Special issue: Review

Analytical approach, dissipation pattern, and risk assessment of pesticide residue in green leafy vegetables: A comprehensive review

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This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1002/bmc.4134

Abstract

The category of "leafy vegetables" comprises a wide range of plants, including cabbage, lettuce, leeks, spinach, Swiss chard, and kale, and it forms a significant component of the human diet. Typically, leafy vegetables are low in calories and fat, are great sources of vitamins, protein, dietary fibre, minerals (including iron, calcium, and nitrates), and are rich in phytochemicals. To counter the impact of pests on vegetables, a broad variety of pesticides is used. Because of their large surface areas, leafy vegetables are expected to have high residual pesticide levels. As such, a sound analytical approach was necessary to detect and quantify residue levels that are equal to or lower than the maximum residue limits (MRL), thus rendering the products safe for consumption. Overall, leafy vegetables consumed raw (after a tap water wash only), boiled, or steamed contribute 2% of total vegetable consumption globally, and they might have a comparatively greater influence on health than that of cereal ingestion. Consequently, in this review paper, we highlight the importance of leafy vegetables, the pesticides that are commonly used on them, and various analytical techniques, including sample preparation, extraction, clean-up, and final detection. The effects on dissipation patterns, pre-harvest residue limits, and safety/risks imposed by various pesticides are also reviewed and discussed. In conclusion, environmentally-friendly extraction methods coupled with high throughput techniques with greater reproducibility and lower uncertainty are needed for quantifying residues in leafy vegetables at very low concentrations. Commercial and household food preparation, such as washing, peeling, blanching, and cooking are effective in removing most of the pesticide residues that are loosely attached on vegetables.

KEYWORDS: Leafy vegetables, analysis, pesticides, maximum residue limit, dissipation pattern, pre-harvest residue limit, risk assessment

1. INTRODUCTION

Use of pesticides to control pests is of enormous importance, both for the reducing crop yield losses and improving food quality (Aktar, Sengupta & Chowdhury, 2009; Cooper & Dobson, 2007; Fantke & Juraske, 2013). Advances in pesticide application have played an important role in increasing agricultural productivity because of the pesticides' significant potency and lower economic costs (Lambropoulou, Sakkas & Albanis, 2002; Olsson, Nguyen, Sadowski & Barr, 2003; Seebunrueng et al., 2011; Sun et al., 2011), thus endowing pesticides with agricultural and economic value (Patil, 1994). However, the health effects of gradual low-level exposure to pesticides that may contaminate agricultural commodities are increasingly attracting more attention (Fantke & Juraske, 2013; Slovic, 2010). Therefore, dietary exposure to pesticides has become an important consideration (Fantke et al., 2011; Fantke, Friedrich & Jolliet, 2012; Fantke & Juraske, 2013). Various programs have been developed and introduced to control pesticide residues in foods of plant origin to ensure compliance with various national and international laws and to guarantee the safety of agricultural foods (Walorczyk, 2008). Nonetheless, an increasing number of pesticides are being used with specific plant species, making the analysis of residual pesticides quite challenging for researchers. These pesticides belong to various chemical classes and exhibit a broad spectrum of distinct physicochemical characteristics, presenting additional challenges (Walorczyk, 2008). In this review article, potential eligible studies (in English) were identified through an electronic search of the PubMed database (up to date) (http://www.ncbi.nlm.nih.gov/pubmed) and Google. We used the main search term "leafy vegetables" combined with the terms "biological importance", "pesticide residue", "maximum residue limit", "sample preparation", "chromatographic determination", "dissipation pattern", "pre-harvest residue limit", "risk assessment", and "safety", to find the relevant literature. We screened titles, keywords, and abstracts of the publications obtained from the database. If deemed appropriate, a full copy of

the article was obtained for further assessment. The present review covers "green leafy vegetables" and their diverse importance, pesticide application and residues, maximum residue limits (MRL), and various extraction and analytical techniques. In addition, dissipation patterns, pre-harvest residue limits (PHRL), and risk assessments are covered. Lastly, a conclusion and future perspectives are suggested based on the authors' experience.

2. GREEN LEAFY VEGETABLES AND THEIR DIVERSE IMPORTANCE

Green leafy vegetables are recognized as major contributors to a healthy diet because of the diversity of their nutritional composition (McMahon et al., 2013; Wołejko et al., 2016). Overconsumption of nutritionally poor, highly caloric, rapidly absorbable, and highly processed foods may lead to systemic inflammation, reduced insulin sensitivity, and a cluster of metabolic abnormalities, including obesity, hypertension, dyslipidemia, and glucose intolerance (generally termed metabolic syndrome). Overconsumption of these foods occurs in virtually all developed nations (Ford, Giles & Dietz, 2002). Human consumption of green leafy vegetables has increased worldwide because they offer multiple health benefits (Fabiani et al., 2017). Low in cost and easily accessible (Van Duyn & Pivonka, 2000), green leafy vegetables are sources of important vitamins, minerals, fibres, and essential amino acids (McMahon et al., 2013; Pandey et al. 2016; Walorczyk, 2008), and they also provide non-nutrient biologically active components such as phytochemicals (e.g., polyphenols and flavonoids) (NSW, 2003; Roberts & Moreau, 2016; Van Dokkum et al., 2008). These components act as antioxidants and prevent the influence of free radicals on biomolecules such as fats, deoxyribonucleic acids, and proteins (Farina et al., 2017). Antioxidant activity levels vary depending on the type of green leafy vegetable (Tarwadi & Agte, 2003). Green leafy vegetables also constitute good sources of nitrates and play a significant role in healing wounds, reducing high blood pressure, and reducing the chance of cardiovascular disease

(Farina et al., 2017; Łozowicka, Jankowska & Kaczyński, 2012). Furthermore, their dietary fibre content contributes to weight loss (because of low caloric content), lowers the risk of cardiovascular diseases (via reducing blood pressure and low-density lipoprotein cholesterol), and reduces the risk of diabetes (by improving glucose metabolism) and colon cancer (owing to the protective role of dietary fibre) (Institute of Medicine, 2002; Jenkins, Vuksan & Jenkins, 2001; O'sullivan & Cho, 1998). Overall, high utilization of fruits and green leafy vegetables is believed to prevent or lower the risk of a broad spectrum of diseases, including several types of cancers (Nomura et al., 2008; Riboli & Norat, 2003; Stevenson & Hurst, 2007; Wu et al., 2009), cardiovascular diseases (Hu, 2003), and stroke (Larsson, Virtamo & Wolk, 2013). Furthermore, fatalities are lower when compared to persons consuming fewer fruits and vegetables (Agudo et al., 2007). Such synergistic affects may be associated with bioactive phytochemicals, including tocopherols, polyphenols, ascorbic acid, glucosinolates, and carotenoids, in both vegetables and fruits (Williamson, Faulkner & Plumb, 1998). It is notable that the predominance of diseases, such as heart disease and cancer, is comparatively low among Asians, and this could be attributed to their custom of high fresh vegetable and fruit consumption (Tarwadi & Agte, 2003). A recent meta-analysis revealed that regular high consumption of leafy green vegetables could remarkably decreases the incidence of several types of cardiovascular disease (Pollock, 2016).

3. PESTICIDE APPLICATION AND RESIDUE IN GREEN LEAFY VEGETABLES

It is estimated that global food production will need to increase 70% by the year 2050 to feed the increasing human population (Farina et al., 2017). To guarantee the food supply, the use of pesticides in modern agriculture is crucial. Wide use of pesticides in the agricultural sector

is encouraged to boost crop production (Farina et al., 2017). However, extensive pesticide use has caused substantial environmental problems because of their ability to penetrate vegetable tissue, thus affecting the natural food chain (Abdulra'uf & Tan, 2013; 2014) as well as human health. The latter is especially true when farmers do not control the time between last pesticide application and harvest (Wang et al., 2013). The World Health Organization (WHO) stated that pesticide poisoning could affect approximately 3 million people annually, resulting in 220,000 deaths worldwide (WHO, 1992). The effects of pesticides on human health range from small disorders such as nausea, allergies, and headaches, to chronic disorders such as neurological disorders, cancer, and reproductive malfunction (Abdulra'uf & Tan, 2015; Farina et al., 2017; Li et al., 2014).

According to the rules of integrated pest management (IPM), the determination of pesticide residues is necessary to predict pesticide concentrations and the required pesticide applications, as well as to determine the pre-harvest interval (PHI). The implementation of the rules of IPM and good agricultural practices (GAP) will generally result in a decrease in the use of pesticides, thus minimizing the effects of those that are environmentally dangerous (Grimalt & Dehouck, 2016). Consequently, the number of commonly used pesticides and the number of residues in plant tissues under cultivation is typically lower than the total number of registered pesticides in all countries.

There are many kinds of pesticides, including insecticides, acaricides, herbicides, fungicides, and more. Determining the fate of pesticides in crops is important for GAP and is reported to be influenced by the efficiency of applied pesticides on the plant surface, the PHI, and the amount of residual pesticide on crops at harvest time (Metwally, Osman & Al-Rushaid, 1997; Rahman, 2012). Insecticides and miticides are efficient for controlling various insects and mite pests in a number of vegetable crops (Tomlin, 2009). These compounds include chlorfenapyr (registered in 19 countries), a pro-insecticide that converts to an active

metabolite in the midgut area of insects and mites. It has been used on cotton, ornamentals, and many other crops (Cao et al., 2005). Its efficiency has been reported to be related to specific species, such as *Spodoptera* sp., *Heliothis* sp., *Pseudoplusia* sp., *Tetranychus* sp., and *Trichoplusia* sp. It has been used in place of pyrethroid insecticides, carbamates, and organophosphates, as well as chitin synthesis inhibitors (Black et al., 1994; Cao et al., 2005; Lovell et al., 1990; Mascarenhas & Boethel, 1997). Fungicides kill moulds, as well as impede their spread and thus save plants (Cornell University, 2008; Park et al., 2016; Tomlin, 2009). Many fungicides (e.g., procymidone, azoxystrobin, dimethomorph) are widely used in leafy vegetables (e.g., *Pimpinella brachycarpa*, Chinese chive, lettuce, Korean cabbage, spinach, *Perilla* leaves, *Aster scaber*, winter-grown cabbage, young radish, and crown daisy). The use of fungicide is high in leafy vegetable cultivation in the Republic of Korea (Park et al., 2016). Thiamethoxam (TMX) and clothianidin (CLO) are neonicotinoid insecticides, which are widely used for the persistent control of a broad range of immature stages of insect pests, including those of Diptera, Lepidoptera, Thysanoptera, Coleoptera, and Hemiptera, with remarkable systemic action involving various processes (Uneme, 2010). Along with their usefulness in pest control, neonicotinoids may assist in the development and safety of plants facing abiotic and biotic stressors (Ford et al., 2011). In general, different kinds of pesticides have different effects and various degrees of usefulness. In addition, various types of vegetables exhibit different MRL values. The commonly used pesticides and their efficacies in leafy vegetables are given in Table 1.

Pesticides differ in active ingredients and are formulated to control pests through various mechanisms (Ng, Fleet & Heard, 2005). Prior to application, they are commonly diluted in water, and crop application is by aerial or ground spray in a specific volume and dilution to attain the suggested application rate of the active ingredient (Dobhal et al., 2014). Generally, vegetables are affected by pest infestations such as aphids and grasshoppers (Amarasekare &

Edelson, 2004; Gecan & Bandler, 1990), which necessitate the regular application of pesticides to increase productivity, quality, and storage longevity. However, improper and excessive pesticide use can result in environmental pollution (Köck-Schulmeyer et al., 2013) and increased human health risks (Hernández et al., 2013). Human intake of pesticide residue from vegetables may be higher than the toxin intake associated with air inhalation or water consumption (Elgueta et al., 2017). This occurs because fresh leaves can be eaten in a raw state or cooked alone or with other foodstuffs (Kim et al., 2016). Moreover, baby green leaves, a form of nutrient-dense vegetables, are consumed in salad products (D'Antuono, Elementi & Neri, 2009) and can be used in both cold and hot dishes (McMahon et al., 2013). The health of numerous consumers is threatened by pesticides, owing to the direct consumption of processed or raw leafy vegetables (Kim et al., 2016). Different cultivars of leafy vegetables may have distinguishing shapes, leaf forms, growth characteristics, or size; therefore, individual pesticides may not behave the same in various cultivars of the same crop (Ripley et al., 2003). For example, Sances, Toscano & Gaston (1992) found that the highest concentrations of cypermethrin occurred on the exterior leaves and basal area of lettuce heads. In most cases, retained pesticides adhere loosely to the plant surfaces, and washing with plain water (either stagnant or running) can help to remove both dust and adsorbed residue in and on leafy vegetables. Increasing the frequency of washing could greatly reduce residue levels. The effect of washing in reducing pesticide residue, however, is highly variable and depends on surface area, thickness, the amount of wax on the cuticle, age of the residue, pesticide characteristics, and washing conditions (Kim et al., 2016). Variable washing processes used during household chores or commercial preparation proved to be very effective in removing loosely attached residues on various fruits and vegetables (Street, 1969). Conversely, if pesticides are translocated internally, washing will not remove the residue, but boiling and parboiling could possibly reduce residual levels. Care must be taken to prevent

the loss of important food constituents, such as β-carotene (Kim et al., 2016). Commercial and household food preparation such as washing, peeling, blanching, and cooking are effective in removing most of the pesticide residues that are loosely bound to or that have penetrated into raw crops (Kim et al., 2016). Rawn et al. (2008) evaluated the decrease in captan residues in apples from rinsing and peeling. In addition, chlorpyrifos residue on rice grains was reduced 60% by water washing (Lee, Mourer, & Shibamoto, 1991; Kim et al., 2016).

4. MAXIMUM RESIDUE LIMITS

Even with an appropriate pre-harvest interval between the last pesticide application and harvest time, residues may exist and pose a potential risk. Therefore, residual levels of pesticides in foodstuffs are often regulated to reduce the harmful or unnecessary intake of pesticides by consumers (Na et al., 2012). Several countries have specifically established their own MRLs for pesticides in various foodstuffs. However, conflicts between countries regarding acceptable levels may hinder international trade; therefore, the standardization of MRLs is necessary. In this regard, two organizations, the European Union (EU) at the European level and the Codex Alimentarius Commission of the Food and Agriculture Organization (FAO) (Codex Alimentarius, 2012) have set reference MRLs. To control pesticide residues in agricultural commodities, each country follows two different approaches: (1) regulatory observation aimed at raw agricultural commodities, measuring the residual levels of specific matrices in agreement with the MRL (Arias et al., 2014; Bempah et al., 2012; Berrada et al., 2010; Cho et al., 2009; Neidert, Trotman & Saschenbrecker, 1994), or (2) total diet research, in which dietary consumption of pesticides are determined via analysis of consumed foodstuffs (Kim et al., 2016; Leoni et al., 1995; Seo et al., 2013; Yang et al., 2012). For both research and regulatory purposes, an effective methodology is needed to screen and

quantify residues at a concentration equivalent to or lower than the MRL, and to confirm the identities of the compounds in agricultural commodities. Such methodologies usually involve multi-residue methods (MRM) or single-residue methods (SRM) based on sample-homogenization with an appropriate solvent, separation of the liquid portion of the sample from the insoluble material, purification, and clean-up by solid-phase extraction (SPE), followed by a terminal chromatographic determination step. MRMs and SRMs involve the same fundamental steps. However, MRMs are usually applied to monitor or screen various classes of pesticides in commodities, whereas SRMs are applied for individual pesticides in specific crops to determine half-lives, dissipation patterns, PHI, and PHRL (Jang et al., 2014; Park et al., 2013; Rahman et al., 2012; 2013a; Siddamallaiah & Mohapatra, 2016). Therefore, for the purposes of safety and dietary risk assessment, an effective analytical method must be developed to quantitatively determine the parent compound and its metabolites, if any, in specific crops, even though the metabolites are not included in the definition of residue.

5. ANALYTICAL METHODS

Analytical methods play a key role in determining MRLs, from sample homogeneity to instrument detection limits. Significant effort is given to develop and test novel analytical tools and methodologies in pesticide science. If the experimental sample is not sufficiently large to represent the original lot or unit, the total cost, time, and efficiency associated with using the advanced analytical instruments and methodologies will not provide useful conclusions, and may result in confusing data (Farha et al., 2015a; Lehotay & Cook, 2015). Therefore, the Ministry of Food and Drug Safety (MFDS, 2014) of the Republic of Korea endorsed a sample weight of 20-25 g for extraction to lessen sampling error, to ensure experimental sample homogeneity, and to secure low detection limits and quantification with higher precision, thereby enabling MRL compliance. The quick, easy, cheap, effective, rugged, and safe (QuEChERS) technique, with efficient sample preparation, does not require

the proposed sample amount (20-25 g) (Farha et al., 2015a). Moreover, QuEChERS can be used with MRMs associated with mass spectrometry, which have not been previously used in most laboratories because of the combined expense (Anastassiades et al., 2003; Farha et al., 2015a).

5.1 Sampling and sample preparation

The following discussion involves sub-sampling in the laboratory. Appropriate sample preparation methods and sub-sampling are required to ensure the safety of a homogeneous and experimental sample (Grimalt & Dehouck, 2016). Typically, the initial ingredient consists of 1-5 kg of the leafy vegetable, the sample size that reaches the laboratory for analysis. The leaves are detached from the stem, then cut and blended with a food processor or blender. Occasionally, the leaves are preserved by freezing and the frozen samples are homogenized by cryogenic milling (Anastassiades et al., 2003; Farha et al., 2015a; Rahman et al., 2015). Once the sample is homogenized, a small amount of the sub-sample, approximately 0.5-100 g (but typically 10-20 g), is used for extraction and analysis (Fang et al., 2015; Farha et al., 2015b; Rahman et al., 2013a;b; Sances, Toscano & Gaston, 1992).

5.2 Sample extraction

The physicochemical characteristics of the analyte, including the polarity of the pesticide, should be considered (Grimalt & Dehouck, 2016). Improvement in extraction processes, together with the development of analytical methodologies, has lessened the complexity of sample treatment and has increased analytical accuracy and precision (Grimalt & Dehouck, 2016). As a fundamental MRM, organochlorine pesticide analysis in foodstuffs (fruits and vegetables) was developed in 1963 using acetonitrile and petroleum ether (Mills, Onley & Gaither, 1963). To analyse pesticides with greater polarity than the organochlorines, Luke et

al. (1975) developed an acetone-dependent method followed by the use of dichloromethane and petroleum ether, partitioning, and clean-up with Florisil. An acetone-based extraction method was also developed in 1983 by the Dutch Food and Consumer Products Safety Authority-Food Inspection Service (General Inspectorate for Health Protection, 1996), which recommended pesticide monitoring over a 25-year period (Grimalt & Dehouck, 2016). The Swedish National Food Administration introduced an analysis using ethyl acetate, associated with clean-up by gel permeation chromatography, in 1989 (Grimalt & Dehouck, 2016; Pihlström et al., 2007). Acetone (polarity index 5.1) has greater polarity than ethyl acetate (polarity index 4.4) and therefore, polar pesticides partition more in acetone. When forcing polar pesticides into an organic solvent, large quantities of anhydrous sodium sulfate (Na_2SO_4) are introduced into the water phase (Grimalt & Dehouck, 2016). Anastassiades et al. (2003) developed a new method based on acetonitrile extraction, with a clean-up using dispersive-solid phase extraction (d-SPE) with a primary-secondary amine (PSA) and octadecylsilyl (C_{18}). This sample treatment procedure was called QuEChERS, and has become popular because it uses fewer conventional analytical steps, and less glassware and solvent (Grimalt & Dehouck, 2016). In the last decade, in the analytical field of multiple pesticide residue of fruits and vegetables, the QuEChERS method has been distinguished as the Official Method of AOAC International (Lehotay et al., 2007) (Fig. 1). Current studies on pesticide analyses use acetonitrile as an extraction solvent because of its ability to extract less lipophilic material from vegetables and its greater solvating ability. Acetonitrile can obtain a maximum extraction efficiency of analytes in vegetables containing moisture of approximately 80-95% (Lee et al., 1991). Partitioning by $MgSO_4$ helps in obtaining a clean chromatogram, reducing the aqueous phase by saturation, and generating heat around 40°C, which aids in the extraction of non-polar analytes (Anastassiades et al., 2003; Diez et al., 2006; Podhorniak, Negron & Griffith, 2001; Rizzetti et al., 2016).

The search for optimal conditions for pesticide analysis is very challenging because many factors must be considered to generate a method that is fast, easy, and can obtain high recovery rates with adequate selectivity. Factors, such as sample size and the volume of extraction solvent, and procedures such as blending or shaking for extraction, the addition of the correct quantity of salts, and materials used for clean-up, are all very important for optimization. Therefore, many strategies have been employed to obtain the best conditions for the maximum response. A number of extraction methodologies have been introduced for identification of pesticides in vegetable samples, including homogenization (Fenoll et al., 2007; Ishimitsu et al., 2002; Mol, van Dam & Steijger, 2003), dispersing extraction (Ueno et al., 2003; Ueno et al., 2004), solid-phase microextraction (Berrada, Font & Moltó, 2004), microwave-assisted extraction (Barriada-Pereira et al., 2007; Singh, Foster & Khan, 2007), and supercritical fluid extraction (Kaihara et al., 2002). Adou, Bontoyan & Sweeney (2001) and Barriada-Pereira et al. (2007) selected SPE, prioritizing graphitized carbon for the GC-electron-capture detection (ECD). Okihashi and Obana (1998) used SPE with carboxylic acid for HPLC determination. In addition, the extraction solvent was evaluated by Tanaka et al. (2007) by studying desorption efficiencies for spinach after the initial extraction cycle. Dichloromethane, ethyl acetate, and cyclohexane were considered as extraction solvents. These data are presented in Table 2. Ethyl acetate provided appreciable recoveries of 70-117%. As a result, ethyl acetate was endorsed as the extraction solvent for the target pesticides (Tanaka et al., 2007).

Multivariate approaches have been considered that require few experimental runs, but provide both qualitative and quantitative mathematical models for the relationship between factors and responses (Abdulra'uf & Tan, 2013; 2014; Candioti et al., 2014). Multivariate studies have involved selection, screening, response surface methodology, modelling, and optimization. Table 3 lists the various extraction methods applied for leafy vegetables.

5.3 Instrumentation

The complexity in sample treatment corresponds with the existing matrix interferences and the use of separation techniques, most commonly gas chromatography (Al Mahmud et al., 2013; D'Antuono, Elementi & Neri, 2009; Farina et al., 2017; González-Rodríguez et al., 2008; Ikeura, Kobayashi & Tamaki, 2011; Park et al., 2016; Srivastava et al., 2011; Rahman et al., 2013b; Tanaka et al., 2007; Van Dyk et al., 2010; Walorczyk, 2008) or liquid chromatography (Farha et al., 2015a, b; Kim et al., 2016a, b; Lehotay & Cook, 2015; Liu et al., 2011; Pan, Xia & Liang, 2008; Park et al., 2012; 2016; Rahman et al., 2015; Wołejko et al., 2016).

Leafy vegetables typically have higher residue concentrations because of their greater surface area and higher area-to-mass ratios (Ripley et al., 2003). The presence of chlorophyll and chromophyll in some leaves, such as in those of *Perilla* and lettuce, may result in greater residues than in the leaves of other vegetables. For example, residues were higher in lettuce cultivar than in cabbage (Ripley et al., 2003). All these factors lead to complex matrices. To overcome the inference problems, liquid chromatography with ultraviolet absorbance detection (LC/UVD) is best (Farha et al., 2015a, b; Liu et al., 2011), and to tackle suppression problems, the appropriate method might be LC associated with tandem mass spectrometry (LC-MS/MS) (Kim et al., 2016a, b; Na et al., 2012; Pan et al., 2008; Park et al., 2012; Rahman et al., 2015; Yang et al., 2012). Suppression problems increase limits of quantification (LOQ) to equal to MRL, which is expected to be $1/10^{th}$. This leads to strong purification, followed by poor recovery.

So far, GC (GC-μECD, GC-NPD) (Al Mahmud et al., 2013; Rahman et al., 2013b), GC-MS, GS–ion trap mass spectrometry (GC-ITMS; Tao et al., 2009), and GC-MS/MS (Vidal, Arrebola & Mateu-Sánchez, 2002a;b) have advanced the field of pesticide monitoring

because of high selectivity, separation power, and identification capability of MS. However, GC-MS/MS and GC-ITMS are very expensive, which leads to less usage (Srivastava et al., 2011). The latest progress in the MRMs associated with GC and MS/MS has been the development of an analysis that replaces traditional GC detectors. Nonetheless, because of inadequate sensitivity for a few compounds, traditional GC detectors are still in use. The need for clean-up has been decreased or eliminated. The method has been simplified, making it possible to recover all analytes in many different matrices via a single extraction and to detect them with either GC-MS/MS or LC-MS/MS.

The advanced approach, associating GC with triple quadrupole MS/MS detection, has additionally simplified the system by replacing regular GC detectors that required numerous injections of each sample extract. The necessity for clean-up has lessened. Moreover, the LOQ for many compounds has lessened. Currently, several publications regarding the analysis of pesticide residuals using GC and LC with MS detection have been published (Alder et al., 2006; Al Mahmud et al., 2013; D'Antuono et al., 2009; Ferrer et al., 2005; Hernández et al., 2006; Kim et al., 2016a, b; Leandro et al., 2006; Na et al., 2012; Pan et al., 2008; Park et al., 2012; Rahman et al., 2015; Yang et al., 2012). Pihlström et al. (2007) presented a novel MRM for determining pesticide residue in fruit and vegetable samples using ethyl acetate extraction and determination by means of LC-MS/MS or GC-MS/MS. The technique was approved for 309 analytes, of which 187 can be detected by LC-MS/MS adopting positive and negative modes, and 122 by GC-MS/MS, in which recoveries are being studied at two non-identical concentration levels with four matrices.

6. DISSIPATION PATTERN

By definition, the observed rate of pesticide residual disappearance is a first-order kinetic reaction (Chang et al., 2011). Therefore, the reaction velocity is linearly proportional to the

concentration of the reactant. Hence,

$$\mathbf{C_t = C_0 e^{-kt}}$$

In addition, the dynamic pattern was well described by first-order kinetics by Rahman et al. (2015),

$\ln C_t = \ln C_o - kt$,

where

C_t = Residual concentration at time t,

C_0 = Residual concentration at time $t = 0$, and

k = Dissipation constant reflecting the degradation potential of the pesticide (Putnam, Nelson & Clark, 2003).

The biological half-life (i.e., the time needed for the pesticide concentration to decline to half of its initial concentration) was computed as $\ln 2/k = 0.693/k$ (Putnam et al., 2003). The dynamics are illustrated in Fig. 2 (Farha et al., 2015a, b, Rahman et al., 2015).

Pesticide endurance was described by its half-life ($t_{1/2}$), which was determined as 0.693/k, where the residual levels were observed to be high compared to the MRL (10 mg/kg) (Farha et al., 2015b; MFDS, 2013). Regarding humidity, corresponding variation was observed between various sites (Fig. 2). The results were in agreement with the studies by Garau et al. (2002) and Fantke and Juraske (2013). However, the primary concentrations of mandipropamid in and on *Perilla* leaves were higher than the MRL (25 mg/kg) (Farha et al., 2015a; MFDS, 2014). Although the decay in various sites was comparable, the differences in temperature between the sites were proposed as a possible reason for the results (residual reduction rate) (Fig. 2) (Farha et al., 2015a).

A study by Khay et al. (2008) showed that, the mean residual level of lufenuron was between 2.72 ± 0.4 and 4.4 ± 0.1 ppm for the recommended and double the recommended application doses, respectively. The residual dissipation rate was 39.7% and 28.4% after one

day, exhibiting residues of 1.64 ± 0.0 and 3.15 ± 0.4 ppm, respectively. The observed residue concentrations were greater than the MRL (KFDA, 2005; Khay et al., 2008). The residual data for the lufenuron dissipation pattern is shown in Fig. 3. Accordingly, the degradation of lufenuron residues was regarded as a monophasic first-order kinetic reaction representing dissipation differing during the 10-day periods with half-lives of 4.6 and 5.8 days, successively, demonstrating minimal loss of lufenuron for the Chinese cabbage sample. Greenhouses provide high temperatures throughout the summer, which plays a major role in this process.

7. PRE-HARVEST RESIDUE LIMIT

PHRLs have been established by the National Agricultural Products Quality Management Service (Gimcheon, Republic of Korea) and incorporated with pesticide MRLs to prevent the distribution of agricultural products that exceed the MRLs (Chang et al., 2011). The PHRL uses an estimate of the pesticide residual quantity applied during the pre-harvest period and determines the pesticide residual quantity at the time of harvest by calculating the biological half-life and dissipation constant (Al Mahmud et al., 2013; Chang et al., 2011; Chung et al., 2017; Kabir et al., 2016a, b; Kim et al., 2016a; Farha et al., 2015a, b; Namgung et al., 2014; Park et al., 2013; Rahman et al., 2013a; 2015; 2016; Troung et al., 2016). The PHRL was computed by an automatic computational method using a statistical procedure in SPSS (SPSS, 2010). The automated system calculates the decay constant required in estimating the PHRL. Because of the upper and lower bounds of the regression coefficient ($-k$) at 95% confidence level, the aforementioned method was used for calculating the decay constant with the help of the upper bound of the agricultural products (Park et al., 2013).

The PHRL was determined by:

$$PHRL = MRL \times e^{k_{min} t}$$

Where,

MRL = Maximum residue limit,

k_{min} = Minimum dissipation constant [automatically calculated in the SPSS program (SPSS, 2010)], and

t = Time before harvest.

The dissipation constant is a proportional variable with respect to the decay of the pesticide residual quantity existing in agricultural production after application (Rahman et al., 2015). Usually, pesticides are not recommended for specified crops unless an analysis of the changes observed for the pre-harvest intervals is conducted to prevent violating the MRL.

8. RISK ASSESSMENT

The increase in pesticide use in crops and plants has become a global constant in the agriculture world. The appearance of pesticide resistant pathogen races is a consequence, and it can cause significant food safety risks for consumers (Wołejko et al., 2016). Accordingly, the risk assessment for carbocyamides and strobilurin ranged from 0.4 to 64.8 % on day 1, but after 14 days, it ranged from 0.0 to 20.9 % for children and adults, respectively(Wołejko et al., 2016). Exposure to any pesticides or their association with yeast and effective microorganisms did not indicate any risk of detrimental effects (Wołejko et al., 2016). As shown by Wołejko et al. (2016), consumer exposure to pesticides did not exceed the acceptable daily intake (ADI) value. Carboxamide and strobilurin have been reported to exhibit deleterious effects on the skin, eyes, and respiratory tract, whereas no evidence was found regarding mutagenic capability or impacts on the endocrine system, with the smallest ranges of ADI equal to 0.04 and 0.03 mg kg^{-1}, respectively.

Globally, leafy vegetables constitute the majority of vegetable production. Many people enjoy consuming vegetables raw more than in the cooked state. Washing does not completely

remove pesticide residue from leafy vegetables. Therefore, consuming leafy vegetables raw with large amounts of pesticide residue is unsafe (Park et al., 2016). Park et al. (2016) studied 31 types of pesticides exceeding MRLs via exposure evaluation. Using the estimated daily intake (EDI) and ADI, a risk assessment was conducted (Park et al., 2016). ADI is defined as the criterion for risk assessment depending on the chronic toxicity levels of the pesticide for humans (Jeong, Lim & Cho, 2012). Generally, EDI is determined by the average intake/person/day and from the residual data for the pesticide (Lee, 2010). The calculated results are available in Park et al. (2016). EDIs ranged from 2.0×10^{-5} to 7.7×10^{-2}. The EDI:ADI ratios ranged from 0.003 to 30.4%. The winter-grown cabbage specimens had EPN ratios of 30.4%, the highest among the total 230 tested pesticides. The estimated daily intake can be calculated by:

Estimated daily intake (EDI) = Residue amount × average uptake (Kabir et al., 2016a; Lee et al., 2009)

The ADI% was calculated as EDI/ADI × 100 (Kabir et al., 2016a; Lee et al., 2009). Foodstuffs with EDI: ADI ratios greater than 100% are considered potentially unsafe for consumption (Chun & Kang, 2003). Hence, the above studies indicated that pesticides exceeding the MRLs were not detrimental to consumer health. Pesticide residues in produce must be managed since the elderly and those with compromised immune systems could be severely affected (Park et al., 2016).

9. CONCLUDING REMARKS AND FUTURE PERSPECTIVES

Pesticides have contributed immensely to agricultural worldwide, enhancing not only the agro-economic field, but also the human diet. Leafy vegetables comprise a major portion of our dietary system and can be consumed fresh or raw, thus it is necessary to ensure their safety for consumption. The determination of residuals in leafy vegetable juices and

advancements in processing are encouraging. To date, the leaf matrix can effectively deactivate active sites inside the GC system and aid problematic compounds in travel from the injector to the detector via the capillary column without any undesired interaction; thus, acting as a natural analyte protectant. Because of the potential risks, leafy vegetable analysis must be conducted using suitable analytical methodologies. Greener analytical approaches for determining impurities in the complex matrices, such as plant material, included higher efficiency, minimized clean-up, concentrated steps, fewer toxic solvents, and upgraded exposure limits all represent substantial improvements. High throughput techniques for screening a large number of LC-amenable pesticides in leafy vegetables with greater reproducibility and lower uncertainty are necessary for quantifying residues at very low concentrations. Further research and development efforts are needed to prove the applicability of immunoassay- and biosensor-based techniques in routine monitoring of pesticide residues in leafy vegetables. Care must be paid to metabolites transformed from the parent compound in plants, which might pose a health problem to consumer. Analytical method should be able to detect and quantify the parent as well as the metabolites, to fulfill the criteria established by regulatory authorities for residue definition.

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Table 1. Pesticides and their efficacy in the control of pests on leafy vegetables.

Sample-Matrix	Pesticides	Efficacy[a]	MRL (mg/kg^{-1})[b]	References
lettuce	Acetamiprid	Insecticide	0.7	Ford et al., 2011; Im et al., 2015; Ko et al., 2014; Namgung et al., 2014; Park et al., 2016; Uneme, 2010; Wołejko et al., 2016
	Azoxystrobin	Fungicide	3.0	
	Bifenthrin	Insecticide, Acaricide	-	
	Boscalid	Fungicide	40	
	Chlorothalonil	Fungicide	-	
	Cyazofamid	Fungicide	-	
	Cypermethrin	Insecticide	0.7	
	Dimethomorph	Fungicide	10	
	Diniconazole	Fungicide	-	
	Endosulfan	Insecticide, Acaricide	-	
	Ethaboxam	Fungicide	-	
	Fenpyroximate	Acaricide	-	
	Fluazinam	Fungicide	-	
	Fludioxonil	Fungicide	40	
	Flufenoxuron	Insecticide, Acaricide	-	
	Fluquinconazole	Fungicide	-	
	Imidacloprid	Insecticide	-	
	Iprodione	Fungicide	25	
	Kresoxim-methyl	Fungicide	-	
	Lufenuron	Insecticide, Acaricide	-	
	Mepanipyrim	Fungicide	-	
	Methoxyfenozide	Insecticide	30	
	Procymidone	Fungicide	-	

	Pyraclostrobin	Fungicide	-	
	Teflubenzuron	Insecticide	-	
	Thiamethoxam	Insecticide	3.0	
	Tiadinil	Fungicide	-	
	Tricyclazole	Fungicide	-	
Spinach	Azoxystrobin	Fungicide	3.0	Dogheim, Ashraf, Alla, Khorshid & Fahmy, 2004; Park et al., 2016
	Bifenthrin	Insecticide, Acaricide	-	
	Boscalid	Fungicide	40	
	Carbofuran	Insecticide, Acaricide, Nematicide	-	
	Chlorantraniliprole	Insecticide	20	
	Chlorfenapyr	Insecticide	-	
	Chlorothalonil	Fungicide	-	
	Cinosulfuron	Herbicide	-	
	Cyazofamid	Fungicide	-	
	Dimethoate	Insecticide, Acaricide	-	
	Dimethomorph	Fungicide	30	
	Ethaboxam	Fungicide	-	
	Fenpropathrin	Insecticide, Acaricide	-	
	Flufenoxuron	Insecticide, Acaricide	-	
	Fluquinconazole	Fungicide	-	
	Fenpyroximate	Acaricide	-	
	Lufenuron	Insecticide, Acaricide	-	

	Methoxyfenozide	Insecticide	-	
	Pendimethalin	Herbicide	-	
	Procymidone	Fungicide	-	
	Profenofos	Insecticide	-	
	Tebufenozide	Insecticide	10	
	Teflubenzuron	Insecticide	-	
Perilla leaves	Acetamiprid	Insecticide	0.7	Farha et al., 2015a, b; Park et al., 2016
	Azoxystrobin	Fungicide	3.0	
	Amisulbrom	Fungicide	-	
	Bifenthrin	Insecticide, Acaricide	-	
	Boscalid	Fungicide	40	
	Chlorothalonil	Fungicide	-	
	Chlorpyrifos	Insecticide	-	
	Cyazofamid	Fungicide	-	
	Cypermethrin	Insecticide	0.7	
	Diethofencarb	Fungicide	-	
	Dimethomorph	Fungicide	-	
	Diniconazole	Fungicide	-	
	Ethaboxam	Fungicide	-	
	Fenpyroximate	Acaricide	-	
	Flubendiamide	Insecticide	-	
	Fludioxonil	Fungicide	-	
	Flufenoxuron	Insecticide, Acaricide	-	
	Indoxacarb	Insecticide	-	
	Lufenuron	Insecticide, Acaricide	-	
	Mandipropamid	Fungicide	25	

	Methoxyfenozide	Insecticide	-	
	Procymidone	Fungicide	-	
	Pyraclostrobin	Fungicide	-	
	Pyrimethanil	Fungicide	-	
	Trifloxystrobin	Fungicide	-	
Crown daisy	Azoxystrobin	Fungicide	3.0	Kim et al., 2016; Park et al., 2016
	Boscalid	Fungicide	40	
	Carbofuran	Insecticide, Acaricide, Nematicide	-	
	Chlorothalonil	Fungicide	-	
	Chlorpyrifos	Insecticide	-	
	Diazinon	Insecticide, Acaricide	-	
	Diethofencarb	Fungicide	30	
	Dimethomorph	Fungicide	-	
	Diniconazole	Fungicide	-	
	Endosulfan	Insecticide, Acaricide	-	
	Flufenoxuron	Insecticide, Acaricide	-	
	Methidathion	Insecticide, Acaricide	-	
	Procymidone	Fungicide	-	
	Pyraclostrobin	Fungicide	-	
	Pyrimethanil	Fungicide	-	
	Thiacloprid	Insecticide	-	
	Thiamethoxam	Insecticide	3.0	
Kale	Azoxystrobin	Fungicide	3.0	Park et al., 2016

	Boscalid	Fungicide	40	
	Diazinon	Insecticide, Acaricide	0.05	
Leek, cabbage	Nonylphenol	Alkylphenols	-	Dogheim et al., 2004; Fang et al., 2015; Rahman et al., 2012
	Profenofos	Insecticide	-	
	Chlorfenapyr	Insecticide	-	
Celery, Green parsley	Malathion	Insecticide	-	Dogheim et al., 2004

a: (source) Pesticide Manual, 15th edition (Tomlin, 2009)

b: all MRL data were obtained from the CODEX index (Codex Alimentarius, 2012)

Table 2. Influence of solvent extraction on pesticide recovery in spinach samples (Tanaka et al., 2007).

Pesticide	Analytical recovery (%)[a]		
	Cyclohexane	**Dichloromethane**	**Ethyl acetate**
Chlorpyrifos-methyl	55	62	85
Pirimiphos-methyl	< 5	< 5	70
Malathion	6	61	84
Chlorpyrifos	44	67	84
Thiobencarb	27	68	86
Isoprothiolane	16	58	92
EPN	49	87	117
Permethrins	53	106	114

Pesticides (2 µg each) were spiked to 2 g of vegetable.

a = Value after one extraction at 100°C for 5 min (n = 3).

Table 3. Overview of the recognized multi-residue methods (MRM) for the analysis of pesticides in leafy vegetables.

Number of analytes	Matrix	Number of Pesticides applied	Sample treatment	Determination technique	Reference
8496 samples (26 kinds, 6782 samples)	Leafy vegetables	230	**SLE:** 50 g sample + 100 mL acetonitrile + 15 g NaCl **GC Analysis:** evaporation to dryness and dissolution in 4 mL hexane:acetone (8:2 v/v) SPE: -Florisil cartridge, -Eluted with 5 mL Hx:Ac (8:2) -Dissolvation in 2 mL Acetone **LC Analysis:** evaporation to dryness and dissolution in 4 mL DCM:methanol (99:1 v/v) SPE: -NH_2 cartridge, -Eluted with 7 mL DCM:methanol (99:1 v/v) -Dissolvation in 2 mL Methanol	GC-NPD, GC-ECD, GC-MS; LC-MS/MS	Park et al., 2016
3 samples (3 kinds, total 75 samples)	Leafy vegetables (lettuce, Swiss chard and spinach)	23 (12 insecticides, 12 fungicide	-extracted with acetonitrile -cleanup by (GCB/PSA) - SPE cartridge:	GC-ion trap mass spectrometry	González-Rodríguez et al. 2008

		s)	acetonitrile:toluene (3:1, v/v) - re-dissolved with 0.5 mL of acetone		
835 samples	Leafy vegetables (Cabbage, celery, gape leaf, coriander, dill, mint, parsley, lettuce, molokia, spinach and watercress. A)	26 (organophosphorous and organonitrogen pesticide residues)	The AOAC Official Methods of Analysis (1995) was followed with some modifications.	GC	Dogheim et al., 2004
2 kinds of samples	Leafy vegetables	1 (Spinetoram)	-extracted with 450 mL H_2O, 50 mL sat. NaCl sol., CH_2Cl_2 -NH_2 cartridge: -5 mL loaded, -Eluted with 10 mL *n*-hexane:ethylacetate (8:2, v/v) - dissolved with 1.0 mL of acetonitrile-methanol (450:450:100, v/v/v)	LC-tandem mass spectrometry	Liu et al., 2011
-	Green Leafy vegetables	129	-extracted with acetonitrile -cleanup by (GCB/PSA) - SPE cartridge	GC-MS/MS	Walorczyk, 2008
-	Amaranth, Parsley	1 (Spinetoram and metabolites)	-QuEChERS (Minor modification) -10 g sample + 50 mL MeCN -(6 g $MgSO_4$ + 1.5 g NaCl), 1 min vigorous shaking	LC-ESI-MS/MS	Park et al., 2012

			-1.5 mL upper layer (0.15 g $MgSO_4$ + 0.05 g PSA, 1 min vigorous shaking) -Centrifugation		
-	Amaranth, Crown daisy	5 (Clothianidin and 4 metabolites)	-QuEChERS (Minor modification) -10 g (±0.1 g) sample + 20 mL MeCN -(6 g $MgSO_4$ + 1.5 g NaCl), 1 min vigorous shaking -15 mL (crown daisy)/10 mL (amaranth) upper layer (1.5 g $MgSO_4$ + 0.05 g PSA, 1 min vigorous shaking) -Centrifugation -Reconstitution: 2 mL [mixture of 1% aq. acetic acid solution and methanol (70:30, v/v)]	LC- MS/MS	Kim et al., 2012
150 samples (75 spinach, 75 lettuce)	Spinach, Lettuce	Nitrates, and nitrites	-4 g homogenized sample + 200 mL ultrapure water (placed at 70 ◦ C for 5 min) -Cooling, filtration -3 mL of the filtrate purified using Alumina Neutral Cartridges, previously activated by 3 mL ultrapure water -The purified extract was filtered (Anotop 10 LC, 0.2 µm, 10 mm filters) prior to chromatographic analysis.	Ion chromatography (suppressed conductivity detection method)	Iammarino, Di Taranto & Cristino, 2014

-	Crown daisy	Diethofencarb	-QuEChERS (Minor modification) -10 g (±0.1 g) sample + 20 mL MeCN -(4 g $MgSO_4$ + 1 g NaCl), 1 min vigorous shaking -6 mL upper layer (500 mg anhydrous $MgSO_4$ + 100 mg C_{18} +100 mg PSA, 1 min vigorous shaking) -Centrifugation -1.5 mL of extracted & clean sample solution collected prior to analysis	LC- MS/MS	Kim et al., 2016a
-	Cabbage	18 preservatives	-10 g (±0.1 g) sample + 20 mL hexane:ethyl acetate (1:2, v/v) + 4 mL 2 mol/L ammonium acetate solution -extracted with oscillator -5 g (±0.1 g) NaCl, 1 min vortexed -10 mL of the supernatant (500 mg anhydrous $MgSO_4$ + 100 mg PSA, 2 min vortexed) -Centrifugation -Supernatant was concentrated & dissolved with 1 mL MeCN-3 mmol/L ammonium acetate (1:1, v/v) -Content filtered through 0.22 µm nylon membrane filter prior to analysis	UHPLC–QTRAP	Zhou et al., 2015

3 replicates, 20 samples	Leafy vegetables	Biomethane production	-Tested in reaction bottles (total volume = 500 mL) - batch feeding set to 5 g/L, the feedstock to inoculum ratio was 1 -O_2 was discharged from the digesters by filling with N_2 gas, & then placed in an incubator at 37°C for 25 days -Bottles shaken; (manually) 1 min twice a day - Methane content analyzed by kinetic analysis	GC (thermal conductivity detector, He carrier gas)	Yan et al., 2017
3 replicates, each vegetable samples	Lettuce, Spinach	20 Organochlorine pesticides	-100.0 mg aliquots of freeze-dried samples, spiked with OCPs standards in acetone to the desirable concentration - Pre-extracted with 2 mL of MeOH by sonication (twice) - supernatants diluted to 30 mL with ultra-pure water, stir bars were placed -Analytes desorbed by sonication -Concentrated & re-dissolved with 120 µL hexane, prior to LVI-GC-MS analysis	GC-MS(SIM)	Barriada-Pereira, Serôdio, González-Castro & Nogueira, 2010

3 replicates, 2 different fortification concentrations	Perilla leaves	Abamectin (Abamectin B1a and B1b)	-QuEChERS (Minor modification) -10 g (±0.1 g) sample + 30 mL MeCN -6 g $MgSO_4$, 30 s vigorous shaking -15 mL upper layer evaporated to dryness, dissolved in 5% acetone in n-hexane SPE: -Silica cartridge, -Washing with 10 mL 20% acetone in n-hexane -Eluting with 10 mL 30% acetone in n-hexane -Evaporated to dryness -Reconstitution in 3 mL MeCN & 1 mL ethyl acetate	HPLC (Fluorescence detector)	Rahman et al., 2013

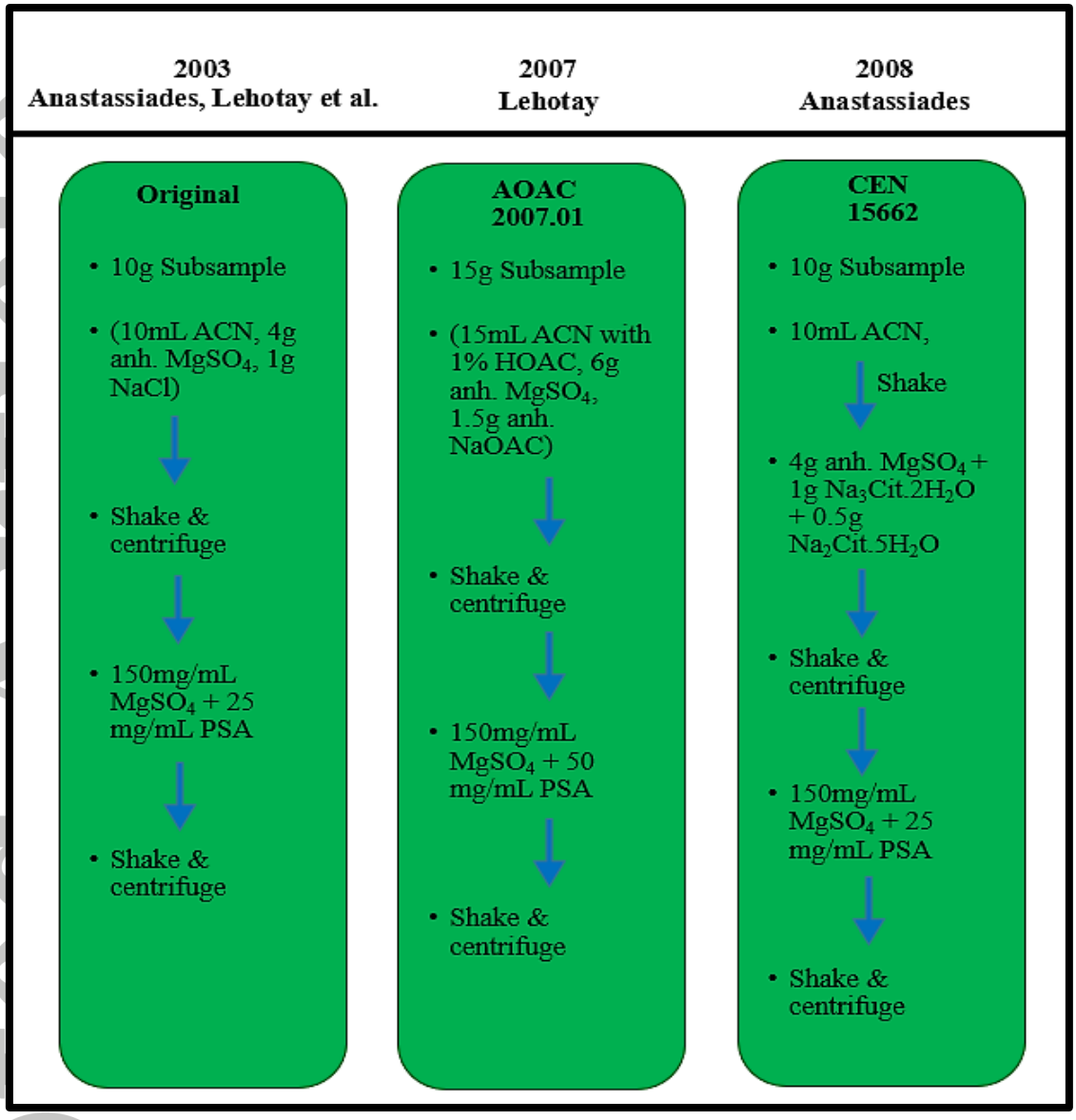

Fig. 1. Three different versions of the QuEChERS method (Anastassiades et al., 2003; Anastassiades, 2007; Lehotay et al., 2007).

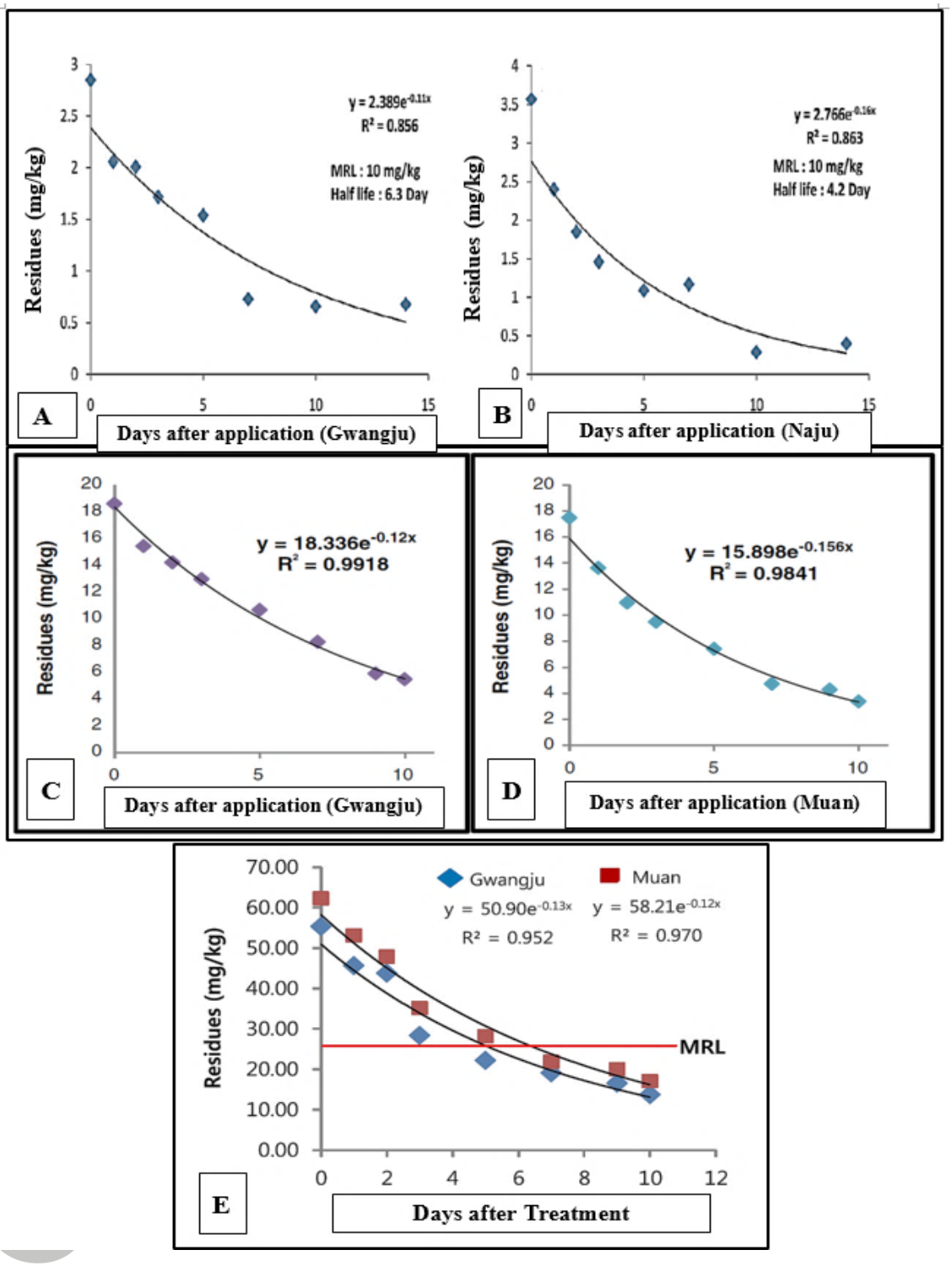

Fig. 2. Degradation curve of total thiamethoxam (TMX + CLO) in Swiss chard grown under greenhouse conditions at Gwangju (A) and Naju (B); and of pyraclostrobin (C, D) and mandipropamid (E) for *Perilla* leaf samples grown in the Gwangju and Muan areas during the experimental period (Farha et al., 2015a,b; Rahman et al., 2015).

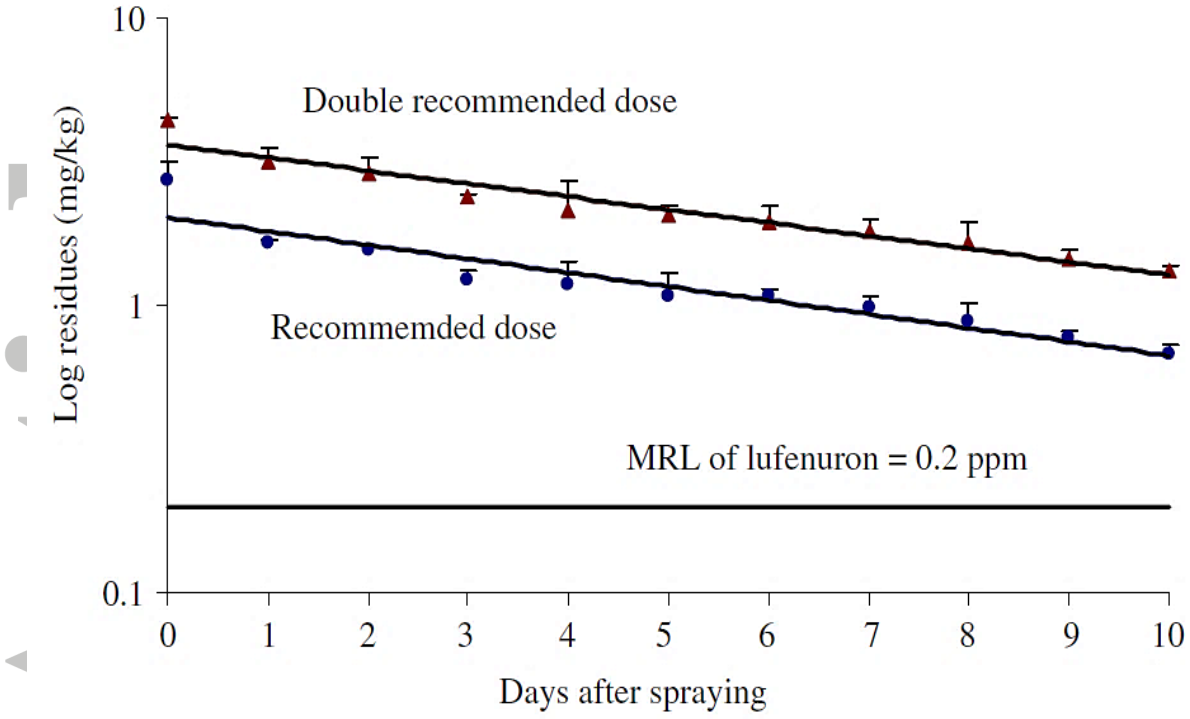

Fig. 3. Decline in lufenuron residue over time following its application to cabbage (Khay et al., 2008).