 µm (p/n: 959963-312)

Flow rate: 0.3 mL/min

Column temperature: 30°C

Injection volume: 10 µL

Mobile phase: A: 5 mM ammonium acetate, pH 5.0 in 20:80 MeOH/H₂O;
B: 5 mM ammonium acetate, pH 5.0 in ACN

Needle wash: 1:1:1 ACN/MeOH/IPA/H₂O w/0.2% FA.

Gradient:	Time	% B	Flow rate (mL/min)
	0	20	0.3
	0.5	20	0.3
	8.0	100	0.3
	10.0	100	0.3
	10.01	20	0.5
	13.0	STOP	

Post run: 4 min

Total cycle time: 17 min.

GC conditions

Inlet: Splitless

Inlet liner: Helix double taper, deactivated (p/n: 5188-5398)

Carrier gas: Helium

Inlet pressure: 19.6 psi (constant pressure mode) during run
1.0 psi during back flush

Inlet temperature: 250 °C

Injection volume: 1.0 µL

Purge flow to split vent: 30 mL/min at 0.75 min

Oven temperature program: 70 °C (1 min), 50 °C/min to 150 °C (0 min),
6 °C/min to 200 °C (0 min), 16 °C/min to 280 °C (6 min)

Post run: 3 min

Capillary flow technology: Purged Ultimate Union (p/n: G3186B) – used for backflushing the analytical column and inlet.

Aux EPC gas: Helium plumbed to Purged Ultimate Union

Aux EPC pressure: 4.0 psi during run, 80.0 psi during backflush

Column: Agilent J&W HP-5ms Ultra Inert
15 m × 0.25 mm, 0.25 µm (p/n: 19091S-431UI)

Connections: Between inlet and Purged Ultimate Union (p/n: G3186B)

Restrictor: 65 cm x 0.15 mm, 0.15 µm DB-5 ms Ultra Inert.

Connections: Between the Purged Ultimate Union and the MSD.

For the instrument acquisition data of MS/MS in LC/MS/MS and MS in GC/MS relating to the analytes, please refer to the acquisition data table in the previous Agilent publications. [4, 11]

Sample Preparation

The sample preparation procedure includes sample comminution, extraction/partitioning and dispersive SPE clean-up. The QuEChERS method employing spinach as the vegetable matrix is similar to the method described in detail in previous application notes [4,5], with the exception of the dispersive SPE step which includes a toluene addition.

The frozen chopped organic spinach was homogenized thoroughly. Fifteen grams (± 0.1g) of homogenized sample was placed into 50 mL centrifuge tubes. Samples were fortified with appropriate QC spiking solutions (100 µL) if necessary, and then 100 µL of IS spiking solution (15 µg/mL of TPP). After vortexing the samples for 30 s, 15 mL of 1% HAc in ACN was added to each tube. An Agilent Bond Elut QuEChERS AOAC extraction salt packet (p/n 5982-5755) was added directly to each tube. Sample tubes were sealed tightly, and hand-shaken vigorously for 1 min. Tubes were centrifuged at 4,000 rpm for 5 min.

A 1 mL aliquot of the upper ACN layer was transferred into an Agilent Bond Elut QuEChERS dispersive SPE 2 mL tube (p/n 5982-5222); or an 8 mL aliquot was transferred into an Agilent Bond Elut QuEChERS dispersive SPE 15 mL tube (p/n 5982-5258). The 2 mL tube contained 50 mg of PSA, 50 mg of GCB and 150 mg of anhydrous $MgSO_4$, while the 15 mL tube contained 400 mg of PSA, 400 mg of GCB and 1200 mg of anhydrous $MgSO_4$. Subsequently, 375 μ L of toluene were added to the 2 mL tubes, and 3 mL of toluene were added to the 15 mL tubes. The tubes were tightly capped and vortexed for 1 min. The 2 mL tubes were centrifuged with a micro-centrifuge at 13,000 rpm for 2 min, and the 15 mL tubes centrifuged in a standard centrifuge at 4,000 rpm for 5 min. An 825 μ L amount of extract was transferred into a 15 mL centrifuge tube and dried by N_2 flow. Samples were reconstituted with 600 μ L of ACN containing 0.1% FA, vortexed and soni-

cated. A 200 μ L aliquot of the extract was transferred into an autosampler vial, and 800 μ L of water or appropriate standard solutions (prepared in water) were added. The samples were capped and vortexed thoroughly prior to LC/MS/MS analysis. For samples analyzed by GC/MS, a 600 μ L reconstituted sample was either transferred directly to an autosampler vial or used to prepare the calibration curves.

In order to determine toluene's affect on the dispersive SPE procedure, another aliquot of ACN extracts was processed following the original dispersive SPE clean-up procedure.

Figure 1 shows the dispersive SPE procedure scheme according to the original method (w/o toluene) and the modified method (w/ toluene).

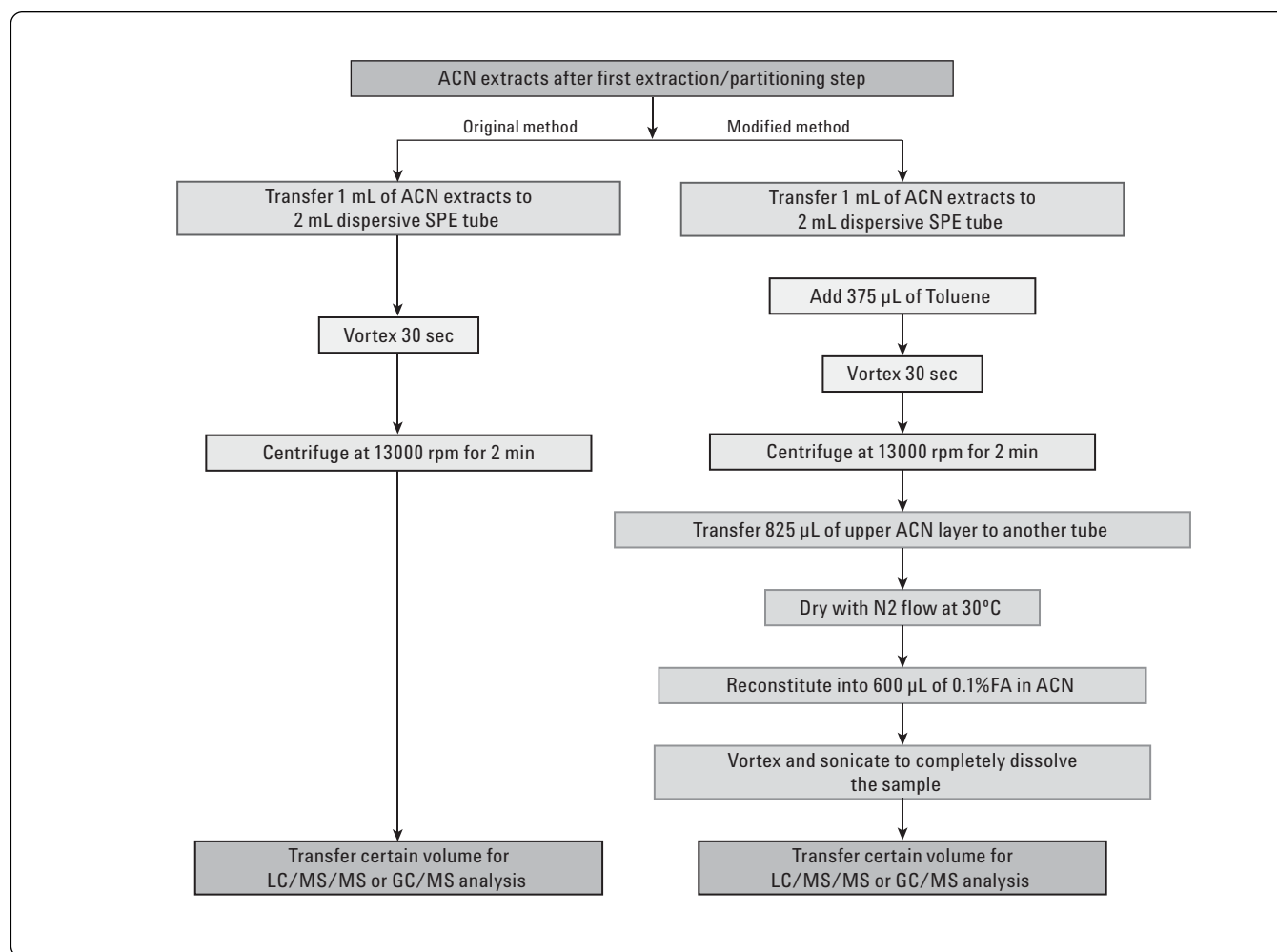


Figure 1. Dispersive SPE procedures of original method (w/o toluene) and modified method (w/toluene).

Results and Discussion

Impact on the Clean-up of Matrix

The QuEChERS methodology for pesticide residue analysis provided high-quality results with a fast, easy, inexpensive approach. For pigmented fruits and vegetables, the addition of GCB in the dispersive SPE tube can greatly remove pigments and sterols. This was clearly shown by the color of the extracts. The spinach ACN extract after the first salt extraction step was very dark green in color. When a dispersive SPE kit for pigmented produce (with GCB) was employed for dispersive SPE clean-up, the upper ACN extract layer became

clear with an almost colorless to very light yellow color. On the contrary, when a dispersive SPE kit for general fruits and vegetables was used without GCB, the upper layer was still a dark green to black color. The dispersive SPE extracts modified by the addition of toluene gave a bright yellow color after vortexing and centrifuging. The increase of color for the extracts suggested that the addition of toluene either reduced the affinity of GCB for those pigment molecules, or back-extracted those molecules from the GCB. The addition of toluene resulted in more impurities in the final extracted sample which is demonstrated by the comparison of the UV chromatograms ($\lambda = 254$ nm) for the two matrix blanks as shown in Figure 2.

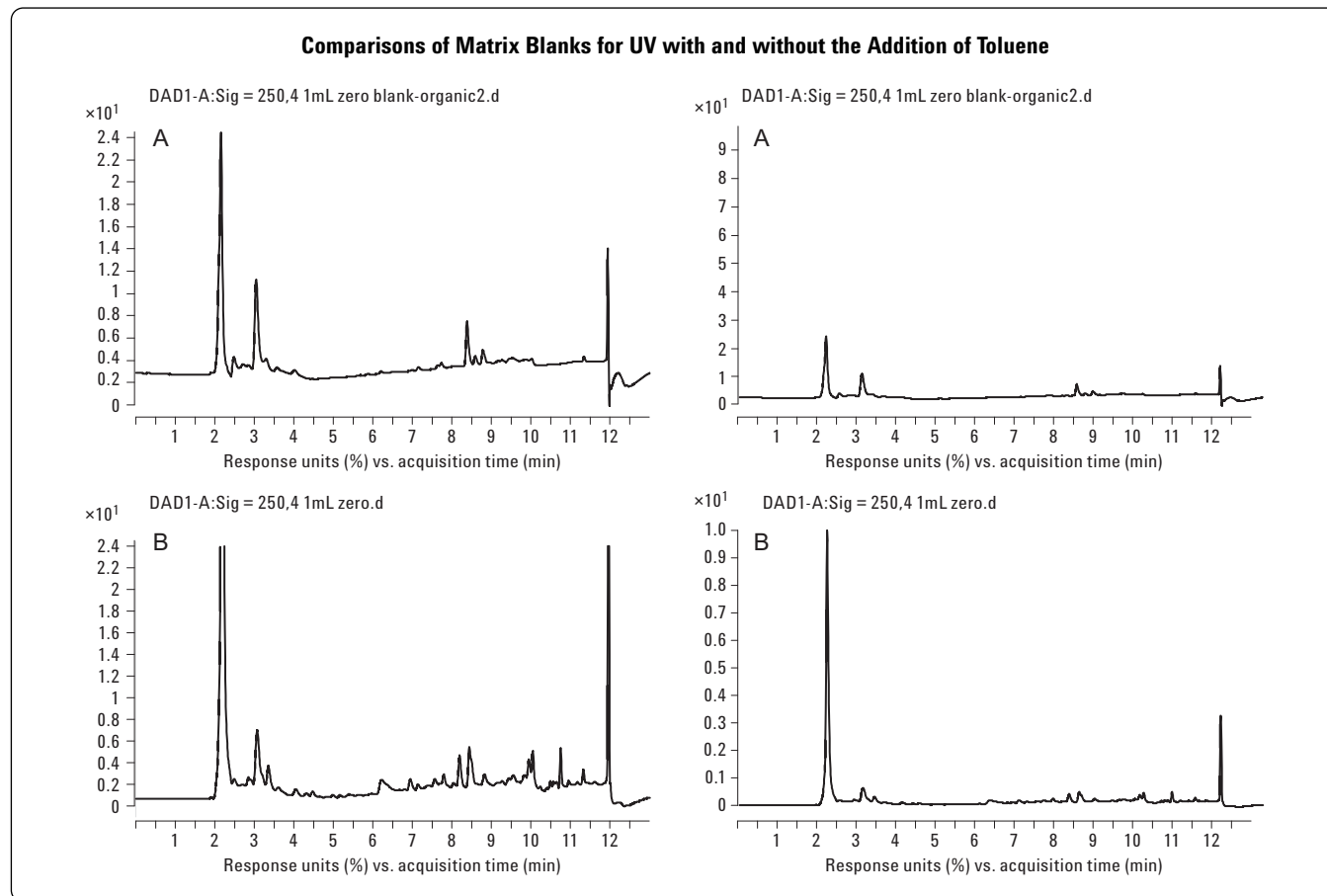


Figure 2. UV chromatogram ($\lambda = 254$ nm) comparison of matrix blank obtained with original method without toluene (A) and modified method with addition of toluene (B). Left chromatograms shown in small scale for detail comparison, and right chromatograms shown full scale for big interference peaks comparison. In both cases, the same scale was used for blank A and B chromatograms.

However, the increase in matrix impurities didn't affect the LC/MS/MS or GC/MS pesticide analysis. Figure 3 shows the spinach matrix blank LC/MS/MS chromatograms processed by the modified method with addition of toluene (A) and the original method without addition of toluene (B). With the enhanced selectivity of LC/MS/MS, the two blank samples

(A and B) showed similarly clean chromatograms. Figure 4 shows the spinach matrix blank GC/MS chromatograms processed by the modified method with the addition of toluene (A) and the original method without the addition of toluene (B). The two blank chromatograms show some minor differences, but similarities are confirmed.

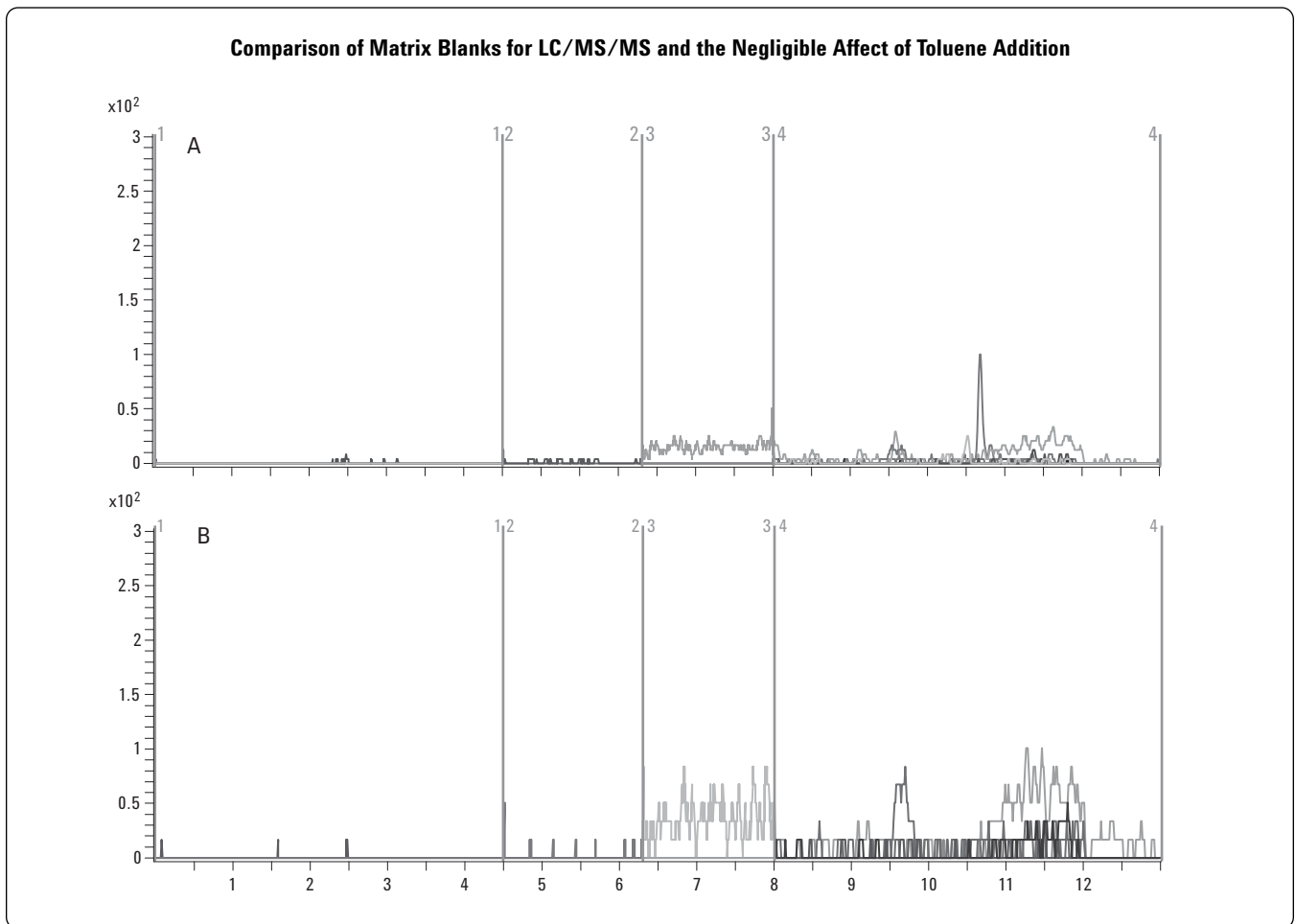


Figure 3. Spinach matrix blank LC/MS/MS chromatogram. A. Spinach matrix blank processed by modified method (w/toluene); B. Spinach matrix blank processed by original method (w/o toluene).

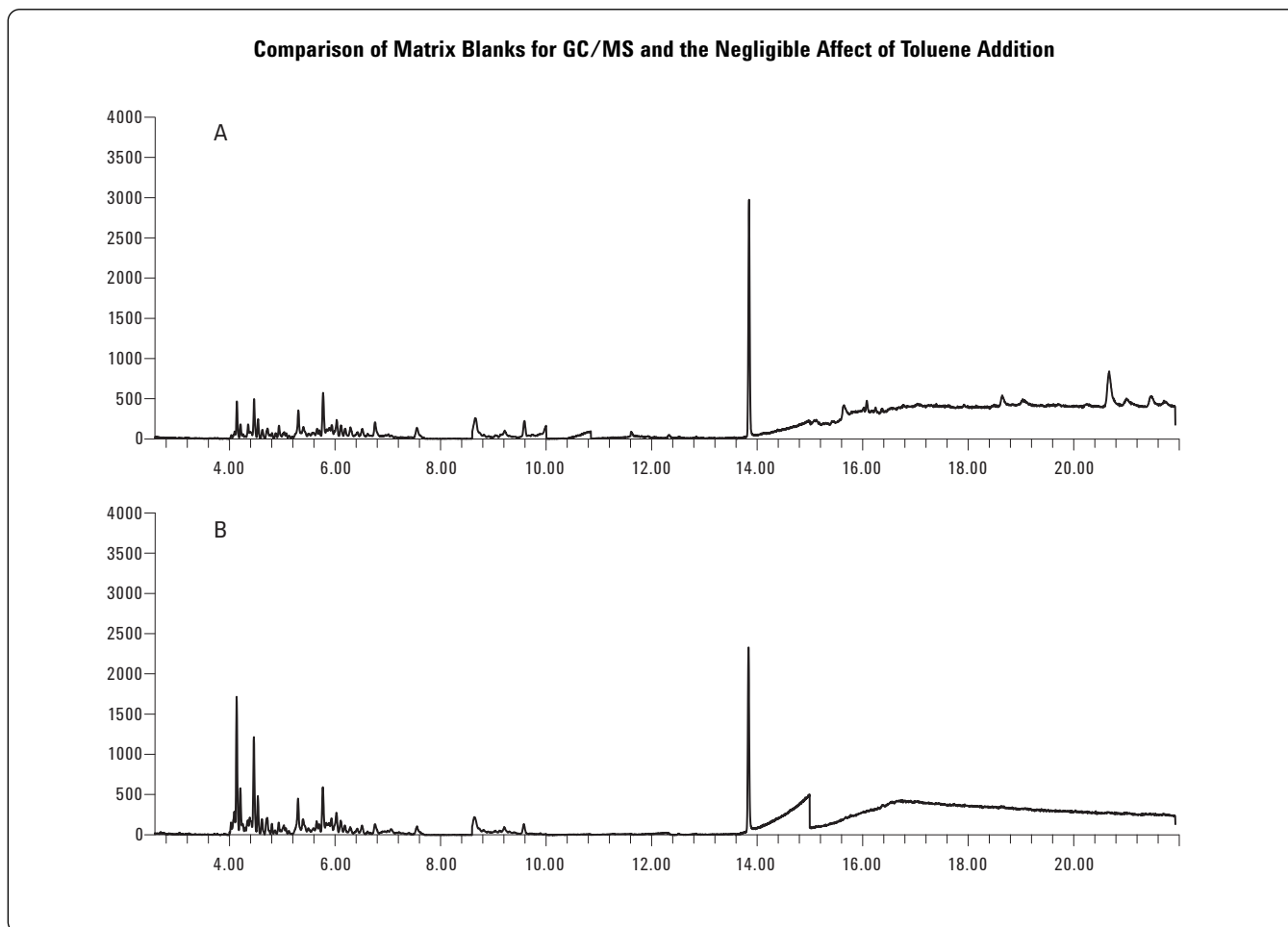


Figure 4. Spinach matrix blank GC/MS chromatograms. A. Spinach matrix blank processed by modified method (w/toluene); B. Spinach matrix blank processed by original method (w/o toluene).

Significant Improvement Made on Some Pesticides

The improvements made by the addition of toluene on certain pesticides was very significant (50% to 300% increase in recovery). Because GCB adsorbs planar compounds, the method produced very low recoveries (20% to 60%) of pesticides with planar compounds and poor precision (>14% RSD). These problematic pesticides determined by the original method included carbendazim, thiabendazole, pymetrozin, cyprodinil, chlorthalonil, coumaphous, dichlorobenzophenone, and folpet. The first four pesticides were analyzed by LC/MS/MS, and the second four pesticides by GC/MS.

The optimum volume of toluene addition was determined by parallel spinach samples spiked at the same concentration level and subjected to buffered salt extraction. An 8-mL

aliquot of ACN extract was transferred into a 15 mL dispersive tube. Different volumes of toluene were added according to the following ratios: 8:1, 8:2 and 8:3 (ACN extracts/toluene, n = 3). Samples without the addition of toluene were also processed for comparison. The final samples were analyzed by LC/MS/MS, and an average of analyte responses (peak area of analyte/peak area of IS) were used for response comparison. As shown in Figure 5, the addition of toluene increased the extraction efficiency, as indicated by a 200% to 300% higher analyte response. In general, the more toluene added, the higher the responses obtained. Therefore, the addition of toluene at a ratio of 8:3 was selected for both the LC/MS/MS and GC/MS experiments. This ratio is comparable to the ratio of 3:1 ACN/toluene that Schenck recommended. [7]

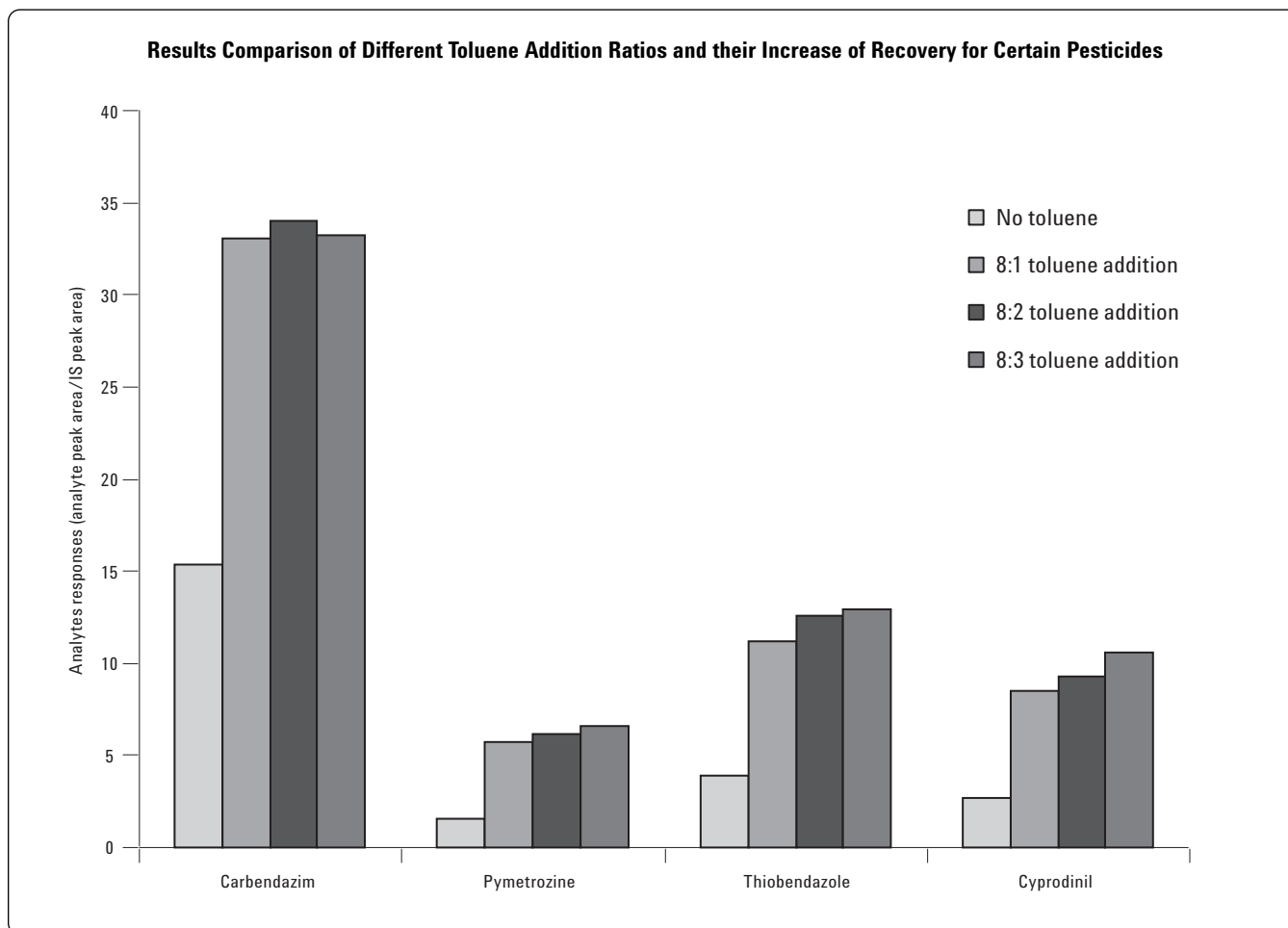


Figure 5. Results comparison of different toluene addition volumes. First column: results generated with no toluene addition; second column: results generated with toluene addition at ratio of 8:1 (ACN extracts/toluene); third column: results generated with toluene addition at ratio of 8:2; fourth column: results generated with toluene addition at ratio of 8:3.

The two different sizes of dispersive SPE (1 mL and 8 mL) were also compared for toluene addition. According to the ratio of 8:3, 3 mL of toluene were added to the 8 mL tubes; while 375 μ L of toluene were added to the 1 mL tubes. The results obtained by the modified method were also compared to those from the original method. As shown in Figure 6, both dispersive SPE volumes incorporating the modified method significantly increased the recovery of the difficult pesticides by 200-300%, and gave a substantial improvement in precision. The 1 mL volume dispersive SPE provided slightly higher recovery compared to the 8 mL volume dispersive SPE,

especially for pymetrozine and thiabendazole. Processing a single sample with the buffered salt extraction and partitioning step produced about 14 mL of ACN extract, which is enough to process dispersive SPE by both the original and modified methods at a 1 mL volume simultaneously. Additionally, a smaller amount of toluene was required. Therefore, the use of the 1 mL volume dispersive SPE kits with the modified method is recommended for problematic pesticides. This eliminates the need for another buffered salt extraction, saving analyst time, labor and additional sample and solvents.

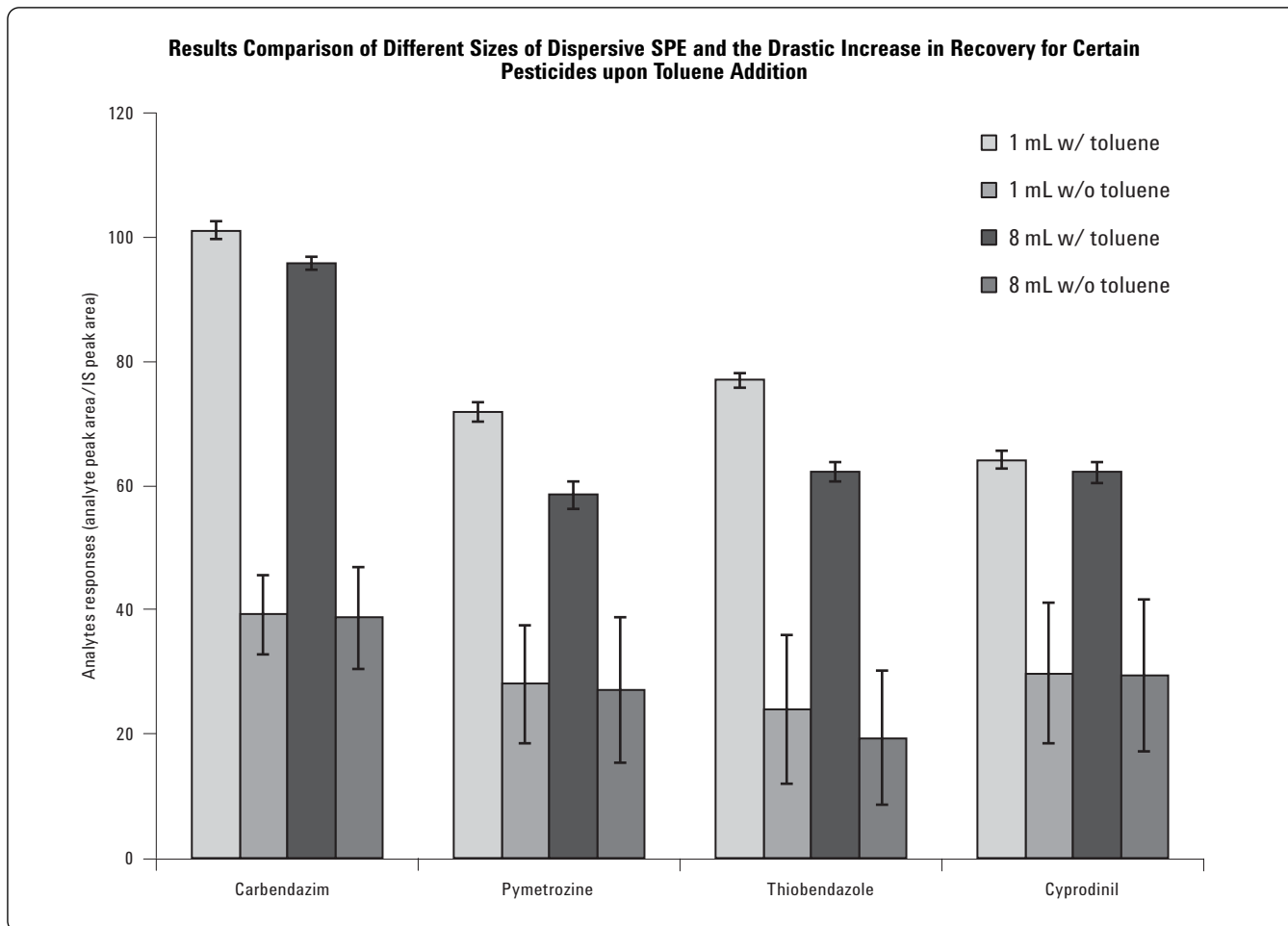


Figure 6. Results comparison of 1 mL and 8 mL dispersive SPE with the modified method (w/ toluene) and the original method (w/o toluene).

Impact on Other Pesticides

The impact of toluene addition on other pesticides was monitored and the results used to classify these pesticides into three groups. The first group of pesticides showed the same recovery and precision from both the original method and modified method. The second group of pesticides were those in which the addition of toluene generated about 10% to 15% less recovery, but still showed acceptable precision. The third group included only one pesticide, dichlorvos, from the 34 pesticides screened by LC/MS/MS or GC/MS. For this

pesticide, the addition of toluene adversely affected the analysis of dichlorvos producing much lower recovery and unacceptable precision. In general, these negative impacts were observed more on GC amenable pesticides than LC amenable pesticides, and may be linked to the additional drying step in the modified method.

Table 1 shows the impact the addition of toluene made on the modified dispersive SPE analysis of representative pesticides.

Table 1. The Impact on Certain Pesticides by the Modified Dispersive-SPE with Addition of Toluene

Analytes	Original method (w/o toluene)		Modified method (w/ toluene)		Impact with modified method	Detection method
	Recovery	RSD (n=6)	Recovery	RSD (n=6)		
Carbendazim	38.9	14.6	98.5	2.5	Positive	LC/MS/MS
Thiabendazole	21.8	19.7	69.7	2.7	Positive	LC/MS/MS
Pymetrozine	27.6	21.2	65.2	3.7	Positive	LC/MS/MS
Cyprodinil	29.6	23.4	63.1	3.2	Positive	LC/MS/MS
Chlorothalonil	21.1	16.4	47.3	5.9	Positive	GC/MS
Coumaphos	30.1	24.0	87.9	6.1	Positive	GC/MS
Dichlorobenzophenone	53.7	4.5	77.7	6.1	Positive	GC/MS
Folpet	62.0	14.6	88.2	6.3	Positive	GC/MS
Dichlorvos	88.8	6.0	20.4	89.8	Greatly negative	GC/MS
<i>o</i> -Phenylphenol	88.6	4.6	73.7	7.4	Slightly negative	GC/MS
Diazinon	94.9	5.9	81.3	4.0	Slightly negative	GC/MS
Chlordane	103.9	4.5	101.3	4.5	None	GC/MS
Permethrin	81.4	7.2	83.3	5.1	None	GC/MS
Acephate	95.5	5.6	99.8	4.7	None	LC/MS/MS
Carbaryl	108.0	2.5	109.1	1.9	None	LC/MS/MS
Propoxur	97.0	3.1	96.7	2.5	None	LC/MS/MS

Conclusion

This application note discusses the impact of the addition of toluene on the QuEChERS AOAC method for the analysis of multiclass pesticide residues using Agilent Bond Elut AOAC buffered extraction kits and Bond Elut AOAC dispersive SPE kits for pigmented fruits and vegetables. The addition of toluene at a ratio of 8:3 (ACN extracts/toluene) to the dispersive SPE step can significantly increase the recovery of problematic pesticides with planar structure by 50% to 300% and improve precision. The addition of toluene can also generate some negative effects, by introducing more matrix impurities, and reducing the recovery of certain pesticides. Therefore, the modified method should not be considered a direct

replacement for the original method. It does provide an option for problematic pesticides affected by GCB in the analysis of a highly pigmented matrix. The extraction will not have to be repeated from the beginning. The ACN extracts after the first buffered salt extraction step can be processed by both the original and modified AOAC methods simultaneously with Agilent Bond Elut 2 mL dispersive SPE kits for pigmented matrix, saving the analyst additional sample preparation and solvent usage. By combining the results from the original and modified methods, analysts can obtain extremely impressive results and analyze a greater variety of multiclass pesticides in pigmented fruits and vegetables relative to the original method.

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