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Article

Development and Validation of a Quick Easy Cheap Effective Rugged and Safe-based Multi-residues Analysis Method for Persimmon, Grape and Pear using Liquid Chromatography-tandem Mass Spectrometry

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Association of official agricultural chemists official method was adapted and used to develop analytical method for determination of 76 pesticides residues in persimmon, grape, and pear by concurrent use of liquid chromatography-tandem mass spectrometry. Despite few exceptions in particular matrix, two fortified spiking (100 and 400 ppb) in three matrices gave satisfactory results in terms of accuracy, repeatability, precision, and linearity. Method detection limits (MDLs) were determined using five low spiking and eight replicate samples. MDLs were calculated by multiplying the standard deviation with student t-value 2.998 for n-1 (7) degree of freedom at 99% confident level. limit of quantification were obtained by multiplying standard deviation with 10. Experimental results indicate grape was the most problematic matrix among tested fruits and persimmon the least. Etoxazole is the most problematic pesticide and not applicable in this method. Developed method was successfully applied for the determination of residual pesticides in blind-incurred samples.

Key words: grape, multi-residue analysis, pear, persimmon, quick easy cheap effective rugged and safe

Along with the increasing use of pesticides to increase food production, there had been a marked development of different types of pesticides. Not only pesticides itself but also additional environmental pollution emitted during pesticide production have resulted in the occurrence of those chemicals in air, water, and soil along with those in the crops, fruits, and vegetables [Bai *et al*., 2006]. In many cases, pesticides are repeatedly applied during the entire growing season and sometimes even at the fruiting stage. Once these pesticides are absorbed by the fruits and vegetables, humans who consume them feel noxious [Kumari *et al*., 2004]. It is reported that over 1000 compounds are applied to agricultural crops to control undesirable pests [Ortelli *et al*., 2004]. The wide spread concern for food safety of society has led to the strict regulation of maximum residue limit (MRL) of pesticide residues in food. Although fresh vegetables, fruits and pulses are important part of a healthy diet due to the presence of significant amount of nutrients and minerals, they can, at the same time, be a source of toxic substances such as pesticides [Knežević and Serdar, 2009].

Normally, preparation for eating fruits is completed just after washing with water. Rasmussen *et al*. [2003] reported that none of the residual pesticides, chlorpyrifos, deltamethrin, fenitrothion, fenpropathrin, iprodione, and kresoxim-m, was significantly reduced when apples were subjected to simple washing. It is therefore very important to determine the amount of pesticides contained in fruits and vegetable, and to develop multi-residue analysis method for a safe consumption of fruits and vegetables.

Although a number of studies have developed methods for the analysis of pesticide residues from fresh fruits and vegetables, the results may vary depending on the varieties of fruits and vegetables. Such kind of variety differences lead to the differences in, for example, sugar contents, thickness of lipid layer, and acidity of extract that have been shown to affect analytical results [Koesukwiwat *et al*., 2010; Lehotay *et al*., 2010]. Therefore, it is still necessary to develop a method for the multi-residue analysis of pesticides in market fruits and vegetables available in the particular region. As described above, a variety of fruits from one region cannot

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represent that from another region. For example, residue analytical results of persimmon conducted in china cannot represent fruits in Korea. Even in the same country, the results may vary when samples are taken from different places, for example, samples taken from mountain region may give different results when compare to those taken from low land. Therefore, the analytical results of samples that are taken from the region of interest can only represent this particular region.

Due to the simplicity and flexibility of QuEChERS (**qu**ick, **e**asy, **ch**eap, **e**ffective, **r**ugged and **s**afe)-based sample preparation method [Anastassiades *et al*., 2003], it serves as a template for modification of multi-residue analysis method [Asensio-Ramos *et al*., 2010; Koesukwiwat *et al*., 2010; Gonzalez-Curbelo *et al*., 2011]. A number of modified versions have been successfully applied for extraction of pesticides from a variety of foods, mainly from fruits and vegetables [Lesueur *et al*., 2008; Nguyen, 2008; Húšková *et al*., 2009]. Lehotay *et al*. [2005] modified the original unbuffered QuEChERS method using acetate, and Anastassiades *et al*. [2007] modified the method using citrate buffering conditions to avoid the degradation of pH-sensitive pesticides under basic conditions. Both methods met statistical criteria for acceptability from independent scientific standards organizations, and are thus recognized as association of official agricultural chemists (AOAC) international official method 2007.01 for the acetate-buffering method and European Committee for Standardization (CEN) standard Method EN 15662 for the citrate-buffering method. This makes QuEChERS a suitable standard method for pesticide residue analysis in fruits and vegetables, with applicability to other foods with proper validation.

Since its development in 1989, electrospray ionization (ESI)-based liquid chromatography-mass spectrometry (LC-MS)/MS was intensively used for the analysis of thermally labile and/or nonvolatile organic molecules including pharma-ceutically active compounds, veterinary drugs, antibiotics, and pesticides [Alder *et al*., 2006; Kuster *et al*., 2006; Hao *et al*., 2007]. Simultaneous analyses of as many as 100-300 pesticides with better data quality and efficiency can be achieved using LC-MS/MS, which is designed to have superior sensitivity. On the other hand, the development of data acquisition software such as DMRM (dynamic multiple reaction monitoring), Scheduled MRM (multiple reaction monitoring), and Timed Selected Reaction Monitoring provided the effective determination of many pesticides on LC-MS/MS. Target pesticides analyzed in this experiment are widely used for controlling pests on fruits grown in the southern Korean peninsula. MRL for those pesticides are also already set in Korea. In the present study, QuEChERS-based multiclass, multi-residue analysis for the determination of 76 pesticides in persimmon, grape, and pear using LC-MS/MS in DMRM mode is described. Analytical method was validated by determining linearity, recovery of pesticides from two fortified spiking levels, standard deviation and relative standard deviation (%). Proficiency of the method was investigated using blind-incurred samples of grape, pear, and persimmon. Also documented are method performance and validation data such as the LC-MS/MS short-term stability and, MDLs (method detection limits), and LOQ (limit of quantification) that were determined by using the U.S. EPA protocol (U. S. EPA, Federal Register).

Analytical results obtained from the Dynamic MRM data acquisition mode were the results of two MRM transitions to identify target pesticide compounds in order to meet the European Union criteria for the mass spectrometric identification of target compounds [European Commission Council Directive 96/23/EEC and 2002/657/EC].

Materials and Method

Chemicals and Standard Stock Solution Preparation. All pesticides used were obtained from Dr. Ehrenstorfer GmbH (Augsberg, Germany). Gradient-grade acetonitrile, methanol, and glacial acetic acid (100%) were purchased from Merck KGaA (Darmstadt, Germany). Formic acid and ammonium acetate (>98 and 99% purity) were purchased from Sigma Aldrich (St. Louis, MO). Distill water was deionized by using Milli-Q system from Millipore (Bedford, MA). For sample extraction and dispersive solid phase extraction steps, QuEChERS products, Restek Q-sep Q 150 containing 6 g anhydrous $MgSO_4$ and 1.5 g anhydrous NaOAc were added into a 50-mL plastic centrifuge tube, and Rstek Q-sep Q 251 containing 150 mg anhydrous $MgSO_4$, 50 mg PSA (primary secondary amine), and 50 mg C_{18} were supplied by Restek (Bellefonte, PA). Persimmons, pears, and grapes were obtained from local suppliers.

To prepare stock solutions of individual standards, all standards were dissolved in acetonitrile to reach 1000 ppm except carbendazim, which was dissolved in methanol to reach 200 ppm due to its solubility. The appropriate volume of each stock solution was mixed together and diluted with acetonitrile to obtain 20 ppm mixture of all 76 target pesticides. Working standard solutions were diluted from 20 ppm mixed standard using acetonitrile.

Sample Preparation. Chopped samples were homogenized by Artlon gold mix homogenizer (Artlon,

Seoul, Korea) and kept in a freezer at −20°C until used. Fifteen grams each of homogenized samples were weighed into a 50-mL empty polypropylene centrifuge tubes, and 100 μL of desire concentration of pesticide mixture and 100 μL of acetonitrile were spiked for spiked samples and matrix blanks, respectively, which were mixed with sample using vortex mixer for 30 s to thoroughly spread pesticide in the sample prior to pipetting 15 mL of 1% acetic acid (HOAc) in acetonitrile into the samples. The centrifuge tubes were then vortex mixed for 1 min using Touch Mixer, model 232 (Fisher Scientific, Hampton, UK). After the contents were pour into 50-mL polypropylene centrifuge tubes containing 6 g anhydrous $MgSO_4$, and 1.5 g sodium acetate (NaOAc), the tubes were shaken vigorously by hand for 2 min and then centrifuged at 3000 rpm for 5 min. The resulting supernatant (1 mL) was pipette into 2-mL mini-centrifuge tubes containing 150 mg anhydrous $MgSO_4$, 50 mg PSA, and 50 mg C_{18} was vortex-mixed for 30 s and centrifuged at 12,000 rpm for 5 min. A 500-μL aliquot of supernatant was pipette into appropriate test tubes and mixed with 50 μL of 5 ppm TPP (triphenyl-phosphate), 50 μL standard solution for matrix matched standard, and 50 μL acetonitrile for reagent blank, matrix blank, spiked sample, and 1 mL of 0.1% formic acid. Then the contents were transferred into 2-mL vials after filtered through a 0.2-μL PTFE (polytetrafluoroethylene) filter (Pall Corporation, Ann Arbor, MI) and used for the LC-MS/MS analyses. A 15-mL aliquots of distill deionized water were used as sample for reagent blank and passed through all the procedure mentioned above. In this case, 100 μL acetonitrile was spiked instead of spiking with pesticide standards. Schematic diagram of sample preparation method is presented in Fig. 1.

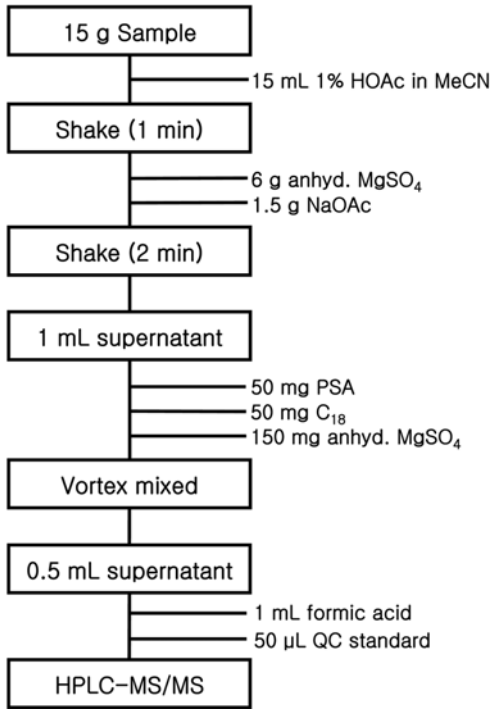

Fig. 1. Schematic diagram of sample preparation method.

All fruit samples were screened for pesticides using the above sample preparation method except spiking of standard pesticide solution. The same volume of acetonitrile was spiked instead of standard solution. The samples free from pesticides were selected for use as control sample. However, in the cases of peach and persimmon, finding pesticide-free samples was difficult. Some of target pesticides were found to be considerably highly concentration. For example, detector signals for dinotefuran and pyraclostrobin in persimmon were as high as the signals for 0.005 ppm-spiked matrix matched standard. Experimental concentration of 0.005 ppm-spiked matrix matched standard was 0.00016 ppm. However, the other persimmon samples contained higher pesticides both in number and concentration. Therefore, sample containing dinotefuran and pyraclostrobin was selected as control sample. For recovery test and MDL determination of these pesticides, another persimmon sample which is free from dinotefuran and pyraclostrobin but contains other target pesticides was used as control sample.

Eight replicates were prepared for MDL determination and four replicates for fortification study. For MDL determination, samples were spiked with 100 μL of desired concentration of standard solutions to achieve 0.5, 2, 5, 10, and 50 ppb spiking concentrations. For fortification study, 150 μL of standard solutions were spiked into samples to achieve 100 ppb and 300 μL was spiked to obtain 400 ppb spiking concentration, because the highest concentration of mixed pesticide standard was 20 ppm. The same volume of acetonitrile was spiked into samples for matrix blank and reagent blank. Matrix-matched standard solutions were prepared using respective matrix extract of persimmon, pear, and grape. Calibration standards were prepared for each spiking concentration using 4-point calibration. New calibration standards were prepared and used for each set of run. TPP was used as internal standard to check the stability of instrument between each run and also among a series of run. Detector signals for internal standard were not used for calibration curves and calculation of recoveries of pesticides, but were used for elimination of outliers along with signals for matrix and reagent blanks.

Instrumentation and data analysis. LC-MS/MS analysis was performed using an Agilent Technologies (Palo Alto, CA) model 1200 series HPLC coupled to an Agilent 6410 triple-quadrupole mass spectrometer.

Instrument parameters were: gas temperature 350, gas flow 10 L/min, and nebulizer 50 psi. N_2 gas was used as nebulizer gas. Ionization was performed by electrospray ionization at positive mode. Analyte separation was achieved by YMC-Pack Pro C18 RS, 3 μm, 100×3 mm i. d., (YMC Co., Ltd., Kyoto, Japan). Column temperature was set at 40°C. Mobile phase consisted of water buffered with 10 mM NH_4OAc and 0.1% formic acid (solvent A) and MeCN (solvent B). The LC gradient for the separation was: from 0 to 2 min, a linear increase of B from 5 to 70%; isocratic from 2 to 10 min (30% A: 70% B); from 10 to 15 min, a linear increase of B from 70 to 90%; from 15 to 20 min, a linear increase of B from 90 to 95%. Initial conditions were re-established at 3 min and was maintained for 2 min. Post running time was set for 5 min, resulting a total run time of 30 min. The flow rate of mobile phase was 0.2 mL/min. Sample injection volume was 10 μL. Data processing was performed in MassHunter Workstation data acquisition software using DMRM mode. Cycle time was 500 ms and min/max dwell was 10.39 ms/246.5 ms with dwell time of 1.2 min. Peak area of each pesticide was directly used as the signal for quantitative analysis using matrix-matched calibration standards.

Results and Discussion

Instrument performance. Standard mixtures of 76 pesticides and internal standard were initially determined for mass spectra, retention times, optimum voltages for fragmentor and collision cell, and precursor and product ions at scan mode. Acquisition time, flow rate, and gradient were also optimized using standard mixtures at MRM mode. The fragmentor voltage that gave the most intense peak of precursor ion was selected first. Subsequently, the most intense ion pairs were selected as quantifier and qualifier ions, and the collision energy that gave the most intense area of those ions were selected for the analysis. After satisfactory results were determined, standard mixtures were tested again at DMRM mode for 1.2 min of delta retention time. Use of DMRM mode improved performance of the instrument. Better peak shape, and higher response were observed. When data acquisition was performed at MRM mode, cycle time was found to be 1200 ms even though dwells for all pesticides were set at 2-5 ms. When performed at DMRM mode, cycle time was reduced to 500 ms and dwell for each pesticide range from 10.39 to 246.5 ms as described above, resulting in the improved chromatographic data (peak area, peak shape etc.). Fragmentor voltage, collision energy for product ions, and retention time at DMRM mode for each pesticide are described in Supplementary Table 1 along with MRM transitions and pesticides' information. Supplementary Fig. 1 A-D shows total ion chromatograms for the pesticides, with target pesticides possessing wide range of polarity with retention time ranging from 5 to 20 min.

Instrument within-run precision was obtained from

Table 1. Instrument Stability and Performance

Run	Performance	Grape	Pear	Persimmon
0.5 ng/g	avg-response	196369.88	344603.27	216920.62
	SD	5145.21	15286.98	5523.04
	%RSD	2.62	4.44	2.55
2 ng/g	avg-response	173947.85	214038.29	212847.29
	SD	5766.45	2467.65	8411.05
	%RSD	3.32	1.15	3.95
5 ng/g	avg-response	145160.96	141881.27	330155.82
	SD	4773.72	4051.23	10162.16
	%RSD	3.29	2.86	3.08
10 ng/g	avg-response	207765.74	338444.53	319732.25
	SD	7020.12	6906.10	10349.90
	%RSD	3.38	2.04	3.24
50 ng/g	avg-response	215025.41	361739.67	399537.31
	SD	6252.07	19416.11	13999.03
	%RSD	2.91	5.37	3.50
All Runs	avg-response	182491.25	281034.69	297978.08
	SD	26539.48	88408.51	71663.61
	%RSD	14.54	31.46	24.05

detector response to internal standard (TPP) at each set of run. Instrument performance was stable at within-run giving relative standard deviation from 2.55 to 3.95% for all tested fruits at each set of run (Table 1). However, instrument performance between runs showed somewhat unstable responses, giving higher relative standard deviations of 14.54, 24.05, and 31.46% in runs for grape, persimmon, and pear, respectively.

Method Validation. To determine MDL and LOQ, five spiking levels (0.5, 2, 5, 10, and 50 ppb) with eight replicates for each level were determined using all fruit samples of persimmon, pear, and grapes. Spiked samples (n=8) were passed through all steps described earlier and analyzed in LC-MS/MS. Data were organized in excel spread sheet and calculated for recoveries, SD (standard deviation), % RSD (relative standard deviation), S/N (signal to noise ratio), MDLs, and LOQs. Matrices matched standard solutions were used for 4-point calibration. S/N was obtained by dividing mean recovery (ppm) by SD. MDLs were calculated by multiplying SD with student t-value (2.998) for n-1 (7) degree of freedom at 99% confidence level. For calculation of LOQs, SD was multiplied by 10. The results of MDLs were counter-checked using the following criteria: 10×MDL > spiking level > MDL; 120% > recovery % > 70%; S/N between 5 and 10. Most cases met these criteria. However, in the case of S/N, some pesticides showed S/N higher than 10 even at the lowest spiking level (0.5 ppb), implying that MDLs of these pesticides could be lower than the calculated values with the spiking concentration still remaining high. Due to complexities of pesticides properties and responses to MS detector, determination of the exact S/N ratio of such a large number of pesticides was difficult; thus, further experiments to obtain correct S/N ratio were not carried out. According to analytical detection limit guidance, the S/N ratio is a useful test for MDL validity, but a high S/N ratio does not necessarily indicate that the MDL is invalid. Therefore, calculated MDLs results were selected for respective pesticides, although S/N was higher than 10. Similarly, some pesticides showed unsatisfactory recoveries and/or linearity accompanied by S/N ratio lower than 5 even at the highest spiking level of 50 ppb, which is due to their high LOQs that require higher spiking concentration to obtain satisfactory results. The results showed that MDLs and LOQs are comparable to previously reported results (Supplementary Table 2) [Venkateswarlua *et al*., 2007; Wong *et al*., 2010].

Most pesticides gave satisfactory results in terms of recovery, linearity, % RSD, and S/N (at MDL spiking) in three fruit matrices (Supplementary Table 2). Only a few pesticides (bifenazate, deltamethrin, etoxazole, and spiromesifen) showed poor linearity, low recovery, and/or not-applicable (NA) in MDL spiking (Supplementary Table 2) or fortified spiking (Supplementary Table 3). The reason why these pesticides are non-applicable in this method can be attributed to matrix effect, which exerted ionization suppression, analyte instability, and low ionization efficiency or combine effect of these factors [Wong *et al*., 2010]. Bifenazate showed good results at low spiking in grape, but gave non-applicable results at fortified spiking, indicating both analyte instability and matrix effect take part in the process of ionization. In addition, linearity of solvent-only calibration standard showed good linearity (data not shown), implying analyte instability is initiated by the matrix effect. Spiromesifen also gives results very similar to those of bifenazate. Satisfactory results for bifenazate in persimmon at both MDL spiking and fortified spiking, and spiromesifen at MDL spiking in persimmon and fortified spiking in pear also confirmed this assumption. Previous study also reported that the effect of one specific combination of pesticides and matrices can vary from one time point to another. For example, a pesticide that is affected by 30% suppression on one occasion can be affected by 30% enhancement on another occasion. The matrix effect is compound-dependent, which is often due to interaction of co-eluting matrix components with target pesticide in the ionization step [Jansson *et al*., 2004]. In the case of etoxazole, linearity of both matrix-matched and solvent-only calibration were poor, showing that the instability of analyte was caused by the experimental condition rather than matrix effect.

Poor linearity and low recovery of prochloraz and triflumizole in grape at fortified spiking (Supplementary Table 3) may be due to their very low LOQs ranging from 0.15 to 0.79 ppb in all fruits tested. Spiking of 100 ppb is 100-1000 times higher than the LOQs of these pesticides. Apparently ionization efficiency decreases at higher concentration, thus resulting in poor linearity and recovery. Although unsatisfactory results were observed for deltamethrin in grape, good linearity and recovery were found in both pear and persimmon. According to the results from pear and persimmon, none of the fortified spiking concentration met 10-fold LOQs of deltamethrin, which may have resulted in unsatisfactory results in grape. The recovery of fenazaquin at fortified spiking in grape and persimmon also gave results similar to that of deltamethrin. Considering the results with respect to linearity, recovery, and % RSD, grape appeared to be the most problematic matrix among the tested fruits and persimmon the least. On the other hand, etoxazole, from the point of view of pesticide, was the most problematic and not applicable in this experimental condition.

Table 2. Pesticides found in blind-incurred samples

Persimmon			Grape			Pear		
Pesticide	Code No.	Conc.(ppm)	Pesticide	Code No.	Conc.(ppm)	Pesticide	Code No.	Conc.(ppm)
Carbendazim	PS-1	0.022	Carbendazim	GR-1	0.027	Boscalid	PE-4	0.019
	PS-4	0.038		GR-3	0.002	Chlopyrifos	PE-2	0.017
	PS-5	0.03		GR-4	0.176	Diflubenzuron	PE-2	0.007
	PS-8	0.029	Clothianidin	GR-1	0.051	Pyraclostrobin	PE-1	0.009
Difenoconazole	PS-1	0.004		GR-2	0.011		PE-2	0.005
	PS-3	0.030		GR-3	0.011	Pyrimethanil	PE-9	0.006
	PS-5	0.042		GR-5	0.068	Tebuconazole	PE-5	0.005
	PS-6	0.005		GR-6	0.028	Tebufenozide	PE-10	0.004
	PS-7	0.004		GR-7	0.006			
	PS-8	0.017		GR-10	0.126			
	PS-9	0.014	Difenoconazole	GR-7	0.003			
	PS-10	0.025	Fluquinconazole	GR-1	0.063			
Dinotefuran	PS-1	0.015	Pyraclostrobin	GR-4	0.021			
	PS-2	0.052		GR-5	0.037			
	PS-4	0.016		GR-6	0.078			
	PS-6	0.021		GR-9	0.012			
	PS-7	0.031	Pyrimethanil	GR-1	0.183			
	PS-8	0.047	Spirodiclofen	GR-3	0.018			
Fenazaquin	PS-3	0.163	Tebuconazole	GR-4	0.007			
	PS-4	0.037		GR-10	0.139			
	PS-5	0.335	Thiamethoxam	GR-7	0.004			
	PS-7	0.016	Trifloxystrobin	GR-4	0.004			
Pyraclostrobin	PS-4	0.008						
	PS-7	0.005						
Tebuconazole	PS-3	0.002						
	PS-8	0.031						
	PS-10	0.029						
Tebufenozide	PS-3	0.007						
Thiacloprid	PS-3	0.008						
Thiamethoxam	PS-1	0.007						
	PS-4	0.0005						
	PS-7	0.008						
	PS-8	0.102						
	PS-10	0.029						
Trifloxystrobin	PS-1	0.103						
	PS-7	0.044						
	PS-10	0.151						

PS=Persimmon, GR=Grape and, PE=Pear

Despite a few exceptions, the developed method clearly showed that it can be applied for the analysis of residual pesticides in tested matrices at ppb level. However, using matrix-matched standards for the calibration and, using PSA and C_{18} for dispersive SPE (solid phase extraction) clean-up could not compensate the significant matrix interferences for pesticides bifenazate, etoxazole, and spiromesifen. The results of these pesticides changed from matrix to matrix, indicating that there are exceptional compounds and matrices which still need to be validated using QuEChERS sample preparation method regardless of its approval for multiresidue analysis.

Application to Blind-Incurred Samples. The developed method was applied for the analysis of residual pesticides in blind-incurred samples collected from various regions in Korea. Sample preparation and quantitative analysis were conducted using exactly the same procedure as described earlier. The amount of

pesticide detected was determined using matrix match calibration curve. The results are listed in Table 2. Among the observed pesticides, 10 were found in both grape and persimmon, and 7 in pear. The most frequently found pesticide in grape was clothianidin, which was found in 7 samples out of 10 tested samples. In persimmon, difenoconazole was observed in 8 samples out of 10. All persimmon samples tested (n=10) contained pesticides, 9 samples of grape and 6 samples of pear out of 10, contained pesticides. Trace amounts of pesticides with concentrations lower than the lowest calibration level (0.016 ng) were not listed.

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