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Letter

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Interlaboratory Assessment of Cryomilling Sample Preparation for Residue Analysis

ABSTRACT: The effectiveness of the comminution approach used for bulk field samples limits the size of the subsample that must be extracted and analyzed to ensure an adequately representative and reproducible measurement. In many cases this subsample size restricts the residue method to the use of larger vessel formats, limiting downstream throughput. The introduction of a secondary fine-milling step to this process using a subsample size already known to be representative can further improve sample homogeneity and allow direct method scaling to small high-throughput formats. Dramatic increases in method throughput can then be achieved through the simultaneous processing of numerous samples in parallel. This approach was evaluated across a diverse grouping of crop matrices using two substantially different pesticide types. Both fortified and field-collected samples demonstrated a high degree of precision and reproducibility across laboratories. Additional benefits of this approach include significant reductions in cost and solvent waste generation, as well as improvements in assay quality and transferability.

KEYWORDS: *homogeneity, pesticide residue, sample processing, cryomilling, high-throughput*

INTRODUCTION

Crop residue studies are used to determine the magnitude of pesticide residues that remain in targeted crop commodities following proposed application rates and timing. These studies are key in establishing tolerances for enforcement to ensure that exposure is acceptable based on the pesticide label and use. These types of preregistration studies can generate a significant number of samples for analysis. Samples from these studies are collected throughout a field plot to obtain a representative sampling. This produces sample sizes up to a few kilograms. These large samples are typically individually prepared using a vertical cutter-mixer (VCM) style blender with dry ice, often referred to as cryogenic processing, to reduce sample particle size and improve homogeneity for subsequent extraction and analysis of subsamples.[1] Currently, subsampling to obtain analytical portions of 5−10 g is common and considered representative of the initial homogenized bulk.[2]

This relatively large sample size (i.e., 10 g) limits the throughput for most crop residue methods through the use of larger extraction formats such as 50 mL tubes and 250 mL bottles. Subsequent steps including transfers, additions, partitioning, evaporation, centrifugation, and/or other cleanup techniques require the analyst to address each sample manually in sequence or in small sets. The ability to process a large number of samples in parallel through these various method steps is essential in improving throughput. Smaller formats such as 1.4−2.0 mL tubes in 96-well plates provide the ability to process numerous samples in parallel, but limitations in homogeneity from the bulk VCM−dry ice milling do not provide adequate repeatability at the required 75−100 mg sample sizes needed for this scale-down.

This paper introduces a process by which a 10−20 g subsample of the preliminary homogenized bulk is further refined to improve sample homogeneity to a level that allows scale-down to the 96-well tube format. Although this approach adds an additional step to the overall workflow, the downstream impacts from parallel sample processing have produced dramatic improvements in net sample throughput. Examples are given that demonstrate the effectiveness of this secondary fine-milling process with both field-collected and worst-case simulated samples on a diverse grouping of crop matrices with two substantially different pesticide types.

MATERIALS AND METHODS

Matrices Evaluated. A range of common raw agricultural commodities (RACs) was selected to represent crop groups as defined by SANCO.[3] Corn stover and corn grain (dry), corn forage (wet), soybean grain and cotton seed (oily), and whole oranges (acidic) represent all matrix groups. RACs were collected from residue field test plots at multiple locations using normal regional agricultural harvest practices. Oranges were purchased from a local grocery store (Indianapolis, IN, USA).

Primary Bulk Sample Homogenization. The bulk raw sample material was first homogenized using a Robot Coupe (Ridgeland, MS, USA) model R23 or R30. For this process, samples were combined with dry ice (ca. 25% w/w) to enable more efficient disruption. Resulting samples were placed in a −20 °C freezer overnight to allow sublimation of the dry ice prior to use or further processing.

Fine-Milling of Subsample. Secondary milling was conducted on various SPEX freezer/mills (Spex Sample Prep, Metuchen, NJ, USA) at four corporate locations with large milling vessels. The same procedure was followed by each laboratory. Twenty minutes of precooling, 2 min of milling, and a 1 min pause (cool-down), followed by a final 2 min milling period, were used. Milled samples were transferred to 50 mL centrifuge tubes and maintained on dry ice (including shipping) to keep the samples in a free-flowing, frozen state.

Experimental Design. The study contained two parts, field-treated residue samples and spike recovery samples. The spike recovery samples were fortified to 0.100 mg/kg (ppm) of either glyphosate and aminomethylphosphonic acid (AMPA) or spinosad A and spinosad D using 100 μL of a fortification solution in the large 100 mL freezer/mill vessel prior to milling. The fortification solutions were prepared at one location and shared with the other two laboratories to minimize spiking variability.

Preparation of Spiked Samples. For spiking experiments, matrix samples were coarsely homogenized using VCMs (Robot Coupe,

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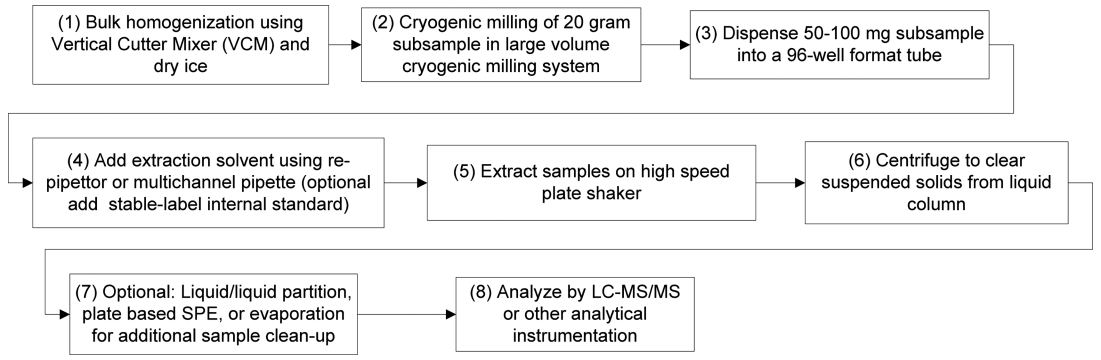

Figure 1. High-throughput plate-based workflow.

Table 1. Accuracy and Variability in Field-Treated Samples

		Monsanto		laboratory A		laboratory B	
matrix	analyte	concn ratio[a] (N = 6)	% RSD	concn ratio (N = 6)	% RSD	concn ratio (N = 6)	% RSD
corn forage	glyphosate	100	11.5	93	12.7	95	11.3
	AMPA	99	8.1	115	9.3	98	9.7

[a]Concentration determined by new high-throughput method divided by concentration determined by the previous method (10 g), expressed as a percentage.

Table 2. Accuracy and Variability in Fortified Samples Comparing Laboratories, Matrices, and Analytes Tested

		Monsanto		laboratory A		laboratory B	
matrix	analyte	av recovery (N = 36)	% RSD	av recovery (N = 36)	% RSD	av recovery (N = 36)	% RSD
corn forage	glyphosate	96	10.1	90	13.6	98	8.7
	AMPA	85	10.9	79	9.4	98	12.9
cotton seed	glyphosate	92	5.8	88	5.8	304[a]	8.0
	AMPA	76	9.1	105	9.1	97	8.9
corn stover	glyphosate	94	10.3	92	10.3	122[b]	7.8
	AMPA	92	10.2	92	10.2	91	11.4
		Dow AgroSciences		laboratory A		laboratory B	
matrix	analyte	av recovery (N = 36)	% RSD	av recovery (N = 36)	% RSD	av recovery (N = 36)	% RSD
corn grain	spinosad A	93	6.9	99	6.7	122[b]	6.7
	spinosad D	87	7.1	92	7.4	123[b]	6.9
soybean grain	spinosad A	94	6.1	99	6.2	90	6.4
	spinosad D	92	7.6	98	7.2	89	7.6
whole oranges	spinosad A	93	12.4	101	11.1	95	11.7
	spinosad D	96	11.4	102	11.7	97	10.5

		Dow AgroScience 10 g data[c] − LOQ (10 ppb)		Dow AgroScience 10 g data[c] − 100X LOQ	
		av recovery (N = 6)	% RSD	av recovery (N = 6)	% RSD
soybean grain	spinosad A	95.0	4.30	98.0	2.89
	spinosad D	94.3	5.15	95.5	8.14
whole oranges	spinosad A	94.8	9.61	94.7	11.0
	spinosad D	95.7	9.09	96.2	10.8

[a]This set of samples has a significantly high bias in recovery. This anomalous result was repeated across all subsamples of the site with consistent precision, and therefore it was concluded that this result is not an artifact of poor homogenization, but is more likely an error in fortification. [b]Average recovery is not within 70−120%. [c]Concentration determined by using a low-throughput method that uses 10 g of sample.

various models). Ten 20 ± 1 g aliquots of each of the primary milled tissues were weighed into plastic tubes. Four of these tubes were retained by the company laboratory, and three were sent to each of two external laboratories along with fortification solutions prepared by the company laboratory. The subsamples were fortified and secondary milled by the procedure described above and then returned to the company laboratory. A single subsample of each matrix was fine-milled without fortification to serve as batch quality controls.

For field-treated residue samples, a primary milled corn forage sample with known levels of glyphosate and AMPA was aliquoted into nine 20 ± 1 g subsamples. Three subsamples were retained by the company laboratory, and three were sent to each of two external laboratories.

After the secondary milling procedure was completed by each study site, the samples were returned to the company laboratory (either Monsanto or DowAgroSciences) for analysis by LC-MS/MS. Multiple 75−100 mg subsamples of each fine-milled sample were dispensed for analysis.

Dispensing Secondary Milled Samples. Samples that have undergone fine-milling were maintained in a frozen state to ensure homogeneity and prevent stratification. This was particularly important for higher water content samples such as forage and

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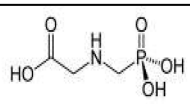
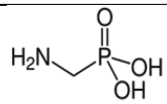
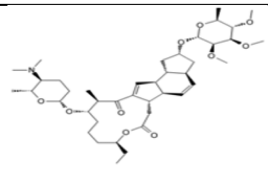
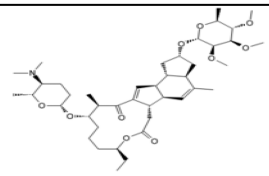
Table 3. Physicochemical Properties of Analytes

Matrix	Glyphosate	AMPA	Spinosad A	Spinosad D
Structure				
Molecular Weight	169	111	732	746
H_2O Solubility, mg/L	10000	5800	235	333
K_{ow}	-3.2	-1.6	55	90

oranges. Sets of samples were placed at the dispensing station on dry ice. Plastic disposable spatulas (VWR, Radnor, PA, USA) used for transferring were also chilled on dry ice prior to use. Subsamples of 75 ± 10 mg (for glyphosate/AMPA) or 100 ± 5 mg for spinosyn A/D were transferred to tared 1.4 mL polypropylene Matrix tubes (Thermo Scientific, Waltham, MA, USA). The exact sample mass was recorded and used to apply the appropriate factor for correcting sample concentration.

Analytical Methods. After homogenization and dispensing into 96-well format tubes, matrix samples were extracted and processed using validated methods. An overview of the workflow is shown in Figure 1. Within-set quality control samples assured the performance of the methods. Samples were analyzed by LC-MS/MS using electrospray ionization (ESI), and the mass spectrometer was operated in multiple reaction monitoring mode using two transitions for each analyte. Glyphosate and AMPA were analyzed in negative ionization mode, whereas spinosads A and D were analyzed in positive mode.

RESULTS AND DISCUSSION

The capability of the two-stage milling process to provide milligram scale subsamples that are representative of the field was tested by examining replicate subsamples in three phases of this round-robin study: (1) field-treated residue samples; (2) fortified samples; and (3) comparison with large-scale (5 g) samples.

The field-treated residue sample phase utilized corn forage as model RAC with field-applied glyphosate. Glyphosate and AMPA residues were previously determined at a 10 g scale using a validated analytical method. These same samples were bulk homogenized, and replicate 20 g subsamples were fine-milled using the secondary process by three independent laboratories. Six 75 mg subsamples of the fine-milled product were analyzed from each independent laboratory preparation. The results from the scaled-down assay are summarized in Table 1. The glyphosate and AMPA concentrations determined according to the new method were compared to those of the previous assay and were nearly identical (91−115%) In addition, the variability obtained using the scaled-down method ranged from 8 to 13%, well within SANCO's acceptable margin of <20%. These results demonstrate that 20 g subsamples are suitable for the secondary fine-milling to represent original primary milled bulk taken from the field in this RAC.

The fortification experiment enabled systematic examination of additional matrices across crop grouping categories using analytes that ranged in molecular weight, polarity, and charge (Table 3) to determine the effectiveness of sample comminution. The fortification approach represents the worst-case scenario, where all of the residue of the 20 g sample is fortified within a very small region, creating a "hotspot". The even distribution of this hotspot is performed solely by the secondary fine-milling process. Precision and recovery data from this interlaboratory study are summarized in Table 2.

Precision for glyphosate and AMPA ranged from 8.7 to 13.6% in corn forage, from 5.8 to 9.1% in cottonseed, and from 7.8 to 11.4% in corn stover across the three laboratories (Table 2). The recoveries of glyphosate and AMPA in cottonseed (oily matrix), corn forage (wet matrix), and corn stover (dry matrix) were mostly within the acceptable range of 70−120%. One result, the glyphosate data from the cottonseed samples prepared by laboratory B, had a significantly high bias in recovery. This anomalous result was repeated across all subsamples from that site with consistent precision (8% CV), and therefore it was concluded that this high result is not an artifact of poor homogenization.

A second pair of analytes, spinosads A and D, were chosen for testing on the basis of their dissimilarity to glyphosate and AMPA (Table 3). The fortification and milling processes were identical to those used for glyphosate and AMPA. The resulting mean recoveries of spinosads A and D in corn grain (dry matrix), soybean grain (oily matrix). and oranges (acidic matrix) were mostly within the acceptable range of 70−120%. There was a small amount of positive bias in the recoveries observed for spinosads A and D in the corn grain samples prepared by laboratory B. However, the precision values for spinosads A and D across all of the matrices tested were all <13%.

Low variability was observed in samples evaluated across laboratory sites, matrices, and analytes. Fortifying a hotspot is a worst-case scenario, yet uniformity in the sample results was still observed when a 75 or 100 mg aliquot was analyzed. Across laboratories, the % RSDs were very consistent, demonstrating the robustness of the technique across laboratories with varying degrees of experience.

The spinosad A and D recoveries and RSDs from samples analyzed in 10 g or 75 mg aliquots were compared to determine if additional variability was observed with the scaled-down assay. The 10 g aliquot samples were prepared by fortification of spinosads A and D into a 10 g aliquot of control matrix that had been primary-milled only. The 75 mg aliquot samples were prepared as described above with primary milling of the control matrix, followed by fortification of a 20 g aliquot of the matrix prior to the secondary milling step. The % RSD obtained at the 10 g scale ranged from 2.9 to 8.1 for spinosads A and D in soybean grain and was comparable to the % RSD obtained from

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the 75 mg scale assay of 6.1−7.6%. In addition, the % RSD obtained at the 10 g scale ranged from 9.1 to 11.0% for spinosads A and D in oranges, which, again, was comparable to the % RSD obtained from the 75 mg scale assay of 10.5−12.4%. This similarity in RSD values across sampling size and milling procedure demonstrates that additional variability was not observed in the 75 mg samples beyond the innate inconsistency of the assay.

The advantages of the fine-milling and scaled-down sample size methods are a significant increase in throughput, improved quality due to a reduced risk of contamination and reduced variability, and a decrease in costs due to fewer FTE hours required due to automating the extraction, reduced reagent use, and reduced reagent disposal. The increase in throughput observed with the milligram-scale sample methods was 10 times for glyphosate and AMPA and 3 times for spinosads A and D when compared to the traditional gram-scale methods. Thus, there is a significant increase in the number of samples that can be processed in the same time interval utilizing the milligram-scale methods.

In conclusion, analysis of the field-treated samples demonstrated that the 10 g scale method and the 75 mg scale method that included a second processing step, fine-milling, gave comparable results in terms of calculated concentrations and variability. The fortified samples were prepared using analytes chosen for their dissimilar physicochemical properties, matrices that encompass the four main crop groupings (wet, dry, oily, and acidic), and laboratories with various degrees of experience with the secondary fine-milling technique. When the resulting concentration data were compared across these variables, excellent agreement in the recovery and variability values obtained was observed. Additionally, the fine-milling technique has significant advantages in increasing throughput, decreasing cost, and increasing assay quality.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS USED

VCM, vertical cutter-mixer; RAC, raw agricultural commodities; RSD, relative standard deviation; LC-MS/MS, liquid chromatography−tandem mass spectrometry

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