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The Benefits of the Geno/Grinder® High-Throughput Tissue Homogenizer to Increase Sample Throughput for Pesticide Residue Analysis by LC/MS/MS

SUBJECT (SP022): Pesticide Residue Analysis

APPARATUS: Geno/Grinder®

APPLICATION: DNA/RNA and Other Extractions

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Summary

The high-throughput analysis of pesticides has been hindered by the slow and laborious sample preparation stage. Using traditional clean-up procedures, over twenty steps are required to get the sample in a convenient form for analysis, which limits sample throughput to typically 8 samples per day. This study evaluates a new approach using an innovative laboratory tissue homogenizer, to extract various pesticide residues from different kinds of plant materials and to identify and quantitate the active ingredients by LC/MS/MS. It shows that by automating the sample preparation procedure, the number of clean-up steps can be significantly reduced, resulting in an increased sample throughput over traditional approaches.

Discussion

Three of the leading pesticides sold by DuPont are Famoxate®, Curzate® and Tanos® to control various fungal diseases in crops. Famoxate is a fungicide containing the active ingredient famoxadone and is mainly used to control early blight tomatoes. It protects from spore germination and fungal growth by its strong adhesion to leaf and stem surfaces. Curzate is a fungicide that contains cymoxanil as the active ingredient and is mainly used for late blight potatoes, but is also applied to grapes, tomatoes, cucumbers and leafy vegetables. Its protection mechanism is to penetrate the surface to induce host defense response to stop lesion growth and sporulation. Tanos is a fungicide containing both famoxadone and cymoxanil and the cumulative effect of these two active ingredients offers even better control of late blight potatoes. The typical pesticide residue set at DuPont contains 8 samples and usually takes an 8 hour workday to complete. The goal of this study was to take advantage of recent developments in sample preparation with the Geno/Grinder® and instrument sensitivity and selectivity using the API 5000 LC/MS/MS system to increase the number of samples analyzed per day by a factor of three. The methodology follows OPPTS 860-1360 Multiresidue Test Method[1], which has been developed by the United States Environmental Protection Agency (USEPA) Office of Prevention, Pesticides and Toxic Substances (OPPTS), for use in the testing of pesticides and toxic substances. Under this method, test data must be submitted to the agency for review under federal regulations and dictates that average recoveries have to be 70-120% with a coefficient of variation (CV) or RSD of less than 15%.

Sample Preparation

Sample preparation for pesticide residue analysis has typically followed DuPont Report No. AMR 3705-95, "Analytical Method for the Determination of Famoxadone and Cymoxanil Residues in Various Matrices".[2] In this procedure, ground-up samples are weighed into extraction jars, followed by the addition of water to allow them to re-hydrate prior to extraction. Acetonitrile is added and samples are ground with a laboratory homogenizer. The plant matrix is then allowed to settle-out and liquid extracts are filtered and collected in mixing cylinders containing sodium chloride. The mixing cylinders are capped, shaken, and inverted to aid in the dissolution of sodium chloride and then allowed to stand while the acetonitrile (upper layer) and water phases separated. Separate acetonitrile aliquots are taken for cymoxanil and famoxadone analysis.

Cymoxanil and Famoxadone Analysis

For the analysis of cymoxanil, the acetonitrile aliquot is back extracted with hexane, concentrated, diluted with water and passed through a conditioned strong anion exchange (SAX) solid phase extraction (SPE) cartridge stacked on top of a conditioned carbon black SPE cartridge. Cymoxanil passes through the SAX cartridge and is retained on the carbon black cartridge. It is then selectively eluted on the cartridge and the concentrated cymoxanil residues are dissolved in a hexane/ethyl acetate mixture. The resulting solutions are passed through silica SPE cartridges.

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Cymoxanil is retained, selectively eluted, and concentrated into a methanol/water mixture adjusted to pH 3. Samples are filtered and analyzed by HPLC with UV detection. For the analysis of famoxadone, the acetonitrile aliquot is back extracted with hexane, the acetonitrile fraction is concentrated to approximately 2 mL and carefully taken to dryness. The residue is dissolved in 10% ethyl ether/90% hexane (v/v) and passed through a glass chromatography column packed with a layer of sodium sulfate, florisil, and sodium sulfate. Each column is washed with additional 10% ethyl acetate/90% hexane (v/v). The eluate is concentrated, carefully taken to dryness, reconstituted in acetonitrile and water and analyzed by LC/UV.

Sample Prep for LC/MS Analysis

There are approximately 20 clean-up steps in the AMR 3705-95 procedure, which limits the number of samples to 8 to 10 per day. A modification to this procedure, laid out in DuPont 13753 – "Analytical Method for the Determination of Cymoxanil and its Metabolites in Leafy Vegetables Using LC/MS"[3], significantly reduces the number of clean-up steps. In this procedure, samples are extracted using an acetonitrile/water mixture as described in DuPont Report No. AMR 3705-9, Revision 2. For cymoxanil, NaCl is added to an aliquot to separate the aqueous phase from the organic phase. The aqueous phase is discarded, and the acetonitrile layer containing cymoxanil is passed through a SAX SPE column. The extract is then cleaned further using a hexane liquid/liquid extraction followed by an Envi Carb SPE column. Cymoxanil is not retained on either of these columns, so the eluent is passed directly into an LC/MS system for analysis. Based on the sensitivity and selectivity of LC/MS, the number of sample clean-up steps is reduced to approximately 10, allowing for the analysis of up to 16 samples per day.

Geno/Grinder®

To further reduce the clean-up steps, a new piece of technology was evaluated, called the Geno/Grinder (Cole-Parmer, Metuchen, NJ). The Geno/Grinder is a laboratory mill and tissue homogenizer specifically designed for vigorous vertical shaking of deep-well titer plates. It was originally designed to prepare plant tissue for extractions of nucleic acids, proteins, and other constituents by shaking the tissue, steel balls and a buffering agent together in each well of a titer plate. The application of this tool can be expanded to include microorganisms when small silica beads are used instead of steel grinding balls. Microbes can also be disrupted in standard 96-well titer plates as opposed to deep-well plates. Sample material that can be prepared includes bacteria, yeasts, molds, seeds, stems, roots, leaves, and certain animal tissue. Due to the strength of the vertical shaking motion of the equipment, many seeds and other forms of plant tissue can also be pulverized dry in plates with the help of one or two grinding balls per well. Another benefit of this technology is that additional time savings are achieved because the sample vials are disposable, which means the laborious process of cleaning the homogenizer probes is eliminated. The Geno/Grinder is shown in Figure 1.

Figure 1. Geno/Grinder High-Throughput Tissue Homogenizer

Sample Clean-Up Using the Geno/Grinder

For the leaves of watery crops like tomatoes and potatoes, 10 g of sample was extracted twice with 20 mL of a 90/10 mixture of acetonitrile/water solution, using SAX and the SPE process described earlier. To ensure complete extraction of the compounds from the plant material, steel balls were added to the samples to pulverize the plant material. The samples were than extracted by shaking at high speeds in a Geno/Grinder at approximately 700–1200 cycles per minute, depending on the sample. The sample extract was brought to 50 mL by adding acetonitrile. Approximately 1 mL of the extract was filtered and 100 µL of sample diluted to 1 mL in an HPLC vial with mobile phase, and analyzed directly by LC/MS/MS. Using the Geno/Grinder has cut down the number of clean-up steps to 3, compared to 20 with the AMR 3705-95 method and 10 with the DuPont 13753 method.

Experimental

Figure 2. LC/MS/MS Instrument

To evaluate the efficiency of the sample preparation and clean-up stage described above, cymoxanil, the active ingredient in many of the fungicides, and its various metabolites, were determined in a number of wet and dry crop/plant materials. All samples generated were analyzed using the API 5000 LC/MS/MS (Applied Biosystems, Foster City, CA). This instrument is coupled with an HPLC system to first separate the biological species of interest, based on their elution times o a column. The eluent is then introduced into the mass spectrometer for ionization to identify and quantitate the species using triple quadrupole MS technology. The principle of this technology is based on confirmation of a particular molecular ion by the generation of its daughter species using collisionally-induced dissociation or fragmentation[4]. This technique, commonly known as multiple reaction monitoring (MRM), uses two resolving quadrupole mass filters, separated by another quadrupole, which is slightly pressurized by the introduction of a collision gas like hydrogen or helium. In the MS/MS mode, the first quadrupole is used in a mass-resolving mode to select the precursor ion. The second quadrupole (or pressurized multipole collision cell) is used to produce fragmentation of the precursor or parent ion. The final quadrupole is used in a mass-resolving mode to provide mass analysis of the resulting fragmented or daughter ions. These species are then compared to reference spectra/data in order to produce unambiguous identification of the biomolecules of interest. The LC/MS/MS instrument used for this study is shown in Figure 2.

The benefits of MS/MS design is that it can also be used in MS mode for quantitation purposes. In this mode, the first quadrupole is typically used in the rf-only mode, as wide mass range. The collision cell is also used in the rf-mode, but this time collision gas is owing, so the cell is just used to transmit ions to the last quadrapole, which is used in the mass-resolving mode. The ability to switch between both modes is very important, in order to maximize the amount of data being generated, particularly when small amounts of sample are being analyzed. There is no question that the higher daughter of fragmented ions that are being identified, will lead to better confirmation of the parent molecules. This is especially important when analyzing data from an LC separation. Cymoxanil, for example, which is an aliphatic nitrogen compound with a formula of $C_7H_{10}N_4O_3$ and molecular weight of 198, typically elutes on the column after 10 to 15 minutes, over the period of 20-30 seconds. For this reason it is very important to be able to switch rapidly between SIM and MRM mode, to collect both the molecular and fragmented information, for positive and unambiguous identification of the species. Once identification is made in the MRM mode, quantitation of unknown samples is normally carried out in the SIM mode.

Instrument Methodology

Reversed-phase liquid chromatography was used to separate cymoxanil and it metabolizes from the rest of the extractants. The LC/MS/MS instrument was operated in the single ion monitoring MS negative ion mode for quantitative analysis. Peak area was used for quantitation. For confirmation of the presence of the analyte in unknown samples, the relative intensities of the fragmented ions were measured using the MRM mode. A brief summary of the HPLC and LC/MS/MS conditions are shown in Table 1.

Table 1. Brief Summary of the HPLC and LC/MS/MS instrumental conditions used for this study.

HPLC System: Agilent 1100 Series HPLC Settings:

Injection volume:	40 µL
LC column:	Agilent Zorbax® XDB-C (15.0 cm x 4.6 mm i.d., 5 µL)
Column temperature:	30 °C
Mobile phases:	0.01 M acetic acid and acetonitrile

LC/MS/MS System: Applied Biosystems API 5000 LC/MS/MS Settings:

Ionization source:	Electrospray
Polarity:	Negative
Mode:	Selected ion monitoring (SIM) and multiple reaction monitoring (MRM)
Monitoring:	197 daltons
Mass/charge:	(Cymoxanil, M-1)
Elution timeframe:	0-17 min

A 0.01 ppm cymoxanil LOQ fortification sample (DuPont-13753) and control (blank) in the SIM mode is shown in Figure 3. It can be seen very clearly that there is a significant peak eluted at about 1 5 minutes, which corresponds to the cymoxanil. Confirmation of the compound is then made by measuring the intensities of the fragmented daughter ions in the MS/MS mode, by comparing them to known ratios in a data base of pesticides. The MS/MS spectrum (60-300 daltons) of cymoxanil in MRM mode is shown in Figure 4, while the resulting calibration curve for 0.2, 0.5, 1.0, 5.0 and 10.0 ug/L is shown in Figure 5.

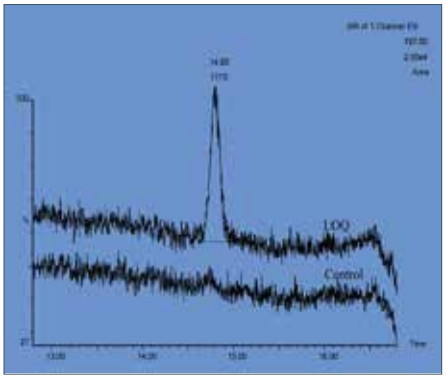

Figure 3. Elution of cymoxanil using LC/MS mode

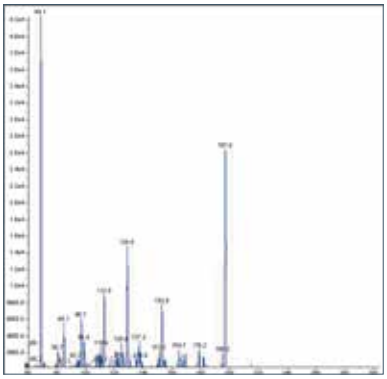

Figure 4. The MRM spectrum of cymoxanil showing the fragmented ions

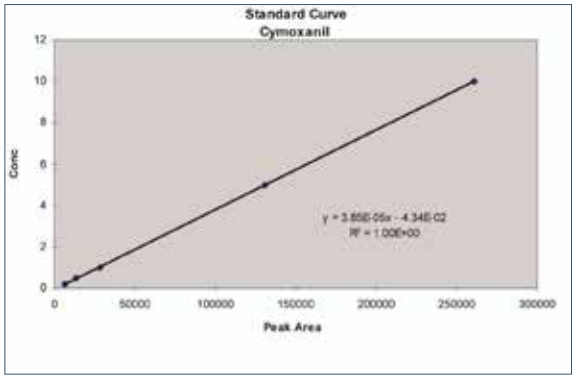

Figure 5. Calibration curve for 0.2, 0.5, 1.0 SD and 100 µg/L cymoxanil

Table 2. % Recovery Data and RSD for 0.05 ppm (LOQ) and 0.50 ppm (10 x LOQ) Reference Samples

Reference value	AMR-3705-95 (% recvery)	DuPont-13753 (% recovery)	Geno/Grinder (% recovery)
0.05 ppm (LOQ)	84.0 ± 3.4 (n=4)	93.0 ± 3.7 (n=5)	78.0 ± 3.1 (n=6)
0.50 ppm (10 x LOQ)	68.5 ± 1.5 (n=2)	89.0 ± 2.4 (n=5)	78.0 ± 6.9 (n=4)

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Sample Preparation

Once the methodology was optimized for the separation and quantitation of cymoxanil, the following Geno/Grinder sample preparation clean-up procedures were used for comparison purposes with traditional sample preparation methods. The basis of this method was then used to investigate an experimental new insecticide (DuP-1) and to identify and quantitate its active ingredient and metabolites in various wet/dry plant and crop materials

- Dilute 1ml of extract to 5ml with H20 (adjust to pH 2.5)
- Filter through SAX SPE and collect extract
- Dilute extract to 10.0m1
- Filter through PTFE membrane into an LC vial
- Analyze by LC/MS/MS

Results

The LC/MS separation of the experimental new insecticide under investigation and its metabolites (A-G) are shown in Figure 6. The MRM (1-298 Daltons) of a 0.2 ppm standard of the metabolites D and E together with a tomato sample spiked at the LOQ is shown in Figure 7. Table 3 compares the Geno/Grinder sample preparation and clean-up method with a traditional homogenizer (Tissuemizer® probe) sample preparation using Dupont-13753 methodology described earlier. It shows quantitative data (ppm in sample) for the experimental new compound and one of its metabolites (D), for various crop samples. This methodology was then used to analyze a variety of wet, dry, oily and acidic crops for the active ingredient together with its metabolites. Table 4 shows spike recovery and RSD data for one of the watery crops (tomato), showing that DuP-1 and all its metabolites are within the guidelines stated in EPA OPP TS 860-1360 Multiresidue Test Method for testing of pesticides and toxic substances, which states that test data must have average recoveries between 70-120% with a precision of less than 15% RSD. Although they will not be presented here, the study also looked at other crops including limes (acidic crop), almonds (oily crop) and wheat straw (dry crop) and achieved similar spike recoveries and precision values.

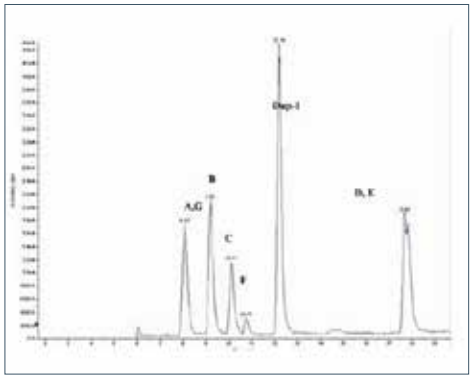

Figure 6. LC/MS separation of the experimental compound (DuP-1) and its metabolites (A-G)

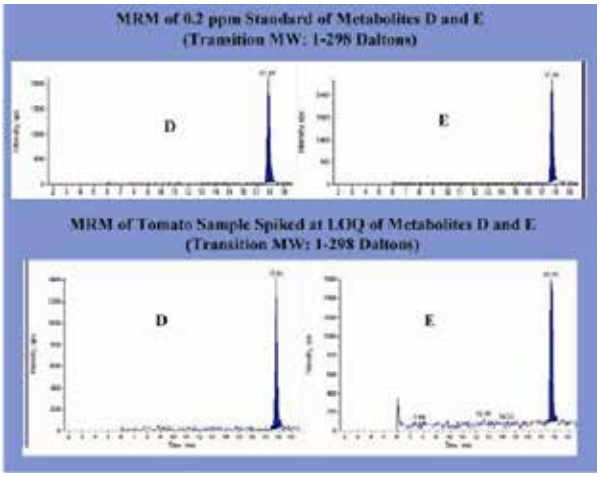

Figure 7. The MRM of a 0.2 ppm standard of the metabolites D and E (top) together with a tomato sample spiked at the LOQ (bottom). The transition is shown at a molecular weight of 1-298\daltons.

Table 3. Comparison between Geno/Grinder and DuPont 13753 sample preparation methods for a variety of wet and dry crop samples.

Crop	DuPont 13753 Method	Geno/Grinder Method	DuPont 13753 Method	Geno/Grinder Method
	DuP-1 (ppm)		Metaboltie D (ppm	
Alfalfa	14.4	15.7	0.15	0.14
Corn Stover	0.003	0.002	N/D	N/D
Cucumber	0.002	0.0015	N/D	N/D
Green Onion	0.012	0.002	N/D	N/D
White Straw	0.009	0.0075	0.003	0.002

Table 4. Spike recovery and precision data for tomatoes showing that DuP-1 and all the metabolites are within the guidelines stated in EPA OPPTS 860-1360 multitude test method.

	DuP-1	Met A	Met B	Met C	Met D	Met E	Met F	Met G
% Recovery & RSD of LOQ Sample	78 + 4.7	93 + 4.6	101 + 6.1	100 + 9.5	98 + 1.8	97 + 7.5	113 + 7.0	107 + 6.6
% Recovery & RSD of 10 x LOQ Sample	80 + 5.8	82 + 6.8	83 + 8.0	85 + 4.8	78 + 8.1	76 + 4.5	82 + 6.9	77 + 9.6

Conclusion

It has been shown that the extraction of pesticide residues with the Geno/Grinder and identification and quantitation of its active ingredients and metabolites using LC/MS/MS, provides an extremely efficient, rugged and high-throughput analytical method. Compared to the traditional sample preparation, clean-up and detection methods, sample throughput was increased by a factor of 3, from 8 samples to 24 samples per day.

References

1. Environmental Protection Agency, Office of Prevention, Pesticides and Toxic Substances (OPPTS) Method 860-1360 "Multiresidue Test Method for Pesticides and Toxic Substances".
2. DuPont Report No. AMR 3705-95: "Analytical Method for the Determination of Famoxadone and Cymoxanil Residues in Various Matrices".
3. DuPont Report No. 13753: "Analytical Method for the Determination of Cymoxanil and its Metabolites in Leafy Vegetables Using LC/MS".
4. B. A. Thomson, D.J. Douglas, J.J. Corr, J. W. Hager, C.A. Joliffe. "Improved collisionally activated dissociation efficiency and mass resolution on a triple quadrupole mass spectrometer system". Analytical Chemistry, 67, 1696-1704 (1995).

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