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Pesticide Analysis in Vegetables Using QuEChERS Extraction and Colorimetric Detection

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ABSTRACT

A novel combination of extraction and detection methods is demonstrated for pesticide residue analysis in vegetable samples. Acetylcholinesterase (AChE) inhibition was used as a simple colorimetric test for organophosphates/carbamates (OP/C), and was tested with extracts from the widely-used QuEChERS extraction method. In the absence of pesticide, diluted (50% with water) acetonitrile did not inhibit enzyme activity, demonstrating the compatibility of this extraction solvent with the AChE inhibition test. QuEChERS extraction of chlorpyrifos-spiked tomato, spinach and lettuce samples indicated a high sensitivity to OP/C, with AChE inhibition occurring in the ppb range. The applicability of this method combination was tested by screening tomatoes from 18 different sources, including private gardens, farmer's market venders, and local supermarkets. Tomatoes from one private garden, three "certified naturally grown" farmer's market venders and two "organic" supermarket source had AChE inhibition significantly above nominally pesticide-free controls, suggesting the presence of OP/C residue. These residues were likely below levels of health concern, as indicated by lack of complete AChE inhibition, and the absence of inhibition upon sample dilution. This study demonstrates that the combination of QuEChERS extraction and AChE-inhibition detection provides a relatively simple and inexpensive alternative for detection of OP/C in vegetable samples.

Keywords: QuEChERS, vegetable, pesticides, acetylcholinesterase

INTRODUCTION

Although organic food production has become more prevalent, the production of vegetables is still largely dependent on the use of pesticides such as organophosphates (e.g., Jaipieam et al. 2009), which can clearly present a health risk to consumers (Kamanyire and Karalliedde 2004). In an attempt to keep consumer exposure to these pesticides below levels of health concern, monitoring programs have been established in many countries. While routine, residue monitoring does remain a relatively expensive and complex process, typically relying on chromatographic techniques for

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detection. This limits the ability to effectively monitor pesticides in situations with inadequate resources, such as in developing countries. Unfortunately, these are locales where risks from pesticide exposure are of greatest concern due to increased use (Nweke and Sanders 2009), weak regulation, poor education about safe application practices (Williamson et al. 2008), and/or increased reliance on crop foods (rather than meat) as a critical dietary component. Studies of pesticide residues in developing countries illustrate situations where pesticide residues are routinely found on market vegetables (e.g., Amoah et al. 2009; Srivastava et al. 2011; Hossain et al. 2015). Thus, pesticide monitoring is most difficult in countries where that monitoring is arguably most needed. As has been noted by other authors, an effective, simple, and inexpensive method is needed to enable environmental analysis in situations of limited resources (Hennion and Barcelo 1998; Mallat et al. 2001; Qian et al. 2009; Xu et al. 2012).

Assessment of pesticide levels is a two-step process: extraction & detection. Extraction is now relatively simple and cheap due to the recent development of the QuEChERS extraction method ("Quick, Easy, Cheap, Effective, Rugged, Safe") (Anastassidoes et al. 2003). As a consequence of its advantages over conventional extraction techniques, QuEChERS has now become the extraction method of choice, and numerous studies exist demonstrating its utility for the extraction of a wide array of chemical compounds from many foodstuffs (e.g., Lehotay 2007; Lesueur et al. 2008; Nguyen et al. 2008; Koesukwiwat et al. 2010). While QuEChERS has simplified the extraction process, the pesticide detection step remains a relatively expensive and complex process. Two detection methods are typically used: gas (GC) or liquid chromatography (LC) coupled with mass spectrometry (MS) (e.g., Lesueur et al. 2008; Nguyen et al. 2008). While routine, specific and precise, these detection methods are less feasible in situations of limited resources or where a faster screening process is desired.

Enzyme-based detection methods, such as ELISA or acetylcholinesterase (AChE) inhibition tests, present an alternative method for monitoring pesticides, and have been used for monitoring pesticides in vegetables (Watanabe et al. 2006; Graber Neufeld et al. 2010), water samples (Mallat et al. 2001), and in human samples (Nweke and Sanders 2009; Worek et al. 2012). In some cases such tests are used as pre-screening tests, reducing the number of samples tested using more complex means (Moris et al. 1995; Hennion and Barcelo 1998). Enzyme-based tests are typically faster and less expensive, and often have high specificity and sensitivity (e.g., Qian et al. 2009; Wang et al. 2011). However, enzyme-based tests with vegetables generally utilize external washes (which do not detect pesticides accumulated inside the plant tissue), or crude extracts which can result in more pronounced matrix effects. Matrix effects vary with both the type of vegetable tested and the specific test kit used, and the resulting dilution of samples to avoid these matrix effects reduces the limit of detection for this assay (Xu et al. 2012). The applicability of enzyme-based tests with unprocessed extracts is thus limited. This limitation could be circumvented by applying a clean-up procedure, such as QuEChERS. However, to date none of the published studies on enzyme-based detection methods for pesticides have utilized these two techniques in concert to simplify the process of pesticide monitoring. The present study verifies the utility of combining the QuEChERS extraction method with the AChE inhibition test, a broad-specificity test for OP/C pesticide, and demonstrates its applicability in the screening of market vegetables.

MATERIALS AND METHODS

Samples

Single samples of supermarket tomato, spinach and lettuce that were certified organic were used as our control samples. These samples were used as nominally pesticide-free samples to assess the effect on enzyme activity of spiking with chlorpyrifos, and to measure enzyme activity from extracts of other organic and nonorganic vegetable samples. Tomato samples for our local survey were purchased from the Harrisonburg (Virginia) farmer's market, from local supermarkets, or collected from local gardens (private residence, and campus garden for Eastern Mennonite University). Samples were frozen (-20ºC) until analysis.

Extraction Method

All samples were extracted using the QuEChERS extraction technique (Anastassiades et al. 2003), which resulted in collection of both surface and internal pesticide residues. In brief, samples were ground in a mortar, and 5g aliquots of the ground sample were then transferred to 50 ml Eppendorf tubes. In rapid succession, 5 ml acetonitrile (HPLC grade), 2 g anhydrous $MgSO_4$, and 0.5 g anhydrous sodium acetate were added to the sample. After capping and shaking the sample vigorously for 1 minute, the sample was spun at 1500 rpm for 2 min. Dispersive solid phase extraction (SPE) was performed by combining 182.5 mg SPE sorbent (Supelco PSA/ENVI-Carb 55233-U) with 1 ml aliquot of the top acetonitrile layer. After vortexing 20 seconds, vials were centrifuged at 3000 rpm for 1 minute. At this point vegetable pigment was no longer visible in the extraction solution (Figure 1). The supernatant was transferred to a clean tube and analyzed immediately with the AChE test (see below).

Detection Method

Pesticide detection was performed using a colorimetric commercial test kit (Organophosphate / Carbamate Screen Kit, PN 550055; Abraxis LLC; Warminster, PA) based on AChE inhibition. The test, a modification of the standard Ellman method (Ellman 1960), produces a yellow color in the presence of AChE activity. Color was quantified with a spectrophotometer; a decrease in absorbance at 405 nm indicated AChE inhibition in the presence of pesticide residue. Two controls (provided with the kit) were run with each sample batch: a negative control (no pesticide), and a positive control (5 ppb diazinon). Percent inhibition was calculated as (absorbance of negative control – absorbance of sample) / (absorbance of negative control – absorbance of positive control). All QuEChERS extractions (in acetronitrile) were diluted to 50% with HPLC-grade water.

RESULTS

Extraction solvent

QuEChERS typically uses acetonitrile as an extraction matrix (Lehotay et al. 2010), whereas the AChE inhibition assay is based the use of a 50% methanol extract. AChE activity was therefore tested in the presence of acetonitrile to establish its compatibility with this typical QuEChERS solvent. Enzyme activity was significantly inhibited ($p<0.05$; paired t-test) by the presence of 100% acetonitrile (Figure 2). When this extract was diluted to 50% with HPLC-grade water, enzyme inhibition was not significantly different from that occurring in the stock solvent (50% methanol). Dilution of QuEChERS extracts to 50% acetonitrile is therefore compatible with using the

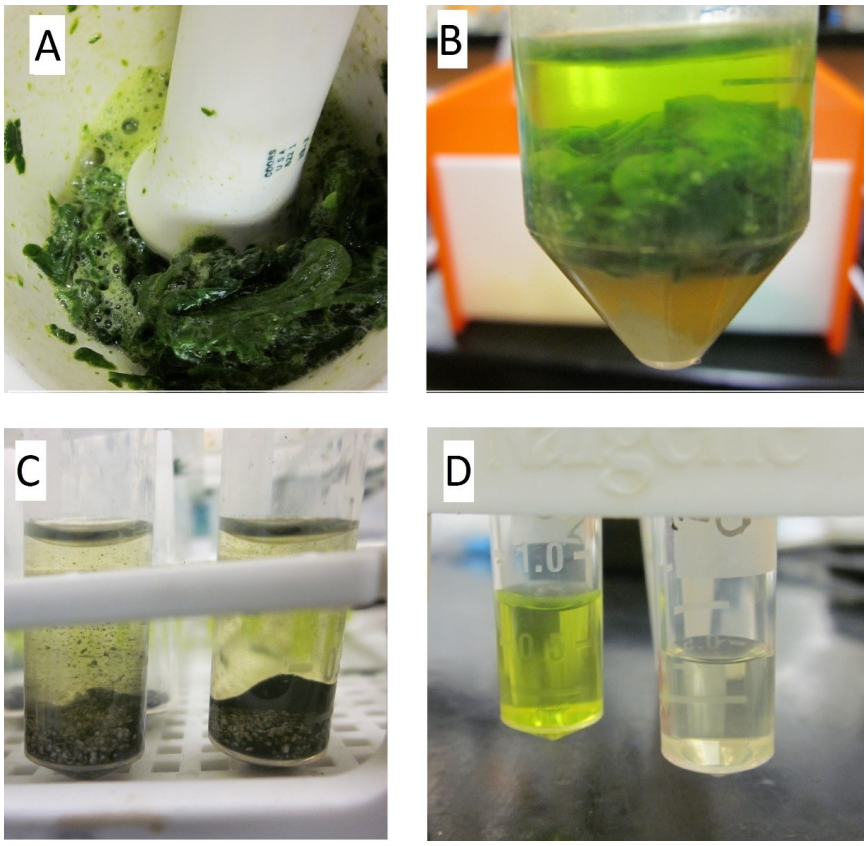

FIGURE 1. Example of QuEChERS procedure as applied to spinach sample. The sample is ground (A), extracted in acetonitrile (B), and placed in SPE sorbent (C). The final extract (D, right tube; cf left tube prior to sorbent exposure) has pigments removed which would otherwise interfere with the colorimetric assay.

AChE inhibition assay, and all subsequent tests (samples, blanks and standards) were performed with 50% acetonitrile.

Assay Sensitivity

Nominally pesticide-free spinach and tomato samples ("certified organic" labeled vegetables from the supermarket) were extracted using QuEChERS and tested with the AChE inhibition assay. A low, but significant ($p<0.05$; paired t-test, extracts compared with negative control), level of enzyme inhibition was observed in these samples (Figure 3; "0 ppb chlorpyrifos"). AChE was inhibited when tomatoes, spinach, or lettuce were spiked (prior to extraction) with chlorpyrifos, a representative OP/C. Significant inhibition ($p<0.05$; Dunnett's test) occurred down to the ppb range (Figure 3).

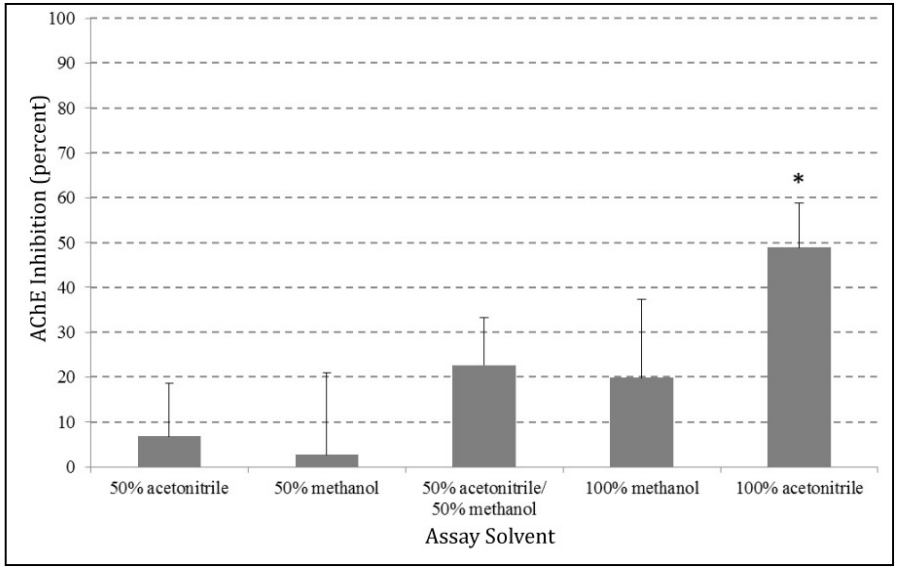

FIGURE 2. Inhibition of acetylcholinesterase activity by extraction solvents, in absence of pesticide residues, demonstrating assay compatibility with typical QuEChERS solvent (acetonitrile) when diluted to 50% with water (Asterisk indicates statistically significant inhibition of enzyme activity in indicated solvent relative to stock negative control, N=3-9 for each category).

Duplicate and triplicate tests of samples suggest that the use of the AChE inhibition assay with QuEChERS samples has a precision comparable to that of QuEChERS used with traditional chromatography techniques, as indicated by calculated relative standard deviation (RSD) and relative percent difference (RPD). The enzyme assay alone had RSDs of 4.75%, 8.57%, and 7.77% when testing 50% methanol negative controls, 50% acetonitrile negative controls, and a 50% methanol positive control, respectively. Market samples of tomatoes measured in duplicate with the enzyme assay had an average RPD of 19.5%. Thus, measured differences in samples (e.g., different tomatoes from a single source) reflect both real differences in those tomatoes, and some difference associated with this estimated level of precision.

Background signal

Nominally pesticide-free samples exhibited inhibition of AChE ("0 ppb" samples; Figure 3). We did not have access to reference standards in which a vegetable sample would be laboratory certified as pesticide free; we therefore could not be assured that pesticides were in fact absent from the nominally pesticide-free samples. However, serial dilutions of tomato samples from three venders that sold certified naturally grown ("organic") tomatoes still had baseline inhibition, even when diluted by 1000x with acetonitrile (Figure 4). Duplicate blank QuEChERS samples (samples processed without the addition of any vegetable) had an inhibition of 19.3%. These results strongly suggest that there is a normal background inhibition of AChE in these samples which is associated with QuEChERS processing (perhaps solvent), and that pesticides

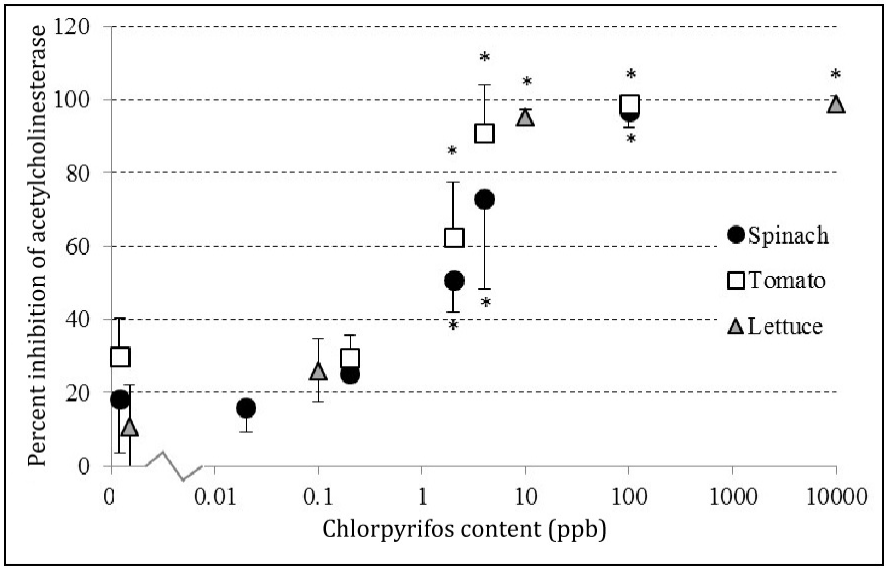

FIGURE 3. Inhibition of AChE by varying concentrations of chlorpyrifos, a representative organophosphate pesticide (Asterisks indicate samples with significantly more enzyme inhibition than unspiked samples; N=3-4 for each category).

were below the detection limit in the nominally pesticide-free samples used for creating a calibration curve (Figure 3). Pesticide presence is therefore indicated by inhibition above a baseline level, rather than simply the presence of any inhibition.

Comparing pesticides in market tomatoes

To demonstrate the applicability of this combination of techniques, tomatoes from several sources in the Harrisonburg, Virginia area were analyzed for pesticide residues. The sampling focused in particular on testing whether pesticide residues would be detected in tomatoes from sources that would be expected to be pesticide free (private gardens where pesticides were not used, from the local farmers market, or organically labeled tomatoes from local grocery stores). Screening of multiple tomatoes from each site indicated that 6 out of the 18 sources had AChE inhibition (Figure 5) significantly above control levels ($p<0.05$, Dunnett's test). Elevated AChE inhibition in 6 "organic" sources suggests the presence of pesticide residues in samples from some growers that do not themselves use pesticides. One sample (campus garden) had a marginally ($p=0.04$) lower signal than control; likely this was due to a single anomalously low sample.

The average relative standard deviation between tomatoes from the same source was 29.8%, suggesting that tomatoes from the same source often have similar levels of pesticides. However, individual tomatoes from a source varied considerably in some cases (e.g., Vender G), suggesting that individual tomatoes can vary in their residue levels even from a single source, and sampling design for screening from vegetable

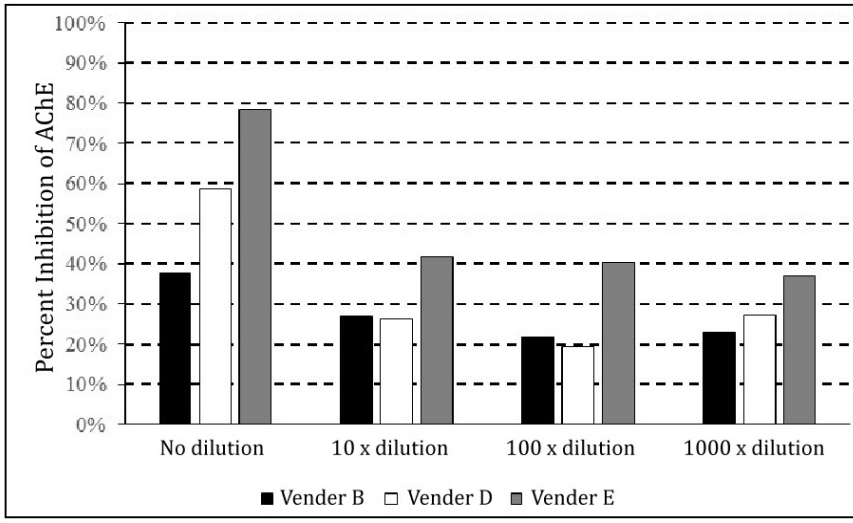

FIGURE 4. Serial dilutions of samples (with acetonitrile) versus percent inhibition, depicting the percent inhibition as samples are diluted. Each data point is the average of duplicate samples.

sources should therefore include multiple samples from each source to account for this variability.

DISCUSSION

This is the first demonstration that the widely-used QuEChERS extraction technique can be utilized in combination with a relatively simple enyzme-based detection assay for a pesticide. The AChE assay was compatible with QuEChERS acetonitrile extracts, and showed a sensitivity and precision comparable to traditional chromatography methods. Clean-up with QuEChERS, followed by dilution to 50% acetonitrile, provided an extract that could be used directly for a relatively quick and inexpensive detection of OP/C using the AChE inhibition test. ELISA assays have been used for a range of specific pesticides (Hennion and Barcelo 1998), and further studies should investigate the similar application of QuEChERS extracts with these tests.

The level of sensitivity is comparable to that of chromatographic methods (Malik et al. 2010; Srivastava et al. 2011), and similar to that found both in pesticide ELISA tests (e.g., Qian et al. 2009) and in other tests of AChE inhibition (Xu et al. 2012). It is also one or two orders of magnitude lower than established maximum residue limits for chlorpyrifos in vegetables (0.05 to 0.5 ppm, European Commission, 2008; 0.01 to 2 ppm, Codex, 2010). Partial inhibition of enzyme occurs over a relatively narrow range of concentrations; total inhibition occurred by the time concentrations reach 10 ppb. Thus, although it is a sensitive technique that easily detects the presence of low concentrations of chlorpyrifos, the lack of a large linear range of response makes it less straightforward for determining exact concentrations. Multiple tests with serial dilution of samples could be used to estimate the concentration in a sample (for example, see

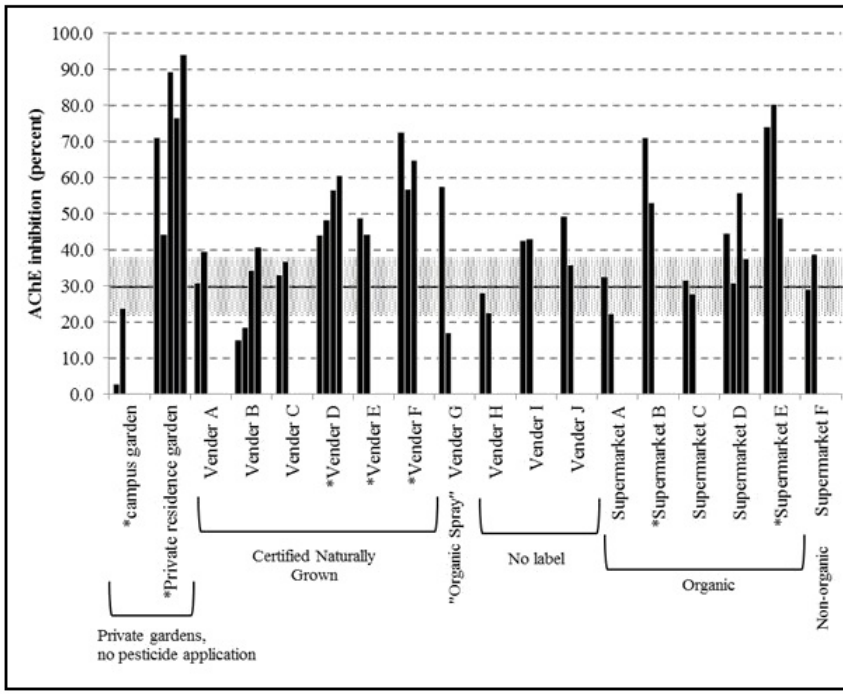

FIGURE 5. AChE inhibition in individual tomatoes from 18 sources in the Harrisonburg area, grouped according to location (private gardens, venders at farmer's market or supermarket), and labelling. The mean and standard deviation for control (nominally no pesticides) is indicated by the horizontal line and shaded box; asterisks indicate sites significantly different from these control samples.

Figure 4), or the technique may be used as a non-quantitative manner either for initial screening (e.g., identifying samples for later more detailed analysis), or for indicating the general presence/absence of pesticide residues. In addition, the precision (as indicated by the RSD) of the current technique is similar to the precision in studies utilizing QuEChERS with GC/MS and HPLC (Lesueur et al. 2008; Lehotay et al. 2010). Taken together, the combined QuEChERS and ELISA method is thus comparable to the QuEChERS used with chromatography methods.

Although residues were indicated from six sources, the presence of residues indicated by this test do not necessarily indicate residue levels are of health concern. In fact, none of the samples showed full inhibition of AChE, suggesting that residue levels were actually quite low, less than what would be equivalent to 10 ppb chlorpyrifos. For two sources that had significant residue levels (Venders D and E), we took a semi-quantitative approach to further estimating the residue levels which further suggested relatively low pesticide levels. Dilution of these samples by 10x reduced AChE inhibition to control levels (Figure 4) and dilution to 100x and 1000x the original concentration did not cause any further decrease in inhibition (consistent with the

observation of a background level of inhibition). The results indicate the utility of serial dilutions in using this assay in a semi-quantitative manner that could match screening results with levels of health concern.

It is somewhat surprising to detect pesticides in these tomato samples, given that all samples where pesticides were detected were labeled as being free of pesticides. The route of pesticide contamination for tomato samples in this study is not known, but there are multiple possible sources, including pesticide drift from neighboring fields, transport-related contamination, and inaccurate product labeling. While there is considerable emphasis on production of vegetables without pesticide use, the presence of residue on certified "pesticide-free" tomatoes suggest that there should be more consideration given to other avenues by which pesticide residues may lodge on vegetables. For instance, the large amount of pesticide that does not reach its target (Pimentel 1995) could represent a significant source of contamination for organic vegetables. Overall, our screening of 18 tomato samples demonstrates the successful application of this combination of rapid and inexpensive extraction and detection methods.

There are several potential limitations to the current methodology. The AChE inhibition test is non-specific for OP/C, and the specific identity of residues is therefore not indicated. The assay responds to all OP/C, but to a greater or lesser amount depending on the specific compound (Xu et al. 2012). Specific concentrations can only be reported if the exact pesticide is known, or if concentrations are reported as (for instance) "chlorpyrifos equivalent". However, the degree of AChE inhibition is arguably the more relevant parameter, as an indicator of actual toxicity regardless of the specific compound. A second limitation is the general reliance of QuEChERS on acetonitrile as an extractant. Acetonitrile may be more difficult to obtain in developing countries, especially given the worldwide fluctuations in acetonitrile availability. Further work should be done on other solvents that have been used with the QuEChERS method, such as ethyl acetate, which is more widely available and less toxic than acetonitrile (Lehotay et al. 2010). Finally, additional work might help to refine the technique for additional precision at these low concentrations.

While chromatographic techniques are recognized as the "gold standard" for pesticide analysis, we suggest that enzyme-based assays (AChE or ELISA) with cleaned-up samples provide distinct advantages under certain circumstances. Under conditions where resources are limited (e.g., developing countries), this method has the potential to be used with a lower investment of resources and training. Even in situations where chromatographic detection is possible, initial screening of samples with the assay test would reduce the time and expense associated with monitoring efforts (Mallat et al. 2001). The use of simple and inexpensive analytical techniques such as demonstrated in this study could thus facilitate monitoring in situations where pesticide residues are of concern.

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