EXPERIMENTAL DESIGNS OF QuEChERS-HEXYL-METHYLIMIDAZOLIUM HEXAFLUOROPHOSPHATE METHOD COUPLED WITH LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY FOR THE DETERMINATION MULTIPLE PESTICIDES IN FRUITS AND VEGETABLES

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FACULTY OF SCIENCE
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KUALA LUMPUR

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ABSTRACT

This research targets the development and validation of the best efficient method for sample extraction of pesticide analytes and LC-MS/MS (Agilent G6490A) instrumentation for selected samples of freshly obtained fruits and vegetables. However, the instrument underwent auto-tuning and Mass-Hunter optimization initially, using 1000 µg/kg standard solution of pesticides mixture to obtain product ions, collision energies, and retention times of the respective analytes. Then, the best mobile phase was first selected comparatively among the nine analyzed setups using responses of the default instrumental settings. Subsequently, multivariate optimization was carried out on the main factors of the instrument, screened (Plackett-Burman) and optimized (Box-Behnken) using response surface methodology (RSM) for the design of experiment (DOE) generated by Minitab-17 statistical software. However, the total chromatographic peak area (TCPA) resulted from the multiple reactions monitoring (MRM) scan analysis of 100 µg/kg standard solution of analytes was used for the optimization. After that, a comparative analysis was attempted between the optimized and unoptimized instrumental settings. Similarly, some important parameters in the sample preparation methodologies were also selected for multivariate optimization using the RSM designs. These occurred after the selection of acetonitrile (ACN) and 1-hexyl-3-methylimidazolium hexafluorophosphate ([C_6MIM][PF_6]) ionic liquid-based respectively for extraction and cleanup purposes. Subsequently, individual optimization studies were carried out on the QuEChERS-dSPE, and QuEChERS-IL-DLLME technical factors using Milli-Q-water

(analytical sample) consistently spiked with 200 µL of 100 µg/kg multi-pesticides mixture. Eventually, the optimized factors of the two methods above were combined (QuEChERS-dSPE-IL-DLLME) and comparative studies were conducted with their respective unoptimized conditional methods. Consequently, the optimized QuEChERS-dSPE-IL-DLLME method was selected and validated (SANTE/11813/2017) for the determination of multi-pesticide residues in fruit and vegetable samples. Resultingly, the precision was expressed based on the laboratory repeatability (RSD_r %) (≤ 20%), as well as the accuracy range for the relative (82 – 138%) and absolute (84–101%) recoveries, were satisfactory. The overall matrix effects were very week (≤ -80%). The range of LOD (0.01 - 0.54 µg/kg) and LOQ (0.03 - 1.79 µg/kg) were acceptable. Also, linearity (5 – 400 µg/kg) of the evaluated results and regression coefficient (R^2) were > 0.99. Conclusively, this developed method could potentially be more reliable and suitable for routine determination of multiple pesticide residues in vegetables and fruits.

Keywords: Design of experiment (DOE), Response surface methodology (RSM), QuEChERS-dSPE-ionic liquid-base-DLLME, Pesticides residue in fruits and vegetables, Liquid chromatography-tandem mass spectrometry (LC-MS/MS)

KAEDAH REKA BENTUK EKSPERIMEN QUECHERS-HEKSIL-METILIMIDASOLIUM HEKSAFLUOROFOSFAT DENGAN KROMATOGRAFI CECAIR SPEKTROMETRI JISIM UNTUK PENENTUAN PELBAGAI RACUN PEROSAK DALAM BUAH-BUAHAN DAN SAYUR-SAYURAN

ABSTRAK

Penyelidikan ini menyasarkan pembentukan dan pengesahan kaedah terbaik untuk pengekstrakan sampel analit racun perosak dan instrumentasi LC-MS/MS (Agilent G6490A) dalam sampel terpilih buah-buahan dan sayur-sayuran segar. Walau bagaimanapun, instrumen ini telah melalui pengoptimuman auto pada mulanya, menggunakan standard campuran racun perosak 1000 µg/kg untuk mendapatkan ion-ion produk, tenaga perlanggaran, dan masa penahanan untuk analit berkenaan. Selepas itu, fasa mudah alih yang terbaik dipilih secara relatifnya daripada sembilan tetapan yang dianalisa menggunakan tetapan alat lalai. Selanjutnya, pengoptimuman multivariate dijalankan pada faktor utama instrumen, disaringkan (Plackett-Burman) dan dioptimumkan (Box-Behnken) menggunakan metodologi permukaan tindak balas (RSM) untuk reka bentuk eksperimen (DOE) yang dihasilkan oleh perisian statistik Minitab-17. Walau bagaimanapun, jumlah kawasan puncak kromatografi (TCPA) yang dihasilkan daripada analisis imbasan tindak balas pelbagai (MRM) 100 µg/kg larutan piawai analit digunakan untuk pengoptimuman. Selepas itu, analisis perbandingan dijalankan di antara tetapan alat yang dioptimumkan dan tidak dioptimumkan. Begitu juga, faktor-faktor yang paling penting dalam metodologi penyediaan sampel juga dipilih untuk pengoptimuman multivariat menggunakan reka bentuk RSM. Ini berlaku selepas pemilihan acetonitril dan 1-heksil-3-metilimidazolium heksafluorofosfat ($[C_6MIM][PF_6]$) berasaskan cecair ion (IL) masing-masing untuk tujuan pengekstrakan dan pembersihan. Selepas itu, kajian pengoptimuman individu dijalankan pada faktor-faktor teknikal QuEChERS-dSPE dan

QuEChERS-IL-DLLME menggunakan air Milli-Q (sampel analisis) secara konsisten dengan 200 µL 100 µg/kg campuran racun perosak. Akhirnya, faktor-faktor yang dioptimumkan bagi kedua-dua kaedah di atas dikaitkan (QuEChERS-dSPE-IL-DLLME) dan kajian komparatif dijalankan dengan kaedah bersyarat yang tidak optimum. Oleh itu, kaedah QuEChERS-dSPE-IL-DLLME yang optimum dipilih dan disahkan (SANTE/11813/2017) untuk menentukan sisa-sisa racun perosak dalam sampel buah/sayur-sayuran. Hasilnya, ketepatan dinyatakan berdasarkan maklumat kebolehulangan makmal (RSD_r %) (≤ 20%), serta jarak ketepatan untuk relatif (82 - 138%) dan pemulihan mutlak (84 - 101%), sangat baik. Kesan matriks keseluruhan kurang berkesan (≤ -80%). Julat LOD (0.01 - 0.54 µg/kg) dan LOQ (0.03 - 1.79 µg/kg) boleh diterima. Di samping itu, kelinearan (5 - 400 µg/kg) hasil yang dinilai dan pekali regresi (R^2) adalah >0.99. Kesimpulannya, kaedah yang telah dibentuk ini berpotensi menjadi lebih kukuh dan sesuai untuk penentuan rutin pelbagai residu racun dalam sayur-sayuran dan buah-buahan.

Kata kunci: Reka bentuk percubaan (DOE), Metodologi permukaan tindak balas (RSM), QuEChERS-dSPE-ionik cecair-DLLME, Sisa racun makhluk perosak dalam buah-buahan dan sayur-sayuran, Spektrometri jisim kromatografi cecair (LC-MS/MS)

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Thank you very much, and God bless.

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[beim][DCA]	:	1-butyl-3-ethyl imidazolium dicyanamide
[bmin][DCA]	:	1-butyl-3-methylimidazolium dicyanamide
[bmpyr][DCA]	:	1-butyl-3-methylpyrrolidinium dicyanamide
$[C_4MIM][PF_6]$	:	1-Butyl-3-methylimidazolium hexafluorophosphate
$[C_6MIM][PF_6]$	:	1-hexyl-3-methylimidazolium hexafluorophosphate
$[C_8MIM][PF_6]$	:	1-octyl-3-methylimidazolium hexafluorophosphate
$\sum$	:	Summation symbol
$\leq$ or $\geq$	:	Less than/equal to or greater than/equal to
µg/kg	:	Microgram per kilogram
µg/mL	:	Microgram per milliliter
µL	:	Microliter
ABS	:	Aqueous bi-phasic system
AChES	:	Acetylcholinesterase biosensor
ACN	:	Acetonitrile
AF	:	Ammonium formate
ANOVA	:	Analysis of variance
AOAC	:	Association of official analytical chemists
ApH	:	Average pH reading
AR	:	Absolute recovery
ART	:	Average retention time
ATCPA	:	Average total chromatographic peak area
ATCPH	:	Average total chromatographic peak height
B-B	:	Box-Behnken design
C_{18}	:	Octa-dodecyl bonded silica
CCIM	:	4-chloro-5-p-tolylimidazole-2-carbonitrile
CCR	:	Central composite rotatable
CE	:	Collision energy
CPE	:	Cloud point extraction
CQ & MQ	:	Chlormequat and mepiquat
CTAB	:	Cetyltrimethylammonium bromide
DAD	:	Diode array detector
DDT	:	Dichloro-diphenyl-trichloroethane
DESI-MS	:	Desorption electrospray ionization mass spectrometry
DLLME	:	Dispersive liquid–liquid microextraction
DMRM	:	Dynamic multiple reaction monitoring
DOE	:	Design of experiment
DPT	:	2,9-dimethyl-1,10-phenanthroline
d-SPE	:	Dispersive-solid phase extraction
EMV	:	Electron multiplier voltage
Eqn	:	Equation
ESI	:	Electrospray ionization
ESI-IMS	:	ESI-ion mobility spectrometry
ETU	:	Ethylene thiourea

EU	:	European Union
FA	:	Formic acid
FAAS	:	Flame atomic absorption spectrometry
FAO	:	Food and agricultural organization
GC	:	Gas chromatography
GC-AED	:	Gas chromatography-atomic emission detector
GCB	:	Graphitized carbon black
GC-MS/MS	:	Gas chromatography-tandem mass spectrometry
GC-NPD	:	Gas chromatography nitrogen phosphorus detection
GDPs	:	Gross domestic products
HF-LPME	:	Hollow fiber liquid phase microextraction
HLLE	:	Homogeneous liquid–liquid extraction
HOAc	:	Acetic acid
HPLC	:	High performance liquid chromatography
HPLC-DAD	:	High-performance liquid chromatography–diode array detection
HPLC-LIFD	:	High-performance liquid chromatography laser-induced fluorescence detection
HS-SDME	:	Headspace single drop microextraction
IL	:	Ionic liquid
IM	:	Ionization mode
LC	:	Liquid chromatography
LC_{50}	:	Lethal concentration
LC-MS/MS	:	Liquid chromatography-tandem mass spectrometry
LD_{50}	:	Lethal dose
LLE	:	Liquid–liquid extraction
LOD(s)	:	Limit of detection(s)
log*P*	:	Partition-coefficient in the organic and aqueous solvents
LOQ(s)	:	Limit of quantitation(s)
LPME	:	Liquid phase microextraction
LTTMs	:	Low transition temperature mixtures
MALDI-MS	:	Matrix-assisted laser desorption ionization mass spectrometry
ME	:	Matrix effect
MF	:	Molecular formula
mg/kg	:	Milligram per kilogram
MIM	:	Mono-isotopic mass
MIP	:	Molecular imprinted polymer
mL	:	Milliliter
MNPs	:	Magnetic nanoparticles
m-PFC	:	Multi-plug filtration cleanup
MRLs	:	Maximum residue limits
MRM	:	Multiple reactions monitoring
MS	:	Mass spectrometry
MU	:	Measurement of uncertainties
MWCNTs	:	Multi-walled carbon nanotubes

NaOAc	:	Sodium acetate salt
ND-EESI-MS	:	Neutral desorption-extractive electrospray ionization-mass spectrometry
OCPs	:	Organochlorine pesticides
OFAT	:	One factor at a time
OPPs	:	Organophosphate pesticides
OVAT	:	One variable at a time
PAHs	:	Polycyclic aromatic hydrocarbons
P-B	:	Plackett-Burman design
PBITU	:	N-phenyl benzimidoylthiourea
PDA	:	Photodiode array
PIESI-MS	:	Paired ion electron spray ionization-mass spectrometry
PIN	:	Pesticide identity number
pK_a	:	Acid dissociation constant
PP	:	Pyrethroid pesticide
PPM	:	Parts per million
PSA	:	Primary secondary amine
QQQ	:	Triple quadrupole
QuEChERS	:	Quick, easy, cheap, effective, rugged and safe
QuPPe	:	Quick polar pesticides
R^2	:	Coefficient of simple linear regression
rpm	:	Revolutions per minute
RP-UPLC	:	Reversed-phase ultra-performance liquid chromatography
RP-UPLC	:	Reversed-phase ultra-performance liquid chromatography
RR(s)	:	Relative recovery(ies)
RSD(s)	:	Relative standard deviation(s)
RSM	:	Response surface methodology
RT	:	Retention time
RTILs	:	Room temperature ionic liquids-based
S/N	:	Serial number
SDHI	:	Succinate dehydrogenase inhibitors
SDME	:	Single-drop microextraction
SPE	:	Solid-phase extraction
SPME	:	Solid-phase microextraction
STDEV		Standard deviation
STEYX	:	Standard error
$t_{1/2}$	:	Half-life
TCPA	:	Total chromatographic peak area
TCPH	:	Total chromatographic peak height
TIC	:	Total ion chromatography
TPP	:	Triphenyl phosphate
UHPLC-MS/MS	:	Ultra-high performance liquid chromatography tandem mass spectrometry
USA	:	United States of America
VA-D-µ-SPE	:	Vortex-assisted dispersive micro-solid phase extraction

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CHAPTER 1: INTRODUCTION

1.0 General Introduction

1.1 Food Contamination

Foods are contaminated through various activities performed by man such as the accidental or intentional discharge of chemicals or waste substances from domestic, industrial and agricultural activities into the environment (Chapman, 2007; Prasad & Ramteke, 2013). However, most of these contaminants are non-biodegradable that can be easily transferred from the ground surface to the underground water because of their ability in dissolving sparingly in water (Gong et al., 2016; McCarthy & Zachara, 1989). At long run, the contaminants pollute the foods through their respective circulatory movements in the environment (Lake et al., 2012). The contaminants include inorganic matters (copper, cadmium, manganese, arsenic, lead, etc), organic chemicals such as heat generated compounds [polycyclic aromatic hydrocarbons (PAHs) and acrylamide)], organic polymers (bromodiphenyl ethers, chlorobiphenyls, chlorodibenzodioxins, chlorodibenzofurans etc), mycotoxins (aflatoxins), perfluoroalkyl acids (Chan-Hon-Tong et al., 2013). Other contaminants with emerge-concerns include phthalates, bisphenol A, alkylphenols (Meador et al., 2016), phytosterols, estrogens, phytoestrogens (Ribeiro et al., 2016), pharmaceuticals/veterinary drugs and pesticides (McGrath et al., 2012).

Meanwhile, the increase in population and improved health quality of life has tremendously led to high demands for food materials needed for survival (Trostle, 2010). Thus, agriculture is the primary practices in most countries across the globe due to its significant economic impacts on the countries' survival and gross domestic products (GDPs) (Byerlee et al., 2009). Because, the food crops are grown and protected with effective pesticides (Raven, 2014).

For this reason, Food and Agricultural Organization (FAO) define pesticides as any substances or mixture of substances that is intended for preventing, destroying, attracting, repelling, or controlling any pest including unwanted species of plants or animals during production, storage, transport, distribution and processing of food agricultural commodities, or animal feeds or which may be administered to animals for the control of ectoparasites (Lamikanra & Imam, 2005).

Moreover, pesticides are also used for household and environmental health purposes for destroying vectors (insects or micro-organisms) transmitting deadly diseases such as mosquitoes causing Malaria fever, fleas causing plagues including Cholera disease, etc. (Topalis et al., 2011).

1.1.1 Historical Use of Pesticides in Agricultural Practices

In summary, pesticides have also been used for more than six (6) decades for the steady protection of food crops and animals against infestations and diseases to meet the expectation of governments and the entire global population (Acunha et al., 2016). The historical background shows that the management of pests started gaining effectivity with the use of pesticides after the end of World War II (Gay, 2012).

The highly toxic cyanide compounds of arsenic and hydrogen were the first generated pesticides used. Luckily, they were abandoned because they proved to be less effective towards their targets and very toxic to humans. The second generations are synthetic pesticides that include dichloro-diphenyl-trichloroethane (DDT), which was first produced in 1939 by Paul Muller (Swiss chemist) (Bharati & Saha, 2017). The compound presented its self as the most common synthetic pesticides due to its broad-spectrum activity against wide range of pests. It, fortunately, possesses lower toxicity in handlings and various applications. Consequently, the Swiss chemist was awarded the Nobel Prize in 1948 due to the innovation of DDT (Muir, 2012). As time goes by, the quantitative use

of registered pesticides has been increasing tremendously based on their activity, especially in the developing countries particularly in the continents of Asia and Africa (Arinaitwe et al., 2016).

1.1.2 Pesticide Use around the World

About two (2) million tonnes of pesticides are consumed annually worldwide in agricultural practices, domestic and public health sectors. However, the European countries as well as the United States of America respectively consumed 45 and 24 %. Other countries that include Asia consumes the remaining 31 %. Moreover, the annual average quantity of pesticides consumed in some Asia countries is illustrated in Figure 1.1 (Abhilash & Singh, 2009).

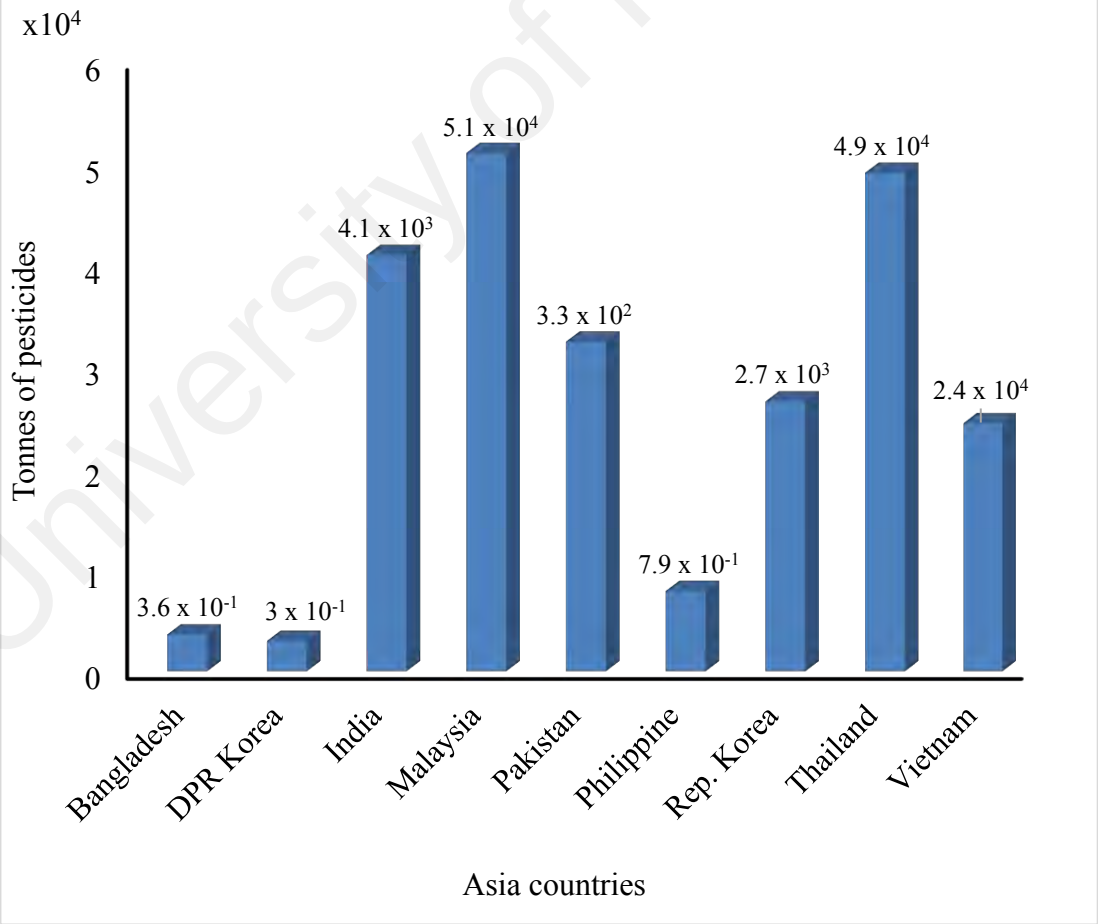


Figure 1.1: Pesticides consumed in some Asian countries. Adapted from "Pesticide residues in fruits and vegetables from Pakistan: A review of the occurrence and associated human health risks", by Syed et al., 2014.

Meanwhile, a report recently showed that Malaysia has been experiencing yearly increment (4 - 5%) of pesticides usage in national (public health) and agricultural practices. However, 4, 5, 16 and 75 % of the pesticides were used as rodenticides, fungicides, insecticides, and herbicides, respectively (Chamhuri & Batt, 2015).

1.1.3 General Classification of Pesticides

1.1.3.1 Based on the target organism

One of the best ways of classifying pesticides is based on their target organisms or specific function (Table 1.1).

Table 1.1: Classification of pesticides based on the target organisms (Fishel, 2014)

S/N	Class of pesticides	Targets/function	Pesticide(s)
1	Termiticide	Termites	Fipronil
2	Silvicide	Trees	Tebuthiuron
3	Rodenticide	Rodents	Warfarin
4	Repellent	Vertebrates & invertebrates	DEET, methiocarb
5	Predacide	Mammal predators	Strychnine
6	Plant growth regulator	Regulates plant growth	Gibberellic acid, etc.
7	Piscicide	Fish	Rotenone
8	Nematicide	Nematodes	Aldicarb, fenamiphos
9	Molluscicides	Snails and slugs	Metaldehyde
10	Insecticide	Insects	Carbaryl, etc.
11	Insect growth regulator	Controls insects growth	Diflubenzuron
12	Herbicide	Weeds	Atrazine, etc.
13	Fungicide	Fungi	Chlorothalonil, etc.
14	Fumigant	Various organisms	Aluminum phosphide
15	Desiccant	Dries farm produce	Boric acid
16	Defoliant	Removes plant foliage	Tribufos
17	Bio-pesticide	Various organisms	Bacillus thuringiensis
18	Bait	Various organisms	Anticoagulants
19	Bactericide	Bacteria	Streptomycin, etc.
20	Avicide	Birds	Avitrol, etc.
21	Attractant	Attracts various pests	Pheromones
22	Algaecide	Algae	Copper sulfate
23	Acaricide and Miticides	Mites	Aldicarb, Bifenazate

1.1.3.2 Based on the chemical structure

The chemical structures of pesticides play a vital role in classification. Hence, pesticides are classified into four groups based on their chemical structures. These classes include:

(a) Carbamate compounds

Carbamic acid serves as the primary source of the organic carbamate pesticides. These pesticides possess a general structural formula (Figure 1.2).

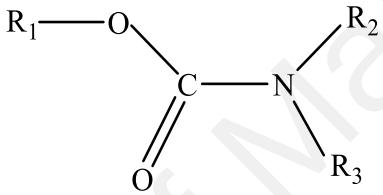


Figure 1.2: The general structural formula for Carbamate pesticides (Zacharia, 2011)

The R_1, R_2, and R_3 represent alcoholic, methyl and hydrogen groups respectively of the carbamate structure. Examples of the widely use carbamate pesticides include ethienocarb, aminocarb, carbaryl, carbofuran, methomyl, aldicarb, carbendazim, and fenobucarb (Wang et al., 2016; Zacharia, 2011).

(b) Pyrethroid compounds

Pyrethroid pesticides are analog products of synthesized pyrethrins which are originated from *Chrysanthemum cinerariaefolium* flowers (Srivastava et al., 2016). Pyrethrin compounds are very useful insecticides, biodegradable with lower toxicity to mammals (Yuxin et al., 2016). Unfortunately, the usefulness of these compounds in the agricultural applications is not encourageable because of their rapid photochemical degradation (Debbab et al., 2014). Circumstantially, this led to the production of pyrethroids pesticides that are classified into the type II (photochemically stable with

higher insecticidal property), and type I (photochemically unstable with lower insecticidal property) groups based on their different chemical structures (Figure. 1.3).

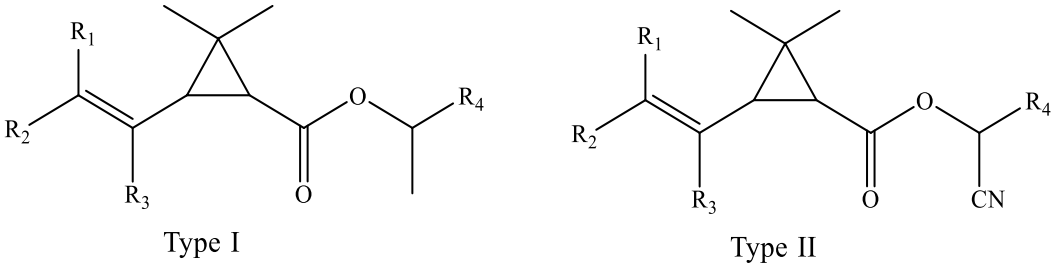


Figure 1.3: The general structural formula of Pyrethroid (type I and II) pesticides (El-Kheir & Shukri, 2004)

(c) Organo-chlorine compounds

Organo-chlorine pesticide compounds (OCP) are made up of four or more atoms of chlorine attached to the carbon and hydrogen groups. These organic pesticides are the first to be synthesized and most widely used insecticides for agricultural production and community purposes (Donham & Thelin, 2016). Some of the most well-known chemical classifications of these pesticides structures include chlorobenzenes (e.g. hexachlorobenzene) and chlorocyclohexanes (e.g. hexachlorohexane), cyclodienes (e.g. aldrin, endosulfan, heptachlor, dieldrin and chlordane), and dichlorodiphenylethanes (e.g. DDT, Perthane, Methoxychlor, and dicofol) (Vargas-Bernal et al., 2012) as presented in Figure 1.4.

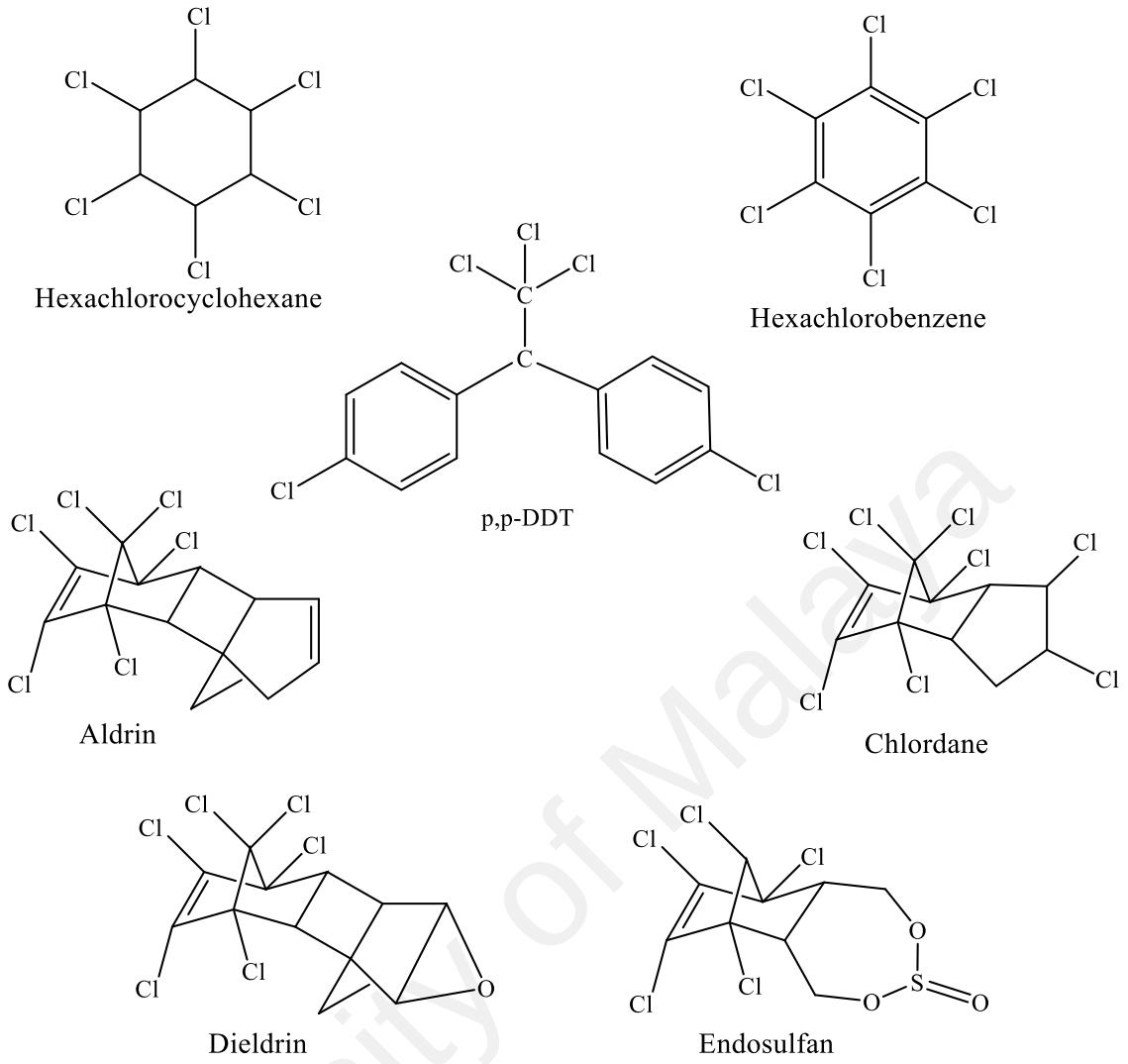


Figure 1.4: The chemical structures of organochlorine pesticides (Zacharia, 2011)

1.1.3.3 Organo-phosphorus compounds;

These chemicals are one of the most diverse pesticide families that are mostly derived from phosphoric acid. The basic structure of the compounds is made up of a phosphate group. The compounds contain an atom of phosphorus (P) bonded to four other atoms at the center. Usually, three single bonds are individually attached to three of the atoms/groups while the 4th atom is attached to the P-atom by double bonds). The structure of most popular basic organophosphate pesticides (OPPs) are shown in Figure 1.5. (Rathnayake & Northrup, 2016).

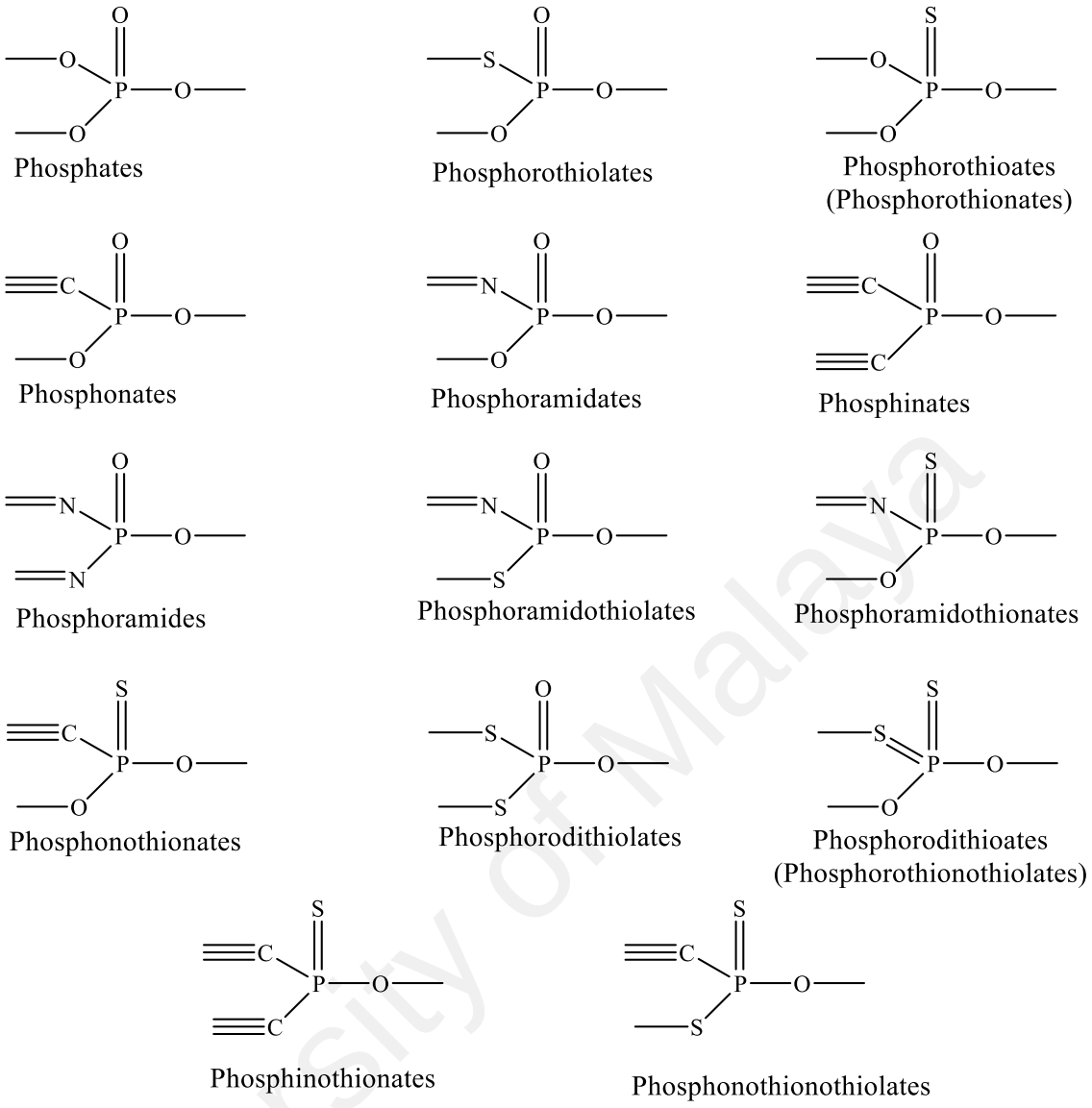


Figure 1.5: Basic organo-phosphorus pesticide structures (Chambers & Levi, 2013)

The examples of the OPPs which are widely used include: Trichlorfon, Tribufos, Triazophos, Tetrachlorvinphos, Terbufos, Temephos, Quinalphos, Pirimiphos-methyl, Phoxim, Phostebupirim, Phosmet, Phosalone, Phorate, Parathion-methyl, Parathion, Paraoxon, Oxydemeton-methyl, Oxon, Omethoate, Naled, Monocrotophos, Mevinphos, Methyl parathion, Methidathion, Methamidophos, Malathion, Lorsban, Isoxathion, Fosthiazate, Ferthion, Fenitrothion, Fenamiphos, Ethoprop, Ethion, Dursban, Disulfoton, Dioxathion, Dimethoate, Diisopropyl, fluorophosphates, Dicrotophos, Dichlorvos, Diazinon, Demeton-s-methyl, Coumaphos, Chlorpyrifos-methyl, Chlorpyrifos,

Chlorfenvinphos, Chlorethoxyfos, Bensulide, Azinphos-methyl and Acephate (Troyer & Leffel, 2014).

1.1.4 Environmental Circulation of Pesticides

The persistence nature of pesticides after their application on land is encouraged by their transportation (circulation) in the environment (Figure 1.6). The circulation is rapid and simultaneous due to the processes of rain wash-off, run-off, plant uptake, leaching, volatilization, etc. (Gavrilescu, 2005). Although, the persistence nature and movement of pesticides in the environment right from the application sites can be affected by characteristics of the soil, soil's moisture (ground-water), climatic condition, pesticidal handlings and the bio-population (Gavrilescu, 2005) as illustrated in Figure 1.6.

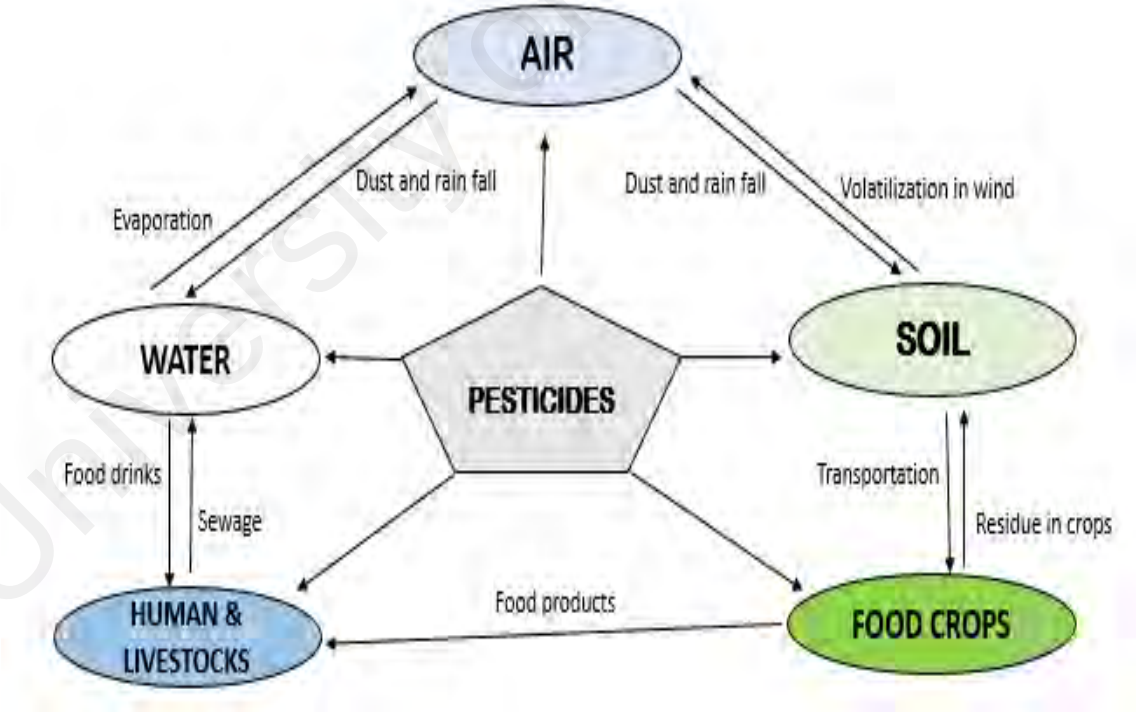


Figure 1.6: Pesticide cycle in the environment. Adapted from "Alternative and biological pest controls", by "CAN", 2016.

Environmental circulation of pesticides is regarded as one of the most life-threatening contamination movement because of the pesticides residual accumulation rendering

themselves persistent in the environment, at long-run polluting foods and drinks thereby causing many problems due to their high concentrations in the body (Kaur et al., 2016).

1.1.5 Problems Caused By Pesticides Usage

In the beginning, the pesticides used show to be more effective against the targeted pests yielding higher agricultural outputs. Unfortunately, the pests of the present day have evolved defensive mechanisms to withstand the chemicals. Consequently, this encourages the extensive application of pesticides in our present agricultural productions. Many kinds of pesticides especially older ones such as carbamate and organophosphate are forbidden in the developing nations due to their health implications. Many farmers use these pesticides illegally because of their economic benefits over the newly excogitated ones (Wilson & Tisdell, 2001). For this reason, humans (farmers) and other animals are being exposed to lots of pesticide pollutions resulting in various health toxicities (Houbraken et al., 2016). Moreover, the health risks are becoming a world problem due to the high rate of food insecurity (Acunha et al., 2016).

In fact, the symptoms of pesticides exposure on human include: loss of vision, irritation of the skin, problems associated to pulmonary and respiratory tracks, damages of the immune, hormone and nervous systems, gene mutations, birth defects and different kinds of cancer which lead to death in most cases (Sim & Gysi, 2015). Thus, there is an urgent need to balance-up the health risks and the expected benefits involved through the effective and efficient usage of the pesticides (Falconer, 1998).

1.1.6 Legislative Rules Guiding the Use of Pesticides

Maximization of benefits and minimization of pesticide effects are the most important goals to be achieved while using the chemicals. Based on the reported health-related problems caused by the continuous usage of pesticides, compels many countries to form legislative organizations, which ensure the regulatory handlings of the chemicals

(Chamhuri & Batt, 2015). Notably, countries within the European Union (EU) and the United States of America (USA) forbids the use of some pesticides unless with official authorization. In 2008, more than seventy thousand (70,000) food samples were analyzed. The result indicated that the EU reported only two hundred (200) samples to contained pesticide residues and 3.5 % of the result were beyond the maximum residue limits (MRLs) (McGrath et al., 2012).

In some Asian countries such as India, which legislatively controls its pesticides use based on "The Insecticides Act, 1968" which ensures importation, manufacturing, transportation, sales and use of qualitative, efficacious and safe pesticides to farmers and domestic users (Abhilash & Singh, 2009). Also, the Indonesian government enacted "Degree No. 7, 1973" to ensure qualitative handling and efficient use of pesticides. The rules are further use for controlling registered pesticides and distribution within and outside the country, signed by Indonesian Agricultural Minister (Chamhuri & Batt, 2015).

Notably, the Malaysian government under the Occupational Safety and Health Order of 1999 prohibited the use of hazardous chemicals that are containing benzene and white phosphorus, etc. Furthermore, the use of pesticide in Malaysia is control or guided by several government-organizations apart from the Ministry of Human Resources, to ensure the health-safety of Malaysian workers and the public at large. Consequently, it led to the enactment of the "Pesticides Act 1974" to control pesticides presence in food production, importation, concentrative levels, and toxicities, as well as to advance their methods of analysis (Rampal & Nizam, 2006).

1.1.7 Some Properties of Pesticides Selected for the On-going Research

These include;

1.1.7.1 Henry's law constant (vapor pressure)

The vapor pressure is defined as the pressure exerted by the gas in equilibrium at a given temperature with liquid or solid in a closed container (Rao, 2010). Also, the vapor pressure describes Henry's law constant, which refers to the volatility measurement of the concentration of a pesticide in moistened soil or water, based on its molecular weight, solubility and at a particular temperature. Thus, Henry's law constant (vapor pressure) of a pesticides compound is directly proportional to its volatilization property (Jantunen & Bidleman, 2000).

1.1.7.2 Degradational property

Pesticides can break down into smaller molecules, which makes them less useful to perform their functions (Tiryaki & Temur, 2010). This process is catalyzed by enzymes (biotic reaction) and sunlight (photochemical reaction) resulting in the chemical reactions such as dechlorination, reduction, oxidation, elimination, rearrangement, isomerization, and conjugation (Chaplain et al., 2011). The degradation rate of a pesticide in the soil is estimated as its half-life ($t_{1/2}$), and it depends on the chemistry (nature) of the pesticide, temperature, pH, and type of soil (Büyüksönmez et al., 1999). Thus, pesticide degradation is directly proportional to the biotic and abiotic reactions, while the $t_{1/2}$ is inversely proportional to the biotic and abiotic reactions (Matouq et al., 2008).

1.1.7.3 Solubility in water

It refers to the ability of pesticides to dissolve in water (mg/L) at a temperature range of 20-25 ºC. This property helps to estimate or study the degradation of pesticides and their movement in the environment at different phases that include body tissue, soil, water, and air. However, pesticides solubility in water is dependent on their polarity, molecular weight, pH, and temperature for the medium of distribution (Ortiz-Hernández

et al., 2014). Thus, the solubility of pesticides in water is directly proportional to their distribution (leaching) in the soil (Hijosa-Valsero et al., 2016).

1.1.7.4 Pesticides partition coefficient in octanol/water

It helps to predict the transporting fates, structural activities and lipophilic property of pesticides in the environment. However, it is based on pesticides' solubility and distribution partition-coefficient in the organic and aqueous solvents which depend on their polarities, densities and molecular weights (Mamy et al., 2015). Moreover, the pesticides partition-coefficient (*P*) in organic and aqueous solvents is expressed mathematically as a ratio of pesticide concentration in octanol to the pesticide concentration in water (Equation 1.1 and 1.2) (Earll, 1999; Finizio et al., 1997).

$$Partition\ coefficient\ (P) = \frac{Concentration\ of\ pesticide\ in\ octanol}{Concentration\ of\ pesticide\ in\ water} \qquad \text{Eqn (1.1)}$$

$$\text{Therefore,} \qquad \log P = \log_{10} P \qquad \text{Eqn (1.2)}$$

Notably, the log*P* results; +1, 0, and -1 indicates 10:1, 1:1, and 1:10 partition-coefficients ratio of Organic/Aqueous respectively. Resultingly, the positive, neutral and negative log*P* indicate high affinity of pesticide compounds in organic, organic/aqueous and aqueous phase respectively (Bhal, 2011).

Meanwhile, log*P* is further estimated to XLogP_3 value based on the facts that different compounds with similar structures may have the same (log*P*) properties (Wang, 2007). Thus, the XLogP_3 value of a pesticide molecule is inversely proportional to it affinity towards the organic partition phase (Bhal, 2011). The higher the log*P* or XLogP_3 value, the lower the affinity of pesticides molecules towards the organic solvent.

1.1.7.5 Acid dissociation constant (pK_a) of pesticides

It refers to the acidity strength possessed by pesticides. However, the acid ionization constant (K_a) is obtained from the aqueous solution of an acid/base ionic dissociation reaction (Equation 1.3). Moreover, the K_a is used for estimation of Acid Dissociation Constant (pK_a), which is expressed as the negative logarithm of base-10 (Equation 1.4). Thus, a lower value of pK_a indicates a stronger acidic property of pesticides (Kortum et al., 2000).

$$AH \rightleftharpoons A^- + H^+ \Leftrightarrow K_a = \frac{[A^-][H^+]}{[AH]} \qquad \text{Eqn (1.3)}$$

$$\text{Therefore, } pK_a = -\log_{10} K_a \qquad \text{Eqn (1.4)}$$

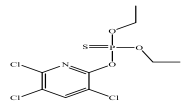
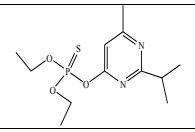
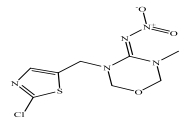
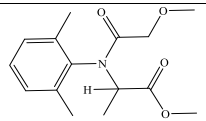
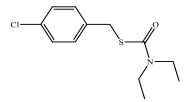
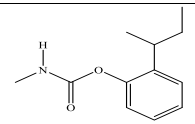
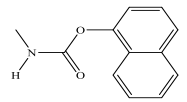
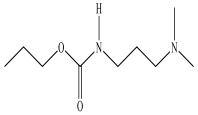
1.1.7.6 Toxicity and tolerance level of pesticides

Toxicity of a pesticide is defined as the deleterious effects caused by pesticide exposure (chronic or acute state), which resulted in some health symptoms. Thus, pesticide toxicity symptoms are directly proportional to the exposure level (dose) and time (Tennekes & Sánchez-Bayo, 2013). Meanwhile, the tolerance level of pesticides could be estimated using experimental animals such as albino rats, etc. The lethal dose (LD_{50}) or concentration (LC_{50}) per body weight (mg/kg) of each animal was exposed orally or dermatologically to 50% population. Thus, the lower obtained values of LD_{50} and LC_{50} signifies higher toxicity effects of the pesticides (Chandra et al., 2014).

1.1.8 Tabular Summary of the Properties of Analyzed Pesticide Compounds

Table 1.2 shows the targeted pesticides for the on-going research with the summaries of their respective properties:

Table 1.2: Summarized properties of the analyzed pesticide compounds

Common name(s)	Molecular formula	Mono-isotopic mass (g/mol)	Function(s)	Vapor pressure (mm Hg at 25 °C)	XLogP$_3$	pK$_a$	Oral LD$_{50}$ of rat (mg/kg)	Structural formula	References
Dursban/ Chlorpyrifos	$C_9H_{11}Cl_3NO_3PS$	348.926	Insecticide/ Nematicide	2.02×10^{-5}	5.3	-4.2	320		"T$_3$DB", 2017a; Ferrell & Aagard, 2003; "PubChem", 2004; Wagner, 1999
Diazinon	$C_{12}H_{21}N_2O_3PS$	304.101	Insecticide	9.01×10^{-5}	3.8	2.6	1340		Eiden, 2000; Ferrell & Aagard, 2003; Leiss, 2004; "PubChem", 2004; "WHO", 1998
Thiamethoxam	$C_8H_{10}ClN_5O_3S$	291.71	Insecticide	4.95×10^{-11}	1.5	0.41	1563		Kumar et al., 2014; "PubChem", 2004; "T$_3$DB", 2017b
Metalaxyl	$C_{15}H_{21}NO_4$	279.147	Fungicide	5.62×10^{-6}	1.6	1.41	669		Arias et al., 2006; Ferrell & Aagard, 2003; "PubChem", 2004
Thiobencarb/ Benthiocarb	$C_{12}H_{16}ClNOS$	257.064	Herbicide	1.80×10^{-5}	3.4	-	1300		"Ceesay & BRANCH", 2000; "PubChem", 2004
Baycarb/ Fenobucarb	$C_{12}H_{17}NO_2$	207.126	Insecticide	3.60×10^{5}	2.8	14.8	350		"Fenobucarb", 2009; "OSHA", 2008; "PubChem", 2004
Carbaryl	$C_{12}H_{11}NO_2$	201.079	Insecticide/ Nematicide	Very negligible	2.4	10.4	230		"FAO", 2007; Ferrell & Aagard, 2003; Kahru & Dubourguier, 2010; Lewis, 1996; "PubChem", 2004
Propamocarb	$C_9H_{20}N_2O_2$	188.152	Fungicide	6.0×10^{-6}	1.2	9.6	2900		"EURL-SRM", 2006; Hotchkiss et al., 2001; "PubChem", 2004

1.2 Vegetables and Fruits

Fruit and vegetable foods are one of the bases that constitute healthy diets worldwide, playing vital roles nutritionally for the attainment of a healthy life. Moreover, fresh fruits and vegetables provide dietary fibers, carbohydrate, vitamins particularly Vitamin C, minerals particularly electrolytes, and bioactive compounds. The bioactive compounds include phytochemicals, which possesses antioxidant, phytoestrogen activities and anti-inflammatory agents (Slavin & Lloyd, 2012). Moreover, the dietary fibers supplies by these foods prevent gastrointestinal cancers and contribute to lowering the cholesterol level in the blood (cholesterolemia) (Lattimer & Haub, 2010).

Consequently, these led to reducing the high peril of cardiovascular diseases and the reduction of high risks of obesity. Meanwhile, the derived nutrients and biological compounds in fruits and vegetables depend on nature, size, geographical locations they were cultivated (Roth, 2013). In the year 2010, it was recommended by the Dietary Guidelines of the United States of America suggested that one-half of a person's plate of food should contain fruits and vegetables (Kaiser et al., 2014).

Unfortunately, the percentage of nutrients in fruits and vegetables has been decreasing over the years due to soil depletion of essential materials caused by intensive modern agricultural techniques. For instance, a report published by Esther and Newark (2015) shows that the percentage contents of calcium, iron, and potassium in twenty (20) fresh vegetables decreased by 19, 22, and 14%, respectively, from the year 1930 to 1980. Similarly, another report by Landsman (2013) shows that there is a dropped in the percentage levels of calcium, iron, vitamin A and C in twelve analyzed fresh vegetables by 27, 37, 21 and 30%, respectively, from the year 1975 to 1997. Approximately, this shows that a man of today has to consume eight (8) oranges to derived vitamin A that will equal that of a single orange consumed by a man five decades ago. Justifiably, this shows

that the consumption of more fruits and vegetables is essential to support the health condition of our body.

Notwithstanding, the fresh vegetables and fruits of today have been accumulated with pesticide residues because of the continuous miss-management and excessive application of pesticides during pre and post-agricultural practices (Kaur et al., 2016). For example, the triazole fungicides, carbamates, pyrethroids and organochlorine pesticides (OCPs) are most well-known for controlling pests in vegetables and fruits (Zhang et al., 2016). Based on this fact, the food quality controllers and the analytical scientists have periodically analyzed the concentration levels of pesticides residue in vegetable and fruit samples to minimize the extent of pesticides' damages onto the body and the ecosystem.

1.3 Research Problem

The continuous usage and mishandlings of pesticides in agricultural and domestic needs have led to many health and environmental problems such as congenital disabilities and various kind of cancers. However, most of the conventional methods such as solid-phase extraction (SPE) (Xie et al., 2015) and liquid phase microextraction (LPME) (Alsharif et al., 2016; Lawal et al., 2016) have been employed for extraction (sample preparation) of multi-pesticide residues in food matrices. Also, lots of instruments such as gas chromatography-atomic emission detector (GC-AED) (Cook et al., 1998) and high performance liquid chromatography (HPLC) (Aulakh et al., 2005) have been used for quantification of pesticide analytes in the prepared samples. Unfortunately, the sample preparation techniques possess poor sensitivity of analytes, while the instruments are poorly sensitive for detection and quantification of pesticide analytes in trace amount due to lack of optimization before carrying out the analysis (Cortada et al., 2009). Moreover, extensive ranges of different chemical properties of pesticides including the acidity that is one of the significant challenges for multi-residue determination of the analytes in food

samples (Yang et al., 2013). In addition, the analytical sample also plays challenging roles for pesticides extraction during sample preparation because of their features that include non-polar, polar, fatty and waxy samples (Majors, 2007; Orso et al., 2014).

Accordingly, these compel food safety analysts to look for better sample preparation and qualitative/quantitative instrumentation techniques. It is hoped that this will overcome the drawbacks and be efficiently used for the sample preparation of pesticides' multi-residue in food samples before quantifying it with a suitable instrument (Banerjee et al., 2007). Alternatively, coupling the methods of quick, easy, cheap, effective, rugged and safe (QuEChERS) dispersive-solid phase extraction (d-SPE) (Anastassiades et al., 2003) with dispersive liquid-liquid microextraction (DLLME) (Zhang et al., 2016) could solve the drawbacks of the previous techniques after multivariate optimization. Moreover, using ionic liquid-based (IL-based) as a cleanup solvent in DLLME technique could provide better results of pesticides determination in food matrices regarding analytes recovery and precision (Zhang et al., 2012). Finally, the optimized LC-MS/MS instrument aims to provide better analytical performances for the routine multi-pesticides residue determination in a sample of fruits and vegetables prepared by the developed QuEChERS-dSPE-IL-based-DLLME.

1.4 Design of Experiment

Design of experiment (DOE) or experimental design is defined as series of tests in which purposeful changes are made to the input variables of a system or process and the effects on response variables are measured (Telford, 2007). DOE can be classified into univariate and multivariate.

1.4.1 Univariate and Multivariate Design of Experiment

The univariate DOE is one of the commonly used design for most extraction techniques that study one factor or variable at a time (OFAT or OVAT). It occurs by

varying one factor out of a set of factors while keeping other factors fixed at a particular experiment or analysis. Thus, this process is repeated on the other factors individually before selecting the set of factors (values) that provide the best response or result. Unfortunately, this kind of designs depend on intuition, guesswork, experience, luck and lacks interactions among the factors. Moreover, this design requires large resource inputs, which could lead to unreliability, inefficiency and time consuming (Anthony, 2014; Montgomery, 2013).

Based on the fact above, this research will be carried out using multivariate DOE to determine and estimate the possible factors or variables' interactions at the best specific levels. The interactions of the factors strongly affect performances of the technical processes to yielding satisfactory (optimum) results. The response surface methodology (RSM) of DOE used for the research are Plackett-Burman and Box-Behnken designs. Consequently, the results obtained in most cases after employing RSM will be better than the univariate results (Anthony, 2014; Montgomery, 2013).

1.4.1.1 Plackett-Burman designs

The design is used for screening factors, which contributes significantly to particular confidence or significant level to achieve excellent results. The design is used to obtain experimental responses after carrying out some experimental runs (4, 8, 12, 16, 24, 32, 64, etc.) depending on the level of variables [low (-) and high (+)] (Anthony, 2014).

1.4.1.2 Box-Behnken designs

The resulted significant factors from Plackett-Burman (screening) design experimental runs will undergo optimization using the Box-Behnken design of the experiment. However, the experimental runs depend on the number of factors [minimum of three (15 runs) and maximum of ten (170 runs) for unblocked experiment]. Moreover, it depends on the levels of variables [low (-), medium (0) and high (+)] (Montgomery, 2013).

1.5 Justification and Significance of the Research Study

The Malaysian cultivated and imported food materials especially fresh fruits and vegetables could be contaminated and accumulated with pesticide residues due to mismanagement by farmers and excessive (uncontrolled) usage during cultivation and preservation (Schreinemachers et al., 2012). Significantly, there is an urgent need to develop a better sample treatment method with more sensitivity of targeted analytes and use of a more sensitive instrument that can be used more efficiently for the trace determination of pesticides in a sample of fresh fruits and vegetables. Also, the knowledge acquired from this research will help to control excessive use of pesticides or consumption of highly contaminated fruits and vegetables, which are above the maximum residue limits (MRLs) based on the guideline commission of the European Union (EU). The study will also contribute to the global control of food qualities to reduce the illnesses and side effects caused by pesticides in human and animal tissues, as well as to serve as a reference platform for future studies.

1.6 Objectives of the Research

i. To increase the sensitivity of LC-MS/MS by optimizing the instrumental operation using the design of experiment.

ii. To optimize QuEChERS-dSPE and QuEChERS-IL-DLLME sample treatment methods.

iii. To compare the performance between the default, RSM optimized QuEChERS-dSPE, QuEChERS-IL-DLLME, and the combined QuEChERS-dSPE-IL-DLLME method.

iv. To validate the best sample treatment method according to EU Commission guidelines for the determination of multi-pesticide residues in fruits and vegetables.

CHAPTER 2: LITERATURE REVIEW

2.1 Experimental Design

Historically, R.A. Fisher pioneered the use of Experimental design in the field of Agricultural Sciences in the 1920s – 1930s to improve its production (Rolph, 1995).

Later on, the experimental design was employed by the industries as well as the military during the Second World War in the 1940s. At long run, the design developed by George Box was later introduced for the optimization of chemical processes (Telford, 2007). The performance influence and optimization processes are essential to experimental design in the chemometrics approach. For instance, experimental design helps to ascertain the optimal condition of the experiment, and it expresses the best interaction model among the multiple factors or variables (Mousavi et al., 2018). Since then, a series of researches have been conducted and published concerning the use of experimental design.

El-Atrache et al. (2013) documented the use of experimental design to model the carbamates pesticide recovery through the influence of the four parameters that include the type of sorbent, the mass of sorbent, the volume of sample and volume of elution. The result shows that the volume of sample and eluent of the solid phase extraction (SPE) are the significant parameters (P-value < 0.05 statistical level) based on a full (2-level) factorial design experiments. Furthermore, a 2^3 factorial experimental (randomized-block) design was successfully carried out to examine the effects of salting, temperature and time on the pesticides extraction in samples of apple using solid phase microextraction (SPME). The result demonstrated that the three factors were significant (P-value < 0.05 statistical level) on the analytes extraction (Abdulra'uf & Tan, 2013). Also, the central composite rotatable (CCR) experimental design have recently been reported for the optimization of supercritical (fluid) carbon dioxide to determine the

optimal levels of temperature and pressure used for the extraction of pesticides in a sample of banana flour. The results of the investigated variables were significant (P-value < 0.05 statistical level) on the pesticides extraction (Sartori et al., 2017).

2.2 Liquid Chromatography-Tandem Mass Spectrometry

Historically, lots of mass spectrometry instruments have been produced from the periodical series modification trailing to the mass spectrometer, pioneered by Francis Aston in 1919 (Sharma, 2013). Similarly, several modified chromatographic columns used for liquid and gaseous separation have been produced over the years. These columns were trailed to the previous attribution made by Mikhail Tsvet in 1903 (Lundanes et al., 2013). Thus, the liquid (LC) and gas chromatography (GC) instruments are respectively connected with different kind of detectors. The detectors include diode array (DAD), photodiode array (PDA) detector and mass spectrometry (MS) detector. However, the mass spectrometry is supported by electrospray ionization (ESI) in many cases (Parejo et al., 2004). These instruments include high-performance liquid chromatography (HPLC) (Aulakh et al., 2005) and gas chromatography-atomic emission detector (GC-AED) (Cook et al., 1998). Furthermore, the recently used MS detection instruments such as gas chromatography-tandem mass spectrometry (GC-MS/MS) (Chang et al., 2016) and liquid chromatography-tandem mass spectrometry (LC-MS/MS) (Rajski et al., 2013) are also included.

2.2.1 Electrospray Ionization

Banerjee and Mazumdar (2012) described the electrospray ionization (ESI) technique as a soft ionization that involves the thermal breakdown of large supramolecules to produce gaseous phase ions without fragmentation. Historically, Fenn introduced the ESI mass spectrometry (MS) technique in 1989 which was used for the analysis of protein molecules. For this reason, Fenn and Tanaka shared the fourth Nobel Prize in 2002 for

the development of ESI-MS and matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS) respectively.

Fortunately, ESI-MS has been developing over the last three decades in instrumental modifications from microspray to nanospray and applications for the analysis of many chemical and biochemical compounds. Also, ESI-MS is one of the essential technique emerged which is frequently used in clinical laboratories applications because of its sensitivity, robustness, and reliability (Ho et al., 2003). Accordingly, anionic surfactants were determined in the samples of water using an advanced triple quadrupole instrument that is made up of ESI-tandem mass spectrometry (ESI-MS/MS). The obtained results were satisfactory according to validation studies of the developed method (Santos et al., 2015). Similarly, dynamic multiple reaction monitoring (DMRM) of liquid chromatography-ESI-MS/MS was employed for the determination of 57 multiple pesticides in a sample of tomatoes that were extracted using QuEChERS method and the obtained results were acceptable after validation (Andrade et al., 2015).

Moreover, ESI-MS has been undergoing a series of modification to improve its efficiency toward the analysis of various kind of compounds. The report of Jafari (2009) shows that ESI-MS technique has been modified to ESI-ion mobility spectrometry (ESI-IMS) cell to increase the ionization rate by increasing the droplets of electrospray for the desolvation of the molecules after increasing the flow rate. The modified technique of the instrument successfully increased from 15-30 % by resolution after the analysis of codeine and morphine molecules. The fact that, forensic and pharmaceutical analysis has been carried out using desorption electrospray ionization mass spectrometry (DESI-MS) since it was introduced in 2004. Moreover, the recent review revealed the successes of DESI-MS technique in the analysis of adulterations, food forensics, food additives, veterinary drugs, natural toxins and pesticides in food samples (Nielen et al., 2011).

Similarly, two kinds of carbamate and three kinds of organophosphorus insecticides were analyzed using the neutral desorption-extractive ESI-MS (ND-EESI-MS) technique in samples of honey without carrying out the sample treatment. The obtained results of the analysis were satisfactory (Deng et al., 2017). Ultra-trace level of nineteen acidic pesticides was determined in the ground and surface water after modification of ESI-MS to paired ion ESI-MS (PIESI-MS). The developed method successfully enhanced the sensitivity that overcame the setbacks of the ESI-MS technique, provided by the less sensitive negative ion mode (Xu & Armstrong, 2013).

The use of LC-MS/MS instrument has been helpful in the field of analytical sciences, food quality controllers for more than a decade in determining pesticide residues (Frenich et al., 2014). The instruments are used to run under the initial factory settings (auto-tuning and Mass-Hunter optimization) without fine-tuning the parameters associated with the ion source (Szerkus et al., 2016). Although, further optimization will increase the peak areas of resulting analytes (Leito et al., 2008). In other words, the sensitivity of the analytical method will be increased if the ESI of the instrument is optimized (Szerkus et al., 2016), specifically for multi-pesticides analysis at the lowest concentration level at various matrices (Kittlaus et al., 2011; Lawal et al., 2018).

Recommendatory, optimization considering the essential parameters of triple quadrupole LC-MS/MS instrument such as starting % organic mobile phase, column temperature (ºC), flow rate (mL/L), injection volume (µL), gas temperature (ºC), gas flow (L/min), nebulizer (psi), sheath gas temperature (ºC), sheath gas flow (L/min), capillary voltage (V) and delta$^{(+)}$ electron multiplier voltage (EMV) (V). Optimizing these parameters plays essential roles for the achievement of a favorable ESI, thereby increasing the sensitivity and efficiency of an analytical method through excellent responses of the instrumental mass spectrometry.

2.3 Liquid Phase Microextraction

Liquid phase microextraction (LPME) techniques are one of the best methods used for sample preparation of any kinds of contaminants in food and drink samples. The techniques are simple, rapid and robust with economic advantages towards successes in food analyses (Abdulra'uf et al., 2012; Farajzadeh et al., 2014). Moreover, LPME is a revered novel technique for food analyses. The methods are enjoying significant modification as reported in the literatures (Abolhasani et al., 2015; Goudarzi et al., 2015; Kailasa & Wu, 2010; Liu et al., 2015; Qin et al., 2015). It would ensure more conveniences leading to enhancement of extraction efficiencies such as lowering the limit of detection (LOD), relative standard deviation (RSD), increasing the relative recovery (RR) and enrichment factor (EF). Research on several modifications of LPME techniques is being carried out to improve the robustness of the methods.

The recent documentation of Pena-Pereira et al. (2010) shows a series of periodical modifications of LPME techniques. These include; single-drop extraction (1995), drop-in-drop extraction (1996), dynamic liquid stage microextraction (1997), microsyringe drop for supporting the dynamic system (1997), use of fiber in LPME (1999), headspace-solid phase microextraction (HS-SPME) (2001), IL-base as the extracting agent (2003), water used as solvent in LPME (2005), ultrasound as factor supporting LPME (2006), microwave radiation as factor supporting LPME (2007), automation of single-drop microextraction (SDME) (2007), combining LPME with flame atomic absorption spectroscopy (FAAS) (2008), using Ionic liquid-based in LPME and dispersion of analytes by thermal desorption device (2009). These LPME techniques are mainly classified into SDME, hollow fiber liquid phase microextraction (HF-LPME) and dispersive liquid-liquid microextraction (DLLME) for various analyses of food samples (Figure 2.1).

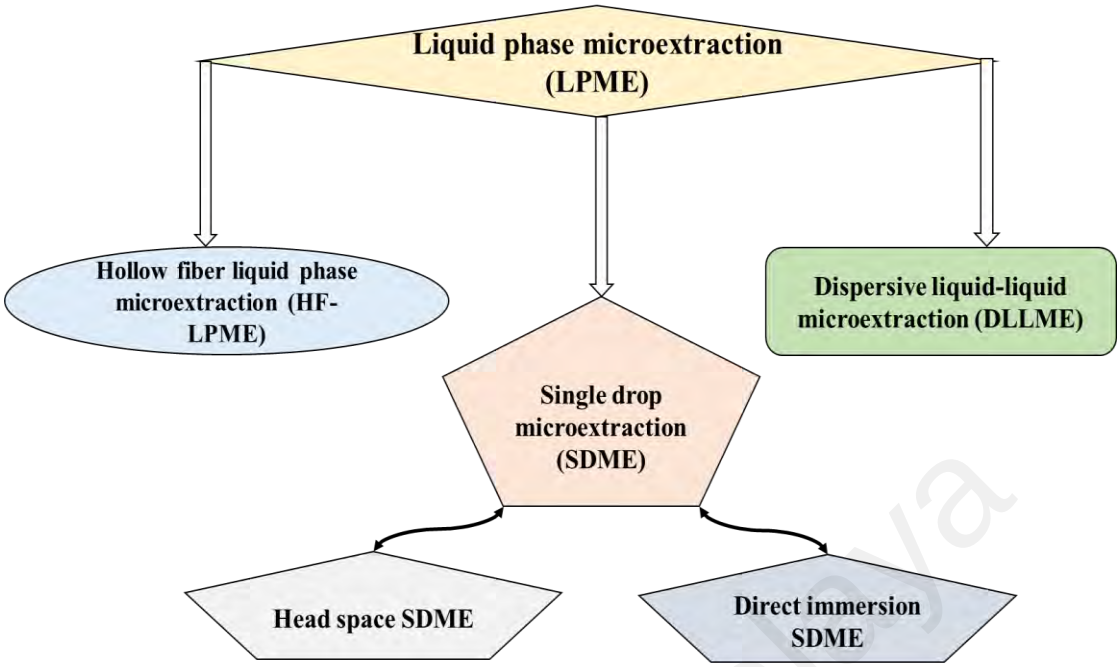


Figure 2.1: Classification of LPME techniques. Adapted from "Recent advances in analysis of pesticides in food and drink samples using LPME techniques coupled to GC-MS and LC-MS: A review", by Lawal et al., 2016.

2.3.1 SDME/HS-SDME

In (1996), Jeannot and Cantwell introduced the single drop microextraction (SDME) methodology. The extraction technique is based on the analytes distributional principle between a single micro-drop of the extracting liquid inclined at the needle's tip into the aqueous phase (analyte solution), or the needle's tip could be placed some few millimeters above the aqueous solution (headspace). Immediately the extraction finished, the micro-drop will be drawn back into the microsyringe to carry out advance electrophoresis or chromatographic investigation (Socas-Rodríguez et al., 2014). The analytes diffused from the sample solution to extracting liquid of SDME and headspace single-drop microextraction (HS-SDME) techniques are illustrated in Figure 2.2 and Figure 2.3 with the permission of Han and Row (2012), and Sarafraz-Yazdi and Amiri (2010), respectively. The diffusion rate depends on the equilibrium distribution constant, time, the volume of analyte solution & extracting liquid, temperature as well as the stability of

the extracting micro-drop (viscosity) during a known agitation degree (Jeannot et al., 2010).

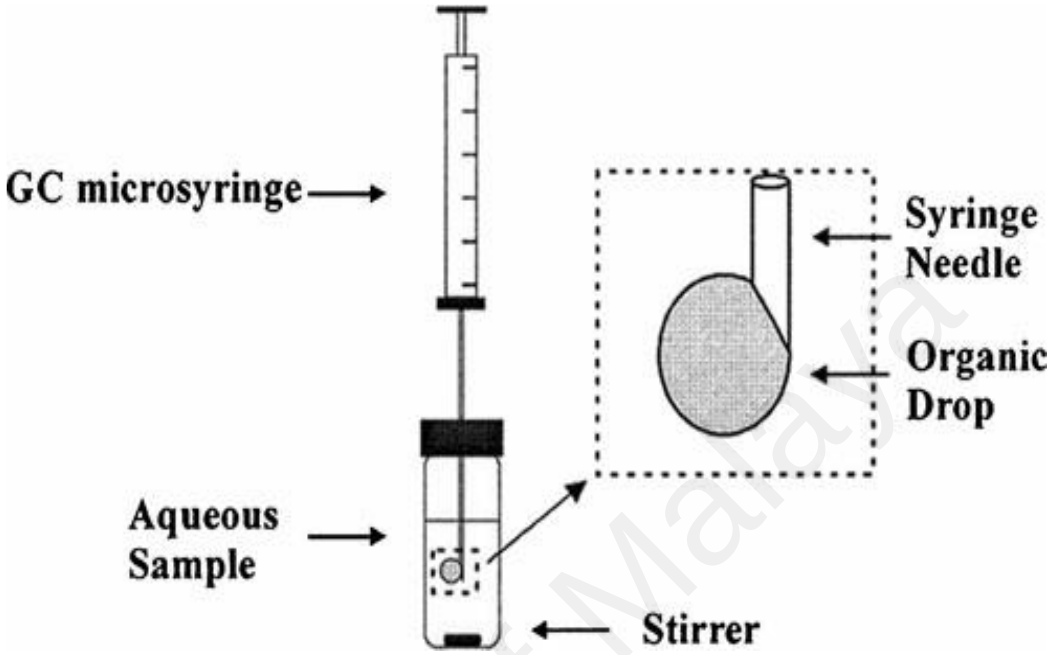


Figure 2.2: The SDME technique of Han & Row (2012), reprinted with permission.

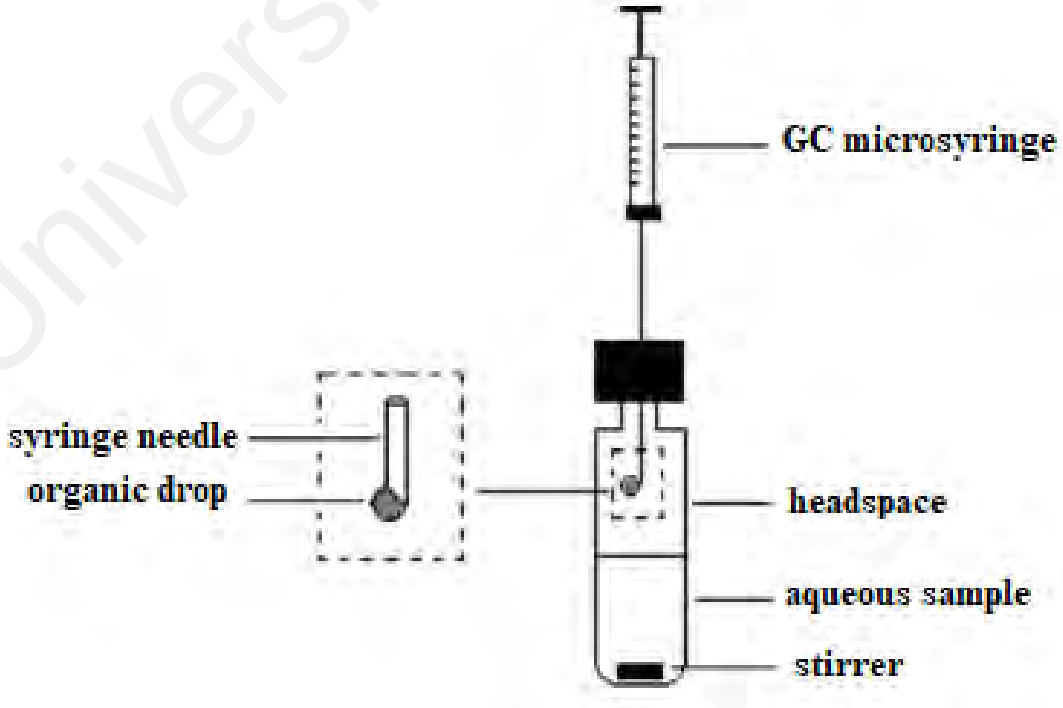


Figure 2.3: The HS-SDME technique of Sarafraz-Yazdi & Amiri (2010), reprinted with permission.

2.3.2 HF-LPME

Hollow fibers are produced from organic polymers (polyesters, polyethersulfone & polypropylene) and inorganic materials (zirconia and titania). The materials are configured in a rod-like shape to increase the extraction rate of the sample analytes (Kobayashi et al., 2000; Tan et al., 2001). The rate of extraction is supported by an optimized revolution per minute (rpm) speed of a magnetic stirrer, and the best organic solvent is selected to penetrate the hollow fiber pores for successfull extraction (Limian et al., 2010). Figure 2.4 illustrated the hollow fiber liquid phase microextraction (HF-LPME) methodological setup.

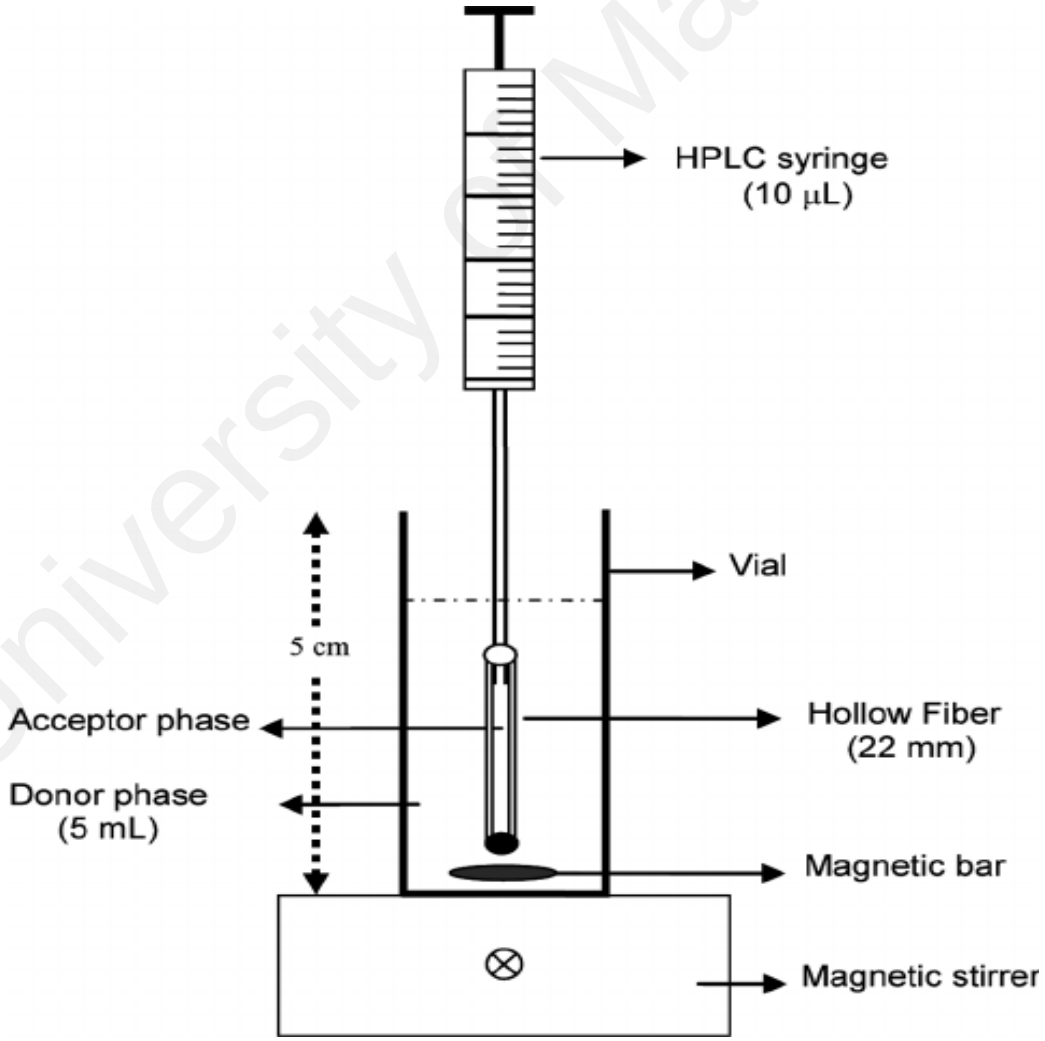


Figure 2.4: The HF-LPME setup of Demirci & Alver (2013), reprinted with permission.

2.3.3 DLLME

In an early study, Assadi and the co-workers introduced the use of DLLME for determination of analytes in a sample (Rezaee et al., 2006). Moreover, Rezaee et al. (2010) further described DLLME as a method that depends on the treble component system of solvents such as cloud point extraction (CPE) and homogeneous liquid-liquid extraction (HLLE). It depends on the selection and use of a suitable solvent (extractant) which provide fastness and simplicity of the microextraction. For instance, there is a need for high-density organic solvents such as chloroform, carbon disulfide, chlorobenzene or Ionic liquid-based, and a disperser solvent that is highly miscible with both aqueous and extractant phases, for example; acetone, acetonitrile (ACN) or methanol. The sample will be accommodated in a conical screw test-tube, followed by the rapid injection of disperser and extractant phases into the test-tube content and later admit for centrifugation. However, this leads to the production of the high amount of turbulence arising to smaller droplets formation that disperses in the aqueous phase. The cloudy solution will be formed shortly creating large surface areas between the extractant and analyte solution, which signifies the achievement of the equilibrium state. Furthermore, sedimental phase appears at the bottom of the test-tube. The benefit of DLLME technique includes; low cost, environmentally friendly, high RRs and EFs (Berijani et al., 2006). The graphical expression steps involved in DLLME method is illustrated in Figure 2.5 designed by Zhang et al. (2013).

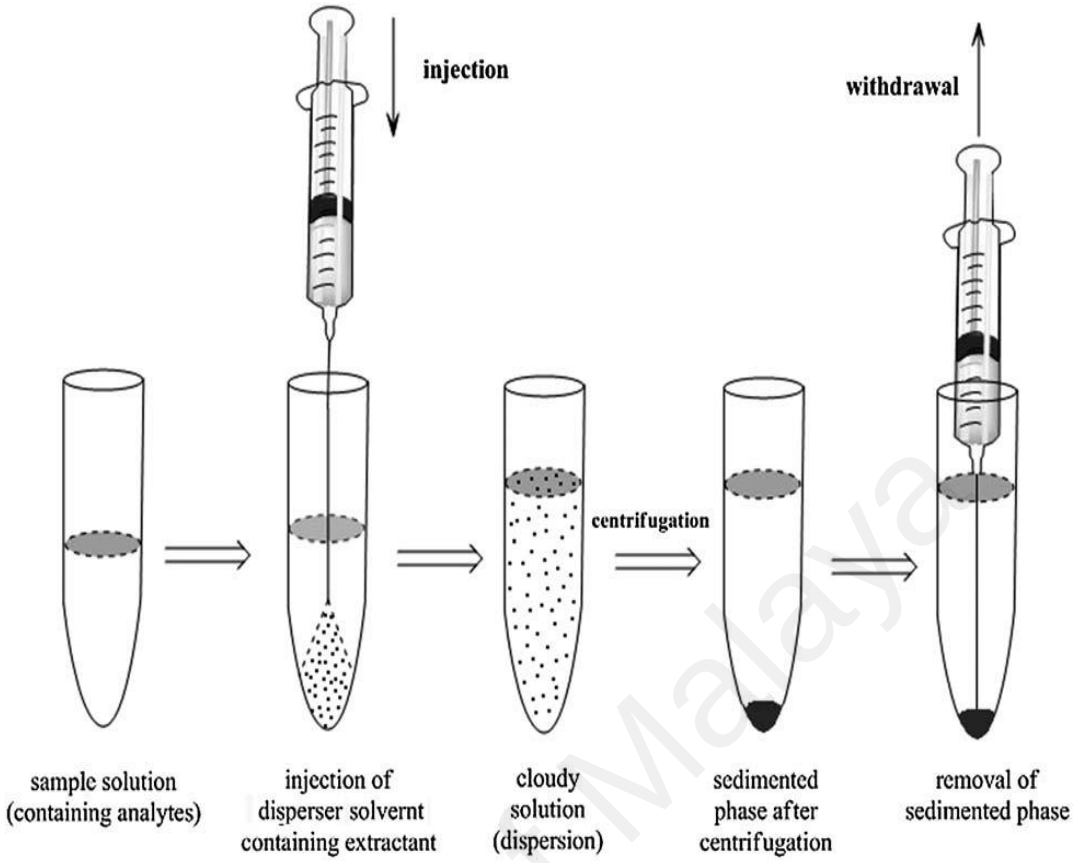


Figure 2.5: DLLME technique of Zhang et al. (2013), reprinted with permission.

2.3.3.1 Ionic liquid-based Extraction

The ionic liquid is a molten salt or solvent having its melting point below or close to room temperature and having poorly coordinated ions. The ionic liquid is prevented from forming a stable crystal lattice due to the delocalization of the charged ions coupled with the organic components (Wasserscheid & Keim, 2000). The historical synthesis of ionic liquid has been dated back to 1914 when ethylammonium nitrate was first synthesized. Since then, there have been a series of synthesis and publications regarding the applications of ionic liquid over the years (Wasserscheid & Keim, 2000). However, the room temperature ionic liquids (RTILs) have been useful for the extraction of targeted analytes in both laboratory and industrial applications. It could also serve as a potential substitute for organic solvents in liquid-liquid extractions of analytical chemistry because of their distinctive properties (Kokorin, 2011). The properties include low flammability, high thermal and chemical stability, vapor pressure negligibility, broad liquid range with

high solvation as well as ability to extract and select organic and inorganic compounds (anions and cations) efficiently (Dimitrijević et al., 2017). The extraction and quantification of carbaryl, carbofuran, fenazaquin, hexythiazox, iprodione, tebuconazole and thiophanate pesticides in banana samples were carried out using RTILs as an extractant in DLLME method coupled with high-performance liquid chromatography-diode array detection (HPLC-DAD) and analytical performance were satisfactory (Ravelo-Pérez et al., 2009).

Similarly, ionic liquid DLLME technique was employed for the extraction of organophosphorus pesticides (OPPs) in samples of water and gave valid results via GC-MS (Cacho et al., 2018).

Furthermore, You et al. (2018) documented the determination of fungicide compounds in a sample of juice using an ionic liquid-based air assisted liquid-liquid microextraction method coupled with HPLC-MS instrument. Also, a strong interaction was reported for the one-step simultaneous extraction of differently polarized acetamiprid, simazine, imidacloprid, tebufenozide and limuron pesticides using aqueous bi-phasic system of 1-butyl-3-ethyl imidazolium dicyanamide ([beim][DCA]) was found better than 1-butyl-3-methylimidazolium dicyanamide ([bmin][DCA]) and 1-butyl-3-methylpyrrolidinium dicyanamide ([bmpyr][DCA]) ionic liquids after computational and experimental approach (Dimitrijević et al., 2017). The recent modificatory use of cholinium ionic liquid coupled with water and surfactant (Triton x-100) were used for the development of the aqueous bi-phasic system (ABS).

The ABS extractant was very promising for the analysis of atrazine, prometryn and simetryn herbicides (Tian et al., 2018). In another study, a polymeric ionic liquid magnetic adsorbent was used for the SPE of chlorpropham, fenthion, phoxim and quinalphos pesticides in water samples. The SPE adsorbent was made up of the ionic

liquid, and polyelectrolyte multi-layer films wrap on magnetic silica assembled layer-by-layer to provide durability, better extraction capability and reusability (He et al., 2017). The findings of Zheng et al. (2015) showed the modification of ionic liquid with graphene into a nanocomposite (IL-GR) and dispersed with gelatin into acetylcholinesterase (AChE) biosensor that is exceptionally stable and sensitive by cross-linking it with glutaraldehyde (GA). The biosensor was used electrochemically for the detection of monocrotophos and carbaryl pesticides in samples of tomato juice after the biosensor was absorbed by biocompatible matrixes.

Moreover, the documentation of Liu et al. (2018) revealed that pyrethroid pesticides were determined in samples of juice using a dispersive magnetic core dendrimer nanocomposites-solid phase microextraction coated with ionic liquid-based. The technique possesses the ability to retain the dendrimer and cyclodextrin molecules. It broadened the potentials of the developed technique for the absorption of targeted pyrethroid analytes in trace amount.

Moreover, ionic liquid-based DLLME solvents have been reported to be used procedurally for the analysis of broad spectrum of analytes in vortex food and beverage samples.

2.3.3.2 The use of DLLME technique in analyses of food and beverage samples

Gure et al. (2015) proposed the use of vortex-assisted Ionic liquid-based DLLME-HPLC for the analyses of 4 types of sulfonylurea herbicides (SUHs) in selected samples of wine. 15 mL conical extraction test-tube was occupied with 2.5 mL of the sample spiked with few drops of the prepared analyte standards, 0.2 mol/L citrate buffer and 10% NaCl. The mixture was brought to 5 mL by addition of ultrapure water. Then, extractant solvent of 80 mg Ionic liquid-based 1-hexyl-3-methylimidazolium hexafluorophosphate ($[C_6MIM][PF_6]$) and disperser solvent of 700 µL methanol was rapidly injected into the

extraction test-tube and centrifuged at 9000 rpm for 5 mins. The sedimental phase droplet was further mixed with 500 µL methanol/water (1:1 v/v) and 0.01% HOAc. The mixture was filtered into 1.5 mL vial through a 0.2 µm membrane, and 3 µL of the filtrate was injected into an HPLC instrument.

Similarly, selected studies of OPPs in herbal medicines, vegetables and fruits was performed and revealed by Yee-Man et al. (2013) using the technique of DLLME. Each of the freshly purchased samples of fruits and vegetables from the local Chinese market was homogenized and refrigerated at −20 °C, similarly for the dried herbs were purchased from Chinese clinic of traditional medicine. The samples were treated individually; ground, sieved through 0.85 mm mesh and spiked with the prepared OPPs standards. 0.1 g for each of the sample analyte was mixed with 1.2 mL ACN (dispersant) in a 15 mL test-tube at 50 °C, vortexed and centrifuged for 3 mins at 3300 rpm. The supernatant was mixed with 80 µL tetrachloromethane (extractant) in a 15 ml conical test-tube, and 5 mL of deionized water was added. The test-tube was vortexed for 30 secs and centrifuged (3300 rpm) for 5 mins. Then, 1 µL of the extract (sedimental phase) was injected into the GC–MS.

It has been demonstrated that the DLLME technique could also be used for determining triazole fungicides possessing lipophilic property (Kmellár et al., 2010). Farajzadeh, Mogaddam, et al. (2014) reported the use of a newly developed method based on temperature elevation in DLLME coupled with gas chromatography nitrogen-phosphorus detection (GC-NPD) for determination of triazole pesticides in samples of honey. The samples were purchased from the local market at Eastern Azerbaijan, Iran, as well as a sample which was obtained from a non-agricultural mountainous region at Kale bar (East Azerbaijan), Iran. 15 g for each of the sample was transferred to a 50 mL volumetric flask, homogenized and diluted with deionized water up to the mark. The

analyte solution was equilibrated for 15 mins, transferred into 70 mL conical extraction test-tube, spiked with 25 µg/kg of the prepared pesticide standards, and placed in a water-bath for 4 mins at 75 ºC. Afterward, 1.5 mL dimethylformamide (dispersant) and 130 µL 1,2-dibromoethane (extractant) were rapidly introduced into the extraction test-tube and allowed to cool for 3 mins under a running tap. Centrifugation was performed at 4000 rpm for 5 mins. Finally, 1 µL of extraction (bottom) phase was retracted and injected into a GC instrument.

PAHs are other kinds of chemical analytes, which could also be analyzed by a modified DLLME technique. Kamankesh et al. (2015) showed the use of microwave-assisted DLLME-GC/MS for the determination of 16 PAHs in grilled meat. 1 kg of the ground sample with 2% fats was prepared by homogeneous mixing it with an appropriate quantity of flavoring agents including salt and grated onions, and it was preserved in the fridge for an hour. Then, the specified quantity for each of the analyte standards was spiked onto 150 g of the prepared sample before being skewered and grilled over red-hot charcoal for 10 mins. Afterward, 10 mL of 50:50 mixture of ethanol and KOH was used to hydrolyze 1 g of the spiked sample in the test-tube and microwaved for 1.5 mins. The microwaved sample was centrifuged (≈ 2700 rpm) for five mins. The supernatant was introduced into another test-tube containing 1 mL each of carrez solution I and II to precipitate out the soluble carbohydrates and proteins. Then, the test-tube was re-subjected to centrifugation at ≈ 2700 rpm for another five mins. 10 mL of the supernatant was decanted into an extraction test-tube and mixed with 15 % NaCl solution, 80 µL ethylene tetrachloride (extractant), 300 µL acetone (dispersant) and 2 µL of 40 mg/kg biphenyl (internal standard). The test-tube content was subjected to centrifugation that lasted for five mins at ≈ 2700 rpm. Lastly, 2 µL of the sedimental phase was retracted and injected into a GC-MS instrument.

Mycotoxins such as aflatoxins and ochratoxin-A have also been analyzed using the DLLME technique. Arroyo-Manzanares et al. (2012) described the use of DLLME coupled with capillary High-performance liquid chromatography laser-induced fluorescence detection (HPLC-LIFD) in the determination of ochratoxin-A in samples of red, rose and white wines, purchased from the township market of Granada, Spain. After series of DLLME optimization using the multivariate experimental design for the selection of the best extracting (500–700 μL), dispersing (800–1000 μL) solvents, and percentage of ionic strength (0 - 5% NaCl) needed for the extraction. Under optimized conditions, 5 mL for each of the sample aliquot and 0.25 g of the NaCl (5%; w/v) were transferred into the 10 mL conical extraction test-tube. 940 and 660 μL of ACN (disperser) and chloroform (extractant) solvents, respectively was rapidly injected into the test-tube and centrifuged for 1 minute at 5000 rpm. At this juncture, the ochratoxin-A analyte settling at the bottom of the test-tube was retracted and evaporated to dryness using nitrogen streaming after it was transferred into another test-tube. Then, it was diluted with 1 mL methanol/water (v/v), filtered and analyzed using capillary HPLC–LIFD instrument.

A novel technique was recently developed and used for the determination of aflatoxin B1, B2, G1 & G2 in pistachios nuts using DLLME after SPE (Rezaee et al., 2014). The pistachio nuts were purchased from the local market of Rafsanjani, Iran. For each sample, 5 g was homogenized and transferred into a 50 mL centrifugal test-tube. Then, 1 g NaCl, 10 mL n-hexane, 10 mg/kg of the prepared aflatoxin standards (spiking agent), and methanol/water (4:1, v/v) were sequentially added into the test-tube and subjected to 20 mins sonication, followed by 4 mins centrifugation (5000 rpm). The resulted supernatant was decanted, and the sedimental phase was diluted to 60 mL with distilled water and loaded into an SPE system. 5 mL of the partially oven-dried extract was transferred into a 10 mL conical test-tube, which contained 200 μL chloroform (extractant) and 1.5 mL

methanol (dispersant). The analyte undergoes centrifugation at 5000 rpm for three mins. The obtained sedimental phase was water-bath evaporated, and the residue was dissolved with a 30 µL methanol and injected into an HPLC instrument.

Furthermore, heavy metals such as copper could also be determined using the DLLME approach, for instance, Shrivas and Jaiswal (2013) proceeded with the analysis of copper in vegetables and cereals using FAAS after DLLME. The cereals and vegetables were sourced from various local markets in India. The samples were stored after they were oven dried for 10 hours at 100 ^{o}C and finely ground into powder. 2.5 g of each sample was ashed and transferred together with 3 mL of H_2O_2 and 7 mL of nitric acid into a 50 mL beaker. The mixture was heated to dryness, and the residue was collected with 10 mL of 1M HCl. 0.5 mL of 1% NaCl, ascorbic acid, 0.0006 M 2,9-dimethyl-1,10-phenanthroline (DPT), buffer solution and 13 mL of 5 ng/mL standard aqueous solution of Cu (II) were transferred into a conical extraction test-tube. Then, 0.5 and 0.2 mL of chloroform and 0.02 M N-phenyl benzimidoylthiourea (PBITU) were injected into the tube as dispersing and extractant solvents respectively. Finally, centrifugation (755 rpm) was carried out for two mins, and the organic phase was carefully introduced into the test-tube and diluted with 400 µL ethanol before being nebulized into FAAS.

Therefore, the results of the reviewed DLLME technique for the analyses of food and beverage samples are presented in Table 2.1.

Table 2.1: Various DLLME applications on the analysis of food and beverage samples

Samples	Analyte	Ext. Solvent	ESV (µL)	SS (rpm)	ET (min)	LODs (µg/kg)	LOQs (µg/kg)	RRs (µg/kg)	RSD (%)	Detection	Ref
Wine	4 Sulfonylurea herbicides	IL-based	80	9000	5	≤ 6.6	≤ 22	80–104	≤ 6.9	HPLC	A
Fruits, vegetables and herbs	OPPs	Tetrachloro methane	80	3300	5	≤ 0.0005	≤ 0.0014	70–119	≤ 10	GC-MS	B
Honey	5 fungicides	1,2-dibromoethane	130	4000	5	≤ 0.21	≤ 1.1	97-100	≤ 4	GC-MS	C
Grilled meat	16 PAHs	Ethylene tetrachloride	80	2683	5	≤ 0.3	≤ 1	85-104	≤ 9	GC-MS	D
Red, rose and white wines	Ochratoxin A	Chloroform	660	5000	1	0.006	nr	92–98	≤ 4.1	HPLC–LIF	E
Pistachios	4 aflatoxins	Chloroform	200	5000	3	0.04	nr	85–93	≤ 13	HPLC	F
Cereals and vegetables	Copper concentration	Chloroform	200	755	2	0.05	≤ 0.16	94–98	≤ 3.5	FAAS	G

ESV, extraction solvent volume; ET, extraction time; IL-based, ionic liquid-based; SS, stirring speed; LOD, limit of detection; LOQ, limit of quantitation; RR, relative recovery; RSD, relative standard deviation; nr, not reported; PAHs, polycyclic aromatic hydrocarbons; OPPs, organophosphate pesticides; Ref, references; A, Gure et al. (2015); B, Yee-Man et al. (2013); C, Farajzadeh et al. (2014); D, Kamankesh et al. (2015); E, Arroyo-Manzanares et al. (2012); F, Rezaee et al. (2014); G, Shrivas and Jaiswal (2013)

2.3.4 Limitations of LPME Techniques and Recommendation

The techniques of LPME are useful for extraction of various analytes in food matrices subjected to creative modifications. Such modifications ensure more conveniences and enhancements of extraction efficiency by lowering the LOD, RSD, and increasing EFs and RRs.

LPME techniques and the various antecedently reviewed modifications are justifiably reliable preconcentration methods for multi-targets analysis of samples, which consumed low organic solvent with high simplicity, sensitivity, fastness, precision, accuracy, and showing low LODs, high EFs, and RRs. We hope that the LPME techniques reviewed will serve as a reference for providing useful (positive) management tools in solving problems such as regulatory enforcement in controlling the quality of food materials globally.

The limitations of LPME techniques are such that, the majority of the organic solvents used by these techniques are toxic, i.e., not 100% compatible with green chemistry. Also, the selection of the best solvent is difficult as well as the appropriate volumes to be used for the analysis because they depend on the nature of the sample and analyte. Moreover, other crucial requirements for the preliminary stages before proceeding with the main extractions include; the best agitation speed (rpm), ionic strength of the extraction medium (%), extraction time (min), and temperature. Moreover, instability of the extracting micro-drop during agitation may affect the SDME method, and HF-LPME showed to be inefficient towards the extraction of high polar analytes in a sample.

Recommendatory, chemometrics optimization of essential parameters in LPME techniques could take care of the setbacks. Also, the use of non-toxic Ionic liquid-based is recommended for the microextraction of analytes considering its results, which reportedly proves to be more efficient and environmentally friendly than the organic

solvents. Moreover, other non-toxic alternative green solvents could be used in LPME techniques such as; the supercritical and liquid CO_2 is the recently developed natural and renewable low transition temperature mixtures (LTTMs).

Meanwhile, attention has been drawn recently towards the use of simple glassware in sample preparation (Orso et al., 2014). The method that is quick, easy, cheap, effective, rugged and safe technique (QuEChERS) couple to dispersive solid phase extraction (d-SPE) to overcome the setback challenges of the previous techniques for pesticides determination in fruits and vegetables (Grimalt & Dehouck, 2016).

2.4 QuEChERS-dSPE

Primarily, solid phase extraction (SPE) is a developed technique from the LLE method that is made up of many kinds of sorbent materials such as polymeric solids and porous carbon. The materials could also exist as particles of carbon nanostructures, e.g., nanodymonds, nanotubes, nanohorns, nanocones, etc. (Valcarcel et al., 2008). A simple SPE is miniaturized by devices that include; coated fibers, membranes, and stirrers. These were transformed into a cartridge known as conventional SPE (Lawal et al., 2018b).

On the other hand, d-SPE is used as an alternative and modified form of the conventional SPE, which was initially suggested as a method used for cleaning matrix substances by adding a small quantity (≈50 mg) of the sorbent material into the extraction sample without conditioning it (Anastassiades et al., 2003). The step involves the addition of ACN usually as the extracting solvent (buffering at pH 5 – 5.5). The significant characteristics of ACN over the use of other extraction solvents such as acetone and ethyl acetate are compatible with gas chromatography (GC) and very applicable in the reverse-phase of liquid chromatography (LC) (Anastassiades et al., 2003). Also, the solvent is very suitable for extracting polar and non-polar analytes (“RESTEK”, 2015). In addition, the solvent is not favorable for the extraction of highly lipophilic materials such as fats,

waxes, and pigments (Anastassiades et al., 2003). Extraction salt can be added to a small (weighed) sample size in a centrifuge tube before the tube undergoes series of vortexing (shaking) and centrifugation. Subsequently, partitioned phases will occur after centrifugation at different levels depending on their densities. Notably, the base-sensitivity and stability of pesticides can be improved if octadodecyl bonded silica (C_{18}), primary secondary amine (PSA), and graphitized carbon black (GCB) in d-SPE to cleanup the interferences in the organic phase (Biziuk & Stocka, 2015).

The most important property of C_{18} as a sorbent material for the cleanup purpose is its excellent ability to remove the non-polar interferences such as lipids and fats (Aranzana, 2010). This property of C_{18} helps to improve the detection of analytes such as pesticide residues in the extracts of complex (sample) matrices without significant adverse effects on their responses ("Waters", 2011). Meanwhile, PSA aids to eliminate sugar molecules, polar, organic and fatty acids but the recent report shows that PSA is sometimes not capable of removing excessive interferences in a complex sample of fruits and vegetables (Zhao et al., 2012). Besides, GCB helps to take-off pigments such as chlorophyll and steroids in analyte solutions. Unfortunately, limited use of GCB in d-SPE cleanup since it can circumstantially eliminate 50 % of the targeted pesticides with a planar aromatic group such as hexachlorobenzene, thiabendazole and cyprodinil fungicides (Łozowicka et al., 2017).

Moreover, a reliable and efficient d-SPE cleanup methodology can also be achieved, if the appropriate amount of salts are added to the homogenized sample. This is because of their crucial roles; e.g., magnesium sulfate ($MgSO_4$) aids in the absorption of water molecules that are mixed with the analytes in the organic phase, and sodium chloride (NaCl) helps in moving the analytes to the organic phase, and it further helps to separate

the organic phase from the aqueous phase (containing carbohydrates and sugars) (Aranzana, 2010; "RESTEK", 2015).

2.4.1 QuEChERS-dSPE Methodology

The QuEChERS methodology is based on the modified feature of d-SPE, which was initiated by Anastassiades et al. (2003) in the determination of pesticide residues. The method has been successfully used for sample treatment due to its flexibility and extraction efficiency of targeted analytes (Johnson, 2012). Moreover, the technique provides more acceptable extraction cleanups of analyte interferences to yield excellent results after chromatographic instrumentation (Petrarca et al., 2016). Comparatively, such method is simpler, with less time, less labor and less consumption of organic solvent than the traditional or conventional SPE method. Also, multiple SPE analysis will be carried out to capture a similar amount of residues in a single QuEChERS-dSPE analysis (Liu et al., 2014). Thus, the QuEChERS-dSPE methodology is illustrated in Figure 2.6.

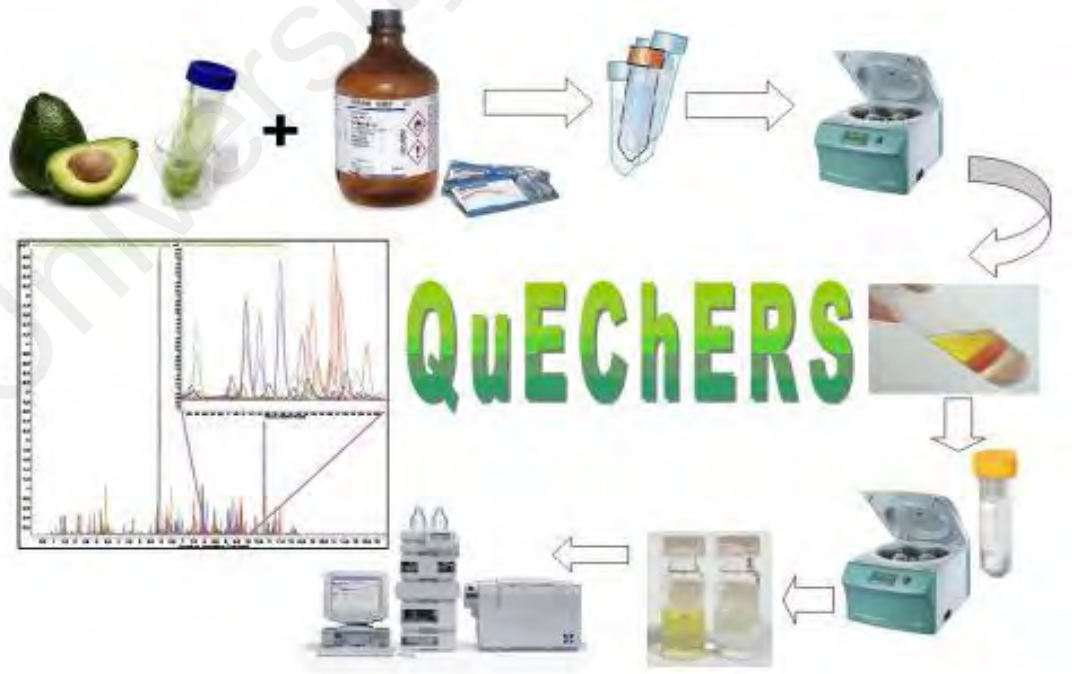


Figure 2.6: QuEChERS-dSPE methodology of Arroyo-Manzanares et al. (2013), reprinted with permission.

Recently, QuEChERS-dSPE technique was regarded as one of the best alternative methods endorsed by the Association of Official Analytical Chemists (AOAC) International for determining residue of multi-pesticides in vegetables and fruits (Lehotay et al., 2007). The most commonly employed kits and experimentations related to QuEChERS-dSPE methodology are developed under the AOAC official 2007.01. (Method A) and European EN 15662 (Method B) as illustrated in Figure 2.7. These kits are used based on the nature and type of food sample, for example, there are special kits meant for general food samples, the samples with extremely colored extracts, the samples with waxes or fats extracts and the samples with fats and pigment extracts ("RESTEK", 2015).

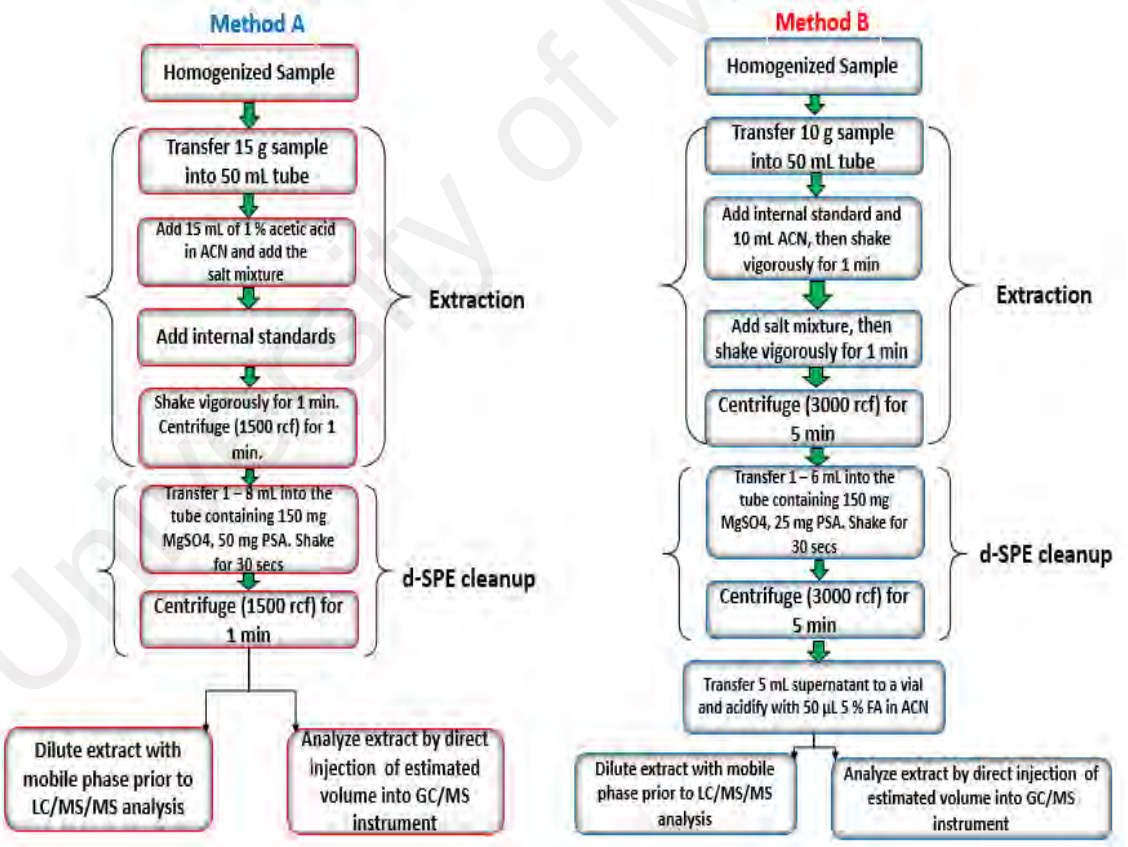


Figure 2.7: Schematic QuEChERS-dSPE methods. Adapted from "Recent modifications and validation of QuEChERS-dSPE coupled to LC-MS and GC–MS instruments for determination of pesticide/agrochemical residues in fruits and vegetables. Review", by Lawal et al., 2018.

The technique continues to gain popularity through various modifications by developing appropriate methodological kits for either QuEChERS extraction or cleanups (Oshita & Jardim, 2015).

2.4.2 The Various Modifications of QuEChERS-dSPE Techniques for Determination of Pesticide Residues in Vegetable and Fruit Samples

Over the years, there have been increasing research interests in the original (traditional) and a modified method of QuEChERS-dSPE for sample preparations for the determination of pesticides residue in fruits and vegetables. It is mainly based on the use of ACN (as extractant), salts (for partitioning), sorbent materials (for cleanups) and technical modifications (Rizzetti et al., 2016).

The continuous application of organic and bio-pesticides in agricultural practices leads to close monitoring of their residual levels in the sample of fruits and vegetables (Lamichhane et al., 2016). In this regard, Romero-González et al. (2014) reported the use of QuEChERS methods for analysis of 14 commonly used bio-pesticides in vegetable and fruit samples. These samples include cucumber, orange, pepper, strawberry, and tomato, purchased in Spanish supermarkets (Almeria). 50 mL conical test tube containing 10 g of each blended sample and 10 mL ACN with 1 % HOAc (v/v). 1 g of sodium acetate (NaOAc) and 4 g of anhydrous $MgSO_4$ were added into the tube after it was shaken for 1 min. Centrifugation was carried out on the tube for 5 min at 5000 rpm after shaking the tube for 1 min. 2 mL autosampler containing 1 mL of the resulting supernatant was introduced into ultra-high performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) for analysis. The technique is more efficient when compared with other reviewed methods based on the resulted LODs (≤ 3 µg/kg), LOQs (≤ 10 µg/kg), RSD (≤ 28 %) and average RRs (70 – 112 %). The method shows its

potential applicability in the determination of bio-pesticides to a great variety of vegetables and fruits.

The health implication of cyazofamid (agrochemical) was recently documented and shown to cause respiratory problems (Jackson et al., 2012). Thus, the ultra QuEChERS (extraction kits) was employed for extraction/determination of cyazofamid and its metabolic compound of 4-chloro-5-p-tolylimidazole-2-carbonitrile (CCIM) in apple, cabbage, mandarin, green pepper, and potato (Lee et al., 2014). The samples were procured randomly from the markets (Republic of Korea) and homogenized individually. Then, 10 mL ACN was transferred to a centrifuge tube (50 mL) containing 10 g of the blended sample (spiked with 10 – 100 µg/kg analyte standards). Ten min was sufficient to agitate the tube at 250 rpm before the addition of the extraction kits. The tube was shaken for 2 min before subjecting it to 5 min centrifugation at 3,500 rpm. 1 mL of supernatant was transferred into a 2 mL centrifuge tube containing d-SPE cleanup salts, and the tube was centrifuged (15,000 rpm) for 2 min. Then, 400 µL supernatant was mixed with the 50 µL solution- mixture (1 % formic acid in ACN) to mash-up the matrix. The mixture was analyzed with an LC-MS/MS instrument. The method is useful and proved to be quick, robust, sensitive and selective in comparison with other reviewed methods based on the obtained LOQs (2 – 5 µg/kg) and RRs (75.1 – 105.1 %). The method is potentially applicable to the analysis of cyazofamid and CCIM in diverse food materials.

Recently, a study argued that the pesticide residues in Colombian (Bogota) cultivated tomatoes had not been extensively characterized (Arias et al., 2014). Based on this reason, Arias et al. (2014) monitored 24 pesticides belonging to the class of fungicides and insecticides using QuEChERS-dSPE (Restek Q-Sep kits) for the extraction and cleanup of the analytes. In this method, a 50 mL centrifuge tube containing 10 g of homogenized

samples was shaken vigorously for 1 min after adding a 15 mL mixture of 1 % HOAc in ACN. Then, 1 g NaOAc and 6 g anhydrous $MgSO_4$ were added to the mixture of the centrifuge tube and was shaken for another 1 min before centrifugation at 4500 rpm for 5 min. Subsequently, 10 mL supernatant, 150 mg anhydrous $MgSO_4$ and 25 mg PSA collectively, were introduced into a 15 mL centrifuge tube. The mixture was centrifuged for 2 min at the rate of 4500 rpm after being shaken for 30 secs. Then, a 0.22 µm filter was employed to filter the supernatant before injection into the UHPLC-MS instrument. The method provided RRs (71.3 - 112.3 %), LODs (1 – 200 µg/kg) and LOQs (10 – 800 µg/kg). The technique could well be utilized in an optimum condition to provide excellent results in other food materials apart from fruits and vegetables.

Furthermore, the high usage of fungicides and insecticides during cultivation or storage of fresh fruit and vegetables has become a significant concern that requires analytical attention (López-Fernández et al., 2012). Bilehal et al. (2014) studied 5 pesticides (fungicides and insecticides) in Indian pomegranate and mango using the QuEChERS-dSPE method. 15 g of each blended sample was extracted with 15 mL ACN after addition of 10 g anhydrous sodium sulfate (Na_2SO_4) for 3 min at 2000 rpm. Then, the d-SPE salt (25 mg PSA) was used to clean up 1 mL supernatant (aliquot) in a 10 mL centrifuge tube. The resulting extract was slightly evaporated (at 50 ^oC) to dryness using a stream of nitrogen flow and filtered through the 0.2 µm membrane. Finally, a reversed-phase ultra-high performance liquid chromatography (RP-HPLC) was used to analyze the filtrate. The method is simple, rapid but could be less effective as compared with other reviewed methods based on the obtained results of RRs (87.0 - 96.0 %) and RSD (0.8 - 20.5 %).

Moreover, Carneiro et al. (2013) have demonstrated the use of QuEChERS technique for the determination of 128 pesticides in banana samples. The samples were collected from the pesticide-free areas of Brazil (Minas-Gerais); the extraction occurred in a 50 mL

centrifuge tube containing 10 g of homogenized sample and spiked with estimated analytes standard solutions. Then, 15 mL ACN was mixed with the tube's content, followed by the addition of 1 g NaOAc and 4 g anhydrous $MgSO_4$. The mixture was shaken and agitated for 1 and 9 min (4000 rpm) respectively. Then, d-SPE was carried out on the obtained supernatant in a 50 mL centrifuge tube which contained 1.5 g anhydrous $MgSO_4$. The tube was shaken for 1 min, centrifuged (4000 rpm) for 9 min and the resulting supernatant was introduced into a 2 mL autosampler vial before analysis using ultra-high performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) instrument. The simple modified technique is more efficient as compared with other methods reviewed because it provided excellent analytical performances; RRs (70-120 %), LODs (≤ 5 µg/kg), LOQs (≤ 10 µg/kg) and RSD (≤ 20 %). These results demonstrated the feasibility and applicability of the method for the routine analysis of pesticide residues and other contaminants in samples containing a large quantity of water.

In a similar approach, Jadhav et al. (2015) reported the use of a modified QuEChERS technique, which involved the use of 10 mL ethyl acetate (EtOAc) containing 1% HOAc as an extraction solvent. The solvent was used for the determination of some agrochemicals in 10 g (homogenized) sample of Indian fruits and vegetables. The samples include bitter gourd, capsicum, curry leaves, drumstick, grape, mango, and okra, respectively. 10 g anhydrous Na_2SO_4 and 0.5 g NaOAc was added to a 50 mL centrifuge tube containing each of the samples. The tube was vortexed for 2 min and centrifuged (5000 rpm) for 5 min. The 5 mL supernatants underwent d-SPE cleanup with 25 mg PSA in a 10 mL centrifuge tube and shaken for 30 secs before centrifugation. The 2 mL of the cleaned extract was transferred into 10 mL test tube containing 10 µL of 10 % diethylene glycol (DEG), and the mixture was evaporated to dryness at 35 °C under nitrogen stream flow. Then, methanol was added to dissolve the obtained residue (1:1). The solution was

mixed with 2 mL ammonium formate (20 mM in H_2O), ultrasonicated for 1 min, vortexed for 30 secs, followed by 5 min centrifugation (10,000 rpm). The extracted aliquot was filtered through 0.2 μm pores of the nylon-66 filter before analysis with LC-MS/MS instrument. The results obtained by this method are satisfactory with RRs (70 – 120 %), low RSD (< 20 %) and LOQs (0.2 – 1 μg/kg). This method could be potentially applicable as a standard regulatory tool for the routine analysis of agrochemical residues (basic or acidic compounds) in fruits and vegetables. In another report, pesticides residue analysis was carried out on tomato samples, as it is one of the most widely consumed vegetables (Golge & Kabak, 2015).

The fact that, Turkey is ranked fourth worldwide in tomatoes cultivation. Unfortunately, there is no record of pesticides residue monitoring in the product (Karaağaç, 2015). Hence, Golge and Kabak (2015) have determined 109 residues of pesticide in the tomatoes cultivated in the areas of Antalya and Mersin (Turkey). QuEChERS method was employed in which 15 g from the blended 1 kg (representative) sample was placed in a 50 mL centrifuge tube. 15 mL ACN/HOAc (99:1 v/v) was added and shaken until the solvent was uniformly mixed followed by addition of NaOAc (1.5 g) and $MgSO_4$ (6 g) before the tube was centrifuged (5000 rpm) after it was vortexed for 1 min. d-SPE was carried out on the supernatant (4 mL) after it was mixed with PSA (0.2 g) and $MgSO_4$ (0.6 g) in 15 mL centrifuge tube. Then, vortexed for 1 min and centrifuged the tube's mixture at 5000 rpm. Finally, the resulting supernatant was analyzed using an LC-MS/MS instrument. The developed method yielded satisfactory results with RRs (77.1 - 113.2 %), LODs (0.5 - 10.8 μg/kg), LOQs (1.3 - 30.4 μg/kg) and RSD (< 20 %). The method could be potentially applicable to the analysis of other fruit and vegetable samples with high water content.

The recent recommendatory report shows that the determination of ethylene thiourea (ETU) (precursor of highly effective ethylenebisdithio-carbamate fungicides) in food materials is highly demanding because it has been known to cause thyroid cancer (Singh & Srivastava, 2013). Thus, Zhou et al. (2013) revealed the use of the QuEChERS-dSPE technique for the extraction of ETU in samples of cucumber and potato. A 10 g of the homogenized sample was transferred into a 50 mL centrifuge tube, and the sample was spiked with ETU standard solution before adding 5 mL alkaline ACN (containing 1 % ammonia monohydrate). The mixture was centrifuged (3800 rpm) for 5 min after it was vortexed for 2 min. The extraction process was repeated on the same tube, and the resulting supernatants were transferred into another 50 mL centrifuge tube containing 4 g anhydrous $MgSO_4$ and 1 g NaCl. The mixture was vortexed for 1 min before centrifugation for 5 min. 1 mL of supernatant was introduced into a 2 mL centrifuge tube containing $MgSO_4$ (100 mg) and PSA (50 mg). The tube was centrifuged for 5 min after shaken it for 1 min. Finally, the supernatant obtained was analyzed with LC-MS/MS instrument after filtration (0.22 µm pore). The method resulted in low LODs (0.025 - 0.15 µg/kg), LOQs (0.1 - 0.5 µg/kg) and RSD (< 18 %) with good RRs (60 – 110 %).

Modification of the QuEChERS-dSPE method was also employed for the determination of quaternary ammonium pesticides. It was based on the environmental concerns, which shows that the high residues of such compounds can cause disruption of endocrine glands and could affect the reproductive system in animals (Hoy et al., 2015). The modified technique was documented by Gao et al. (2015) for the determination of chlormequat and mepiquat pesticides; 5 g for each of the homogenized samples of potatoes and pears were weighed and placed into a 50 mL centrifuge tube, respectively. It was then vortexed for 30 sec after the addition of 3.5 mL ACN and 35 µL of the internal standard triphenyl phosphate (TPP). Then, the tube was centrifuged at 6000 ×g (≈ 7300 rpm when the rotor's radius is 100 mm) for 10 min after adding 3 g anhydrous $MgSO_4$

and vortexed for 1 min. d-SPE was carried out on 1 mL of the supernatant in a 2 mL centrifuge tube containing 125 mg anhydrous $MgSO_4$, 25 mg GCB and 25 mg sorbent of PSA. The tube was shaken for 1 min and centrifuged at 13,300 ×g (≈ 10900 rpm when the rotor's radius is 100 mm) for 10 min. The extract was filtered through a 0.22 μm pore membrane before the LC-MS/MS analysis. The results obtained were satisfactory [RRs (83.4 - 119.4 %), LOQs (70 – 700 μg/kg), RSD (< 7.0 %) and LODs (21 – 210 μg/kg)] when compared with other reviewed methods. Thus, the method can be used for the routine analysis of CQ and MQ in fruits and vegetables.

In another recent report, Abad-Fuentes et al. (2015) used modified QuEChERS method for determining succinate dehydrogenase inhibitors (SDHI) fungicide (Isopyrazam, Penthiopyrad, and Penflufen) residues in Spanish samples of vegetables and fruits. 15 g of the homogenized sample was mixed with 150 μL of 50 mg/L of TPP, 6 g anhydrous $MgSO_4$ and 1.5 g NaOAc in a 50 mL centrifuge tube. 15 mL ACN/HOAc (99:1 % v/v) extracting solvent was added into the tube's content and vortexed (1 min) before centrifugation at 2200 rpm for 5 min. 1 mL of the supernatant was cleanup with d-SPE salt [PSA, and C_{18}), 150 mg anhydrous $MgSO_4$ and GCB] in a 2 mL centrifuge tube, vortexed (1 min) and centrifuged (2200 rpm) for 5 min. Finally, the supernatant was filtered through 0.22 μm Teflon paper before analyzing it with UPLC–MS/MS instrument. The method could be used for monitoring different kinds of pesticides in a variety of food samples because of the excellent results obtained [LODs (0.8 – 2 μg/kg), LOQs (≤ 10 μg/kg), RRs (80 – 136 %) and RSD (< 20 %)].

Multi-walled carbon nanotubes (MWCNTs) is a category of carbon nanotubes, which has been used recently in the modification of QuEChERS-dSPE technique. The MWCNTs is used explicitly as a reversed d-SPE sorbent for cleanup of samples with a high proportion of pigments. It is because the MWCNT materials possess a large surface

area and have a unique structure (Lee et al., 2015). The use of MWCNTs as a d-SPE cleanup tool after QuEChERS extraction was recently reported by Wu et al. (2015) for determination of 16 fungicides (amide) in the samples of strawberry, grape, celery, and cabbage. In this method, 5 g of homogenized sample was added to a 50 mL centrifuge-tube followed by the addition of the 500 µL analyte (spiked) standard solutions. The mixture was vortexed for 15 secs and allowed to stabilize for an hour. 9.5 mL ACN was introduced into the tube and was shaken before addition of 2 g NaCl, followed by 1 min vortex and 3 min centrifugation (5000 rpm). 50 mL volumetric-tube containing 1 mL supernatant was diluted to 5 mL with water to yield 20% ACN. Then, the solution was mixed with HOAc to adjust the pH range (3-6). The mixture underwent extraction after addition of 10 mg MWCNTs, shaken for 1 min and centrifuged (9,000 rpm) for 3 min. Later on, 10 mL acetone was introduced into the mixture after the supernatant was thrown away. Then, 2 min centrifugation (9,000 rpm) was further carried out after 1 min vortexing. Evaporation to dryness was conducted on the resulting supernatant (5 mL) under the flow of nitrogen stream. The resulting residue was dissolved in 2.5 mL with a combined solution of ACN/H_2O (20:80 v/v) plus 0.1 % methanoic acid. Finally, filtration using 0.22 µm pore membrane filter was carried out on the resulting solution, and the 10 µL of the filtrate was analyzed with a UHPLC–MS/MS instrument. Likewise, the use of MWCNTs sorbent material for sample cleanup has more advantages compared to PSA because it successfully provided lower LOQs (≤ 10 µg/kg), LODs (≤ 3 µg/kg) and RSD (< 10 %) as well as acceptable RRs (72.4 - 98.5 %).

In another study, Han et al. (2015) documented the use of MWCNTs for the determination of 70 residues of pesticides in garland chrysanthemum, lettuce leaves, and leek. 50 mL of centrifuge tube containing each homogenized sample (10 g) was mixed with 10 mL ACN, and the mixture was shaken for 2 min. The tube was centrifuged (3800 rpm) for 5 min followed by the addition of NaCl (1 g) and $MgSO_4$ (4 g). The mixture was

shaken (1 min), and 1 mL of the supernatant was poured into a 2 mL centrifuge tube containing anhydrous $MgSO_4$ (150 mg) and 10 mg of the MWCNTs sorbent. Then, the tube was vortexed (1 min) and subjected to 3 min centrifugation (10,000 rpm). Finally, 0.22 µm filter (nylon-syringe) was used to filter 1 mL of the supernatant before LC-MS/MS analysis. The method provided lower LOQs (0.3 - 7.9 µg/kg) and LODs (0.1 - 2.4 µg/kg) at RSD (< 14.2 %), with acceptable RRs (74 – 119 %) and could be used for routinely determination of pesticides in foods.

Glufosinate is a non-selective, broad spectrum and post-emergence herbicide known to inhibit the synthesis of enzyme glutamine which causes health-related issues (Rojano-Delgado et al., 2014). Thus, a newly modified QuEChERS technique [quick polar pesticides (QuPPe)] was developed by the reference laboratories of the European Union for the analysis of these pesticides (Anastassiades et al., 2015). The developed method utilized methanol and the sorbent of MWCNTs as extracting solvent and cleanup material, respectively, for the highly polar pesticides. The method was employed by Han et al. (2016) for extraction of glufosinate pesticide in 10 g homogenized samples of apples, bananas, celeries, eggplants, grapes, leeks, papayas, and tomatoes purchased from the local market (Beijing, China). The homogenized samples were individually transferred into a 50 mL centrifuge tube, and 10 mL of methanol was introduced into the tube and vortexed for 2 min. The tube was centrifuged for 5 min at 4,000 rpm. Then, 1 mL of the resulting supernatant was transferred to a 2 mL centrifuge tube containing 5 mg of MWCNTs. The tube was vortexed (1 min) before centrifugation (10,000 rpm) for 1 min, and the resulting supernatant was filtered through a 0.22 µm membrane before analysis with an LC-MS/MS instrument. The method can be used efficiently for monitoring glufosinate routinely in plant (sourced) foods because of its accuracy, sensitivity, reliability, and efficiency, as it showed acceptable LOQs (1 – 10 µg/kg), LODs (0.3 - 3.3 µg/kg) and RRs (80 – 108 %) at RSD (0.6 – 9.8 %).

Blue and green molds (fungi) cause many types of diseases to citrus fruits during transportation or storage, which resulted in high rates of continuous usage of post-harvest fungicides such as Imazalil (Altieri et al., 2013). Based on this fact, Uclés et al. (2015) replaced the cleanup technique with sorbent mixtures that include yttria-stabilized zirconium dioxide and MWCNTs for determination of 16 commonly use post-harvest fungicides in pear and orange samples. Each sample was homogenized, and 10 g of it was mixed with ACN (10 mL) in an automatic axial-extractor and shaken for 4 min. The extract was mixed with 1 g each of trisodium citrate dihydrate and NaCl, 4 g anhydrous $MgSO_4$ and 0.5 g disodium hydrogen citrate sesquihydrate in a 50 mL centrifuge tube. The tube was placed in an automatic axial-extractor and shook for another 4 min before 5 min centrifugation (3500 rpm). Then, 5 mL of the acquired supernatant was introduced into a 15 mL centrifuge tube containing MWCNTs (50 mg), PSA (125 mg), yttria-stabilized zirconium dioxide (175 g), and anhydrous $MgSO_4$ (750 mg). The mixture was centrifuged (3500 rpm) for 5 min after it was vortexed for 30 sec. Finally, the resulting supernatant was diluted with a known amount of ACN/H_2O mixture before spiking it with 10 µL dimethoate-d6 (2.5 µg/mL) to obtain 0.05 mg/kg. Then, 5 µL (aliquot) was injected for analysis with an LC-ESI-MS/MS. The performances [RRs (77 – 120 %), LOQs (≤ 10 µg/kg) and RSD (< 10 %)] obtained from the developed technique were satisfactory.

Furthermore, Qin et al. (2016) showed the application of MWCNTs sorbent cleanup material in a more advanced technique for removal of sample matrix interferents using a multi-plug filtration cleanup (m-PFC). Thus, m-PFC is made up of a column composing of sorbent materials including MWCNTs, $MgSO_4$, and PSA (Zhao et al., 2013). The technique was used to determine the residue of pesticides in purchased samples of kiwi fruit and juice (Beijing, China).

10 mL ACN was transferred into a 50 mL centrifuge tube containing 10 g of the ground sample or juice sample. The tube was vortexed for 1 min before introducing 1g of NaCl and 4 g anhydrous $MgSO_4$, while 3 g NaCl was added to the juice sample. Water-bath containing ice was used for cooling the tube before shaking it for 1 min and 5 min centrifugation (3,800 rpm). Then, the m-PFC procedure was carried out onto 1 mL of the collected supernatants which were contained in a 2 mL microcentrifuge tubes and placed in the automatic equipment. 10 mL syringes were attached to the m-PFC tips, and their needles were directly placed inside the 2 mL microcentrifuge tubes. Notably, the set-up involves 3 cycles of automated pulling and pushing the extracted samples through the m-PFC (sorbent) tips at 6 and 8 mL/min respectively. It was done with the aid of a piston, which was automatically controlled by the equipment. Finally, the cleaned aliquots were filtered through a 0.22 µm membrane after removing the needles before GC/MS analysis. In fact, the technique provided good and acceptable performances with LOQs (3 – 10 µg/kg), LODs (1 – 4 µg/kg), RRs (71 – 120 %) and RSD (< 20 %). The automated method is more effective when compared to the reviewed methods above. It can be used in a wider approach for analysis and monitoring of pesticides. Moreover, it has shown to be easier, robust, less laborious and less time-consuming as it does not require an additional step for centrifugation.

Another developed technical modification of a QuEChERS technique was the use of magnetic nanoparticles (MNPs) to replace the commonly used d-SPE cleanup salt/kit. It is because of the good surface area, adsorption, mechanical, magnetic and optical properties of the magnetic nanoparticles (Latham & Williams, 2008). Li et al. (2014) reported the use of modified QuEChERS-dSPE with MNPs of $Fe_3O_{4(s)}$. The adsorbent material was utilized for the determination of 101 pesticides residues in the samples of apples, cucumber, oranges and tomatoes purchased from Tai'ans supermarket (China). 50 mL centrifuge tube containing 10 g homogenized sample was spiked with the standard

analyte solutions before the addition of 10 mL ACN. The tube was agitated for 30 secs before adding 4 g anhydrous $MgSO_4$ and 1 g NaCl. The tube was shaken for 1.5 min and centrifuged (5000 rpm) for 5 min. 1 mL supernatant was introduced into 2 mL centrifuge tube containing MNPs (40 mg), PSA (50 mg), GCB (10 mg) and anhydrous $MgSO_4$ (100 mg) and the mixture was vortexed for 1 min. A magnet was employed externally during collection of the extracted analytes (supernatant) into 1.5 mL Eppendorf-vial before GC–MS/MS analysis. The method meets the requirements for multi-residue determination of pesticides in fruits and vegetables with RSD (< 10.5 %), LODs (0.03 - 2.17 µg/kg), LOQs (0.1 - 7.25 µg/kg) and RRs (71.5 - 111.7 %). The method could be applied broadly for analysis of various analytes in food samples.

Similarly, Zheng et al. (2015) recently documented the use of MNPs adsorbent in one-step QuEChERS extraction method for the determination of eleven residues of pesticides in juice and pomace samples obtained from blended and squeezed cucumber. 2 g of a pesticide-free (blank) sample of cucumber was transferred into a 10 mL centrifuge tube. Then, another 2 g sample was transferred into another 10 mL centrifuge tube. The 100 µg/kg of TPP and analyte standard solutions were respectively added to the centrifuge tubes for validation. Then, each tube was treated with 2 mL ACN and vigorously shaken for 1 min before the addition of 1840 mg MNPs adsorbent. The tube was shaken vigorously for another 1 min, and 0.8 mL supernatant was collected into 1.5 mL Eppendorf-vial (containing 0.1 g $MgSO_4$) after the matrix was conglomerated in the tube due to an external magnetic force. The vial was vigorously shaken and allowed to settle down for 0.5 min. The 1 mg/mL D-sorbitol (analyte-protectant) was added to the collected extract. Finally, the 1 µL of it was injected for analysis with GC-MS. The modified method showed acceptable performance [RRs (70.3–114.1 %), LOQs (2 - 49.6 µg/kg) and RSD (8.5-13.5 %)] when compared with other reviewed methods. The method may

serve as an alternative when rapidness is required in place of the commonly used QuEChERS-dSPE technique for analysis of pesticide residues in vegetables and fruits.

Some other adsorbent materials have recently been reported and used as cleanup material after the QuEChERS extraction (Salisaeng et al., 2016). These materials include the newly prepared sorbent material of vortex-assisted dispersive micro-solid phase extraction (VA-D-μ-SPE) based on the cetyltrimethylammonium bromide (CTAB)-modified zeolite (Patdhanagul et al., 2010). This material was successfully used by Salisaeng et al. (2016) for the extraction and removing interferences during the determination of carbamate pesticides in fruit and vegetable samples. The samples of cabbage, cauliflower, cucumber, dragon fruit, grape, rambutan, and watermelon were purchased in Khon Kaen, Thailand. Each of the homogenized samples was weighed (7 g) into 50 mL centrifuge tube and 10 mL ACN containing 1 % HOAc (v/v) was added. The mixture was vortexed for 1 min before 10 min centrifugation (4000 rpm). Furthermore, 0.4 g of sodium acetate and 2 g $MgSO_4$ were respectively added to the mixture followed by 10 min centrifugation (4000 rpm). The supernatant was evaporated (45 ºC) to dryness under the flow of nitrogen stream. The residue was dissolved in 7 mL of purified water in a 15 mL centrifuge tube, which contained the sorbent material of CTAB-modified zeolite NaY. The mixture was vortexed for 2 min after forming a suspension and filtered through 0.45 μm membrane. Finally, the absorbed analytes were eluted with 500 μL methanol, and the eluate was dried under a stream of nitrogen flow. HPLC analysis was carried out after re-dissolving the analyte residue with methanol (100 μL). The modified technique proved sensitive, rapid and achieved excellent extraction efficiency without an additional centrifugation step that gives rise to low LODs (4 – 4000 μg/kg), good RRs (79.5 – 124 %), LOQs (15 – 5000 μg/kg) and RSD (0.1 – 15.7 %). The method could be authentically used for broader analysis of carbamate pesticides in food samples.

Therefore, the analytical performances such as RRs, LODs, LOQs, and RSD for the reviewed literatures of QuEChERS techniques are highlighted in Table 2.2. These show the advantages of appropriate modifications in determining the residues of pesticides in selected samples.

Table 2.2: The analytical performance of QuEChERS coupled with advance cleanups methods for pesticides analysis in fruits and vegetables

Analyte	LODs (µg/kg)	LOQs (µg/kg)	RRs (%)	RSD (%)	Detection	Ref
14 bio-pesticides	≤ 3	≤ 10	70-112	≤ 28	UPLC-MS/MS	Romero-González et al. (2014)
Cyazofamid and CCIM	nr	≤ 5	75-105	nr	LC-MS/MS	Lee et al. (2014)
Fungicides and insecticides	≤ 200	≤ 800	71-112	nr	UHPLC-MS	Arias et al. (2014)
5 fungicides and insecticides	nr	nr	87-96	< 21	RP-UPLC	Bilehal et al. (2014)
128 kinds of Pesticides	≤ 5	≤ 10	70-120	≤ 20	UHPLC-MS/MS	Carneiro et al. (2013)
20 agrochemicals	nr	≤ 1	70-120	< 20	LC-MS/MS	Jadhav et al. (2015)
109 pesticides	≤ 10.8	≤ 30.4	77-113	< 20	LC– MS/MS	Golge and Kabak (2015)
ETU	≤ 0.15	≤ 0.5	60–110	< 18	LC– MS/MS	Zhou et al. (2013)
Quaternary ammonium pesticides of CQ & MQ	≤ 210	≤ 700	83-119	< 7	LCMS/MS	Gao et al. (2015)
SDHI fungicides	≤ 2	≤ 10	80–136	< 20	UPLC–MS/MS	Abad-Fuentes et al. (2015)
16 amide fungicides	≤ 3	≤ 10	72-98	< 10	UHPLC–MS/MS	Wu et al. (2015)
70 pesticides	≤ 2.4	≤ 7.9	74-119	< 14	LC– MS/MS	Han et al. (2015)
Glufosinate pesticide	≤ 3.3	≤ 10	80–108	≤ 9	LC-MS/MS	Han et al. (2016)
16 post-harvest fungicides	nr	≤ 10	77-120	< 10	LC-MS/MS	Uclés et al. (2015)
33 pesticides	≤ 4	≤ 10	71–120	< 20	GC-MS	Qin et al. (2016)
101 pesticides residues	≤ 2.2	≤ 7.25	72-112	< 11	GC–MS/MS	Li et al. (2014)
11 pesticides	nr	≤ 49.6	70-114	≤ 14	GC-MS	Zheng et al. (2015)
8 carbamate pesticides	≤ 4000	≤ 5000	80-124	≤ 16	HPLC	Salisaeng et al. (2016)

CCIM, 4-chloro-5-p-tolylimidazole-2-carbonitrile; ETU, ethylene thiourea; nr, not reported; CQ & MQ, chlormequat and mepiquat; SDHI, succinate dehydrogenase inhibitors; LOD, limit of detection; LOQ, limit of quantitation; RR, relative recovery; RSD, relative standard deviation; Ref, reference; OCP, organochlorine pesticide; OPP, organophosphorus pesticide; PP, pyrethroid pesticide

2.4.3 The Conclusion of QuEChERS-dSPE Methods Reviewed and Recommendation

Despite, the novelty of QuEChERS-dSPE technique documented in 2003 for pesticide residue determination in fruits and vegetables, series of modifications have been carried out to improve the method's analytical performances. The modified techniques enhance extraction/sample preparation to improve analyte recoveries as well as lowering RSDs, LODs, and LOQs. Thus, this review demonstrated the qualitative aspects of the modified QuEChERS-dSPE techniques in providing excellent analytical performance such as range of relative recoveries, quantitation and detection limits of pesticides determined in various sample of fruits and vegetables.

Thus, the obtained results excellently show that QuEChERS-dSPE and its recent modifications are reasonable methods for routine determination and monitoring of pesticide residues in samples of fruits and vegetables. Moreover, modification involving the use of CTAB-modified zeolite NaY, GCB, PSA, yttria-stabilized zirconium dioxide, and florisil as cleanup materials provided the better efficiencies and analyte recoveries when compared categorically with other reviewed methods or cleanup materials. Similarly, the application of MWCNTs provided a better result than PSA and GCB during the cleanup of high pigment samples. Furthermore, the modified (one-step) method employed magnetic adsorbent material without centrifugation during purification of target analytes conveniently, facilitates phase separation of the sample mixtures.

Recommendatory notes include further improvement of QuEChERS and cleanup methods by chemometrics optimization of essential factors such as sample quantity, mechanical setups that include centrifugation speed (rpm) and time (min) and pH of the extraction medium, e.g., % HOAc in ACN for the extraction and cleanup stages. The

optimized factors could play a significant role in achieving optimum condition to increase the sensitivity of the sample treatment method towards targeted analytes.

Furthermore, pesticides determination in other food samples such as the wet samples of cereals and legumes should be encouraged. Moreover, considering the aspects of environmental and green chemistry, it is recommended to replace the commonly used ACN (extractant) in the QuEChERS-dSPE method and other modified methods that involve the use of toxic solvents such as acetone and hexane. Thus, these could alternatively be replaced with the non-toxic, green solvents such as supercritical liquid carbon dioxide, Ionic liquid-based, low transition temperature mixtures (LTTMs), etc.

The QuEChERS-dSPE method pioneered by Anastassiades et al. (2003) is excellently known for cleanup capability in sample preparation (Gunatilake et al., 2014). On the other hand, DLLME is also known for an excellent enrichment capacity since its inception (Zhang et al., 2016). Furthermore, the excellent extraction properties of ionic liquid will suitably serve as a cleanup solvent in DLLME technique which could provide better results of pesticides determination in food matrices (Zhang et al., 2012). Thus, coupling the two methods (QuEChERS-dSPE-IL-DLLME) could solve the setback challenges of the previous techniques used for sample preparation for the determination of pesticide residues in fruit and vegetable samples after multivariate optimization.

On the other hand, the investigation was carried out on the QuEChERS extraction coupled with IL-DLLME method (QuEChERS-IL-DLLME) by skipping the d-SPE cleanup step before the IL-DLLME method. It is to determine the effects of PSA in the proposed method as documented by Rai et al. (2016).

CHAPTER 3: MATERIALS AND METHODS

3.1 Materials

The pesticides standard (100 mg/kg) for thiamethoxam, propamocarb, carbaryl, metalaxyl, baycarb, thiobencarb, diazinon, and dursban were obtained from AccuStandard® (New Haven, USA). Meanwhile, the LC-MS grade organic solvents were used for this research work. The solvents include methanol and ACN (Merck, Germany), ethanol, HOAc, acetone, and formic acid were obtained from Fisher Scientific. The buffer solution for pH 5 – 7 (phosphate) was purchased from Sigma-Aldrich and the ones for 4 (phthalate), 6 (phosphate), and 8 – 10 (borate) were obtained commercially, from Fisher Scientific. The Millipore-filtered (deionized) water was obtained using Merck Millipore water purification system (Billerica, USA). The ProElut™ AOAC 2007.01 QuEChERS and d-SPE kits for general vegetables and fruits were obtained from Dikma Technologies Inc. (Lake Forest, USA), Ammonium formate, 99% (New Jersey, USA), anhydrous magnesium sulfate, sodium chloride and sodium acetate (Hamburg Chemicals, France). The molten salt (HPLC grade) of $[C_6MIM][PF_6]$ ionic liquid-based ($P \geq 97.0$ %) was purchased from Sigma-Aldrich, (Germany).

3.2 Apparatus

3.2.1 Glasswares

HPLC autosampler vials were purchased from Agilent Technologies (USA). The other glassware used for the research work were dried for 3 hours in an oven (105 ºC) after they were cleaned with detergent and rinsed thoroughly with running tap water. Later on, acetone was used to rinse the glassware, dried and dust-protected with aluminium foil in a cupboard.

3.2.2 Analytical Equipment

Dynamica refrigerated centrifuge by CNG instruments (Selangor, Malaysia), Memmert drying oven (Schwabach, Germany), vortexer VTX-3000L by Copens Scientific (Tokyo, Japan) and glass jug blender MX-GX1581WSK (Panasonic, Malaysia) and Supelco HPLC column [Ascentis® Express C_{18} (5 cm x 2.1 mm, 2.7 µm)] (Sigma-Aldrich, USA). The others include weighing balance (Sartorius Technology Park, Germany), pH meter PB (Sartorius group, Germany) and Agilent triple quadrupole LC/MS G6490A [built in Electrosprays ESI (±) MS/MS Sensitivity and Jet stream Technology] instrument (Singapore).

3.3 QuEChERS-dSPE and DLLME Materials

2, 15 and 50 mL polypropylene centrifuge tubes by LabServ Fisher-Scientific (Kuala Lumpur, Malaysia), 100 and 500 µL microsyringe, respectively, were obtained from Agilent (Australia) and Hamilton (USA). Then, 1-10, 10-100 and 1000 µL micropipettes (Eppendorf, Germany).

3.4 Methodology

3.4.1 Stock and Standard Solution

The stock standard solution of 100 µg/mL which is equivalent to 100 mg/kg (i.e. 100000 µg/kg) or parts per million (ppm) ("Data-handling", 2018) for each pesticide was diluted to 10, 1 and 0.1 mg/kg (100 µg/kg) with appropriate volumes of methanol. The appropriate volumes were calculated using the dilution formula as expressed in Equation 3.1 (Koenig, 2010), separately. Afterward, the prepared working standard solutions were preserved in a refrigerator at 4 ºC before carrying out the LC-MS/MS analysis.

$$C_1 C_2 = V_1 V_2 \qquad \text{Eqn (3.1)}$$

Where

C_1: The concentration of the stock standard solution,

C_2: The concentration of the working standard solution

V_1: The volume of the stock standard solution

V_2: The volume of the working standard solution

3.4.2 ANOVA for the Plackett-Burman and Box-Behnken Designs

The analysis of variance (ANOVA) is the statistical partitioning by which total variation for sets of observation are divided into distinct or parts of a component such as the *P*- values. The *P*-value is one of the critical value in the multiple regression model of a hypothesis, which is a mathematical evaluation or expression of the relationship between the dependent variables and responses. Thus, the *P*-value is the significant level that could lead to rejecting the null hypothesis "H_o" in the postulated model of the Plackett-Burman and Box-Behnken design experimental runs (e.g., if the *P*-value is lower than the significant level, then the model is said to be significant and vice-versa). The general regression (polynomial) model for Plackett-Burman (Linear) and Box-Behnken (Quadratic) designs could be expressed as shown in Equation 3.2 (Anthony, 2014; Vallejo et al., 2010).

$$\underbrace{Y = \left(\beta_0 + \beta_1 X_1 + \beta_2 X_2 \ + \cdots + \beta_{12} X_1 X_2\right)}_{\text{Linear}} + \cdots + \underbrace{\left(\beta_{11} X_1^2 + \beta_{12} X_2^2\right)}_{\text{Quadratic}} + \cdots + \varepsilon \quad \text{Eqn (3.2)}$$

Where

Y: The dependent variable

β_0: The average response in factorial expression

$X_1, X_2, X_1^2 \,\&\, X_2^2$: The response variables

$\beta_1, \beta_2, \beta_{11} \,\&\, \beta_{12}$: The regression coefficients

ε: The random error

3.4.3 Conditioning of the LC-MS/MS Instrument

3.4.3.1 Auto-tuning and Mass-Hunter optimization of LC-MS/MS Instrument

The triple quadrupole LC/MS G6490A instrument was optimized using Mass Hunter to determine the optimum fragmentor voltage, identification of the four-fragmentor product ions with their respective retention times (RT) and collision energies (CE). Accordingly, the technical processes provided a better condition for the ESI to encourage responses of peak areas for the concentration of the resulting analyte in a multi-pesticides mixture of standard solutions.

3.4.3.2 Initial Settings of the LC-MS/MS Instrument

The LC-MS/MS instrumentation was initially carried out using the initial (default) settings of the contributory factors. The factors were; analyte injection volume (3 µL), column temperature (30 ºC), flow rate (0.15 mL/min), gas temperature (200 ºC), gas flow (14 L/min), nebulizer gas (45 psi), sheath gas temperature (300 ºC), sheath gas Flow (11 L/min), capillary voltage (3000 V) and delta$^{(+)}$ EMV (150 V). The factors were used for Mass-Hunter optimization of the instrument using 1 mg/kg multi-pesticides mixture of standard solutions. The optimization helps to determine the optimum fragmentor voltage, identification of the four-fragmentor product ions with their respective retention time (RT) and collision energy (CE). Moreover, the instrumental default settings were further used for the selection of the best mobile phase and the development of the best gradient program runs (Table 3.1).

Table 3.1: The gradient program run

Time (min)	% Organic mobile phase (B)	Flow rate mL/min	Pressure (bar)
0.00	15	0.15	600
1.60	15	0.15	600
10.40	100	0.15	600
12.00	15	0.15	600

In the first place, the gradients run used by Rajski et al. (2013), and Vázquez et al. (2015) for analysis of similar multi-pesticide compounds were adopted and the running time program was modified to obtain the best shortest elution time. The gradient program that resulted in the best total ion chromatography (TIC) peaks resolution was considered for the LC-MS/MS instrumentation. Notably, the TIC resolution helps to provide an optimum condition for attaining higher total chromatographic peak area (TCPA) (Scientific, 2014). The TCPA can be mathematically expressed in Equation 3.3 (Bramston-Cook, 2009).

Therefore,

$$TCPA = \sum CPA \qquad \text{Eqn (3.3)}$$

Where

$TCPA$: The total chromatographic peak area

CPA: The chromatographic peak area

Besides, the simultaneous quantification of multiple pesticide analytes using triple quadrupole LC-MS instrument which depends on the TCPA generated by the multiple reaction monitoring (MRM) of the product ions for each of the targeted analytes based on their respective masses. Moreover, the TCPA obtained from LC-MS/MS analysis serves as an index used for estimating the number of target analytes that are present in the analyzed (matrix) samples (Abdulra'uf & Tan, 2015; Lawal et al., 2018a). It is because the peak areas of the targeted analytes are correlated and categorically suitable for multiple pesticides analysis using LCMS instrument because of their close similarities range of XlogP_3 (1.2 – 5.3) (Lazartigues et al., 2011).

3.4.3.3 Selection of LC-MS/MS mobile phase

Unfortunately, the use of experimental design will not be favorable for the selection and optimization of the mobile phases. It is because responses for each of the mobile phase is required individually without interaction to estimate the actual effect of the mobile phase setup. Moreover, the two setups of mobile (organic and aqueous) phases are involved with interactive percentage flow of organic/aqueous changes to create an optimum condition of analytes detection. For these reasons, the traditional method was adopted for the selection and optimization of mobile phase using LC-MS/MS instrumentation as a constituent with the report of Sherma (2001).

Comparative analysis was carried out on some selected mobile phases, which were reportedly used for analysis of pesticides in various samples. Experimentally, the comparative analysis was carried out on the multi-pesticide mixture of 0.1 mg/kg standard solutions. The multiple reaction monitoring (MRM) of the instrumental runs for each of the mobile phases (Table 3.2) will result in chromatographic peak separation, height, and area. Eventually, the mobile phase setup that provided the best separation of analytes and the highest TCPA was selected for further optimization by adding 0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 7.5 and 10 % ACN in mobile phase A. Subsequently, the best pH solution was selected based on the results of the average TCPA responses of the LC-MS/MS instrument.

Table 3.2: The comparative study of the mobile phases

	References	Water (A)	Organic Mobile Phase (B)
1.	1st suggested mobile phase	A	ACN
2.	Rajski et al. (2013), Perez-ortega et al. (2012).	A + 0.1% FA	ACN
3.	Nunez et al. (2012), Economou et al. (2009) and Lucas (2013)	A + 0.1% FA	ACN + 0.1% FA
4.	Vázquez et al. (2015)	A + 0.1% FA	ACN + 0.1% FA + 5% A
5.	2nd suggested mobile phase	A	MeOH
6.	Golge et al. (2015)	A + 5 mM AF	MeOH + 5 mM AF
7.	Zanella et al. (2013)	A + 2% MeOH + 0.1% FA + 5 mM AF	MeOH + 0.1% FA + 5 mM AF
8.	3rd suggested mobile phase	A	MeOH/ACN (1:1)
9.	4th suggested mobile phase	A + 5 mM AF + 0.1%FA	MeOH/ACN (1:1) + 0.1% FA + 5 mM AF

3.5 RSM Optimization of LC-MS/MS Instrument

The RSM was carried out to optimize TCPA corresponding to the Agilent (G6490A) LC-MS/MS Instrument setting that was aimed for quantitation of multi-pesticide residues in fruit and vegetable samples. Hence, the optimization process involved the use of Plackett-Burman and Box-Behnken designs (Qian et al., 2017).

3.5.1 Plackett-Burman Design Runs for LC-MS/MS Screening

In the first place, the instrumental factors and their ranges were selected based on the default setup of LC-MS/MS Instrument and the studied literatures with regards to analysis of pesticides using Agilent HPLC coupled with 6490 QQQ MS/MS (ESI) system (Rajski et al., 2013; Vázquez et al., 2015). Accordingly, 11 factors from the instrumental LC and MS components were screened (Table 3.3) at their respective levels using Plackett-Burman to identify the factors that significantly contribute to high TCPA responses. The

design matrix for the 24 runs was generated using the Minitab-17 statistical software. Eventually, the runs were carried out by analysis of 100 µg/kg mixture of multi-pesticide standard solutions and the responses were recorded in the experimental run sheet.

Table 3.3: Plackett-Burman design space for LC-MS/MS instrument

	Factors	Levels	
		Low (-)	High (+)
1.	Starting mobile phase B (%)	10	20
2.	Column temperature (ºC)	25	35
3.	Flow rate (mL/L)	0.1	0.2
4.	Injection volume (µL)	1	5
5.	Gas temperature (ºC)	150	250
6.	Gas flow (L/min)	11	17
7.	Nebulizer (psi)	30	60
8.	Sheath gas temperature (ºC)	200	400
9.	Sheath gas flow (L/min)	10	12
10.	Capillary voltage (V)	2000	4000
11.	Delta$^{(+)}$ EMV (V)	100	200

3.5.2 Box-Behnken design runs for optimization of LC-MS/MS instrument

The design was used for optimizing the significance factors resulted from the Plackett-Burman design (Table 3.4) at three levels. Afterward, the design for the experimental runs was generated by the statistical software which was made up of one replicate, one block, three center points, and a total run number of 27. Similarly, all the runs were analyzed out using 100 µg/kg mixture of standard pesticide solutions.

Table 3.4: The LC-MS/MS Instrumental factors optimized using Box-Behnken design

Factors		Levels		
		Low (-)	Medium (0)	High (+)
1	Flow rate (mL/L)	0.1	0.15	0.2
2	Injection volume (µL)	1	3	5
3	Sheath gas temperature (ºC)	200	300	400
4	Delta$^{(+)}$ EMV (V)	100	150	200

3.6 Development of Sample Preparation Method

Notably, the TCPA from the instrumental (MRM) scans were used as the response in optimization. Simultaneously, Milli-Q-water was used as a blank sample, which was used for the quality control process of the sample preparation method. It is because water can also represent a matrix sample, which could also be determined quantitatively for the accumulation of pesticide residues as reported by Brondi et al. (2011). Thus, the low possession of matrix interferences of Milli-Q-water could yield a reliable and high recovery of the analyte during optimization for the method development. Also, Milli-Q-water does not easily spoil as compared to the use of a matrix sample of fruits and vegetables. For these reasons, Milli-Q-water was frequently used as blank for the RSM optimization because conformity of a sample over a period is essential for the long-term RSM optimization.

3.6.1 The Selection of QuEChERS-dSPE Salts and Ionic Liquid-Based for DLLME Cleanups

3.6.1.1 The QuEChERS-dSPE salts

The official recommended salts of QuEChERS (extraction) and d-SPE (cleanup) kits of AOAC 2007.01 for general fruit and vegetable samples were proposed and used for this research work. Thus, each sachet for the extraction kit is composed of 6.0 and 1.5 g

of $MgSO_4$ and NaOAc, respectively. Meanwhile, each sachet for the cleanup (d-SPE) kit composed of 50 mg of PSA and 150 mg of $MgSO_4$.

3.6.1.2 Selection of QuEChERS extraction solvent

Comparative studies were carried out on four different organics (extraction) solvent used for QuEChERS extraction, independently. However, some of these solvents were previously used for analysis of pesticides. The solvents investigated include; ACN, methanol (MeOH), ACN/MeOH (1:1 v/v) and ethanol (EtOH).

Experimentally, 100 ± 5 g of a fresh apple sample was homogenized and transferred into a glass beaker. Then, a 50 mL centrifuge tube was occupied with 15 g of homogenized apple sample was sub-sampled from the glass beaker because of its homogeneity that could result in consistency and reliability of the compared results. The sample preliminarily represents the real sample of fruits and vegetables as supported by Anastassiades et al. (2003). The sample was spiked with 200 µL of 100 µg/kg mixture of standard pesticides solution. The tube was mixed with 15 mL ACN before addition of a d-SPE agent, covered and vortexed for 1 min. Later on, the tube was centrifuged at 4000 rpm for 5 min. Then, 200 µL of supernatant was transferred into 2 mL HPLC auto-sampler vial containing 800 µL Milli-Q-water (1:5) and vortexed for 1 min before analysis with LC-MS/MS instrument. Comparatively, the method was also used by swapping ACN to either MeOH or ACN/MeOH (1:1 v/v) or EtOH as the extraction solvents.

3.6.1.3 The ionic liquid-based for DLLME technique

The best ionic liquid-based used for extraction of analytes in the DLLME techniques was selected based on the documented literatures. Consequently, the 1-hexyl-3-methylimidazolium hexafluorophosphate $[C_6MIM][PF_6]$ ionic liquid-based was selected and used for this research over other ionic liquid-based such as 1-Butyl-3-methylimidazolium hexafluorophosphate ($[C_4MIM][PF_6]$) and 1-octyl-3-

methylimidazolium hexafluorophosphate ([C_8MIM][PF_6]) (Faraji et al., 2016; Lawal et al., 2018; Xie et al., 2014; Zhang et al., 2016).

3.6.2 Equalization of Dried, Liquid and Fresh Samples Used for QuEChERS Extraction

Notably, it was assumed that approximately Y mL of Milli-Q-water should be added to X g of a dried ground sample to equate it to Z g of a fresh sample based on the literatures (Chen et al., 2014; Kowalski et al., 2012; Lawal et al., 2018b) as tabulated in Table 3.5.

Table 3.5: Equalization of dried, liquid and fresh samples use for QuEChERS extraction

Mass of dry Sample (X)	Vol. of Milli-Q-water Sample (Y)	Mass of fresh Sample (Z)
3.3 g	6.7 mL	10 g
5 g	10 mL	15 g
6.7 g	13.3 mL	20 g

3.6.3 The Unoptimized and RSM Optimized QuEChERS Technique Coupled with d-SPE and DLLME Extraction/Cleanup Methods

The following QuEChERS extraction and cleanup methods were carried out using blank samples of Milli-Q-water comparatively studied to select the best method that yielded the highest TCPA of analyte recoveries.

3.6.3.1 The default QuEChERS-dSPE method

In the first place, the method revealed by "Agilent" (2011) and "DIKMA" (2016) was employed for QuEChERS-dSPE analysis. Experimentally, 10 mL blank (of Milli-Q-water) was measured in 50 mL centrifuge tube. 1 % acetic acid (HOAc) in 15 mL ACN was added after spiking the content with 200 µL of 100 µg/kg multi-pesticides mixture

of standard solutions. The tube was covered and vortexed for 1 min before addition of a sachet of QuEChERS extraction salt. The tube was covered and shaken vigorously for 1 min and centrifuged at 4000 rpm for 5 min. Then, 0.5 mL supernatant (ACN extract) was transferred into 2 mL centrifuge tube containing a sachet of the cleanup agent. The tube was vortexed for 30 sec before centrifugation at 4000 rpm for 5 min. Then, 80 µL of ACN extract was mixed with Milli-Q-water (1:5) in 2 mL HPLC auto-sampler vial and vortexed (1 min) before LC-MS/MS instrumentation.

3.6.3.2 The default QuEChERS-IL-DLLME method

According to the proposed method used by Zhang et al. (2016) and Xie et al. (2014), 0.5 mL ACN extract from the QuEChERS extraction above before the d-SPE cleanup was transferred into 15 mL centrifuge tube containing 5 % NaCl in 9.5 mL of Milli-Q-water. Then, 100 µL ionic liquid-based was carefully added to the mixture before centrifugation at 4000 rpm for 5 min. Then, the 80 µL $[C_6MIM][PF_6]$ ionic liquid-based extract was collected with a microsyringe and transferred into 2 mL HPLC auto-sampler vial and diluted with 400 µL of methanol (1:5).

3.6.3.3 The RSM optimization of the QuEChERS-dSPE method

QuEChERS-dSPE technique underwent RSM screening and optimization using Plackett-Burman and Box-Behnken designs, respectively. Hence, both designs runs were involved in the use of TCPA responses resulted from the analyses of the 200 µL of 100 µg/kg spiked mixture of standard pesticide solutions on the blank sample of Milli-Q-water.

(a) Plackett-Burman design runs for a QuEChERS-dSPE method

The essential factors and levels (Table 3.6) for QuEChERS-dSPE method were first considered before embarking on the RSM optimization. These factors are associated with the acid/base (pH) of the extraction solvent and the mechanical setups such as

centrifugation speed and centrifugation time in both the extraction and cleanup stages. The RSM optimization of these factors could also play a significant role in achieving optimum condition for the sample treatment and cleanup processes. Apart from the previous reported RSM optimization on the influences of the sorbent materials used in QuEChERS-dSPE method such as PSA, octa-dodecyl bonded silica (C_{18}), graphitized carbon black (GCB), and sodium acetate (NaOAc) salts (Li et al., 2016; Melo et al., 2013; Rai et al., 2016; Rizzetti et al., 2016).

Later on, the statistical software was used to generate the experimental designs with 12 runs as reported by Vidal et al. (2007). The screening runs were carried out to analyze the significant factors at 2-level each and 74% confidence interval (0.26 significant level) in order to limit the factors to three. It is because all the six analyzed factors of the QuEChERS-dSPE method were statistically insignificant at the usual 0.05 but were significant at 0.26 significant level.

Table 3.6: The 2-levels factors used in Plackett-Burman design for a QuEChERS-dSPE method

S/N	Factors	Levels	
		Low (-)	High (+)
1	Quantity of Milli-Q-water (sample) for QuEChERS extraction (mL)	6.7	13.3
2	Percentage of HOAc in 15 mL of ACN (%)	0	2
3	Centrifugation speed for QuEChERS extraction (rpm)	1000	7000
4	QuEChERS extraction time (min)	2	8
5	Centrifugation speed for d-SPE (rpm)	1000	7000
6	Cleanup time for d-SPE (min)	2	8

(b) ***Box-Behnken design for optimizing the 3-significant factors of QuEChERS-dSPE method***

The significance factors (Table 3.7) at three levels each underwent optimization using Box-Behnken design with 15 runs, and 3 center points were generated by the statistical software.

Table 3.7: The 3-levels significant factors of QuEChERS-dSPE method

S/N	Factors	Levels		
		Low (-)	Medium (0)	High (+)
1.	Quantity of sample for QuEChERS extraction (mL)	6.7	10	13.3
2.	Percentage of HOAc in 15 mL of ACN (%)	0	1	2
3.	QuEChERS extraction time (min)	2	5	8

3.6.3.4 RSM optimization of the QuEChERS-IL-DLLME method

The statistical screening of the significant factors for a QuEChERS-IL-DLLME method using Plackett-Burman design before the optimization using Box-Behnken design at a specific significant level. Notwithstanding, the runs were carried using 200 µL spiked standard solution of pesticides mixture (100 µg/kg) on the reagent blank.

(a) ***Plackett-Burman design for screening significant factors in QuEChERS-IL-DLLME technique***

The factors and ranges were selected for the RSM screening and optimization of QuEChERS-IL-DLLME technique based on the report of Zhang et al. (2016) and Xie et al. (2014). These considerations include the volume of extractant, the ionic strength of

the extraction medium, agitation and extraction time. The factors were screened at 0.05 significant level, but only one factor was significant. Based on this reason, the significant level for the eight factors of QuEChERS-IL-DLLME technique (Table 3.8) was extended to 0.26 for the Plackett-Burman design which was generated with 12 runs by the statistical software.

Table 3.8: The 2-levels factors of Plackett-Burman design used for QuEChERS-IL-DLLME

S/N	Factors	Levels	
		Low (-)	High (+)
1.	Quantity of sample for QuEChERS extraction (mL)	6.7	13.3
2.	Percentage of HOAc in 15 mL of ACN (%)	0	2
3.	QuEChERS extraction centrifugation speed (rpm)	1000	7000
4.	QuEChERS extraction time (min)	2	8
5.	Percentage of NaCl in 9 mL of water (%)	0	10
6.	The volume of ionic liquid-based (µL)	50	150
7.	Centrifugation speed for DLLME (rpm)	2000	8000
8.	DLLME extraction time (min)	2	8

(b) ***Box-Behnken design for optimizing the 3-significant factors of QuEChERS-DLLME method***

The 15 runs for the Box-Behnken design was employed for optimization of TCPA based on the three significant factors resulted from Plackett-Burman (Table 3.9).

Table 3.9: The 2-levels factors of Plackett-Burman design used for QuEChERS-IL-DLLME

S/N	Factors	Levels		
		Low (-)	Medium (0)	High (+)
1.	QuEChERS centrifugation speed (rpm)	1000	4000	7000
2.	Percentage of NaCl in 9 mL of water (%)	0	5	10
3.	Volume of ionic liquid-based (µL)	50	100	150

3.6.3.5 The combined default QuEChERS-dSPE to default IL-DLLME method

In this regard, it is a modified setup combination of the default (medium) settings for both QuEChERS-dSPE and QuEChERS-IL-DLLME methods to the QuEChERS-dSPE-IL-DLLME method. The procedure occurred by transferring 10 mL reagent blank into 50 mL centrifuge tube and spiked the content with 200 µL of 100 µg/kg multi-pesticides mixture of standard solutions. 1 % acetic acid (HOAc) in 15 mL ACN was added before covering and vortexing the tube for 1 min. A sachet of QuEChERS extraction salt was added to the tube's content, covered, shaken vigorously (1 min) and centrifuged (4000 rpm) for 5 min. 1 mL supernatant was transferred into 2 mL centrifuge tube that was occupied with a sachet of the cleanup agent. The tube was centrifuged (4000 rpm) for 5 min after vortexing it for 30 sec. Subsequently, 0.5 mL supernatant from the d-SPE cleanup was transferred into 15 mL centrifuge tube containing 5 % NaCl in 9.5 mL of Milli-Q-water. The tube was covered, shaken vigorously (1 min) and centrifuged (4000 rpm) for 5 min after addition of 100 µL ionic liquid-based. Then, the 80 µL $[C_6MIM][PF_6]$ ionic liquid-based extract was diluted with 400 µL of methanol (1:5) in 2 mL HPLC auto-

sampler vial and vortexed for 1 min. Finally, the analyte solution was analyzed with LC-MS/MS instrument.

3.6.3.6 The RSM optimized QuEChERS-dSPE combined with IL-DLLME method

The procedure used for the RSM modified QuEChERS-dSPE-IL-DLLME method was similar to that of the default QuEChERS-dSPE-IL-DLLME technique except, that the significant ones replaced the three highlighted factors in QuEChERS-dSPE and QuEChERS-IL-DLLME methods. These include the quantity of sample (13.3 mL Milli-Q-water), 0% HOAc in 15 mL ACN, 2 min QuEChERS centrifuged time, 10% NaCl in 9 mL of Milli-Q-water, ≈ 130 µL $[C_6MIM][PF_6]$ ionic liquid-based and 7000 rpm DLLME centrifuged speed. The extraction performance and cleanup of the combined method were carried out sequentially.

3.6.4 The Comparative Studies of Default and RSM Optimized QuEChERS Technique Coupled with d-SPE and DLLME Extraction/Cleanup Methods

The above techniques were compared based on the resulted average TCPA analyzed in triplicates after spiking the blank sample with 200 µL of 100 µg/kg multi-pesticides mixture of standard solutions. Accordingly, the best method with high average TCPA (analyte recoveries) was selected for the determination of multi-pesticide residues in the fresh sample of fruits and vegetables.

3.7 Sampling and Sample Preparation

A sample of fruits and vegetables were purchased from Tropicana shopping mall, Kuala Lumpur. The samples were respectively analyzed quantitatively for multi-pesticide residues using the developed QuEChERS-dSPE-IL-DLLME method.

Whereby, each of the chopped and homogenized sample was weighed (20 ± 0.1 g) into a 50 mL centrifuge tube for QuEChERS extraction. Then, the content was spiked with

200 µL of 100 µg/kg multi-pesticides mixture of standard solutions and vortexed for 1 min before adding 15 mL ACN into the centrifuge tube, covered and vortex for 1 min. A sachet of QuEChERS extraction salt was added to the content of the tube and shake vigorously for 1 min. Later on, the tube was centrifuged at 4000 rpm for 2 min.

Subsequently, the 1.5 mL supernatant from QuEChERS extraction (ACN extract) was transferred into 2 mL centrifuge tube containing a sachet of d-SPE cleanup salt. The tube was vortexed for half a minute and centrifuged at 4000 rpm for 5 min.

Then after, 1 mL supernatant from d-SPE cleanup step was introduced into 15 mL centrifuge tube containing 10 % NaCl in 9 mL of Milli-Q-water for the IL-DLLME cleanup process. Furthermore, the ≈130 µL $[C_6MIM][PF_6]$ ionic liquid-based was carefully added to the content of the tube and was shaken vigorously for 1 min before centrifugation (7000 rpm) for 5 min. 100 µL of the sedimental extract was retracted with microsyringe into 2 mL HPLC auto-sampler vial and diluted with 0.5 mL MeOH and vortexed for1 min. Finally, the analyte solution in the vial was analyzed using the RSM optimized Agilent (G6490A) LC-MS/MS instrument. The developed sample preparation method is illustrated in Figure 3.1.

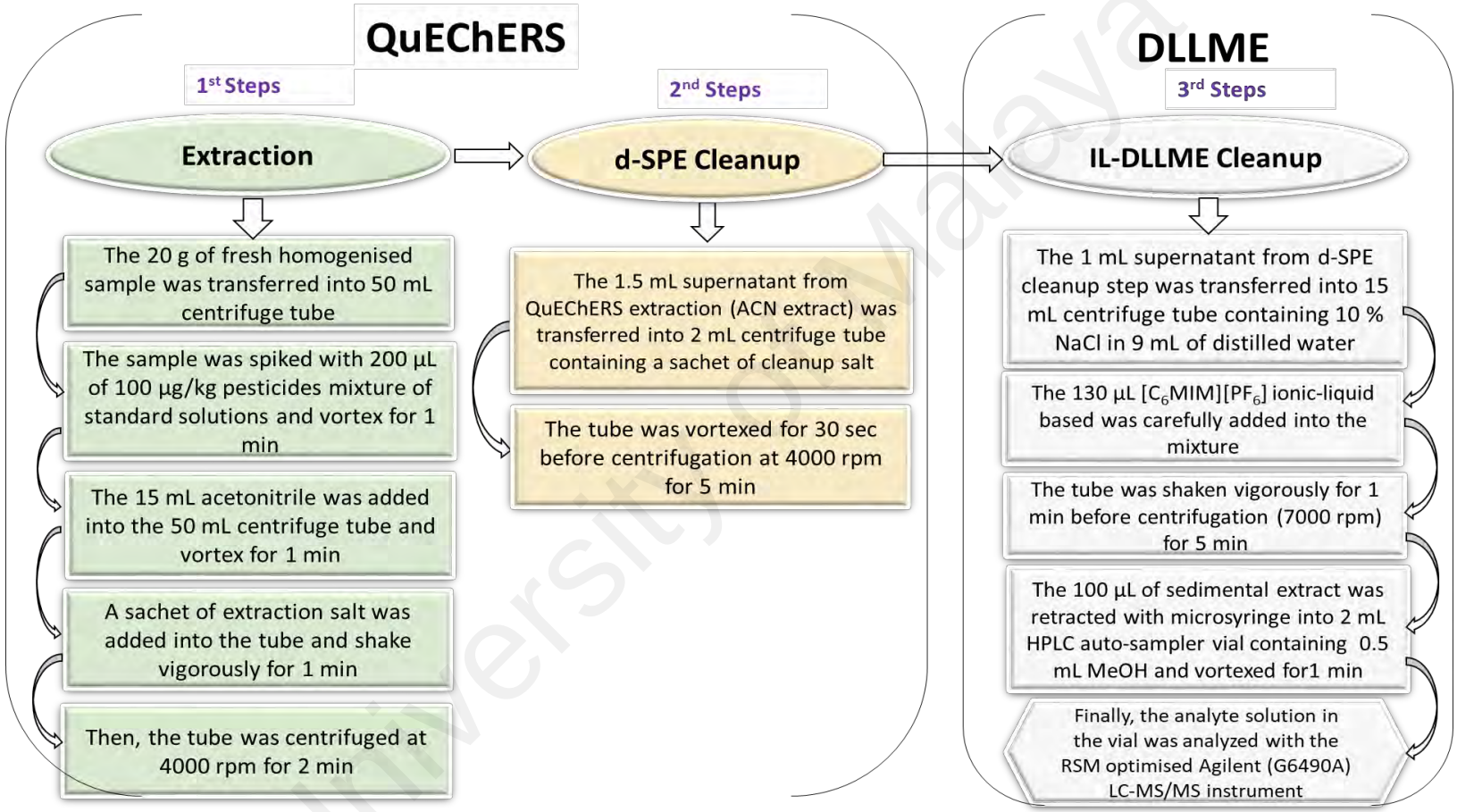


Figure 3.1: The procedure of the developed QuEChERS-dSPE-IL-DLLME technique used for sample preparation

3.8 Statistical Software for Data Analysis

The computations and graphical illustrations were carried out using the Microsoft Excel® (2013) software. The instrumental responses (TCPA) used for Plackett-Burman and Box-Behnken designs analyses were carried out by Minitab-17 software.

3.9 Validation of the Developed Sample Preparation Method

Validation shows the effectivity and desirability of the developed method for the determination of multi-pesticides residue in fruit and vegetable samples using RSM optimized LC-MS/MS instrument (Jovanov et al., 2015). Accordingly, the developed method was validated based on the guidelines of quality control and methodologies for pesticides residue analysis in food and feed published by the European (EU) Commission (Jovanov et al., 2015). The validation parameters include accuracies (RR), precision (RSD_r), limit of detection (LOD) and limit of quantitation (LOQ), matrix effect (ME), linearity and measurement of uncertainties (MU). However, the calibration curve plays a vital role in the estimation of most of the validated parameters.

3.9.1 Calibration Curve

The calibration curve is a standard curve used for the determination of analyte concentration in an unknown sample which is compared with the sets of the concentration of a standard sample (Barwick, 2003). Experimentally, the blank matrix sample for each fruit and vegetable was spiked with an appropriate volume of the prepared multi-pesticides mixture of standard solution (1000 µg/kg) to obtain 5, 100, 200, 300, and 400 µg/kg, individually (Lozano et al., 2018).

3.9.2 Accuracies and Precision for Sample Preparation Method

3.9.2.1 Accuracies for sample preparation method

Accuracy is defined as the differences between measurements or estimated results of analyses from the actual (standard) value (Rodrigues, 2007). However, the accuracy of

the developed method is estimated as absolute recoveries (AR) and relative recoveries (RR). The accuracies were estimated in triplicates (n = 3) at three concentration levels each (5, 100 and 300 µg/kg). Therefore, AR (Equation 3.4) is expressed as the percentage ratio between the concentration of an analyte found in the spiked reagent black (W) and the spiked concentration without extraction (Z) (Yang et al., 2017).

$$\text{Absolute recovery } (AR\ \%) = \frac{W \times 100}{Z} \qquad \text{Eqn (3.4)}$$

While, the RR (Equation 3.5) is expressed as the percentage ratio between the concentration of an analyte found in the spiked matrix blank undergone extraction (Y) and the spiked concentration (Z) (Yang et al., 2017).

$$\text{Relative recovery (RR \%)} = \frac{Y \times 100}{Z} \qquad \text{Eqn (3.5)}$$

3.9.2.2 Repeatability for sample preparation method

On the other hand, precision refers to the closeness of measurements or estimated results of three or more repeatable analyses. Hence, the precision of the ongoing research is expressed based on the laboratory repeatability in term of relative standard deviation (RSD_r %) (Equation 3.6). Importantly, precision supports accuracy by giving confidence to the future of results to be obtained (Rodrigues, 2007). Thus, the precisions were estimated from the repeatable analysis (n = 3) carried out on the homogenized samples prepared by spiking the matrix blank sample of fruits and vegetables at three concentration levels (5, 100 and 300 µg/kg) each.

$$RSD_r\ \% = \frac{Standard\ deviation\ \times 100}{Mean} \qquad \text{Eqn (3.6)}$$

3.9.3 LOD and LOQ for Sample Preparation Method

The LOD and LOQ are the least quantity or concentration that can be determined or measured analytically. The LOD is the signal or response produced by the smallest concentration of an analyte, which is statistically different within a specific confidence level from the background level of noise. Meanwhile, LOQ is the lowest determined analyte concentration (with accuracy and precision) under certain conditions of analysis. Equation 3.7 expresses the LODs and LOQs of each analyte using the developed method for sample preparation of multi-pesticide residues in a homogenized sample of vegetables and fruits. The LODs and LOQs were calculated from the calibrated linear graph based on matrix match calibration standards and the slope based on the signal-to-noise ratio corresponding to a factor of 3 and 10, respectively and standard error ("LODs & LOQs", 2016; Shrivastava & Gupta, 2011).

$$\text{LOD or LOQ}\left(\mu\frac{\text{g}}{\text{kg}}\right) = \frac{F \times \text{STEYX}}{m} \qquad \text{Eqn (3.7)}$$

Where

F: The LOD and LOQ are having the factors of 3 and 10, respectively.

STEYX: The standard error estimated from Microsoft Excel 2013.

m: The slope of the linear regression

3.9.4 Matrix Effect

Matrix effect (ME) is the measurement of the performance capability of a sample preparation method, indicating the impacts of matrix interferences in the analyzed samples, which relates with the analyte recoveries (Wang et al., 2017). The matrix effect was calculated (Equation 3.8) based on the percentage ratio between the slope of analyte recovery (sensitivity) in both the matrix and the ACN minus one (Dias et al., 2016). Eventually, the matrix effect enhances or suppresses analyte recoveries when it is greater

than 100% (ME > 100 %) or less than 100% (ME < 100 %), respectively. However, the ME is ineffective when it is equal to 0 % (ME = 0 %) (Kruve et al., 2008).

$$\text{ME (\%)} = \left[\left(\frac{\text{The sensitivity of analyte in the matrix}}{\text{The sensitivity of analyte in ACN}}\right) - 1\right] \times 100 \qquad \text{Eqn (3.8)}$$

3.9.5 Linearity

The linearity (linear) of the calibration curve was plotted graphically to estimate analytes' concentration (interpolation) from the recovered average TCPA (ATCPA). Meanwhile, the ATCPA responses of the pesticide analysis are directly proportional to the concentration of the standard working solution of the analytes respectively, or the concentration of analyte in the fresh sample (spiked) matrices within a specific range (Cuadros-Rodríguez et al., 2007). Therefore, the linearity of the developed method for the analysis of pesticides residue in fruit and vegetable samples were evaluated from their calibration curves, respectively. The matrix blanks were spiked at five concentration levels ranging from 5 – 400 µg/kg and their respective coefficient of simple linear regression (R^2) were recorded. Thus, the linearity results were examined from the calibration curve for each of the targeted analyte (Kroll & Emancipator, 1993).

3.9.6 Measurement of Uncertainties (MU)

The MU refers to the attribution for the obtained results of analyses at a particular confidence level to the quantity measured (Stevenson, 2015). Hence, MU test was conducted on the validated parameters to be further certified with the obtained results. Thus, the MU was estimated at 95% confidence level based on the empirical model and coverage factor (k = 2) as indicated below (Equation 3.9) (Kaczyński, 2017; Kmellár et al., 2008).

$$\text{MU (\%)} = k \times pooled\ RSD_r \qquad \text{Eqn (3.9)}$$

Meanwhile, the uncertainties due to bias were handled by corrections to avoid contribution toward uncertainties.

3.9.7 The Concentration of Multi-Pesticide Residues in Blank Matrix Samples of Fresh Fruits and Vegetables

Ultimately, the developed QuEChERS-dSPE-IL-based-DLLME sample preparation method was used for the quantitative determination of the multi-pesticide residues in the blank homogenized sample of fruits and vegetables. The studies were carried by analyzing three portions of each sample to estimate the residual level of pesticides in the analyzed samples. Moreover, the quantified residue of multi-pesticides was compared with the maximum residue limits (MRLs) set-up by the European Union (EU) commission (Barroso, 2011; "EU", 2016). Consequently, the results obtained provided awareness on the health risk of consuming the analyzed kind of fruits and vegetables.

CHAPTER 4: RESULTS AND DISCUSSION

4.1 The Result of Auto-tuning and Mass-Hunter Optimization of the Instrument

Mass-Hunter Optimizer provided the optimum fragmentor voltage, four fragmentor product ions with their respective RT and CE for instrumentation of multi-pesticides mixture of standard solutions. Table 4.1 shows the two selected product ions for each of the target pesticides. The optimization presented the analytes' qualifier (MRM_1) and quantifier (MRM_2) according to their abundances, CE and RT at a fixed fragmentor voltage of 380 V as literarily accorded (Alharbi et al., 2016; Naz et al., 2017). Also, Figure 4.1 and 4.2 illustrated the TIC and MRM scans of the multi-pesticide analytes respectively.

Table 4.1: Auto-tuning and Mass-Hunter optimization results of the instrument

PIN	Pesticide	MF	MIM	TOP	COC	IM (ESI)	PI	MRM_1/MRM_2	CE_1/CE_2	ART
8	Dursban (Chlorpyrifos)	$C_9H_{11}Cl_3NO_3PS$	349	Insecticide & Nematicide	Organophosphorus	$[M+H]^+$	350	96.8/197.9	34/22	11.36
7	Diazinon	$C_{12}H_{21}N_2O_3PS$	304	Insecticide	Organophosphorus	$[M+H]^+$	305	96.9/169.1	42/22	10.22
6	Thiamethoxam	$C_8H_{10}ClN_5O_3S$	292	Insecticide	Neonicotinoid	$[M+H]^+$	292	132/211	26/10	2.68
5	Metalaxyl	$C_{15}H_{21}NO_4$	279	Fungicide	Xylylalanine	$[M+H]^+$	280	160.1/220.1	26/10	7.33
4	Thiobencarb	$C_{12}H_{16}ClNOS$	257	Herbicide	Thiocarbamate	$[M+H]^+$	258	89.1/125	54/26	10.34
3	Baycarb (Fenobucarb)	$C_{12}H_{17}NO_2$	207	Insecticide	Carbamate	$[M+H]^+$	208	77/95	42/10	8.34
2	Carbaryl	$C_{12}H_{11}NO_2$	201	Insecticide & Nematicide	N-Methyl Carbamate	$[M+H]^+$	202	127.1/145	30/6	7.16
1	Propamocarb	$C_9H_{20}N_2O_2$	188	Fungicide	Other Carbamate	$[M+H]^+$	189	74/102.1	26/14	1.36

PIN, pesticide identity number; MF, molecular formula; MIM, mono-isotopic mass; TOP, type of pesticide; COC, class of chemical; IM, ionization mode; ESI, electrospray ionization; PI, precursor ion (m/z); MRM, multiple reactions monitoring; CE, collision energy (eV); ART, average retention time (min)

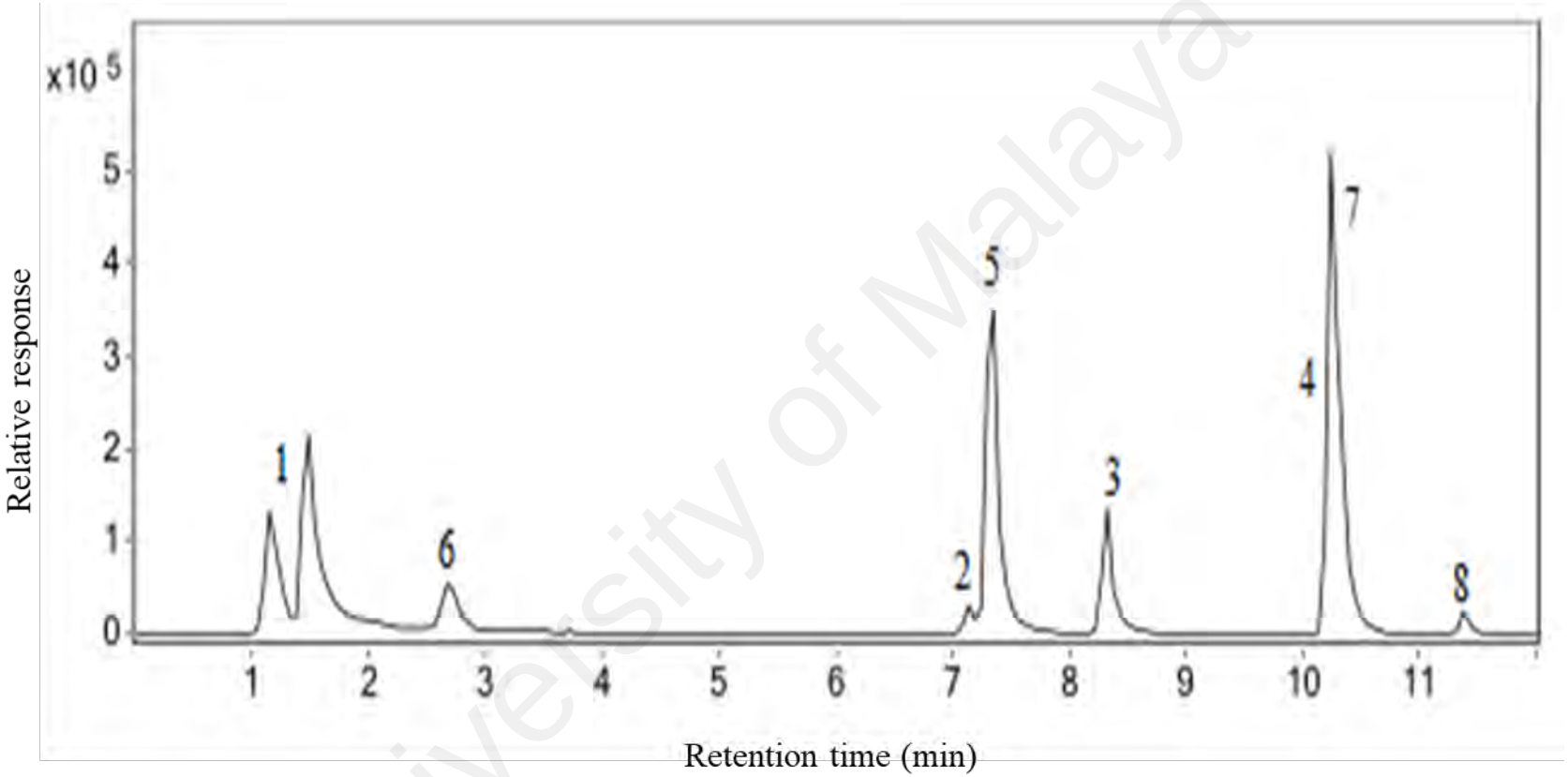


Figure 4.1: The chart of the Total Ion Chromatography (TIC) of the 1 mg/kg multi-pesticide analytes

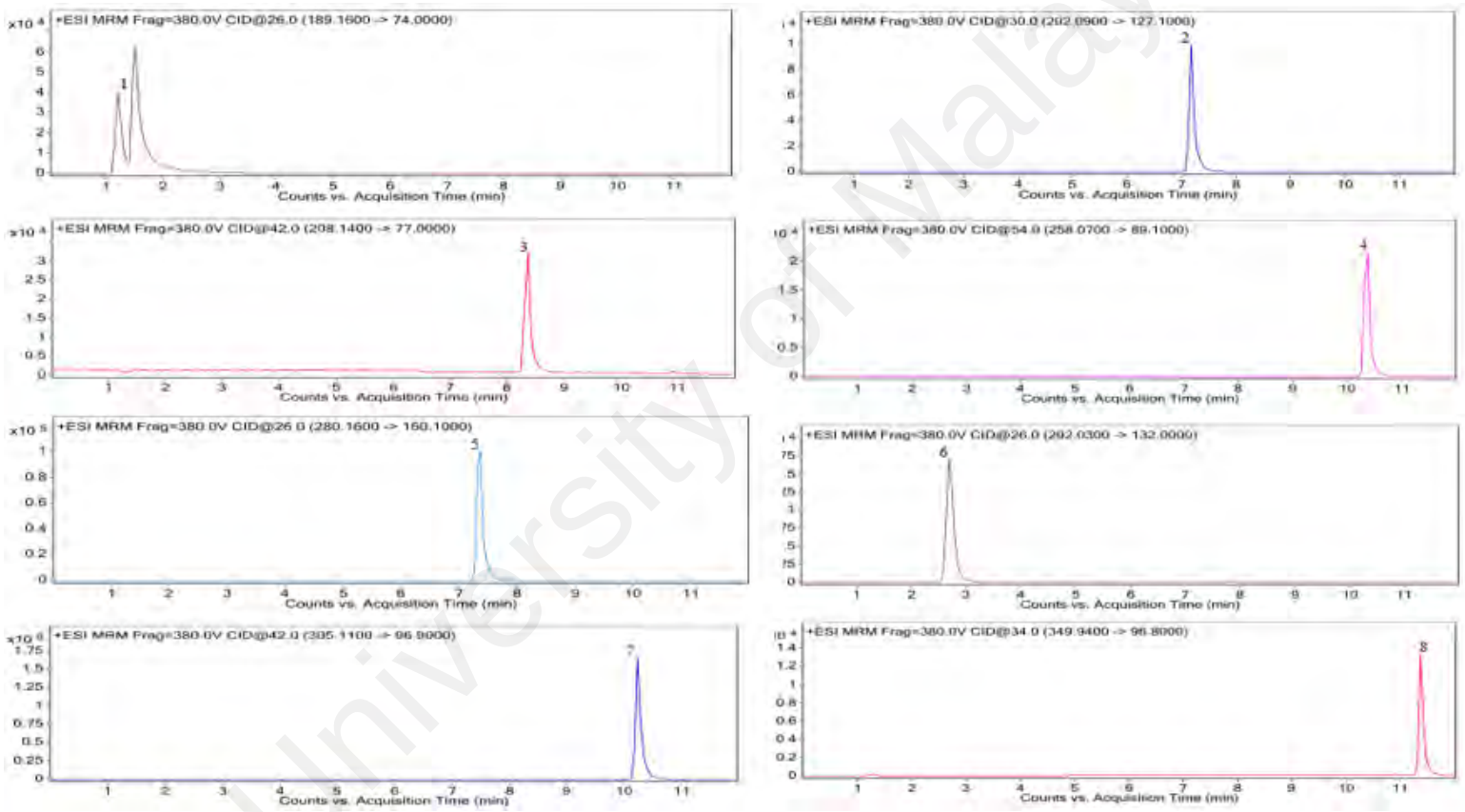


Figure 4.2: MRM illustrations for the multi-pesticides mixture of standard solutions

4.2 Selected and Optimized Mobile-Phases Setup for LC-MS/MS Instrumentation

After the screening and comparative studies of some selected mobile phases used for multi-pesticides residue determination in different kinds of matrices. Therefore, the mobile phase setup [0.1 % formic acid in Milli-Q-water (A) and 0.1 % formic acid in ACN (B)] was selected for this research. It was based on the results obtained for the highest ATCPA ± standard deviation (STDEV) and total chromatographic peak height (TCPH) or average TCPH (ATCPH ± STDEV) (Abdulra'uf & Tan, 2015) of three replicates as tabulated and illustrated in Table 4.2 and Figure 4.3 respectively. This result was supported by other findings using the mobile phase for pesticides analysis (Chen et al., 2015; Pastor-Belda et al., 2016) apart from the analyzed references.

Table 4.2: Selection of mobile phase for LC-MS/MS instrumentation

Ref codes	Ref	Water (A)	Organic M/Phase (B)	% M/Phase B	ATCPH ± STDEV	ATCPA ± STDEV
A	1st suggested mobile phase	A	ACN	25	(361 ± 2) x10^5	(47 ± 3) x 10^7
B	Rajski et al. (2013), Perez-ortega et al. (2012)	A + 0.1% FA	ACN	30	(349 ± 3) x 10^5	(46 ± 1) x 10^7
C	Nunez et al. (2012), Economou et al. (2009) and Lucas (2013)	A + 0.1% FA	ACN + 0.1% FA	15	(50 ± 1) x 10^6	(72 ± 9) x 10^7
D	Vázquez et al. (2015)	A + 0.1% FA	ACN + 0.1% FA + 5% A	30	(31 ± 2) x 10^6	(38 ± 1) x 10^7
E	2nd suggested mobile phase	A	MEOH	30	(17 ± 1) x 10^6	(23 ± 2) x 10^7
F	Golge et al. (2015)	A + 5 mM AF	MEOH + 5 mM AF	30	(26 ± 2) x 10^6	(30 ± 1) x 10^7
G	Zanella et al. (2013)	A + 2% MEOH + 0.1% FA + 5 mM AF	MEOH + 0.1% FA + 5 mM AF	10	(58 ± 3) x 10^6	(60 ± 7) x 10^7
H	3rd suggested mobile phase	A	MEOH/ACN (1:1)	30	(27 ± 1) x 10^6	(30 ± 4) x 10^7
I	4th suggested mobile phase	A + 5 mM AF + 0.1%FA	MEOH/ACN (1:1) + 0.1% FA + 5 mM AF	25	(36 ± 5) x 10^6	(32 ± 3) x 10^7

ATCPH, average total chromatographic peak height; ATCPA, average total chromatographic peak area; RT, retention time; AF, ammonium formate; FA, formic acid; STDEV, standard deviation; Ref, reference

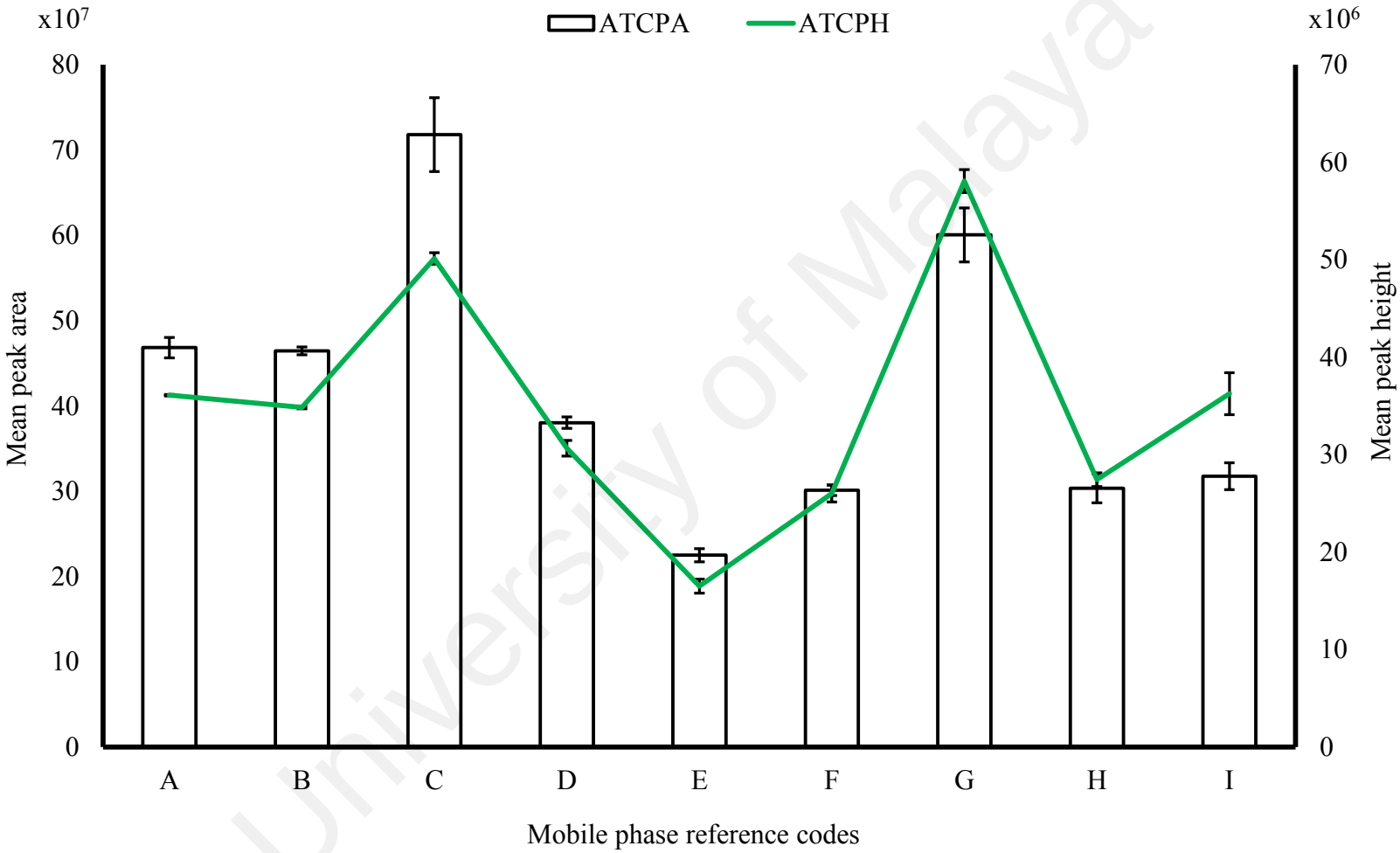


Figure 4.3: The comparative studies of ATCPA and ATCPH results for the analyzed mobile phases

The addition of organic solvent into aqueous mobile phase could provide the optimum condition of log*P* or XLogP_3, which contributes to the attainment of good condition for the multi-pesticide residues analysis in food samples using LC-MS/MS instrument as revealed (Zanella et al., 2013). For this reason, optimization was carried out by serial addition of ACN into the aqueous mobile phase (0.1 % FA milli-Q-water). Consequently, the optimized result revealed that addition of 1 % ACN and 0.1 % FA Milli-Q-water at an average pH of 3.50 ± 0.07 STDEV (mobile phase A) coupled with 0.1 % FA in ACN at pH 6.56 ± 0.04 STDEV (mobile phase B) provided the highest ATCPA (Table 4.3). The result was supported by their respective pH readings (Table 4.3). The two tables were graphically illustrated in Figure 4.4.

Table 4.3: Optimization of selected mobile phase used for LC-MS/MS instrumentation

Solution	% ACN in Aqueous Mobile Phase	ApH reading ± STDEV	Organic Mobile Phase	ATCPA ± STDEV
1	H_2O + 0.1% FA + 0% ACN	3.36 ± 0.00	ACN + 0.1% FA	(27 ± 2) x 10^6
2	H_2O + 0.1% FA + 0.5% ACN	3.37 ± 0.08	ACN + 0.1% FA	(27 ± 1) x 10^6
3	H_2O + 0.1% FA + 1.0% ACN	3.50 ± 0.07	ACN + 0.1% FA	(28 ± 2) x 10^6
4	H_2O + 0.1% FA + 1.5% ACN	3.48 ± 0.04	ACN + 0.1% FA	(27 ± 2) x 10^6
5	H_2O + 0.1% FA + 2.0% ACN	3.45 ± 0.01	ACN + 0.1% FA	(261 ± 3) x 10^5
6	H_2O + 0.1% FA + 2.5% ACN	3.47 ± 0.00	ACN + 0.1% FA	(265 ± 6) x 10^5
7	H_2O + 0.1% FA + 3.0% ACN	3.46 ± 0.01	ACN + 0.1% FA	(2652 ± 4) x 10^4
8	H_2O + 0.1% FA + 3.5% ACN	3.48 ± 0.00	ACN + 0.1% FA	(26 ± 1) x 10^6
9	H_2O + 0.1% FA + 4.0% ACN	3.45 ± 0.04	ACN + 0.1% FA	(26 ± 1) x 10^6
10	H_2O + 0.1% FA + 4.5% ACN	3.41 ± 0.00	ACN + 0.1% FA	(262 ± 5) x 10^5
11	H_2O + 0.1% FA + 5.0% ACN	3.38 ± 0.07	ACN + 0.1% FA	26 x 10^6 ± 0
12	H_2O + 0.1% FA + 7.5% ACN	3.37 ± 0.03	ACN + 0.1% FA	(259 ± 4) x 10^5
13	H_2O + 0.1% FA + 10.0% ACN	3.37 ± 0.03	ACN + 0.1% FA	(256 ± 4) x 10^5

FA, formic acid; ApH, average pH reading; ATCPA, average total chromatographic peak area; STDEV, standard deviation

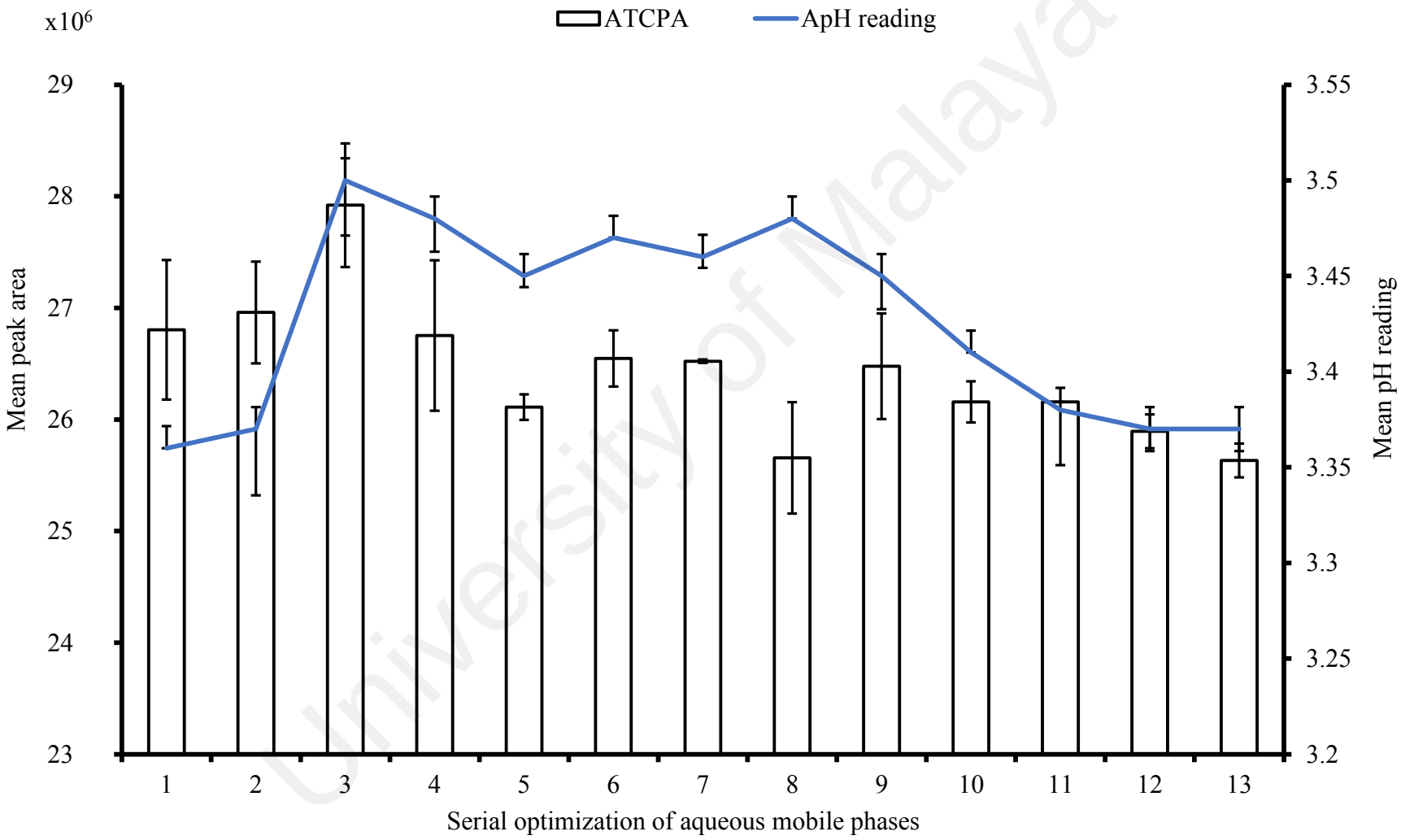


Figure 4.4: Comparative illustration for the optimization of the selected aqueous mobile phase by ATCPA and ApH readings

Moreover, the retention time (min) of the pesticide analytes were less than the results reported by the literatures such as thiamethoxam, 2.68 < 2.87 (Friedrich et al., 2016); propamocarb, 1.36 < 1.47 (Martínez-Domínguez et al., 2015); carbaryl, 7.16 < 16.0 (Morais et al., 2018); metalaxyl, 7.33 < 17.90 (Miliadis et al., 2017); thiobencarb 10.34 < 10.76 (Rebelo et al., 2016), and dursban, 11.36 < 12.30 (Bordin et al., 2016). But the retention time (min) of baycarb (8.34) and diazinon (10.22) were more than 6.73 (Zheng et al., 2017) and 7.09 (Lopez et al., 2016) respectively. Fortunately, the optimized mobile phase contributes towards shortening the total run time (min) for the multiple pesticides analysis using the LC-MS/MS instrument.

Justifiably, the result (ATCPA ± STDEV) of the optimized (selected) mobile phase (51%) setup [(28 ± 1) x 10^6] was slightly (2%) better than the unoptimized mobile phase (49%) setup [(27 ± 1) x 10^6] after comparative studies. Eventually, the optimized mobile phase setup [1 % ACN and 0.1 % FA in Milli-Q-water (mobile phase A) coupled with 0.1 % FA in ACN (mobile phase B)] was used in the LC-MS/MS instrumentation. This process was carried out using the gradient run for the determination of multi-pesticide residues in a sample of fruits and vegetables.

4.3 The Response Plots for Plackett-Burman and Box-Behnken Design

The significant level of Plackett-Burman design is expressed using responses of Normal plot and Pareto chart of standardized effects. The Pareto chart of standardized effects is an illustration of horizontal bars for the screened factors and the red vertical line across the bars indicating the level of significant difference.

Meanwhile, the responses of the surface plot were used for the expression of Box-Behnken design. The plot is illustrated in a three-dimensional view that may provide a clearer picture of the TCPA responses. Furthermore, the interception of TCPA against two significant factors leads to the production of a surface plot. Thus, the highest spot on

the three-dimensional response view of the surface plot signified the best-optimized value that could provide the maximum TCPA.

4.3.1 RSM Optimized LC-MS/MS Instrument

4.3.1.1 Plackett-Burman design responses for the LC-MS/MS optimization

Virtually, 24 runs (Table 4.4) were involved in the Plackett-Burman design generated, for analysis of 11 critical factors of the Agilent (G6490A) LC-MS/MS Instrument as reportedly used (Coscolla et al., 2008). Out of which 23 runs were successfully carried out, but only one run was not successful because the setup was not compatible with the instrumental condition for operation (instrumental error). Consequently, Minitab software was capable enough to carry out the statistical screening at a 95% confidence interval (0.05 significant level) of all the responses except the unsuccessful run. The Minitab software for the estimation of the significant factors did not use the unsuccessful run.

Table 4.4: Plackett-Burman design responses for eleven factors for the LC-MS/MS instrumentation

RO	A	B	C	D	E	F	G	H	J	K	L	TCPA
1	20	35	0.1	1	150	11	60	200	12	2000	100	6623319
2	20	35	0.1	1	250	17	30	400	10	4000	200	12506025
3	10	35	0.2	1	150	17	60	200	12	2000	200	*
4	20	35	0.1	5	150	17	60	400	12	4000	100	52297183
5	20	25	0.2	1	150	17	60	200	10	4000	200	7533311
6	20	25	0.2	1	250	17	60	400	12	2000	100	5309048
7	20	25	0.1	1	150	17	30	400	10	2000	200	18647683
8	10	35	0.2	5	250	17	30	200	10	2000	200	21393759
9	10	35	0.1	5	150	11	60	400	10	2000	200	112836048
10	10	25	0.2	5	150	17	30	400	12	4000	200	34570903
11	20	25	0.1	5	250	11	30	400	12	2000	200	96951628
12	10	35	0.1	5	250	17	60	400	10	2000	100	50058252
13	10	25	0.2	5	150	11	60	400	10	4000	100	25741308
14	20	25	0.1	5	250	11	60	200	12	4000	200	61048937
15	10	35	0.2	1	250	11	60	400	12	4000	200	4561516
16	20	35	0.2	1	150	11	30	400	10	4000	100	4013727
17	10	35	0.1	1	250	17	30	200	12	4000	100	4433525
18	10	25	0.1	5	150	17	30	200	12	4000	100	27091499
19	10	25	0.1	1	150	11	30	200	10	2000	100	5613458
20	20	35	0.2	5	150	11	30	200	12	2000	200	32956285
21	10	25	0.1	1	250	11	60	200	10	4000	200	11843094
22	20	25	0.2	5	250	17	60	200	10	2000	100	11594866
23	10	25	0.2	1	250	11	30	400	12	2000	100	4766861
24	20	35	0.2	5	250	11	30	200	10	4000	100	13334480

RO, run order; A, starting mobile phase B (%); B, column temperature (ºC); C, flow rate (mL/L); D, injection volume (µL); E, gas temperature (ºC); F, gas flow (L/min); G, nebulizer gas (psi); H, sheath gas temperature (ºC); J, sheath gas flow (L/min); K, capillary voltage (V); L, delta$^{(+)}$ EMV (V); TCPA, total chromatographic peak area; *, unused setup because of instrumental error

Resultantly, the significant factors were Flow rate, Injection volume, Sheath gas temperature and Delta$^{(+)}$ EMV. The results were illustrated in the chart (Figure 4.5).

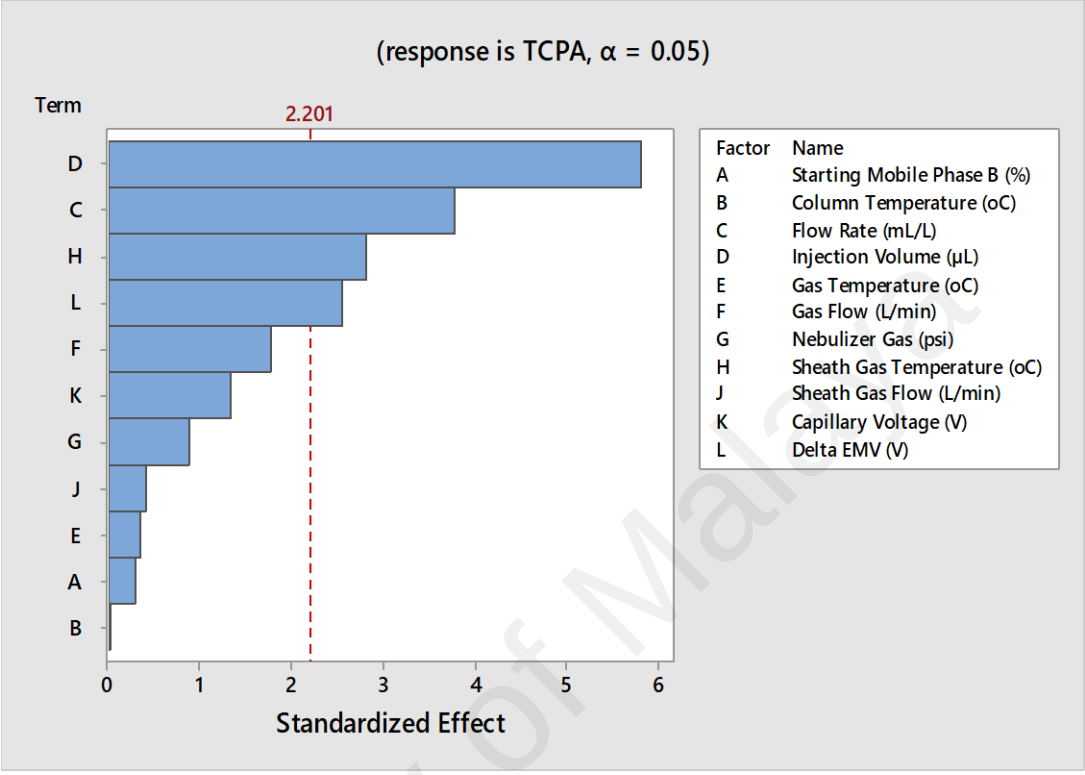


Figure 4.5: Plackett-Burman Pareto chart of 11 screened instrumental factors

The significance of the flow rate agree with the finding of Patel et al. (2017) using an optimized HPLC for quantification of roxithromycin and ambroxol hydrochloride in tablets. The mathematical (regression) model for the screened factors of Plackett-Burman design (Equation 4.1) agrees with the model documented by Vallejo et al. (2010). The *P*-value of the model from the ANOVA results were significant (0.002) which is less than 0.05 statistical level (Table 4.5).

$$TCPA = -472880 + 194492A + 7240B + 248006088C + 9530340D - 22242E - 1920657F + 193706G + 92594H + 1335916J - 4348K + 167116L \quad \text{Eqn (4.1)}$$

Table 4.5: Factorial regression for Plackett-Burman runs for the optimization of LC-MS/MS

TERMS	ANALYSIS OF VARIANCE		UNCODED COEFFICIENTS				
Source	*F*-Value	*P*-Value	Main Effect	Coefficient	SE Coefficient	*T*-Value	*P*-Value
Model	6.42	**0.002**	-	-	-	-	-
Linear	6.42	0.002	-	-	-	-	-
Constant	-	-	-	25928917	3276426	7.91	0.000
A	0.09	0.772	1944916	972458	3276426	0.30	0.772
B	0.00	0.991	72400	36200	3276426	0.01	0.991
C	14.32	**0.003**	-24800609	-12400304	3276426	-3.78	**0.003**
D	33.84	**0.000**	38121358	19060679	3276426	5.82	**0.000**
E	0.12	0.741	-2224168	-1112084	3276426	-0.34	0.741
F	3.09	0.106	-11523944	-5761972	3276426	-1.76	0.106
G	0.79	0.394	5811194	2905597	3276426	0.89	0.394
H	7.99	**0.016**	18518864	9259432	3276426	2.83	**0.016**
J	0.17	0.691	2671831	1335916	3276426	0.41	0.691
K	1.76	0.211	-8695248	-4347624	3276426	1.33	0.211
L	6.50	**0.027**	16711579	8355789	3276426	2.55	**0.027**

Model Summary

S =	15421440	R^2 (adjusted) =	73.04 %
R-square (R^2) =	86.52 %	R^2 (predicted) =	39.27 %

A, starting mobile phase B (%); B, column temperature (°C); C, flow rate (mL/L); D, injection volume (µL); E, gas temperature (°C); F, gas flow (L/min); G, nebulizer gas (psi); H, sheath gas temperature (°C); J, sheath gas flow (L/min); K, capillary voltage (V); L, delta EMV (V); TCPA, total chromatographic peak area

4.3.1.2 Box-Behnken design responses for the LC-MS/MS optimization

Based on the Plackett-Burman screened factors of the of the LC-MS/MS, 27 optimization runs were carried out on the four significant factors that include Flow rate (A), Injection volume (B), Sheath gas temperature (C) and Delta$^{(+)}$ EMV (D) at 0.05 significant level using Box-Behnken design (Dong et al., 2009) as tabulated (Table 4.6).

Table 4.6: Box-Behnken design responses for optimization of LC-MS/MS significant factors

RO	PT	A	B	C	D	TCPA
1	2	0.15	1	300	100	3212880
2	2	0.1	3	300	200	45948949
3	2	0.15	3	400	100	21226211
4	2	0.15	5	300	100	31639197
5	0	0.15	3	300	150	27412061
6	2	0.1	5	300	150	59341689
7	2	0.2	3	300	100	13545963
8	2	0.15	1	300	200	10281586
9	2	0.15	3	200	100	13676817
10	0	0.15	3	300	150	25328649
11	2	0.15	5	400	150	53961209
12	2	0.2	5	300	150	31894779
13	2	0.2	3	400	150	20830312
14	2	0.2	1	300	150	5171605
15	2	0.15	3	400	200	46590534
16	2	0.15	3	200	200	29513573
17	2	0.15	5	300	200	51031714
18	2	0.1	1	300	150	11273447
19	2	0.15	1	400	150	10495997
20	2	0.2	3	200	150	13190386
21	2	0.2	3	300	200	22668595
22	0	0.15	3	300	150	25890246
23	2	0.15	5	200	150	33968671
24	2	0.1	3	400	150	12506025
25	2	0.1	3	300	100	47552062
26	2	0.1	3	200	150	29940610
27	2	0.15	1	200	150	6706898

RO, Run Order; PT, Point Type; C, Flow Rate (mL/L); D, Injection Volume (µL); H, Sheath Gas Temperature (ºC); L, Delta EMV (V)

The optimized factors of the LC-MS/MS instrument could yield more pesticide recoveries (TCPA) if the instrument is set-up at 0.1 mL/L (flow rate), 5 µL (injection volume), 400 ºC (Sheath gas temperature) and 200 V (Delta EMV) when other factors are setup at medium (default) level. In fact, the decrease in flow rate will consequently increase the responses of the detector which at long-run increases the chromatographic peak area of the targeted analytes as mathematically expressed in Equation 4.2 ("Chromatography", 2014).

$$A_x = \frac{K_x M_x}{Q} \qquad \text{Eqn (4.2)}$$

Where

A_x: The peak area of compund x

K_x: The sensitivity of the detector towards compund x

M_x: The injected mass of compund x

Q: The flow rate

Furthermore, the increase in the concentration (injection volume) of analytes onto the column resulted in higher responses of the detector and increased the peak height and area to a certain level. Unfortunately, the responses of the detector decrease when the injection volume is exceeded due to column overload (Dolan, 2015). On the other hand, increasing the value of Delta$^{(+)}$ EMV and the temperature of the nitrogen sheath gas in the mass spectrometry component fundamentally improves the nebulizer spray which increases sensitivity of the detector and ultimately increases the peak areas of targeted analytes (Greco et al., 2014; Yuan et al., 2018). Moreover, Figure 4.6 illustrates the optimized factors.

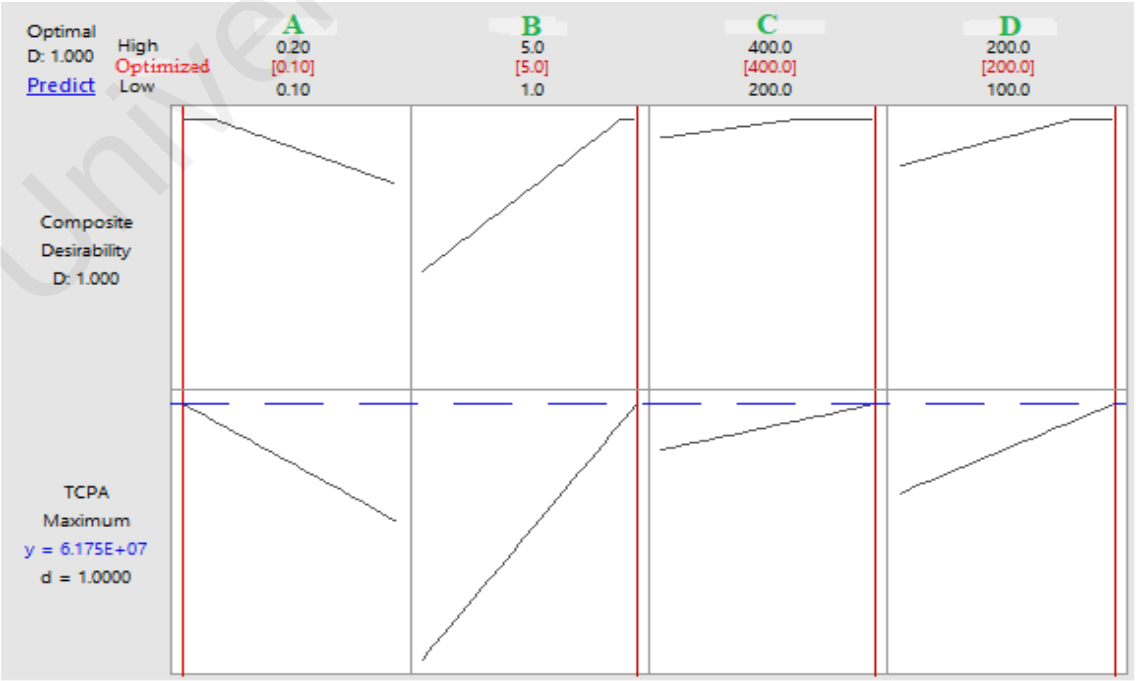


Figure 4.6: Box-Behnken response optimization chart for the instrument

Furthermore, the graphical illustration of the resulted response surfaces (Figure 4.7 – 4.12) has presented the optimized values by taking into account the interactions between significant factors which yielded the optimum TCPA when the insignificant factors were setup at a medium level.

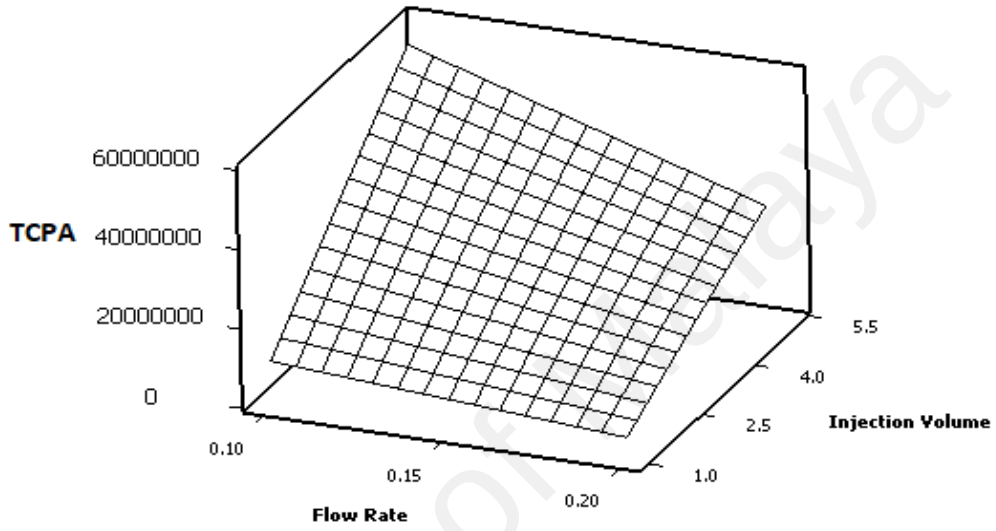


Figure 4.7: Surface plot shows the interaction between Flow rate and Injection volume that yielded highest TCPA

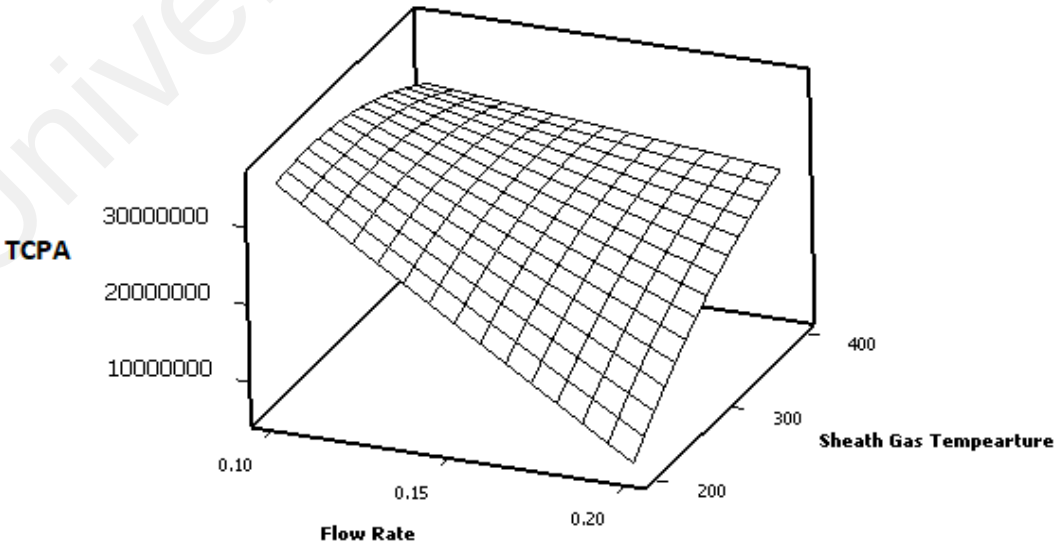


Figure 4.8: Surface plot illustration yielded maximum TCPA when Flow rate interacted with Sheath gas temperature

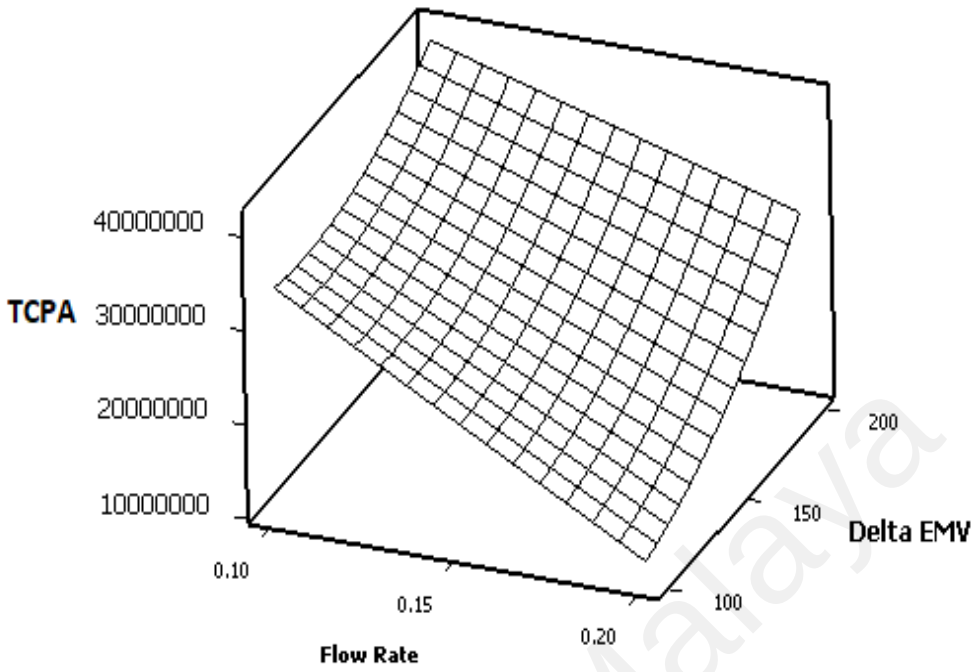


Figure 4.9: Surface plot indicated the highest value of TCPA when Flow rate interacted with Delta EMV

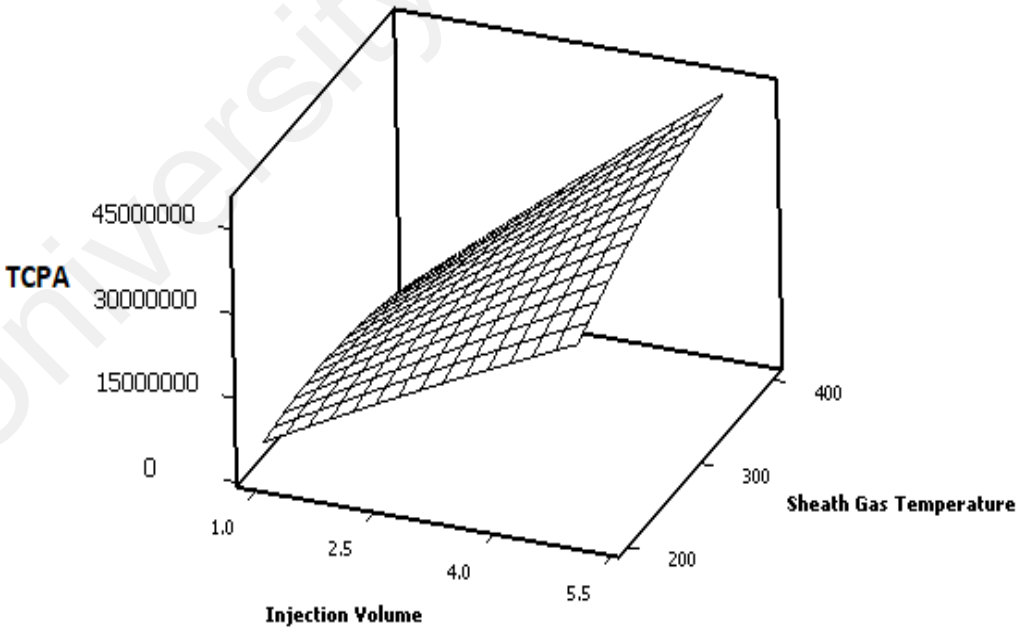


Figure 4.10: Maximum level of TCPA attained on the Surface plot after interaction between Injection volume and Sheath gas temperature

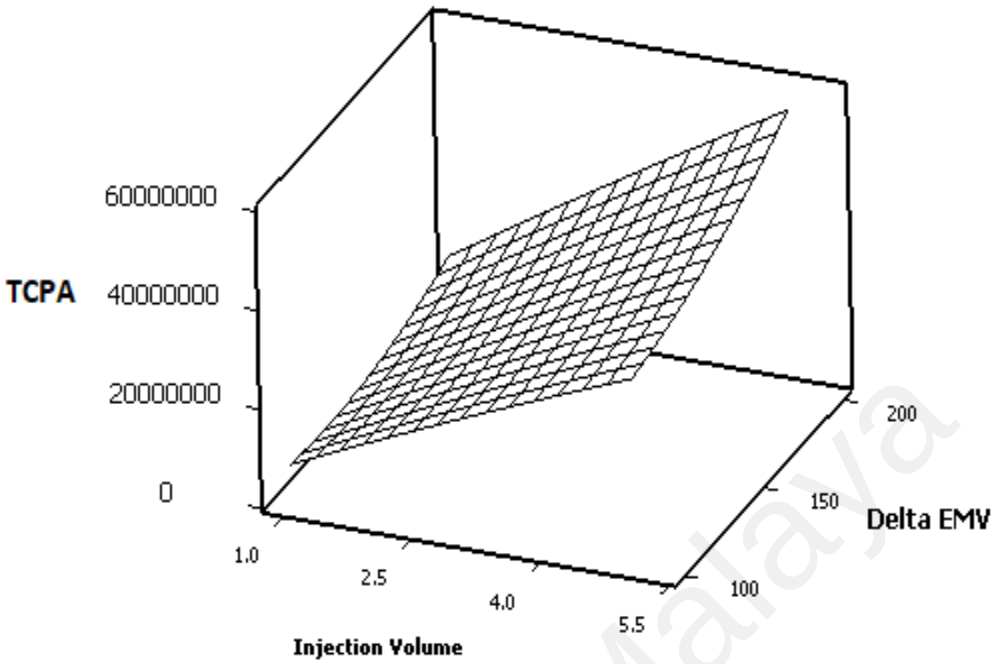


Figure 4.11: Surface plot illustration for Injection volume interaction with Delta EMV, which resulted in maximum TCPA

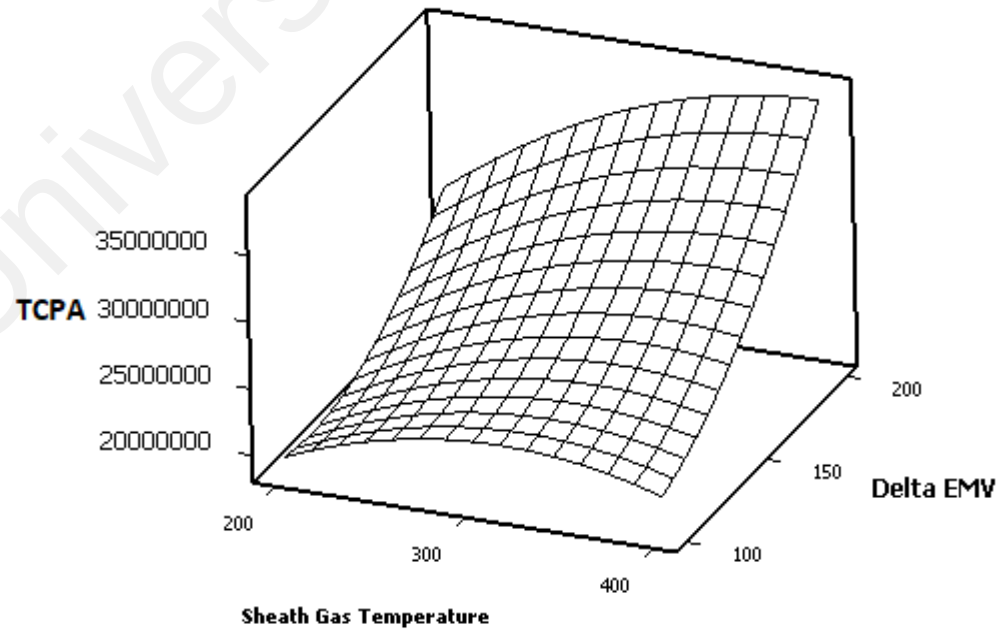


Figure 4.12: Surface plot illustrated the interaction of Sheath gas temperature and Delta EMV that resulted in the highest TCPA

Generally, the mathematical model of the second-degree polynomial for the interactions is expressed in Equation 4.3. The Equation expresses possibilities of having a minimum or maximum quadratic terms among the interacted variables (Hameed et al., 2009; Hibbert, 2012).

$$Y = \beta_0 + \sum_{j=1}^{n} \beta_i X_i + \sum_{i=1}^{n} \sum_{j>1}^{n} \beta_{ij} X_i X_j + \sum_{i=1}^{n} \beta_{ii} X_i^2 + \varepsilon \qquad \text{Eqn (4.3)}$$

Where

Y: The dependent variable

β_0: The average response in factorial expression,

β_i: The linear coefficient

β_{ij}: The interaction coefficient

β_{ii}: The quadratic coefficient

$X_i \; and \; X_j$: The coded values of independent variables

ε: The random error

Besides, the ANOVA (*P*-value) of the response surface regression for Box-Behnken design runs of the optimized LC-MS/MS factors were significant (0.003 < 0.05 statistical level) (Table 4.7). This result is in accordance with the recent report for Box-Behnken design optimization of HPLC which was used for the analysis of pravastatin in pharmaceutical products (Ahmad et al., 2016).

Table 4.7: Factorial regression of Box-Behnken design runs for the optimized LC-MS/MS

TERMS	ANALYSIS OF VARIANCE		CODED COEFFICIENTS			
Source	*F*-value	*P*-value	Coefficient	SE Coefficient	*T*-value	*P*-value
Regression	5.53	**0.003**	-	-	-	-
Linear	0.56	0.698	-	-	-	-
Constant	-	-	107517520	107325799	1.002	0.336
A	0.84	0.378	-546849732	597673447	-0.915	0.378
B	0.32	0.584	7111958	12645837	0.562	0.584
C	0.19	0.669	-130921	298837	-0.438	0.669
D	1.08	0.319	-620779	597673	-1.039	0.319
Square	0.42	0.793	-	-	-	-
A*A	0.00	0.992	14995550	1500711318	0.010	0.992
B*B	0.02	0.881	-143276	937945	-0.153	0.881
C*C	0.49	0.498	-262	375	-0.698	0.498
D*D	0.60	0.452	1166	1501	0.777	0.452
Interaction	0.95	0.498	-	-	-	-
A*B	1.52	0.242	-53362670	43321804	-1.232	0.242
A*C	2.09	0.174	1253726	866436	1.447	0.174
A*D	0.38	0.548	1072575	1732872	0.619	0.548
B*C	0.87	0.368	20254	21661	0.935	0.368
B*D	0.51	0.491	30810	43322	0.711	0.491
C*D	0.30	0.593	476	866	0.550	0.593
Lack-of-Fit	77.33	0.013				

Model Summary

S	R^2	R^2 (adjusted)	R^2 (predicted)
8664361	86.57 %	70.91 %	22.79 %

A, Flow rate; B, Injection volume; C, Sheath gas temperature and D, Delta EMV

4.3.2 RSM Optimized Settings of the LC-MS/MS Instrument

The instrumental parameters were optimized using the RSM designs for Planket-Burman and Box-Behnken. The statistical software generated the designs, and the optimization was carried out on the TCPA responses obtained from integrated multiple reactions monitoring (MRM) scans of 1 mg/kg pesticides mixture of standard solutions. The instrument was operated at 15 % starting organic mobile phase B (ACN + 0.1 % FA) with mobile phase A (deionized H_2O + 0.1% FA + 1% ACN) pushed by a stream of nitrogen gas. The 5 µL analyte solution was injected, passed and runs through a Supelco HPLC column [Ascentis® Express C_{18} (5 cm x 2.1 mm, 2.7 µm)] at 30 ºC and flow rate of 0.1 mL/min. The Mass Hunter Triple quadrupole (QQQ) setup was operated at 200 ºC (Gas Temperature), 14 L/min (Gas Flow), 45 psi (Nebulizer Gas), 400 ºC (Sheath Gas Temperature), 11 L/min (Sheath Gas Flow), 3000 V (Capillary Voltage), and 200 V

(Delta$^{(+)}$ EMV). Eventually, the technical optimization processes provided the best condition for the ESI to encourage higher responses of TCPA for the resulting concentration of the multi-pesticides mixture of standard solutions (Coscolla et al., 2008). It was based on a confirmatory study carried out using 100 µg/kg standard solution of the multi-pesticides mixture. The result of the studies (ATCPA ± STDEV) in triplicate favored RSM optimized LC-MS/MS instrumental setup by 68% ATCPA [(17 ± 2) x 10^7] over the unoptimized 32% ATCPA [(8 ± 1) x 10^7] setup after comparative analysis (Karapinar et al., 2016). Advantageously, the impact of the optimized settings of the instrument successfully increases the instrumental efficiency, which contributed to improving the sensitivity of the sample preparation technique. For instance, the TCPA of the optimized instrumental setup could help to detect and quantifying the targeted analytes at lower concentration level with the provision of satisfactory results of accuracies and precisions in the analyzed sample of fruits and vegetables (Jansson et al., 2004). Later on, the RSM optimized LC-MS/MS settings was employed for the development of the sample preparation technique, which involved sequences of RSM optimizations, combinations and comparative studies of QuEChERS-dSPE and ionic liquid-based DLLME extraction/cleanup methods.

4.4 Optimized Extraction Methods

4.4.1 Selected QuEChERS Extraction Solvents

The selected QuEChERS extraction solvent used for this research was ACN because of its ability to providing the most substantial quantity (volume) and clearest extract solution of the analyte with the lowest matrix interferences. The solvent was selected after comparison with methanol (MeOH), ACN/MeOH (1:1 v/v) and ethanol (EtOH). Also, the sample preparation using the ACN leads to better resolution of analyte chromatograms for the multi-pesticides residue analysis in fresh samples of vegetable and fruit (Anastassiades et al., 2003; Xie et al., 2014).

4.4.2 Selected Ionic Liquid-Based for DLLME Extraction

[C_6MIM][PF_6] ionic liquid-based was selected and for this research. Fortunately, it is the most commonly employed ionic liquid-based for analytes extraction. It is due to its capabilities of high-efficiency cleanup of matrix interferences during sample preparation, excellent chromatographic, hydrophobic, lower volatility and economic benefits as compared to other ionic liquid-based such as 1-butyl-3-methylimidazolium hexafluorophosphate ([C_4MIM][PF_6]) and 1-octyl-3-methylimidazolium hexafluorophosphate ([C_8MIM][PF_6]) (Faraji et al., 2016; Xie et al., 2014; Zhang et al., 2016).

4.4.3 RSM Optimized QuEChER-dSPE Method

4.4.3.1 Screened and optimized significant factors of QuEChERS-dSPE method

*(a) **Plackett-Burman screening***

Plackett-Burman designs with 12 runs (Table 4.8) was used to screen the significant factors as related to the QuEChERS-dSPE method (Fang et al., 2017).

Table 4.8: Plackett-Burman design responses for screening 6-factors of QuEChERS-dSPE method

Run Order	A (mL)	B (%)	C (rpm)	D (min)	E (rpm)	F (min)	TCPA
1	13.3	0	7000	2	1000	2	305490
2	13.3	2	1000	8	1000	2	357928
3	6.7	2	7000	2	7000	2	375391
4	13.3	0	7000	8	1000	8	464061
5	13.3	2	1000	8	7000	2	383030
6	13.3	2	7000	2	7000	8	364323
7	6.7	2	7000	8	1000	8	382003
8	6.7	0	7000	8	7000	2	461944
9	6.7	0	1000	8	7000	8	457133
10	13.3	0	1000	2	7000	8	432234
11	6.7	2	1000	2	1000	8	369337
12	6.7	0	1000	2	1000	2	455224

A, sample quantity for QuEChERS extraction; B, % HOAc in 15 ml of ACN; C, QuEChERS extraction centrifugation speed; D, QuEChERS extraction time; E, centrifugation speed for d-SPE; F, cleanup time for d-SPE

The Plackett-Burman design screening was carried out at 0.26 significant level in order to limit the factors to three. The significant factors include sample quantity, the percentage of HOAc in 15 mL of ACN and QuEChERS extraction (centrifugation) time as illustrated (Figure 4.13).

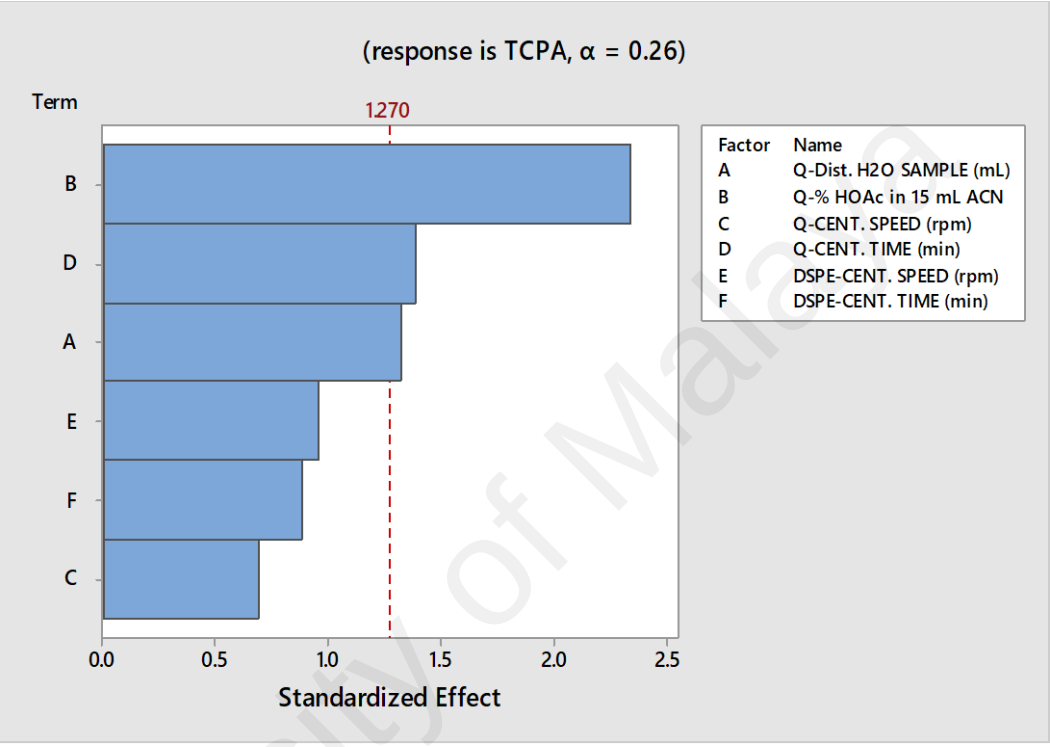


Figure 4.13: Pareto chart of Plackett-Burman design showing the screened factors of QuEChERS-dSPE method

The significance of the QuEChERS centrifugation time (min) is in accordance with the report of Manav et al. (2018) for the determination of pesticides in a sample of diaries and milk after the RSM optimization of QuEChERS method. Table 4.9 shows the ANOVA results that indicated the *P*-value of the model is significant (0.253 < 0.26 statistical level). The regression model for the screened factors of Plackett-Burman design is expressed in Equation 4.4. The equation agrees with the model reported by Vallejo et al. (2010).

$$TCPA = 427655 - 4898A - 28673B - 2.82C + 5669D + 3.89E + 3613F \quad \text{Eqn (4.4)}$$

Table 4.9: Factorial regression of Plackett-Burman design runs for screening the factors of QuEChERS-dSPE method

TERMS	ANALYSIS OF VARIANCE		UNCODED COEFFICIENTS				
Source	*F*-value	*P*-value	Main Effect	Coefficient	SE Coefficient	*T*-value	*P*-value
Model	1.88	**0.253**	-	-	-	-	-
Linear	1.88	0.253	-	-	-	-	-
Constant	-	-	-	400675	12277	32.64	0.000
A	1.73	**0.245**	-32328	-16164	12277	-1.32	**0.245**
B	5.45	**0.067**	-57346	-28673	12277	-2.34	**0.067**
C	0.48	0.521	-16946	-8473	12277	-0.69	0.521
D	1.92	**0.225**	34017	17008	12277	1.39	**0.225**
E	0.90	0.386	23335	11668	12277	0.95	0.386
F	0.78	0.418	21681	10840	12277	0.88	0.418
Model Summary							
S	=	42529.0		R^2 (adjusted) =		32.38%	
R^2	=	69.26%		R^2 (predicted) =		0.00%	

A, sample quantity for QuEChERS extraction (mL); B, percentage of HOAc in 15 mL of ACN for QuEChERS extraction (%); C, QuEChERS centrifugation speed (rpm); D, QuEChERS centrifugation time (min); E, d-SPE centrifugation speed (rpm); F, d-SPE centrifugation time (min)

(b) Box-Behnken optimization

All the 15 experimental runs carried out (Table 4.10) were victorious for optimization (0.26 significant level) of the screened factors of the QuEChERS-dSPE method which were represented by A, B, and C.

Table 4.10: Box-Behnken design responses for the 3-significant factors of QuEChERS-dSPE method

Run Order	Point Type	A (mL)	B (%)	C (min)	TCPA
1	2	6.7	0	5	468556
2	2	13.3	0	5	597884
3	2	6.7	2	5	441682
4	2	13.3	2	5	497001
5	2	6.7	1	2	579964
6	2	13.3	1	2	490067
7	2	6.7	1	8	537592
8	2	13.3	1	8	493233
9	2	10	0	2	523942
10	2	10	2	2	460280
11	2	10	0	8	389043
12	2	10	2	8	423322
13	0	10	1	5	474820
14	0	10	1	5	467664
15	0	10	1	5	468895

A, the quantity of sample (Milli-Q-water) for QuEChERS extraction; B, % HOAc in 15 ml of ACN; C, QuEChERS extraction time; TCPA, total chromatographic peak area

The RSM optimized values are 13.3 mL (≈ 20 g fresh fruit) quantity of sample, 0 % HOAc in 15 mL of ACN, and 2 min of QuEChERS extraction time. However, the 0 % HOAc in 15 mL of ACN is less than the commonly used 1 % HOAc in 15 mL of ACN for the analysis of pesticides, bio-pesticides and agrochemicals (Golge & Kabak, 2015; Jadhav et al., 2015; Romero-González et al., 2014). It could be as a result of high acidic medium (pH) of the default prepared sample (5.37 ± 0.04) coupled with the high pH of the mobile phase A (3.68 ± 0.06) and B (6.56 ± 0.02) which could have diminished the analytes recovery. While the pH of the RSM optimized prepared sample (8.33 ± 0.01) coupled with the mobile phases A and B setup resulted in the higher recovery of analytes. The result agrees with the documentation of Georgakopoulos and Skandamis (2011). Moreover, the optimized setup which favors the reduction of QuEChERS centrifugation time from 5 (default) to 2 min essentially increases the rapidness of the QuEChERS extraction (Hepperle et al., 2015). Thus, Figure 4.14 highlighted the graphical illustration of QuEChERS factors that were optimized to obtain higher TCPA.

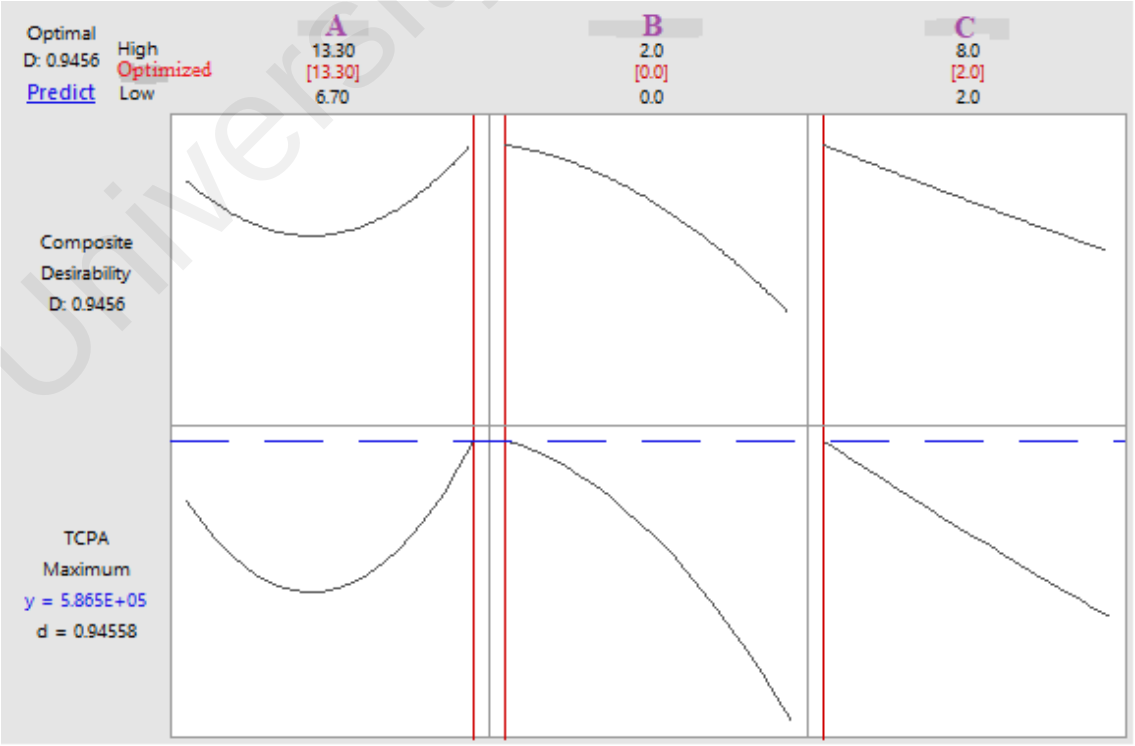


Figure 4.14: Box-Behnken response optimization chart for the 3-significant factors of QuEChERS-dSPE technique

Furthermore, the surface plots (Figure 4.15 – 4.17) illustrated the response surfaces that respectively resulted in the best (optimized) condition to yields more of TCPA collectively, when the insignificant factors were setup at a medium level.

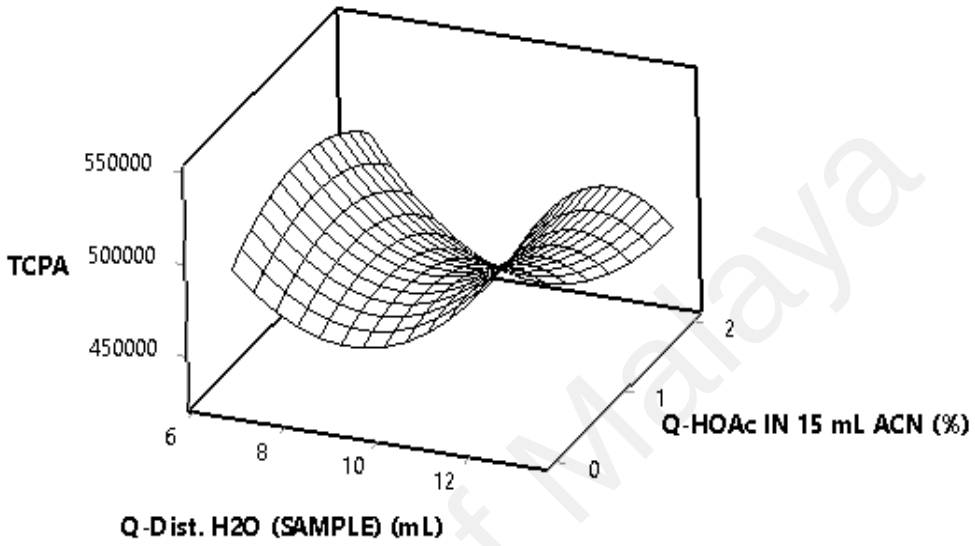


Figure 4.15: Surface plot illustrated the interaction of QuEChERS sample quantity and QuEChERS % HOAc in 15 mL ACN that resulted in the highest TCPA

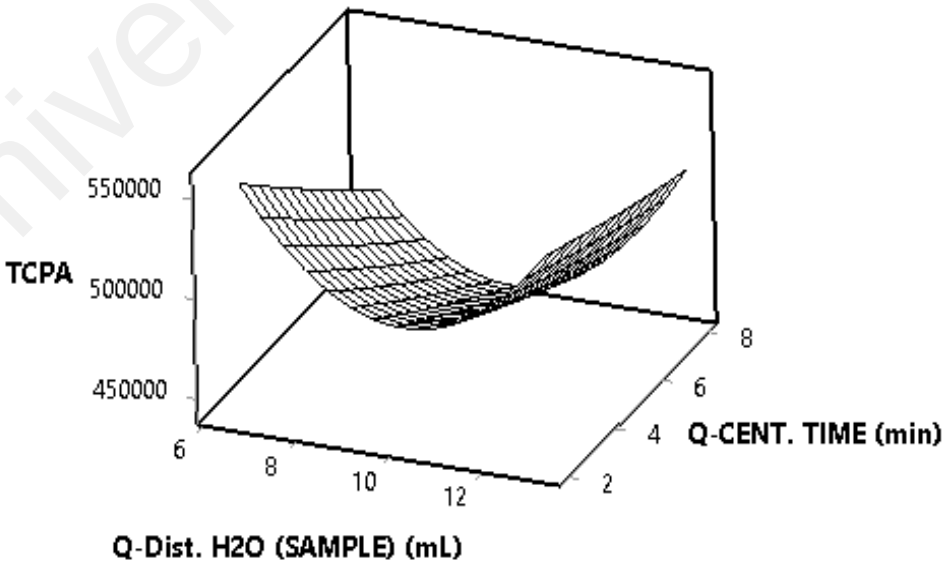


Figure 4.16: Surface plot illustration for QuEChERS sample quantity with QuEChERS centrifuge time, which resulted in maximum TCPA

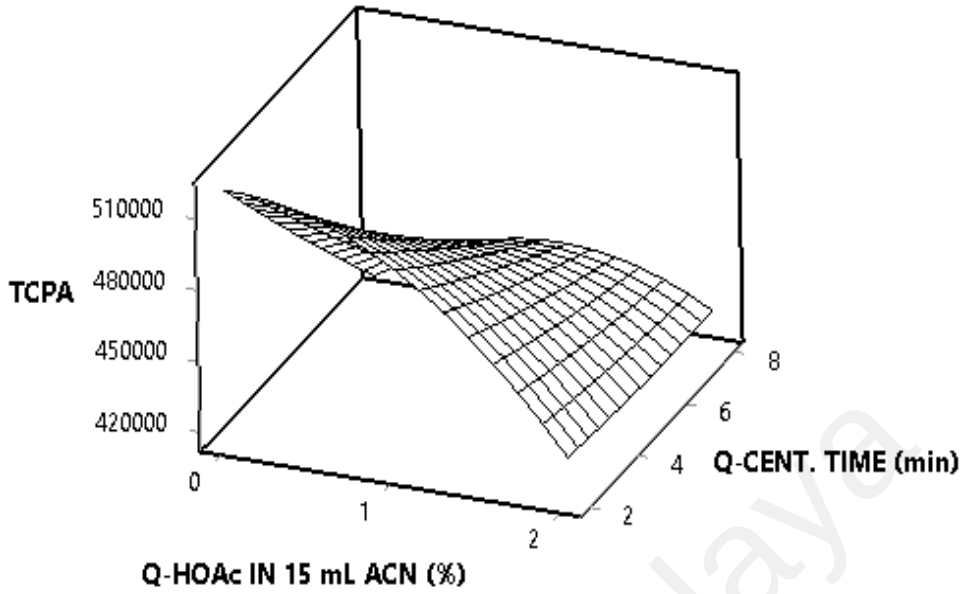


Figure 4.17: Maximum level of TCPA attained on the Surface plot after interaction between QuEChERS percentage of HOAc in 15 mL ACN and QuEChERS centrifuge time

The overall *P*-value of the regression model (Table 4.11) for the Box-behnken design was statistically insignificant (0.576 > 0.26 statistical level).

Table 4.11: Response surface regression of Box-Behnken design runs for the three optimized factors of the QuEChERS-dSPE method

TERMS	ANALYSIS OF VARIANCE		CODED COEFFICIENTS			
Source	*F*-value	*P*-value	Coefficient	SE Coefficient	*T*-value	*P*-value
Regression	0.91	**0.576**	-	-	-	-
Linear	0.93	0.493	-	-	-	-
Constant	-	-	470460	32813	14.34	0.000
A	0.10	0.767	6299	20094	0.31	0.767
B	0.96	0.373	-19643	20094	-0.98	0.373
C	1.72	**0.246**	-26383	20094	-1.31	**0.246**
Square	1.36	0.355	-	-	-	-
A*A	3.26	**0.131**	53444	29578	1.81	**0.131**
B*B	0.59	0.479	-22623	29578	-0.76	0.479
C*C	0.00	0.966	1310	29578	0.04	0.966
Interaction	0.44	0.733	-	-	-	-
A*B	0.42	0.544	-18502	28417	-0.65	0.544
A*C	0.16	0.705	11385	28417	0.40	0.705
B*C	0.74	0.428	24485	28417	0.86	0.428
Lack-of-Fit	367.11	0.003				
Model Summary						
S	R^2		R^2 (adjusted)		R^2 (predicted)	
56834.6	62.10 %		0.00 %		0.00 %	

A: Dist. water sample for QuEChERS extraction (mL), B: Percentage of HOAc in 15 mL ACN for QuEChERS extraction (%), C: QuEChERS centrifugation time (min)

4.4.3.2 Comparative study of the unoptimized and RSM optimized QuEChERS-dSPE-IL-DLLME technique

Both improved and default setups of QuEChERS-dSPE methods were compared based on their ATCPA obtained from the analysis of 100 µg/kg multi-pesticides mixture of standard solution. The data of the comparative studies (ATCPA ± STDEV) shows that the modified method was favored by 56% [(77 ± 3) x 10^3] over the default 44% [(60 ± 2) x 10^3] QuEChERS-dSPE method. Notably, the QuEChERS-dSPE technique reasonably improved the TCPA (56 %) recoveries when compared with the default method (44 %) although its general statistical (ANOVA) model was insignificant (Gall, 2001).

4.4.3.3 Screened and optimized factors of QuEChERS-IL-DLLME method

(a) ***Plackett-Burman design runs for the screened factors of QuEChERS-DLLME technique***

Table 4.12 shows the 12 experimental design points used in Plackett-Burman screening at 0.26 significant level. The design was successfully used to screen eight factors as supported by Fang et al. (2017).

Table 4.12: Plackett-Burman design responses for screening eight factors of QuEChERS-DLLME technique

RO	A	B	C	D	E	F	G	H	TCPA
1	13.3	0	7000	2	0	50	8000	8	61027
2	13.3	2	1000	8	0	50	2000	8	66383
3	6.7	2	7000	2	10	50	2000	2	134956
4	13.3	0	7000	8	0	150	2000	2	1770928
5	13.3	2	1000	8	10	50	8000	2	163949
6	13.3	2	7000	2	10	150	2000	8	1873047
7	6.7	2	7000	8	0	150	8000	2	1763182
8	6.7	0	7000	8	10	50	8000	8	149724
9	6.7	0	1000	8	10	150	2000	8	1715353
10	13.3	0	1000	2	10	150	8000	2	1682070
11	6.7	2	1000	2	0	150	8000	8	1544844
12	6.7	0	1000	2	0	50	2000	2	120666

RO, run order; A, volume of Milli-Q-water for QuEChERS extraction (sample) (mL); B, % HOAc in 15 ml of ACN; C, QuEChERS extraction centrifugation speed (rpm); D, QuEChERS extraction time (min); E, % NaCl in 9 mL of water; F, volume of ionic liquid-based (µL); G, centrifugation speed for DLLME (rpm); H, DLLME cleanup time (min); TCPA, total chromatographic peak area

Consequently, three factors were found significant as illustrated by the Pareto chart (Figure 4.18), which include QuEChERS centrifugation speed (rpm), the percentage of NaCl in 9 mL of water (%) and volume of ionic liquid-based (µL).

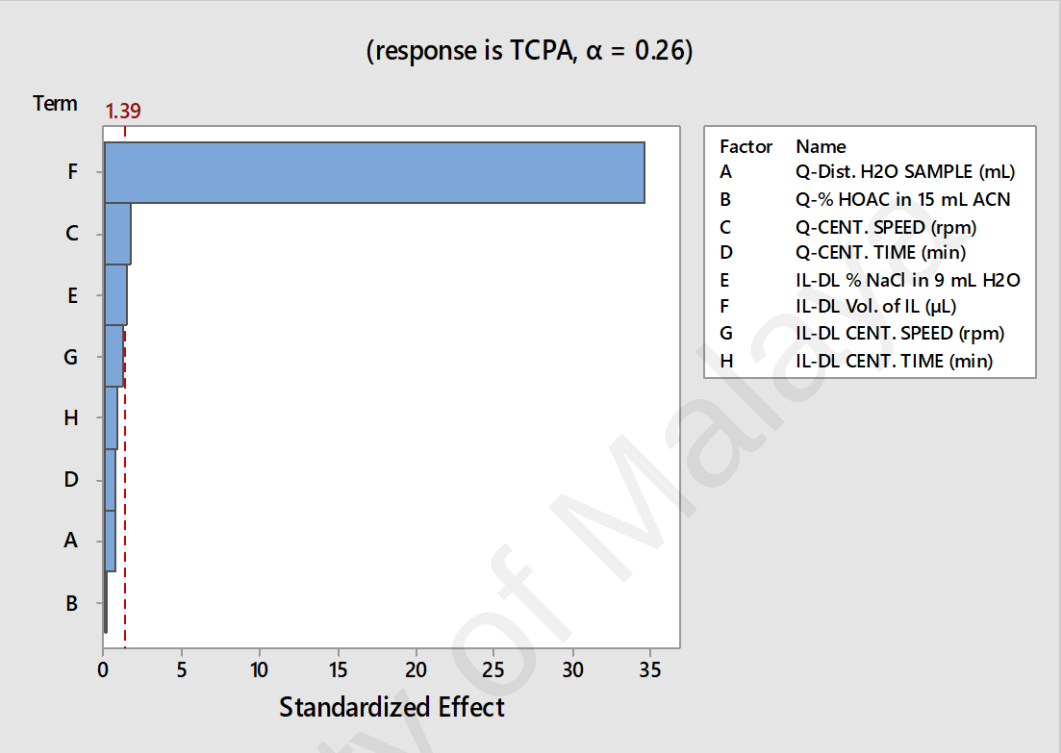


Figure 4.18: Pareto plot of Plackett-Burman design illustrating the eight screened factors of QuEChERS-DLLME technique

The model is mathematically expressed in Equation 4.5.

$$TCPA = -777849 + 4765A + 3883B + 12.77C + 5914D + 6534E + 16088F - 8.79G - 6260H \quad \text{Eqn (4.5)}$$

The overall *P*-value of the model of the ANOVA results is significant (0.001) which is less than 0.26 statistical level as indicated in Table 4.13.

Table 4.13: Factorial regression of Plackett-Burman design runs for the screened factors of QuEChERS-DLLME method

TERMS	ANALYSIS OF VARIANCE		UNCODED COEFFICIENTS				
Source	*F*-Value	*P*-Value	Main Effect	Coefficient	SE Coefficient	*T*-Value	*P*-Value
Model	152.16	**0.001**	-	-	-	-	-
Linear	152.16	0.001	-	-	-	-	-
Constant	-	-	-	920511	23129	39.80	0.000
A	0.46	0.545	31447	15723	23129	0.68	0.545
B	0.03	0.877	7765	3883	23129	0.17	0.877
C	2.74	**0.196**	76600	38300	23129	1.66	**0.196**
D	0.59	0.499	35485	17742	23129	0.77	0.499
E	2.00	**0.253**	65345	32672	23129	1.41	**0.253**
F	1209.52	**0.000**	1608787	804393	23129	34.78	**0.000**
G	1.30	0.337	-52756	-26378	23129	-1.14	0.337
H	0.66	0.476	-37562	-18781	23129	-0.81	0.476
Model Summary							
S	=	80122.3		R^2 (adjusted) =		99.10 %	
R^2	=	99.75 %		R^2 (predicted) =		96.07 %	

A, Milli-Q-water sample for QuEChERS extraction (mL); B, Percentage of HOAc in 15 mL ACN for QuEChERS extraction (%); C, QuEChERS centrifugation speed (rpm); D, QuEChERS centrifugation time (min); E, Percentage of NaCl in 9 mL of water (%) for DLLME; F, volume of ionic-liquid for DLLME; G, DLLME centrifugation speed (rpm); H, DLLME centrifugation time (min)

(b) Box-Behnken design responses for the QuEChERS-DLLME optimization

Box-Behnken optimization design based on the three most significant factors (QuEChERS centrifugation speed, the percentage of NaCl in 9 mL of water, and volume of ionic liquid-based) was successfully carried out on the factors at three levels each (Table 4.14). The design consisted of 15 runs at 0.26 significant level.

Table 4.14: Box-Behnken design responses for the QuEChERS-DLLME method

Run Order	Point Type	A	B	C	TCPA
1	2	1000	0	100	908784
2	2	7000	0	100	923717
3	2	1000	10	100	1053189
4	2	7000	10	100	1254053
5	2	1000	5	50	64190
6	2	7000	5	50	46266
7	2	1000	5	150	1142732
8	2	7000	5	150	1054950
9	2	4000	0	50	70011
10	2	4000	10	50	51068
11	2	4000	0	150	902774
12	2	4000	10	150	1177888
13	0	4000	5	100	1027031
14	0	4000	5	100	1153919
15	0	4000	5	100	1061027

A, QuEChERS centrifugation speed (rpm); B, the percentage of NaCl in 9 mL of water (%); C, the volume of ionic liquid-based (µL); TCPA, total chromatographic peak area

Approximately 130 µL volume ionic liquid-based is the most significant factor among the three factors that contributed to higher recoveries of TCPA. It is because of the more available volume of ionic liquid-based, the more analytes are extracted. Then again, the ionic strength (10% NaCl in 9 mL Milli-Q-water) of the DLLME extraction solution also play a vital role for better TCPA. The optimized setting was found within the range documented for analysis of chlorbenzuron and diflubenzuron insecticides (Pena et al., 2009; Ruan et al., 2015). Although, the TCPA decreases with an increase in the volume of ionic liquid-based after obtaining the maximum recovery of analytes. It could be as a result of high concentration of NaCl in the solution leading to the exchange of ions between chloride and an ionic liquid. Consequently, it resulted in a decrease of ionic liquid in the solution which at long run decreases the TCPA of the analytes due to the poor performance of the extraction (Xu et al., 2011). Thus, the response optimizer illustrates the three optimized factors toward attaining highest TCPA as highlighted in Figure 4.19.

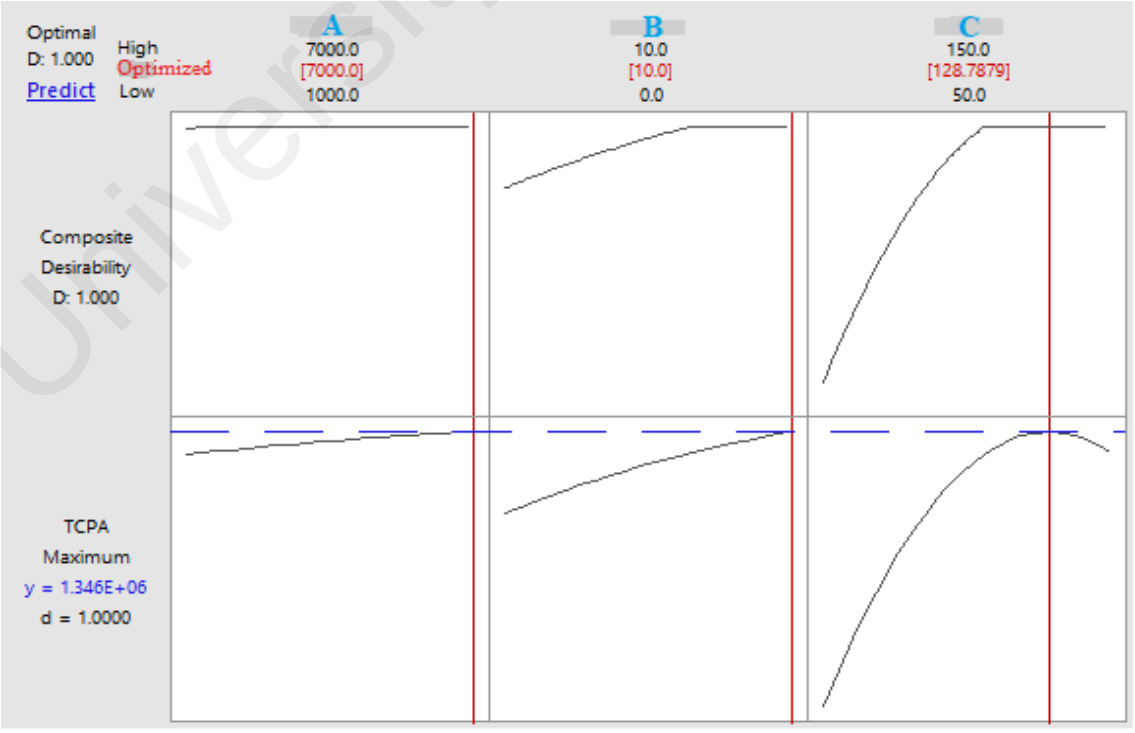


Figure 4.19: Box-Behnken response optimization for the QuEChERS-DLLME

Moreover, the outcomes of the optimization were respectively expressed in the following surface plots (Figure 4.20 - 4.22). The illustrations show that maximization of TCPA was attained when the values of the two significant factors were increased, respectively and the setups of the insignificant factors were setup at a medium level. Figure 4.20 indicated an increase in QuEChERS-centrifugation speed (1000 to 7000 rpm) and the percentage NaCl in 9 mL of water (0 to 10 %) would increase the TCPA. Likewise, an increase in QuEChERS-centrifugation speed and volume of ionic liquid-based (50 to 150 µL) will increase the TCPA in Figure 4.21. Similarly, increasing the percentage NaCl in 9 mL of water and volume of ionic liquid-based increases the TCPA in Figure 4.22.

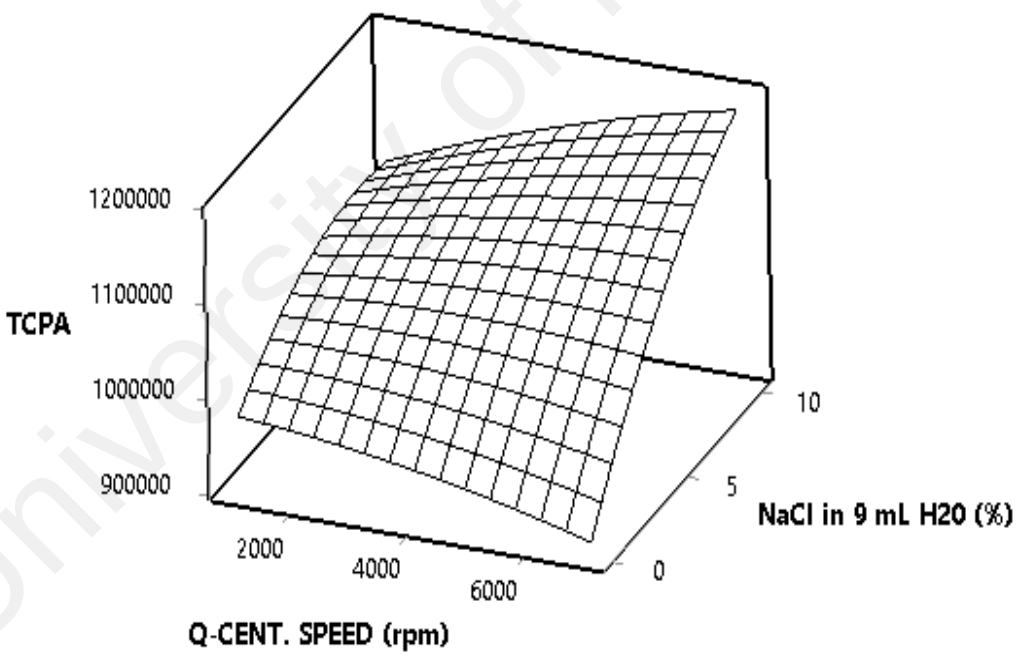


Figure 4.20: Surface plot indicated the highest value of TCPA when QuEChERS-centrifugation speed interacted with % NaCl in 9 mL of water

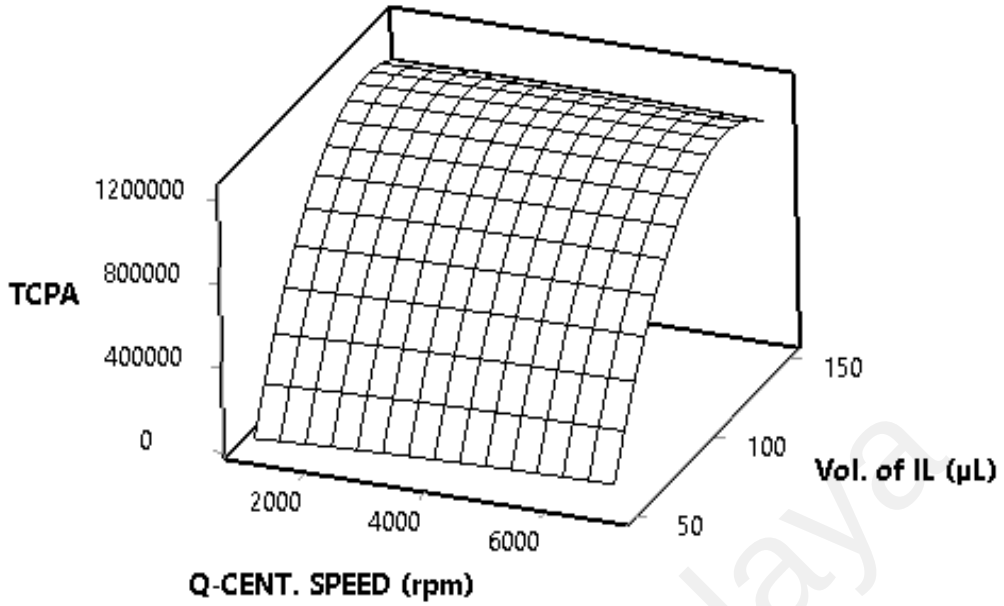


Figure 4.21: Surface plot illustration yielded maximum TCPA when QuEChERS-CENT. SPEED interacted with IL-based Volume

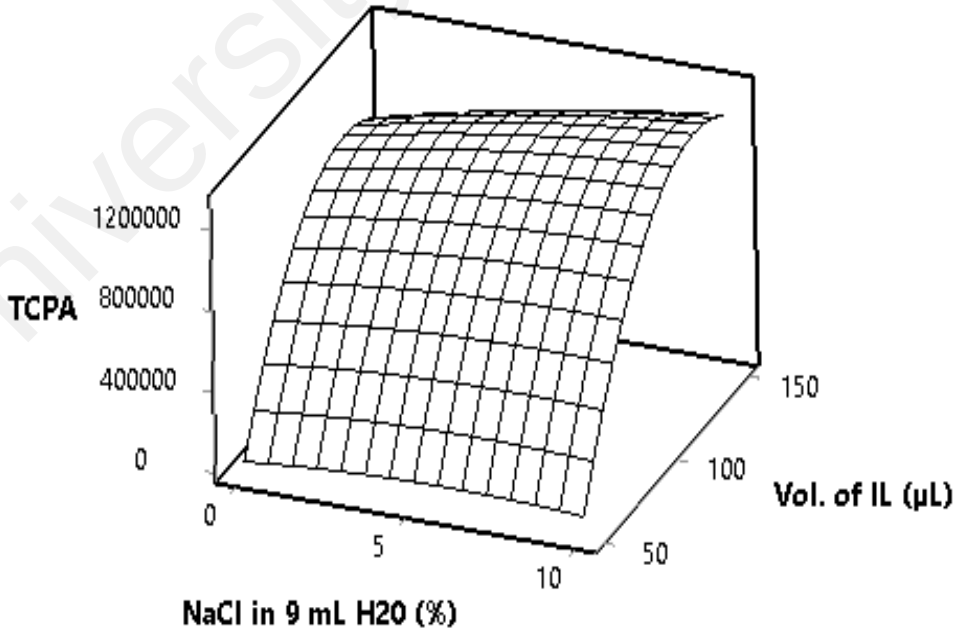


Figure 4.22: Surface plot shows the interaction between % NaCl in 9 mL of water and IL-based volume that yielded highest TCPA

Therefore, the overall *P*-value of the ANOVA results (Table 4.15) for the response surface regression is significant (0.000 < 0.26 statistical level). This result is in accordance with the findings of Zhang et al. (2016) for the determination of triazole pesticides in fruit samples after the RSM optimization of QuEChERS coupled with the ionic liquid-based DLLME method.

Table 4.15: Response surface regression of Box-Behnken design for the QuEChERS-DLLME method

TERMS	ANALYSIS OF VARIANCE		CODED COEFFICIENTS			
Source	*F*-value	*P*-value	Coefficient	SE Coefficient	*T*-value	*P*-value
Regression	57.35	**0.000**	-	-	-	-
Linear	119.28	0.000	-	-	-	-
Constant	-	-	44390	24.34	0.000	0.000
A	0.26	0.634	13761	27183	0.51	0.634
B	11.30	**0.020**	91364	27183	3.36	**0.020**
C	346.29	**0.000**	505851	27183	18.61	**0.000**
Square	50.99	0.000	-	-	-	-
A*A	0.06	0.821	-9562	40013	-0.24	0.821
B*B	0.82	0.408	-36161	40013	-0.90	0.408
C*C	152.46	**0.000**	-494062	40013	-12.35	**0.000**
Interaction	1.78	0.268	-	-	-	-
A*B	1.46	0.281	46483	38443	1.21	0.281
A*C	0.21	0.669	-17465	38443	-0.45	0.669
B*C	3.66	**0.114**	73514	38443	1.91	**0.114**
Lack-of-Fit	1.62	0.404				
Model Summary						
S	R^2			R^2 (adjusted)	R^2 (predicted)	
76885.8	99.04 %			97.31 %	88.50 %	

A, QuEChERS centrifugation speed (rpm); B, the percentage of NaCl in 9 mL of water (%) for DLLME, C: volume of ionic-liquid for DLLME

Also, the mathematical model proposed from the interactive coefficients of the optimized factors is expressed in Equation 4.6.

$$TCPA = 44390 + 91364B + 505851C + 73514BC - 494062C^2 \qquad \text{Eqn (4.6)}$$

Fortunately, the results (ATCPA ± STDEV) of RSM optimized QuEChERS-DLLME [(222 ± 7) x 10^4] was slightly (50.04 %) experimentally better than 49.60 % of unoptimized QuEChERS-DLLME [(222 ± 7) x 10^4] technique after comparative studies were carried out.

4.4.3.4 Comparative study of the unoptimized and RSM optimized QuEChERS-dSPE-IL-DLLME technique

The default QuEChERS-dSPE-IL-DLLME method was setup by selecting the default factors of QuEChERS-dSPE that were significant after the RSM screening and coupling them with that of the default QuEChERS-IL-DLLME technique. Similarly, the RSM optimized factors of the QuEChERS-dSPE method were coupled with the optimized factors of QuEChERS-IL-DLLME technique after setting up the other factors at medium (default) level. Thus, the coupling was modified to the RSM optimized QuEChERS-dSPE-IL-DLLME technique. Later on, the default QuEChERS-dSPE-IL-DLLME method was compared with the RSM optimized QuEChERS-dSPE-IL-DLLME technique by the analysis of 100 µg/kg mixture of multi-pesticide standard solutions. The result of the comparative studies (ATCPA ± STDEV) favored the RSM optimized QuEChERS-dSPE-IL-DLLME [(214 ± 7) x 10^4] which slightly surpassed the unoptimized QuEChERS-dSPE-IL-DLLME [(205 ± 6) x 10^4] technique. Certifiably, this indicates the effectiveness of the involvement of multivariate optimization in the sample preparation methodology used for analysis of multi-pesticide compound (Bedendo et al., 2012).

Eventually, the comparative studies (ATCPA ± STDEV) of the three techniques (Figure 4.23) favored the RSM optimized QuEChERS-IL-DLLME [(222 ± 7) x 10^4] technique by 50.10 % over the 1.73 % RSM optimized QuEChERS-dSPE [(77 ± 3) x 10^3], and 48.17 % QuEChERS-dSPE-IL-DLLME [(214 ± 7) x 10^4] techniques based on the resulted recovery of the ATCPA in the analyzed (Milli-Q-water) samples. However, the modified QuEChERS-IL-DLLME method agreed with the report of Rai et al. (2016) that encourages the use of the QuEChERS-IL-DLLME method by excluding the use of PSA in the cleanup in the analysis of pesticides in a sample of fruits and vegetables.

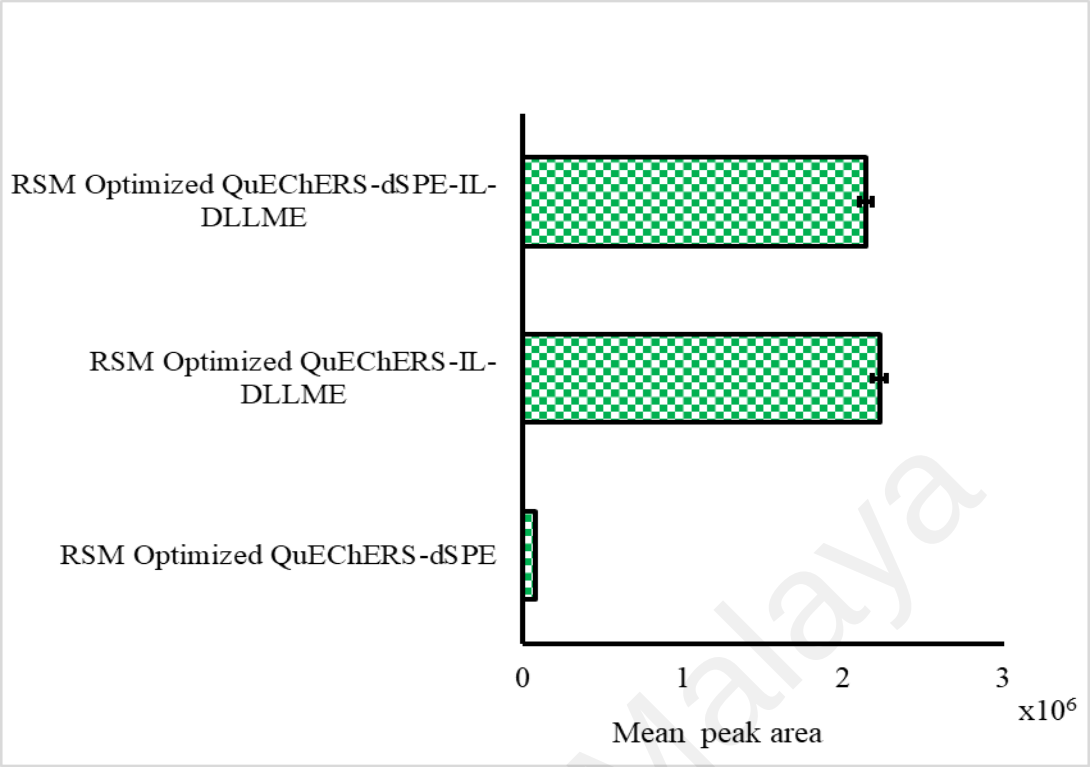


Figure 4.23: The comparative chart of the RSM optimized QuEChERS methods

Even though, the RSM optimized QuEChERS-IL-DLLME technique produced the highest ATCPA as illustrated above but the method could not be used for the pesticide determination in the real sample of fruits and vegetables because of the weak recoveries of analytes. It may be due to the absence of PSA because PSA helps to cleanup matrix interferences of the analyzed samples (Biziuk & Stocka, 2015). Consequently, the RSM optimized QuEChERS-dSPE-IL-DLLME technique was selected and used as the sample treatment method for the determination of multi-pesticides residue in fruits and vegetables after comparative studies. The comparative studies results (ATCPA ± STDEV) of the two techniques favored the RSM optimized QuEChERS-dSPE-IL-DLLME [(341 ± 9) x 10^4] by 65.90 % over the 34.10 % of RSM optimized QuEChERS-IL-DLLME [(177 ± 6) x 10^4] technique after analysis of a real sample of fruits. The sample treatment method provided good results due to its extensive cleanup of matrix interferences with less consumption of organic solvents. The developed method agrees with the recent RSM optimized method which was reported for the analysis of multiple pesticides in a sample

of fruits and vegetables (Lawal et al., 2018). It is also in accordance with the RSM optimized method used for analysis of fungicides residue of triazole pesticides in fruits (Zhang et al., 2016) and a univariate optimized method used for analysis of phthalate esters in edible oils (Xie et al., 2014). However, the method disagrees with the report of Rai et al. (2016) that encourages the exclusion of PSA for the cleanup processes.

4.5 Validation of the RSM Optimized LC-MS/MS Instrument

Validation expresses the desirability, effectivity and certifiable accord of the RSM optimized LC-MS/MS instrument and the sample preparation technique for determination of multi-pesticides residue in the analyzed sample of fruits and vegetables (Li et al., 2017). However, most of the validations carried out for analysis of pesticide residues in food and feed specifically addresses the sample preparation methods but does not lay emphasis on validation of LC-MS/MS instrument (SANTE/11813/2017). It is because a consensus has not been reached on how the validation of the LC-MS instrument should be carried out (Kruve et al., 2015). Based on the above reasons, the RSM optimized LC-MS/MS instrument was not validated because there is no specific (official) guideline for the validation of LC-MS instruments.

4.6 Validation of Sample Preparation Method

The developed QuEChERS-dSPE-IL-DLLME method was validated by the preparation of sample solutions used for the determination of multi-pesticide residues in the sample of fruits and vegetables. The validation (SANTE/11813/2017) was based on the studied and estimated parameters that include linearity range, enrichment factor and accuracies, precisions, matrix effects, LOD, LOQ, and MU.

4.6.1 Accuracies of Sample Preparation Method

The accuracies were expressed as absolute and relative recoveries, which were estimated using the TCPA responses. Even though the responses were obtained from the

triplicate solutions of a prepared standard solution (absolute) and homogenized vegetable and fruit samples (relative) at 5, 100 and 300 µg/kg spiked concentration levels each. Resultingly, the ranges for most of the results obtained for absolute (84–101%) recoveries (Table 4.16 and 4.17) were accurate. Also, more than 90% obtained results of relative recoveries results were 82–138% for fruits and 88-137% for vegetables as tabulated in Table 4.16 and 4.17. The accuracy results were found within the acceptable range (70-120%) in most cases. These results were in the same range when compared to other pesticides residue findings in fruits and vegetables using similar methods and instrumentations (Bedassa et al., 2015; Chen et al., 2013; Dashtbozorgi et al., 2013). Even though, few recoveries at the lowest spiked concentration level (5 µg/kg) were beyond the recommended range (70-120%) for some selected pesticides. These could be affected by matrix-induced chromatographic effect due to the nature of the analyzed samples characterized by compounds that contributed to higher chromatographic responses (Ngan et al., 2015). The most affected samples include bananas, jackfruit, cabbage, and cucumber as presented in Table 4.16 and 4.17. Thus, the results are supported by the documentation of Sivaperumal et al. (2017) that reported the recovery of 121 % of Diazinon pesticide after spiking the raw homogenized sample of mango with 10 µg/kg of the analyte standard solution.

4.6.2 The Repeatability of Sample Preparation Method

The repeatability (RSD_r %) were estimated from the repeated analysis. The analysis was carried out using the homogenized sample of vegetables and fruits at the levels of 5, 100 and 300 µg/kg spiked concentrations. The RSD_r % of spiked fruits and vegetable samples are presented in Table 4.16 and 4.17 respectively, and the results were satisfactorily within the recommended (≤ 20%) ranges. Accordingly, the repeatability agrees with the result ($RSDr \leq 20\%$) documented by Christodoulou et al. (2018) for the analysis of pesticides in vegetables and fruits using LC-MS.

Table 4.16: Accuracies and precision results of pesticides in the analyzed fruit samples

Pesticides			Banana		Orange		Jackfruit		Strawberries		Pear	
	Spike (µg/kg)	AR (%)	RR (%)	RSD_r (%)	RR (%)	RSD_r (%)	RR (%)	RSD_r (%)	RR (%)	RSD_r (%)	RR (%)	RSD_r (%)
Durban	5	90	124	3	101	18	98	5	120	3	117	15
	100	101	98	4	96	8	100	0	98	1	99	9
	300	100	99	1	101	9	98	3	100	2	97	0
Diazinon	5	94	128	2	124	1	101	13	92	7	100	2
	100	100	100	0	100	0	100	1	99	3	101	13
	300	100	100	0	100	0	100	2	99	1	101	1
Thiamethoxam	5	97	93	6	118	3	105	8	119	4	112	8
	100	100	101	2	104	6	99	13	95	2	96	13
	300	100	99	1	100	1	101	2	100	2	98	2
Metalaxyl	5	85	82	6	82	4	131	12	105	4	115	11
	100	101	103	1	97	8	100	7	99	9	100	4
	300	100	99	10	100	4	100	10	98	7	100	6
Thiobencarb	5	96	95	20	98	2	138	25	109	1	93	12
	100	100	101	1	98	2	101	1	100	4	96	6
	300	100	100	3	100	3	100	1	100	1	101	2
Baycarb	5	84	95	1	109	1	120	21	112	24	98	6
	100	101	102	0	100	5	98	2	110	1	102	4
	300	100	98	0	100	1	99	1	95	1	101	4
Carbaryl	5	88	122	2	95	2	124	24	91	14	105	3
	100	101	97	7	97	3	98	1	89	1	97	8
	300	100	99	11	99	3	99	4	98	4	100	5
Propamocarb	5	94	137	9	92	3	96	21	106	13	90	16
	100	100	96	2	102	0	98	5	94	5	101	4
	300	100	101	3	98	0	100	0	100	1	99	5
RANGES	100-300	84-101	82-137	0-20	82-124	0-18	96-138	0-25	89-120	1-24	90-117	0-16

AR, absolute recovery; RR, relative recovery; RSD_r, relative standard deviation of repeatability

Table 4.17: Accuracies and precision results of pesticides in the analyzed vegetable samples

Pesticides			Cabbage		Tomato		Onions		Cucumber		Carrot	
	Spike	AR	RR	RSD_r (%)	RR	RSD_r (%)	RR	RSD_r (%)	RR	RSD_r (%)	RR	RSD_r (%)
Durban	5	90	108	3	101	4	101	5	121	9	107	3
	100	101	99	3	98	3	99	4	97	6	100	1
	300	100	99	1	98	5	98	9	99	13	100	14
Diazinon	5	94	123	1	137	2	108	4	90	7	114	3
	100	100	99	1	100	1	98	6	99	2	101	5
	300	100	101	3	100	2	98	3	99	8	101	5
Thiamethoxam	5	97	135	4	110	2	112	6	101	5	114	3
	100	100	99	1	99	1	94	1	104	2	98	2
	300	100	99	2	100	3	98	7	100	11	94	2
Metalaxyl	5	85	115	3	106	8	99	2	121	10	91	0
	100	101	98	4	97	10	97	11	102	4	100	12
	300	100	99	19	101	14	100	10	98	4	101	9
Thiobencarb	5	96	90	8	112	1	100	6	126	19	111	6
	100	100	100	1	104	2	102	7	103	2	102	4
	300	100	101	2	99	1	98	0	101	4	101	4
Baycarb	5	84	134	1	115	0	120	1	96	23	121	1
	100	101	98	2	106	1	98	2	99	4	99	1
	300	100	100	2	101	2	97	1	101	12	99	2
Carbaryl	5	88	116	3	113	1	118	5	105	13	105	2
	100	101	99	3	101	0	104	3	99	1	105	5
	300	100	99	1	101	8	100	7	98	19	100	16
Propamocarb	5	94	113	3	88	3	112	4	90	6	112	4
	100	100	101	0	98	3	92	3	105	4	112	2
	300	100	100	1	101	1	98	1	100	18	100	18
RANGES	100-300	84-101	98-135	0-19	88-137	0-14	92-120	0-11	90-126	1-23	91-121	0-18

AR, absolute recovery; RR, relative recovery; RSD_r, relative standard deviation of repeatability

4.6.3 LODs and LOQs of Sample Preparation Method

The LODs and LOQs results were estimated respectively using the signal-to-noise ratio that corresponded to 3 and 10 factor from the matrix match calibration curve. Importantly, the two measurements express the performance characteristics of the developed method for pesticide analyses in fruits and vegetables (Shrivastava & Gupta, 2011). Therefore, the LODs and LOQs ranges of results (Table 4.18 and 4.19) for the analyzed fruits [LOD (0.02-0.54 µg/kg) and LOQ (0.07-1.79 µg/kg)] and vegetables [(0.01-0.39 µg/kg) and LOQ (0.03-1.29 µg/kg)] were satisfactory. Virtually, the results were lower than the least calibration level (reporting limit) (5 µg/kg) as well as the maximum residue limits (MRLs) recommended by EU. Fortunately, the resulted LODs and LOQs were better (lower) than the previously documented findings (Ruan et al., 2015; Zhang et al., 2016).

Table 4.18: The LOD and LOQ results of pesticides in the analyzed fruit samples

Pesticides	Banana		Orange		Jack-fruit		Strawberries		Pear	
	LOD (µg/kg)	LOQ (µg/kg)	LOD (µg/kg)	LOQ (µg/kg)	LOD (µg/kg)	LOQ (µg/kg)	LOD (µg/kg)	LOQ (µg/kg)	LOD (µg/kg)	LOQ (µg/kg)
Durban	0.09	0.31	0.14	0.48	0.13	0.45	0.09	0.29	0.18	0.61
Diazinon	0.07	0.25	0.06	0.20	0.04	0.13	0.07	0.24	0.06	0.20
Thiamethoxam	0.06	0.19	0.19	0.64	0.07	0.24	0.15	0.50	0.19	0.63
Metalaxyl	0.10	0.34	0.12	0.39	0.08	0.27	0.13	0.42	0.04	0.13
Thiobencarb	0.03	0.11	0.07	0.22	0.11	0.35	0.02	0.07	0.14	0.46
Baycarb	0.11	0.38	0.03	0.09	0.06	0.21	0.37	1.24	0.07	0.23
Carbaryl	0.11	0.38	0.14	0.47	0.06	0.21	0.54	1.79	0.09	0.31
Propamocarb	0.08	0.28	0.13	0.42	0.07	0.25	0.20	0.67	0.04	0.13
RANGES	0.03-0.11	0.11-0.38	0.03-0.19	0.09-0.64	0.04-0.13	0.13-0.45	0.02-0.54	0.07-1.79	0.04-0.19	0.13-0.61

LOD, limit of detection; LOQ, limit of quantitation

Table 4.19: The LOD and LOQ results of pesticides in the analyzed vegetable samples

Pesticides	Cabbage		Tomato		Onions		Cucumber		Carrot	
	LOD (µg/kg)	LOQ (µg/kg)	LOD (µg/kg)	LOQ (µg/kg)	LOD (µg/kg)	LOQ (µg/kg)	LOD (µg/kg)	LOQ (µg/kg)	LOD (µg/kg)	LOQ (µg/kg)
Durban	0.06	0.20	0.14	0.48	0.11	0.36	0.08	0.26	0.01	0.03
Diazinon	0.10	0.33	0.07	0.22	0.25	0.82	0.07	0.25	0.15	0.48
Thiamethoxam	0.09	0.30	0.02	0.07	0.25	0.84	0.12	0.41	0.39	1.29
Metalaxyl	0.08	0.27	0.12	0.41	0.09	0.29	0.12	0.39	0.08	0.27
Thiobencarb	0.06	0.19	0.14	0.47	0.11	0.36	0.14	0.46	0.10	0.34
Baycarb	0.05	0.16	0.22	0.73	0.22	0.75	0.08	0.28	0.07	0.22
Carbaryl	0.05	0.18	0.07	0.24	0.17	0.55	0.11	0.36	0.16	0.53
Propamocarb	0.06	0.19	0.08	0.28	0.32	1.06	0.16	0.52	0.38	1.27
RANGES	0.05-0.10	0.16-0.33	0.02-0.14	0.07-0.73	0.09-0.32	0.29-1.06	0.07-0.16	0.25-0.52	0.01-0.39	0.03-1.29

LOD, limit of detection; LOQ, limit of quantitation

4.6.4 The Matrix Effects of Sample Preparation Method

The fact that, matrix effects measurement of a sample preparation technique presented the effectiveness of matrix interference in a prepared sample solution towards the enhancement or suppression of analyte recoveries. Thus, the results (Table 4.20 and 4.21) of the matrix effects (ME) for the analyzed sample of fruits (≤ -80%) and vegetables (≤ -86%) were measured. The %ME obtained in all the analyzed samples were very weak as compared to the guideline recommendation (weak ME ≤ -20% suppression and strong ME ≥ 20% enhancement of analyte recovery). It could be due to the qualitative properties of the developed sample preparation method, which was efficiently used for extraction and cleanup of unwanted matrix interferences in the sample solution. Accordingly, this rendered the prepared sample solution very clear for quantitative analysis. Therefore, this is the main reason for having a better recovery of targeted pesticide analytes after using PSA and ionic liquid-based for the cleanups in QuEChERS and DLLME methods, respectively. Nevertheless, the results agreed with the reports of (Ruan et al., 2015; Zhang et al., 2016).

4.6.5 The Linearity Within the Working Range of Sample Preparation Method

The linearity of the analyzed pesticide compounds was evaluated from calibration curves which were prepared from the matrix match standards proportionally between the TCPA responses against five levels of analyte concentrations (5 – 400 µg/kg), respectively. The results (Table 4.20 and 4.21) of the linear calibration curves with R^2 greater than 0.99. The results corresponded with the previous literatures for analysis of pesticides in various food samples (Anagnostopoulos et al., 2012; Prodhan et al., 2015; Shi et al., 2014; Sivaperumal et al., 2015).

Table 4.20: Matrix effect and R^2 results of pesticides in the analyzed fruit samples

Pesticides		Banana	Orange	Jack-fruit	Strawberries	Pear
Durban	ME (%)	-80	-83	-96	-88	-87
	R^2	0.9995	0.9992	0.9993	0.9996	0.9987
Diazinon	ME (%)	-98	-95	-96	-97	-100
	R^2	0.9996	0.9997	0.9998	0.9998	0.9998
Thiamethoxam	ME (%)	-100	-100	-100	-100	-100
	R^2	0.9998	0.9985	0.9997	0.9991	0.9987
Metalaxyl	ME (%)	-100	-99	-99	-99	-100
	R^2	0.9997	0.9995	0.9995	0.9993	0.9998
Thiobencarb	ME (%)	-94	-93	-92	-92	-99
	R^2	0.9999	0.9998	0.9993	0.9998	0.9993
Baycarb	ME (%)	-99	-97	-90	-89	-87
	R^2	0.9995	0.9998	0.9997	0.9954	0.9998
Carbaryl	ME (%)	-100	-100	-100	-100	-100
	R^2	0.9994	0.9993	0.9996	0.9906	0.9996
Propamocarb	ME (%)	-100	-100	-100	-100	-100
	R^2	0.9995	0.9994	0.9997	0.9986	0.9999
RANGES	ME (%)	$\leq$ -80	$\leq$ -83	$\leq$ -90	$\leq$ -88	$\leq$ -87
	R^2	$>$ 0.999	$>$ 0.99	$>$ 0.999	$>$ 0.99	$>$ 0.99

ME, matrix effect; R^2, regression coefficient

Table 4.21: Matrix effect and R^2 results of pesticides in the analyzed vegetable samples

Pesticides		Cabbage	Tomato	Onions	Cucumber	Carrot
Durban	ME (%)	-91	-86	-98	-97	-91
	R^2	0.9997	0.9992	0.9995	0.9996	0.9999
Diazinon	ME (%)	-97	-96	-98	-99	-100
	R^2	0.9995	0.9996	0.9979	0.9998	0.9991
Thiamethoxam	ME (%)	-100	-100	-100	-100	-100
	R^2	0.9995	0.9998	0.9978	0.9994	0.9949
Metalaxyl	ME (%)	-99	-100	-99	-99	-100
	R^2	0.9996	0.9994	0.9997	0.9993	0.9997
Thiobencarb	ME (%)	-96	-93	-98	-98	-98
	R^2	0.9998	0.9992	0.9995	0.9991	0.9995
Baycarb	ME (%)	-98	-88	-96	-97	-97
	R^2	0.9996	0.9983	0.9982	0.9997	0.9996
Carbaryl	ME (%)	-100	-100	-100	-100	-100
	R^2	0.9997	0.9997	0.9989	0.9995	0.9991
Propamocarb	ME (%)	-100	-100	-100	-100	-100
	R^2	0.9997	0.9997	0.9966	0.9992	0.9951
RANGES	ME (%)	$\leq$ -91	$\leq$ -86	$\leq$ -96	$\leq$ -97	$\leq$ -91
	R^2	>0.999	>0.99	>0.99	>0.999	>0.99

ME, matrix effect; R^2, regression coefficient

4.6.6 Measured Uncertainties (MU) of Sample Preparation Method

The MU (Table 4.22) were estimated to further certify the developed method for multi-pesticides analysis in a different sample of foods. The MU was estimated at 95% confidence level based on the empirical model and coverage factor (k = 2). Fortunately, the precision MU results for the analyzed fruits and vegetables were within the acceptable (≤ 50%) range. Comparatively, the MU of the developed method is similar to that of the other literatures that were reported recently (Kaczyński, 2017; Łozowicka et al., 2016).

Table 4.22: Measurement of uncertainty results of pesticides in the analyzed fruit and vegetable samples

Pesticides	Banana	Orange	Jack-fruit	Strawberries	Pear	Cabbage	Tomato	Onions	Cucumber	Carrot
Durban	6	23	5	4	16	5	8	12	18	12
Diazinon	1	0	5	4	6	3	4	9	11	9
Thiamethoxam	6	7	15	5	16	4	4	10	12	5
Metalaxyl	11	10	19	13	14	17	22	15	13	14
Thiobencarb	16	5	18	4	13	7	3	9	16	9
Baycarb	1	4	17	17	10	3	2	2	27	2
Carbaryl	13	5	19	13	10	5	6	10	22	15
Propamocarb	9	2	18	12	17	2	5	5	19	16
RANGES	≤ 16	≤ 23	≤ 19	≤ 17	≤ 17	≤ 17	≤ 22	≤ 15	≤ 22	≤ 16

4.6.7 Multi-Pesticide Residues in Blank Matrix Sample of Fresh Fruits and Vegetables

The quantitative results [mean ± STDEV (µg/kg)] obtained for the concentration level of multi-pesticide residues in the fresh (blank) fruits and vegetables analyzed were successful (Table 4.23 and 4.24). The results are reported based on the EU Commission (SANTE/11813/2017) guidelines. Thus, all the multi-pesticides residue were detected in all the analyzed samples, but not all results were quantified. 65% of the detected results for the quantified residues in the analyzed samples were less than the LOQ of the sample preparation method.

Similarly, 24% of the results were less than or equal to the EU MRLs, respectively. Also, the remaining 11% of the results were less than the reporting limit (RL) of 5 µg/kg which agrees with most of the LOQ results reported by Lawal et al. (2018b) and Zaidon et al. (2018) for analysis of multiple pesticides using QuEChERS methods. On the other hand, all the multi-pesticide residues detected in a sample of carrots were below the quantitation limit. Furthermore, the residual concentration level of pesticide Baycarb in all the analyzed samples was below the LOQ.

Table 4.23: The obtained residue of pesticides in the analyzed fruit samples

Pesticides	µg/kg	Banana	Orange	Jack-fruit	Strawberries	Pear
Durban	EU MRL	3000	300	10	200	10
	ERS ± STDEV	< LOQ	**29 ± 3**	< LOQ	< LOQ	**9 ± 0**
Diazinon	EU MRL	10	10	-	10	10
	ERS ± STDEV	< RL	< LOQ	< RL	< LOQ	< RL
Thiamethoxam	EU MRL	20	150	10	300	300
	ERS ± STDEV	< LOQ	< LOQ	< LOQ	**174 ± 2**	< LOQ
Metalaxyl	EU MRL	50	500	50	500	1000
	ERS ± STDEV	< LOQ	< LOQ	**9 ± 0**	**33 ± 3**	< LOQ
Thiobencarb	EU MRL	10	10	10	10	10
	ERS ± STDEV	**8 ± 0**	< RL	**9 ± 0**	**8 ± 0**	< LOQ
Baycarb	EU MRL	10	10	10	10	10
	ERS ± STDEV	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
Carbaryl	EU MRL	50	10	10	10	10
	ERS ± STDEV	**39 ± 1**	**7 ± 0**	**10 ± 0**	< LOQ	< LOQ
Propamocarb	EU MRL	10	10	-	1000	10
	ERS ± STDEV	**9 ± 0**	< LOQ	< LOQ	< LOQ	<LOQ

EU MRL, European Union maximum residue limit; ERS, the extracted residue of pesticides in the analyzed samples; STDEV, standard deviation; LOD, limit of detection; LOQ, limit of quantitation

Table 4.24: The obtained residue of pesticides in the analyzed vegetable samples

Pesticides	µg/kg	Cabbage	Tomato	Onions	Cucumber	Carrot
Durban	EU MRL	10	300	50	50	100
	ERS ± STDEV	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
Diazinon	EU MRL	50	10	50	10	10
	ERS ± STDEV	< LOQ	< RL	< LOQ	< RL	< LOQ
Thiamethoxam	EU MRL	20	200	10	500	300
	ERS ± STDEV	< LOQ	< LOQ	< LOQ	**192 ± 2**	< LOQ
Metalaxyl	EU MRL	1000	200	500	500	100
	ERS ± STDEV	**86 ± 3**	< LOQ	< LOQ	**83 ± 3**	< RL
Thiobencarb	EU MRL	100	10	10	10	10
	ERS ± STDEV	< LOQ	< LOQ	< RL	**9 ± 0**	< RL
Baycarb	EU MRL	10	10	10	10	10
	ERS ± STDEV	< LOQ	<LOQ	< LOQ	< LOQ	< LOQ
Carbaryl	EU MRL	10	10	20	10	10
	ERS ± STDEV	< LOQ	**10 ± 1**	**18 ± 0**	**8 ± 0**	< LOQ
Propamocarb	EU MRL	700	4000	2000	5000	10
	ERS ± STDEV	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ

EU MRL, European Union maximum residue limit; ERS, the extracted residue of pesticides in the analyzed samples; STDEV, standard deviation; LOD, limit of detection; LOQ, limit of quantitation

CHAPTER 5: CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

The application of chemometrics (multivariate) for the RSM optimization of the Triple Quadrupole (G6490A) LC-MS instrument were successfully carried out. The *P*-values for the general mathematical models (ANOVA) were significant at 0.05 statical level for screening and optimized factors. The impact of the optimized instrumental settings increases the instrumental efficiency through sensitivity, detectability, and quantification of analytes at lower concentration level based on the obtained results. The instrument also improves the sensitivity of the sample preparation technique toward the extraction of pesticide analytes.

The RSM optimization of the default QuEChERS-dSPE and QuEChERS-IL-DLLME sample treatment methods were carried out, independently. The general ANOVA result of the *P*-values for the screened and optimized factors were significant at 0.26 statical level except for the QuEChERS-dSPE method. However, the RSM optimized methods were experimentally or practically useful based on the information obtained from comparative studies. The above methods were combined into the QuEChERS-dSPE-IL-DLLME method and yielded the highest recovery (ATCPA) for determination of multiple pesticides in the studied sample matrix. The method provides efficient cleanup of matrix interferences of analytes which increases the method sensitivity against multi-pesticide residues at lower concentration level.

Eventually, the developed method was validated according to the EU commission guideline (SANTE/11813/2017) for the determination of multi-pesticide residues in a fresh homogenized sample of fruits (bananas, oranges, jackfruits, strawberries & pears) and vegetables (cabbages, tomatoes, onions, cucumbers & carrots). The results obtained were satisfactory; therefore, the developed method would be fit for the routine

determination of multi-pesticide residues in various vegetable and fruit samples when coupled with the optimized LC-MS/MS.

5.2 Recommendations

Recommendations are made based on the research outcomes to enhance its development;

- The developed method can further be validated to estimate the precision of reproducibility (RSD_{WR} %) within a laboratory using different equipment over a period which would be conducted by different analysts for the determination of multiple pesticides in fruits and vegetables as condition provided by SANTE 2017 guideline.
- Since the present study used the European Union guideline (SANTE 2017), other guidelines should be consulted such as CXG 90-2017, CXG 059 or the EURACHEM/CITAC guide for future studies.
- The newly introduced sorbent materials such as graphene and nanomaterials should be encouragingly used as modified cleanup agents in QuEChERS techniques and subjected to experimental design for further development of more sensitive, robust sample preparation methods that would be more helpful for determining traces of contaminants in food samples.

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LIST OF PUBLICATIONS AND PAPERS PRESENTED

Publications

Lawal, A., Wong, R.C.S., Tan, G.H., Abdulra'uf, L.B., & Alsharif, A.M.A. (2018). Multi-pesticide residues determination in samples of fruits and vegetables using chemometrics approach to QuEChERS-dSPE coupled with ionic liquid-based DLLME and LC–MS/MS. *Chromatographia, 81*(5), 759-768.

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Paper Presented

Lawal, A., Tan, G.H., Wong, R.C.S., & Alsharif, A.M.A. (2017, August). *Selection and optimization of mobile phase for multi-standard analysis of pesticides mixture using triple quadrupole LC/MS instrument.* Paper presented at the 6th International Conference for Young Chemists, Penang, Malaysia.