Evaluating the Effectiveness of Different Household Washing Techniques for Removal of Insecticides from Spinach and Chickpea Leaves by Micellar Liquid Chromatography

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Abstract

In the past few decades, the employment of green analytical approaches in chromatographic method development has attracted the analytical community. The greenness of the developed method depends upon the toxicity of solvents and the amount of generated post-analysis waste. In this concern, micellar liquid chromatography (MLC) is a simple and rapid technique that generates very low toxic waste compared to traditional chromatographic pesticide detection methods. Here, MLC method has been validated and applied for the determination of monochrotophos (MCF), imidacloprid (ICP), dimethoate (DM) and profenofos (PFF) in spinach and chickpea leaves. The optimized mobile phase in this method was 0.065 M SDS-2% 1-propanol, 0.01 M NaH_2PO_4 buffered to pH 7. A C_{18} column was used to run the mobile phase at 1 mL/min. The developed method has been validated following the guidelines of SANTE/11,312/2021 and ICH guidelines of; limit of quantification (0.05-0.20 mg/kg), linearity (r^2> 0.997-0.999), precision (<6.3%), accuracy (96.3%–99.8%) and robustness (<6) in real samples. ICP and MCF, apart from DM and PFF, were detected here. After detecting insecticides in spinach and chickpea leaves both were washed with different household chemicals i.e. normal, lukewarm, common salt, lemon juice water and commercial ozonizer. Based on five different washing techniques with time intervals reduction rates were calculated for each washing treatment. The results show that acidic wash, basic wash, and ozonizer can be used as washing techniques for the reduction of superficial and systematic residues of ICP and MCF. But common salt and lemon water have the benefit over vinegar and $KMnO_4$ in the aspect that it will result in a bright colour on the green leafy vegetables after the treatment and are available in every kitchen and also have excess to lower socioeconomic classes which could not afford potassium permanganate and vinegar.

Keywords: Washing, micellar liquid chromatography, residue, insecticide

1. Introduction

In our daily diet, vegetables are included as "protective foods" because they have various health benefits and are rich in vitamins, amino acids, dietary fiber, essential fatty acids, minerals, and many other essential bioactive compounds [1]. Vegetables also have health-promoting constituents like antioxidants and phenolic compounds which are secondary metabolites of plants [2]. The World Health Organization (WHO) recommends that at least 400g of fruits and vegetables should be consumed to meet our recommended daily allowance (RDA) of nutrition [3,4], whereas the American Dietary Guidelines recommends daily five servings of vegetables equivalent to 2,000 calories of daily intake [5]. Globally, crop diversity and the nutritional value of vegetables are particularly important for improving food and its nutrition [4].

Spinach *(Spinacia oleracea* L.*)* is one of the most popular green leafy vegetable (GLV) available worldwide and is commonly known as "paalak" in India [6]. It is either consumed in raw form as salad or cooked into different delicacies. It is widely known as a functional food due to its nutritional value. Apart from this, spinach has other beneficial properties and is considered to be an anti-cancer, anti-obesity, hypoglycemic, hypolipidemic agent, etc. [7]. After common beans *(Phaseolus vulgaris* L.*)* and peas *(Pisum sativum* L.*),* chickpea *(Cicer arietinum* L.*)* is the third most important winter food legume. Based on global production estimates it is mainly grown in India, North Africa, the Middle East, Southern Europe, Asia, America, and Australia [8,9]. Besides the seed of chickpea, the young chickpea leaves locally known as "chanabhaaji" is also consumed in cooked form as GLVs in India and Nepal. Fresh chickpea leaves are considered a good source of minerals [9].

These leafy vegetables are also consumed in cooked or half-cooked forms [11]. Like any other GLVs, these are also prone to attack by pests, insects, weeds, mites, nematodes, etc. which

results in low quality and quantity of yield [12]. To get rid of these pests use of pesticides in agriculture is common and is practiced by the majority of farmers to obtain high-quality yields [13]. Almost all vegetables contain traces of pesticides; when consumed, they reach our bodies, which may cause severe chronic health issues such as neurological, reproductive, respiratory, cardiac, and carcinogenic [14,15].

Due to growing awareness of the harmful effects of pesticides/insecticides, developed and developing countries have fixed the maximum residue limit (MRL) for pesticide application on crops which is generally the legally tolerable level of pesticide in food that comes under good agricultural practices [16]. These good agricultural practices are governed by several pesticide regulatory bodies such as the United States Environmental Protection Agency (USEPA), United States Food and Drug Administration (USFDA), European Union (EU), European Commission (EC), Codex Alimentarius Commission (Codex), etc. Food is considered safe and of good quality when the pesticide residue level is within MRL [17,18]. But in the present day scenario, many developing and under-developed countries are using highly toxic pesticides in agriculture to attain high yields from crop of better quality which include monocrotophos (MCF), dimethoate (DM), imidacloprid (ICP), carbendazim (CBZ), cypermethrin (CYPER), profenofos (PFF), chlorpyrifos (CP) etc. MCF has been banned for use in vegetables in India by the Ministry of Agriculture since 10th October 2005 [19] but is still being used whereas CP is also prohibited by the Environmental Protection Agency (2018) and used in the unabated form. Research on GLVs, fruits, and plants has shown that ICP, MCF, CP and DM penetrate plant, fruit, and vegetable tissues and have residual activity. ICP also exhibits translaminar movement in plants and can penetrate the leaf cuticles. On the other hand, no residual studies have been found for PFF.

To get rid of residual pesticides washing is the most important step [20]. In India, GLVs are generally washed with normal tap water due to which the pesticide residues are not completely removed. In this work to study the effectiveness of washing for the removal of insecticides, two GLVs were selected i.e., spinach and chickpea leaves. Spinach is the most consumed GLV in the world and always remains at a risky level for pesticide treatment due to its higher water content. Chickpea leaves are consumed mainly in rural areas of Central and North India.

In this work, the effectiveness of household washing techniques was employed for the determination of the most commonly used insecticide i.e., MCF, ICP, DM and PFF residues in two GLV, i.e. chickpea leaves and spinach leaves which were collected from the local market of Sagar, Madhya Pradesh (India). Studies on the washing of GLVs using conventional chromatographic techniques have been reported by researchers for about a decade [21–25]. The conventional chromatographic methods are very sensitive, efficient and accurate but they are not counted under green techniques due to the use of toxic solvents like methanol and acetonitrile [26]. Additionally, they are expensive and time-consuming due to the number of analytical steps involved [27].

In the present research work MCF, ICP, DM and PFF were simultaneously separated using micellar liquid chromatography (MLC) with a photodiode array detector (PDA). The MLC technique used here is a green chromatographic technique because the mobile phase used in this technique is an aqueous solution of the micellar phase. Besides its greenness, it is less expensive and less time-consuming due to the direct injection of samples without any sample pretreatment other than filtration. In the present study, two GLVs (spinach and chickpea leaves) were tested for the presence of insecticide residues of MCF, ICP, DM and PFF respectively, after washing with different easy washing methods. Five washing methods were applied on spinach and chickpea leaves i.e., normal water washing (NW), lukewarm water washing (LW), lemon juice water

washing (LJW), common salt water washing (CSW) and commercially available fruit-vegetable ozonizer with normal water (OW). After washing, post-wash water and washed vegetable samples were examined individually for the presence of selected insecticide.

2. Material and method

2.1. Chemicals and instrumentation

The analytical standard 98–99% pure MCF, ICP, DM, and PFF insecticide standard samples were purchased from Sigma-Aldrich, Mumbai (India). To prepare the micellar mobile phase and to maintain the pH of the mobile phase, 99% pure sodium dodecyl sulfate (SDS) and analytical grade sodium dihydrogen phosphate ($Na_2HPO_4.H_2O$) buffer were obtained from Himedia Laboratories Pvt. Ltd., Mumbai (India). HPLC grade solvents 1-propanol, 1-butanol, 1-pentanol and methanol, ≈37% hydrochloric acid (HCl) and >99.0% pure sodium hydroxide (NaOH) for pH balance of the mobile phase were purchased from Rankem, RFCL, New Delhi (India). For the preparation of solutions including mobile phase and analytical samples, HPLC grade water was obtained from Indion Lab-Q Ultra water purification system, Ion Exchange (India) Ltd., Mumbai (India). 0.45 µm nylon membrane filters used for the filtration of solutions were from Micron Separation Inc., Westboro, MA (USA).

Solubilizing of standard analytes was accomplished by ultrasonic bath from Ultrasons-H, Selecta, Barcelona (Spain). An analytical weighing scale was used from Mettler Toledo India Pvt. Ltd., Mumbai (India) to weigh the chemicals and real samples. The pH of the micellar mobile phase was measured using a digital pH meter used was from pH-102/103 Contech, Instruments Ltd., Mumbai (India). The HPLC used was a Shimadzu Corporation (Shimadzu Prominence), Kyoto (Japan) with an isocratic pump LC-20 AT, auto-sampler SIL-20AC, a photodiode array

detector SPD-M20A, 190-800 nm, and a Shimadzu C_{18} analytical column, dimension of 250 mm length × 4.6 mm internal diameter with 5 µm particle size.

2.2. Chromatograph and chromatographic conditions for analysis

For the analysis of selected insecticides, the micellar liquid chromatographic method was used. The HPLC was used with a micellar mobile phase containing 0.065 M SDS-2% 1-propanol and 0.01 M NaH_2PO_4 buffered to pH 7. The sample injection volume was kept at 20 µL. The aqueous micellar mobile phase flow rate was set at 1 mL/min. under an isocratic mode. The software Shimadzu LC Solution software version 1.22 SP1 operated and controlled the chromatographic system. The tables, charts, chromatograms, and diagrams were prepared using MS Excel and Origin Pro 2018 software.

2.3. Standard stock solution preparation

Standard stock solutions of 100 ppm for MCF, ICP, DM and PFF were prepared by weighing 1 mg standard accurately and transferred into 10 mL volumetric flasks. To achieve 100 ppm concentration 10 mL of ultrapure Type-I grade water and methanol were added, respectively. The solutions were ultra-sonicated for 10 min. with an ultrasonic water bath to dissolve standards completely. Then prepared stock solutions were kept under the refrigerator until used.

2.4. Real sample collection and processing

The spinach and chickpea samples were collected from the local vegetable market of Sagar city, Madhya Pradesh (India) following the same strategy as described by Bhamdare et al., 2022 with minor modifications [28]. Only one sample of spinach and chickpea was collected from all four markets consisting of eight samples numbered I-IV for spinach and V-VIII for chickpea. These vegetables were selected because they are the main leafy vegetables consumed in this region

from September to November and pesticide/insecticide treatment is frequent (around 4-5 cycles per harvesting). The collected samples were packed in inert sterile polyethylene bags to prevent further contamination and immediately transferred to the laboratory. Healthy leaves were selected for the best analytical results and the samples were sorted manually with hands by wearing hand gloves. The samples were then prepared for further analysis in the laboratory. The blank samples for all the examined GLVs were grown in the university horticulture garden without any insecticide treatment. Spinach and chickpeas were grown in the University garden through organic farming without pesticides to serve as a control sample.

2.5. Spiked sample preparation for optimization of time and concentration of washing solutions

500 g of fresh hand-sorted blank chickpea and spinach leaves were taken separately and sprayed with 10 ppm of selected insecticides. After spraying, they were left for 1 hour for complete absorbance of insecticide by the leaves. After 1 hour, chickpea and spinach leaves were divided into 50 parts (each part has 10 g). All fifty vegetable samples of spinach and chickpea leaves were washed by dipping and rinsing in five different types of water i.e., NW (100 mL), LW (100 mL) between 37ºC to 40ºC, LJW (100 mL by adding 1 mL lemon juice), CSW (100 mL by adding 1 g common salt) and ozonizer (using 100 mL normal water), and for different period of time i.e, 2, 4, 6, 8 and 10 min respectively. After fixing the washing time, the next step of this analysis was to wash the chickpea and spinach samples in different concentrations of LJW and CSW. After washing, post-wash water remained after all five methods were collected in 100 mL amber glass volumetric flasks separately, labeled, and refrigerated immediately at 4ºC until the chromatographic analysis. Post-wash vegetable samples were also collected after washing.

184 **2.6. Real sample preparation of spinach and chickpea leaves**

185 50 g of fresh hand-sorted chickpea and spinach leaves were taken separately and divided
186 into five parts (each part 10 g). Then, all real samples were prepared following the same strategy
187 as spiked sample preparation.

188 **3. Result and discussion**

189 **3.1. Selection of chromatographic conditions**

190 The analysis in MLC generally starts with the selection of suitable pH. The selection of
191 optimum pH depends on the pKa of the analyte which decides its ionization state. In the present
192 study the pKa for MCF (pKa=4.4), ICP (pKa= 1.6), DM (pKa=NA) and PFF (pKa=1.6) which are
193 ionizable compounds. It is well-established that C18 columns have a working pH range between
194 2.5 and 7.5. Therefore, the pH range for the present study was also set between 3 and 7. During
195 the study, it was found that the neutral form of all the analytes was predominant in the selected pH
196 range which was based on the retention behaviour. MLC conducted using 0.1 M SDS buffered to
197 pH 3, 5, and 7 showed minor variations in the retention time (Rt) of the analyte. Because neutral
198 pH 7 was good for the life of the column, therefore pH 7 was finally selected as the optimum pH
199 for further chromatographic analysis.

200 The hydrophobicity and hydrophilicity of selected analytes were determined based on the
201 octanol-water partition coefficient (log $P_{o/w}$) for MCF; -0.2, ICP; 0.57, DM: 0.78 and PFF; 4.6
202 indicating that MCF is hydrophilic while ICP, DM are slightly hydrophobic and PFF is
203 hydrophobic in nature. The study conducted by Dubey et al., 2014 shows that using a C_{18} column
204 and pure sodium dodecyl sulfate results in broad peaks and poor resolution [29]. To overcome this

problem and improve the chromatographic parameter like efficiency (N) a small amount of short-chain alcohols (1-propanol, 1-butanol, and 1-pentanol) could be added to the mobile phase.

The hybrid mobile phases consisting of 0.05 M SDS- 2% 1-propanol, 0.1 M SDS-2% 1-propanol, and 0.15 M SDS-2% 1-propanol at pH 7 were tested. When 2% 1-propanol was added to 0.15 M SDS, the Rt of the analytes decreased rapidly and the compound eluted in the dead time. On the other hand, a higher amount of 1-propanol was needed to decrease the Rt in the case of 0.05 M SDS. In search of an optimum mobile phase for simultaneous analysis of selected insecticides, 0.065 M SDS with 2% 1-propanol was found adequate because using this mobile phase all the insecticides were well resolved within adequate time. The chromatographic parameters observed while using the optimized mobile phase (Rt, N, B/A) were; MCF (3.5, 1936, 1.2), ICP (6.1 2032, 1.5), DM (7.4, 1968, 1.8) and PFF (11.6, 2267, 2). The mobile phase 0.065 M SDS-2% 1-propanol, 0.01 M NaH_2PO_4 buffered to pH 7 was selected to carry out further chromatographic analysis.

3.2. Method validation

The method was validated following the SANTE/11,312/2021 and International Council for Harmonisation (ICH) guidelines, including limits of detection and quantification, precision and trueness [30], specifically dedicated to the determination of pesticide residues in food and feed samples.

3.2.1. Specificity/selectivity

Under the optimized chromatographic condition, the peaks of selected insecticides i.e. MCF, ICP, DM and PFF were well separated and free from interference from the solvent front eluting at the head of a chromatograph. In GLVs, washed water, and crushed samples, the first peak

corresponding to MCF was eluted at 3.5 min., whereas the matrix from crushed leaves was eluted between 2.5 to 3 min. To evaluate the specificity and selectivity of selected insecticides, spinach leaves washed [**Figure 1 (a)** and **Figure 1 (b)]** and crushed [**Figure 1 (c)** and **Figure 1 (d)]** matrix were spiked with a mixture of MCF, ICP, DM and PFF (10 mg/kg).

3.2.2. Linearity

To study the linearity of MCF, ICP, DM and PFF calibration curves were constructed for individual compounds using the measured area of chromatographic peaks at eight (n=8) increasing concentrations ranging from 0.05 to 10 µg/mL in spiked GLVs (spinach). The slope, y-intercept, coefficient of determination (r^2), and relative standard deviation (RSD%) were calculated using the least square linear regression method and presented in **Table 1**. The calibration curve was linear within the analyzed concentrations. The determination coefficient was in the range of 0.997–0.999 and the RSD% was 3.1-5.8% for selected insecticides which is less than the maximal value indicated by the guideline.

3.2.3. Limits of detection (LOD) and quantification (LOQ)

The limits of detection and limits of quantification for all the selected insecticides were calculated following the 3.3s criterion and 10 s criterion (3 times and 10 times the standard deviation of the lowest concentration solution included in the calibration divided by the slope of the calibration curve) respectively. The obtained results were based on the relative standard deviation of the response and slope of the calibration curve containing selected insecticide in a concentration range close to the limits of quantification. The compounds have limits of detection and limit of quantification in the range of 0.03 - 0.09 mg/kg and 0.05 - 0.20 mg/kg, respectively **(Table 1).**

3.2.4. Trueness and precision

The intra- and inter-day trueness and precision of the proposed method were determined for MCF, ICP, DM and PFF with GLV(spinach) by spiking at three concentration levels lowest quantification level, middle quantification level, and upper quantification level. The intra-day values were determined by analyzing six samples on the same day. Inter-day analysis corresponds to the average of five measurements of the intra-day values taken over 3 month period. Calculations of the closeness of agreement and dispersion were as previously detailed [32]. The obtained results are shown in **Table 2**. Results for relative recovery (96.3–99.8%) and dispersion (<6.3%) were inside the acceptance criteria from the guideline (70–120%) and the value provided by the SANTE/11312/2021 guideline [30].

3.2.5. Robustness

The robustness of the method was examined by replicate injections (n = 6) of 10 µg/mL concentration of MCF, ICP, DM and PFF by introducing small changes in the SDS concentration (0.055–0.075 M), 1-propanol (1.8-2.2%) and pH (6.8–7.2) of the mobile phase. This parameter was examined for each selected insecticide in the optimized mobile phase. Insignificant differences in peak areas and less variability in retention time were observed. The obtained results indicate that the selected factors remain unaffected by small variations in these parameters (RSD <6%).

4. Analysis of real sample

The developed chromatographic method has been applied to know the presence of selected insecticides in all collected samples. During the analysis, MCF was present in spinach, while ICP was found in chickpea leaves. Other investigated insecticides PFF and DM were not detected in any of the samples most probably due to absence, low concentration or they were present in such

a concentration which was beyond the instrumental detection limit. Therefore, further investigation, i.e. washing techniques, were only applied for MCF and ICP in spinach and chickpea leaves respectively.

4.1. Effect of time on the washing of insecticide residue

Two types of foods are commonly used in human dietary plans i.e. vegetarian and non-vegetarian. In India, both are common with around 30% of Indians being pure vegetarian and other being of mixed category. In vegetarian foods, GLVs are acknowledged as a blessing for a safe and healthier life and have been in use for centuries. They are an essential part of the diet to meet the daily nutrient requirements. GLVs are also considered high-risk crops because they can be used fresh as in a salad or can be cooked/processed as per the interest of the consumer. It was also observed in many roadside eateries that they do not wash GLVs and use them directly. As already discussed in the introduction section, different types of pesticides and insecticides are used on GLVs to get rid of insects or pests. The usual process of cleaning GLVs is washing them with normal water. This is not particularly an effective and protective procedure because harmful insecticides may persist on the surface of GLVs.

Therefore, in the present research work, different household methods were applied to wash the GLVs to see their effectiveness in washing. In common household practice, people think that pesticide residues could be reduced by washing with normal water within 5 min. During the preliminary investigation of spinach and chickpea leaves only MCF and ICP were detected in most of the collected samples. Therefore household washing techniques were applied to reduce MCF and ICP insecticide residues in chickpea and spinach leaves. The samples were treated with NW, LW, 1% LJW, 1% CSW and OW for different time intervals i.e. 2, 4, 6, 8 and 10 min. using fresh

water for every wash and the percentage of residual removal was reported [**Figure 2 (a) and Figure 2 (b)**].

During the investigation, it was observed that insecticide residue removal was affected by washing duration for example MCF in spinach samples were removed 22%, 40%, 46%, 42%, 34%, and ICP in chickpea leaves was removed 26%, 44%, 50%, 47%, 37% when washed with NW for 2, 4, 6, 8, and 10 min. using fresh water for each wash, respectively. After washing with LW for 2, 4, 6, 8, and 10 min. using fresh water for every wash, MCF was removed 28%, 41%, 63%, 58%, 46%, and ICP was removed 33%, 44%, 67%, 62%, 49%, respectively. Following a similar procedure, when the samples were washed with ozonizer, it removed 32%, 44%, 70%, 64%, 53% of MCF, and the ICP residual removal was 39%, 47%, 73%, 68%, 56%, respectively. By washing with CSW for the same time interval, the percentage of MCF removal was 25%, 40%, 54%, 46%, 39%, while in the case of ICP it was removed 27%, 44%, 57%, 49%, 43%, respectively. MCF removal was 28%, 40%, 69%, 64%, 58%, and ICP removal was 32%, 44%, 71%, 67%, 53% when washed with lemon water for 2, 4, 6, 8, and 10 min., respectively. Among all five washing techniques, maximum insecticide removal was observed when spinach and chickpea samples were washed for 6 min. Therefore 6 min. washing time was considered adequate for the removal of MCF and ICP from spinach and chickpea leaves, respectively.

4.2. Effect of LJW and CSW concentration on the washing of insecticide residue

Normal water is a common washing method for GLVs in any kitchen. Insecticides are known for their hydrophobic compounds and are not easily washed off with normal water. Therefore, researchers and scientists have reported different washing methods and techniques to see their effectiveness for pesticide or insecticide removal from vegetables. Different methods were reported by researchers which include baking powder ($NaHCO_3$) [32,33], vinegar

316 (CH_3COOH), potassium permanganate ($KMnO_4$) [32], chlorine solution [33], and lemon water
317 ($C_{36}H_{32}Cl_4N_6O_4$) and commercial ozonizer [34], etc. for vegetables but no research group has used
318 these washing techniques in green spinach and chick GLVs. However, no one studied the
319 effectiveness of washing techniques, including LW, 1% LJW, 1% CSW, and OW on spinach
320 leaves. On the other hand, chickpea leaves are the new matrix, and for the first time, they were
321 analyzed to detect the presence of MCF, ICP, DM, and PFF. There are no concentration variables
322 for NW, LW and OW; therefore, the concentration of LJW and CSW was only varied to know the
323 effective concentration of these solutions to get rid of insecticide residues of MCF and ICP in
324 spinach and chickpea leaves, respectively.

325 The concentration of the washing solution was fixed in this experiment. Three
326 concentrations 0.5%, 1%, and 1.5% were selected for LJW and CSW where chickpea and spinach
327 samples were treated with the same for 6 min. (fixed previously) [**Figure 3 (a) and Figure 3 (b)**].
328 When the samples were washed with a 0.5% concentration of washing solutions, it was not much
329 efficient for both insecticides and only reduced 46% and 38% of MCF and 43% and 36% of ICP
330 residues respectively. When the samples were washed with 1% LJW and CSW, 67% and 63% of
331 MCF residues were removed, and the percentage of ICP residue removal was 64 and 61,
332 respectively. Finally, after washing with 1.5% LJW and CSW the pesticide removal rate was
333 slightly decreased as compared to 1% LJW and CSW washing found 69% and 65% for MCF and
334 66% and 62% for ICP respectively. Thus, 1% concentration was considered more efficient than
335 the other two concentrations.

336 Among all treatments, LJW showed better insecticide reduction capability than CSW,
337 while ozone removed more insecticide residues than LJW. Similarly, the results for NW and LW
338 showed that LW reduced more ICP and MCF residues in comparison to NW. Thus, it could be

concluded that common salt has shown better efficiency for removing insecticide residues from spinach and chickpeas compared to LJW. However, it is less efficient in comparison to ozonizer but the effectiveness of CSW is close to it. Therefore, CSW washing method for 6 min. has been fixed to analyse the real samples under the developed chromatographic condition.

4.3. Evaluation of pesticide residues in GLVs

All eight GLV samples were numbered in roman as I-IV for spinach and V-VIII for chickpea were collected from the selected sites and analyzed to detect the presence of MCF, ICP, DM and PFF using the MLC-PDA technique. The chromatographic analysis of analyzed samples revealed that out of eight samples, three samples (II, III, and IV) showed the presence of MCF, while two samples (VI and VIII) were found positive for the presence of ICP. The found concentration of MCF in spinach samples numbered II, III and IV were 1.6 mg/kg, 3.2 mg/kg, and 4.2 mg/kg respectively. On the other hand, chickpea samples numbered VI and VIIIhave shown the presence of ICP at 4.3 mg/kg and 6 mg/kg respectively.

The spinach sample which has shown the maximum concentration of MCF (4.2 mg/kg) and chickpea leaves shown the maximum concentration of ICP (6 mg/kg) have been further treated with different washing solutions to know the percentage removal of insecticide residues.

Therefore in the present study, the research group investigated the effects of washing with NW, LW, 1% CSW, 1% LJW, and OW to wash chickpea and spinach leaves for 6 min. (which has been optimized before in ***sec. 4.1.***). The washed water was collected in a clean container for chromatographic analysis. After filtration with a 0.45 μm filter, different washes of chickpea and spinach were analyzed by an MLC-PDA detector.

The representative chromatograms of 6 min. washing of spinach and chickpea leaves has been shown in the form of a 3D graph for comparative assessments among different washing

techniques [**Figure 4 (a)** and **Figure 4 (c)**]. The effectiveness of washing techniques was cross-checked by crushing the GLV samples obtained after different washings steps. They were also analyzed to know the concentration of insecticide residues (MCF and ICP) left on the surface after washing and systematic residue inside the cuticles of GLVs [**Figure 4 (b)** and **Figure 4(d)**].

The extent of residue reduction by washing depends on the physiochemical properties of the insecticides such as solubility, rate constant, and octanol-water partition coefficient ($P_{o/w}$). Washing leads to the reduction of hydrophilic insecticide residues which are present on the surface of GLVs. Simple washing techniques can wash off surface residues, whereas systemic residues which have penetrated the cuticle layer are slightly affected. Some pesticides and pyrethroids are nonsystemic, adhered only to the upper surface, and easily removed by the water [35]. Thus, washing significantly affects the removal of residues in several vegetables depending on the hydrophilic or hydrophobic nature of the pesticide. Washing GLVs with normal water is a routine practice in every household. In some roadside eateries, this practice is uncommon. People involved there hardly wash vegetables even with normal water. When chickpea and spinach were washed with NW and the water wash was analyzed with an MLC method, it showed the concentration of ICP was 1.22 mg/kg and MCF was 0.32 mg/kg. The concentrations obtained for ICP and MCF in the crushed samples were 1.82 mg/kg and 0.53 mg/kg, respectively. The results show that NW is not effective for washing GLVs. Also, some people do not recommend washing GLVs in CSW as it slightly dulls the GLV colour but less in comparison to potassium permanganate and vinegar solutions. Lemon is the main natural citric ingredient of every household. 1% lemon juice (1 mL) is effective for washing ICP while less effective for the reduction of MCF residues in chickpeas. Around 2.3 mg/kg of ICP was found in chickpeas LJW wash, which is quite high from the maximum residue safety limit of 2 mg/kg. On the other hand, 0.46 mg/kg MCF residues were

detected in the acidic wash of spinach showing its less effectiveness for MCF removal. In the crushed samples of chickpea, the concentration of ICP found was 0.6 mg/kg, while the spinach crushed sample was washed with lemon juice and was found positive for 0.52 mg/kg concentration of MCF.

Talking about ozonizer, it is effective for removing insecticides/pesticide residues from the surface as well as cuticles of GLVs to some extent the rate of ICP and MCF removal was around 1.63 mg/kg and 0.98 mg/kg respectively. In the case of, chickpea leaves and spinach samples which were washed with ozonizer and crushed have shown the presence of ICP in chickpea leaves are 0.67 mg/kg and MCF in spinach leaves was 0.62 mg/kg.

The results obtained in normal water and lukewarm water crushed samples of chickpeas show that both methods are not much effective for washing ICP residues in chickpeas. Similarly, MCF was also not washed off from spinach as it has also shown around a reasonable concentration of MCF in normal and lukewarm water crushed samples.

5. Conclusion

In the present study, MLC coupled with a PDA detector was developed to simultaneously determine MCF, ICP, DM and PFF in spinach and chickpea samples grown around Sagar city (India). The chromatographic examination of marketed spinach and chickpea samples has shown the presence of MCF and ICP, and none of them were found positive for DM and PFF. Therefore washing techniques were only performed for both of them. The developed chromatographic method is green in nature as it uses low-toxicity solvents and also reduces the amount of post-analysis waste, which is very important from an environmental point of view. The obtained GLVs (complex matrix) can be directly injected into the chromatographic system without applying extraction techniques i.e. liquid-liquid extraction, solid phase extraction, QuEChERS, microwave-

assisted, ultrasonic assisted, etc. which are time-consuming as well as costly. The developed method is fundamental for those laboratories which are located in underdeveloped and developing countries and require analytical methodologies with low impact on the environment. All four insecticides were separated and detected in the GLVs within the separation time of 11.5 min. with satisfactory precision and accuracy (< 6.3).

From the results of this study, it could be concluded that acidic wash, basic wash, and ozonizer can be used as washing techniques for the reduction of superficial as well as systematic residues of ICP and MCF. They are as effective as potassium permanganate and vinegar. But common salt and lemon water have the benefit over vinegar and $KMnO_4$ in the aspect that it will result in a bright color on the GLVs after the treatment. Available in every kitchen and also have excess to lower socioeconomic classes which could not afford potassium permanganate and vinegar.

Acknowledgements

The authors are thankful to DST-FIST and DST-PURSE (Department of Science and Technology; Government of India) for supporting with FIST and PURSE scheme (Sl.No.64 Dated 31-05-2016). We also thank the Universitat Jaume I for its support and Hemlata Bhamdare [UGC-Ref.No.3354/(OBC) (NET-DEC.2015)] and Priyanka Pahade [UGC-Ref.No. 3335/(Sc) (NET-DEC.2015)] grateful to University Grant Commission (UGC) for providing a Senior Research Fellowship.

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Figure Captions

Figure 1 (a). Chromatogram of spinach wash without any insecticide treatments (blank)

Figure 1 (b). Chromatogram of spinach matrix spiked with 10 mg/kg of MCF, ICP, DM and PFF

Figure 1 (c). Chromatograms of spinach crushed blank matrix.

Figure 1 (d). Chromatograms of spinach crushed matrix spiked with 10 mg/kg of MCF, ICP, DM and PFF.

Figure 2 (a). Removal of MCF residue with time.

Figure 2 (b). Removal of ICP residue with time.

Figure 3 (a). Removal of MCF residue with concentration.

Figure 3 (b). Removal of ICP residue with concentration.

Figure 4 (a). 3D Chromatograms obtained by the analysis of spinach leaves washed and treated with NW, LW, 1% LJW, 1% CSW, and OW using optimized mobile phase for detection of MCF residue.

Figure 4 (b). 3D Chromatograms obtained by the analysis of crushed spinach leaves treated with NW, LW, 1% LJW, 1% CSW, and OW using optimized mobile phase for detection of MCF residue.

Figure 4 (c). 3D Chromatograms obtained by the analysis of chickpea leaves washed and treated with NW, LW, 1% LJW, 1% CSW, and OW using optimized mobile phase for detection of ICP residue.

Figure 4 (d). 3D Chromatograms obtained by the analysis of chickpea leaves treated with NW, LW, 1% LJW, 1% CSW, and OW using optimized mobile phase for detection of ICP residue.

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