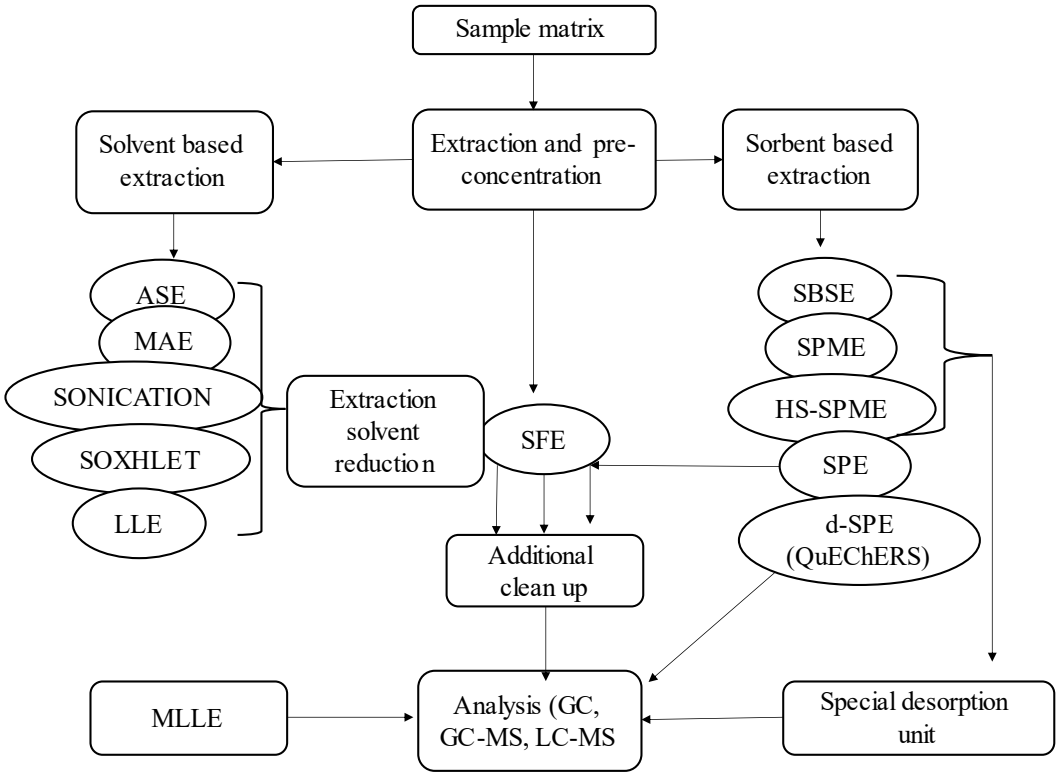
CHAPTER 2

LITERATURE REVIEW

2.1 Multiresidue Methods of Pesticide Analysis

Introducing multiresidue methods has allowed gaining relatively new knowledge of employing thin-layer chromatography (TLC), gas chromatography (GC) and high-performance liquid chromatography (HPLC). In the past few decades, there has been considerable enhancement in clean-up procedures as well as the tools used in analysis. The clean-up procedures have been greatly simplified through the application of gel permeation chromatography (GPC) and the use of solid-phase extraction columns or disks. Beside the conventional methods for pesticides extraction such as liquid-liquid extraction (LLE), pressurized liquid extraction (PLE), superheated water extraction (SHWE) and Soxhlet extraction (SOX), analytical procedures that do not employ organochlorine solvents like dichloromethane have been presented to avoid or minimise environmental and health hazards. Due to this, modern sample preparation trials, like supercritical fluid extraction (SFE), accelerated solvent extraction (ASE), Stir bar sorptive extraction (SBSE), ultrasonic extraction (USE), liquid phase micro-extraction (LPME), solid phase micro-extraction (SPME), liquid-liquid micro extraction (DLLME), solid phase extraction (SPE), dispersive solid phase extraction (DSPE), microwave assisted extraction (MAE) , matrix solid phase dispersion (MSPE) extraction and the quick, easy, cheap, effective, rugged, and safe method (QuEChERS), have been put forward to deal with most of the drawbacks associated with the traditional methods. Instrumental techniques such as ASE, SFE and MAE frequently employ SPME and SPE to purify obtained extracts, and in the case of the SPME method, its

concentration as well. Figure 2.1 explains common steps used in sample extraction, clean up and analysis.



Sources: Rada Đurović and Tijana Đorđević (2011); García-Repetto (2018)

Figure 2.1: Flowchart of Common Steps Used in Sample Extraction, Clean Up and Analysis

SPE is widely used as extraction and clean up technique with many advantages including, small solvent volume, less time, high extraction efficiency and selectivity, good reproducibility, and avoidance formation of emulsions. A wide variety of sorbents were designed to work as stationary phases for SPE such as primary secondary amine (PSA), aminopropyl, diethylaminoprpyl (DEA), polystyrene-divinylbenzene resins, graphitized carbon (GCB), trifunctionally-bonded Silica (C18) and zirconia-derived absorbents namely, Z-sep and Z-sep plus which is a dual silica particle with Zr atom and C18 chains. Recently, nanostructured materials were introduced as SPE sorbent like electrospun nanofibers and carbon nanotubes (Augusto et al., 2013). Chitosan, chitin,

Diatomaceous earth (De Oliveira et al., 2014), laboratory-made sorbents, prepared by depositing poly(methyloctadecylsiloxane) (PMODS), poly(methyloctylsiloxane) (PMOS), aminopropyl-terminated poly(dimethylsiloxane) (APPS) and copolymer of (52–48 %) dimethyl-(48–52 %) methylphenyl-siloxane (DMMPS) onto silica supports (Oshita et al., 2014) and fluorinated sorbent (Manoel et al., 2017).

Numerous factors, like solubility in organic solvents and water, volatility, stability, sample matrix contents (water, pigments, lipids, etc.) and acid-base properties, associated with the physical properties of the tested active ingredients, should be considered when planning for an experiment. The mentioned factors directly influence the choice of sample treatment selected, but can also be used for analysis purpose, i.e., detection limits and quantification accuracy (if the objective is to establish exact concentration value irrespective of the maximum residues limits (MRLs), or just to show whether the result is under or above MRLs) (Rada Đurović and Tijana Đorđević 2011). Considering the complexity associated with these matters, for each specific case, analytical procedure should be selected to reduce problems associated with consumption of solvents and other necessary reagents, analysis duration, and to decrease the number of analytical steps required, which would help to keep in check potential sources of errors. Moreover, it is always preferable that the chosen analytical procedure could allow simultaneous determination of large number of pesticides presents the basic principles involved in modern extraction, determination, and clean-up techniques, which are used by researchers to determine pesticide residues, especially for vegetables and fruit samples.

The Dutch mini-Luke method was introduced in the early 1980s as a very fast and well-organized extraction method to determine large number of pesticides in vegetables and fruits by employing both selective GC detectors (such as nitrogen–phosphorus,

electron capture ECD and flame photometric detector) and LC detectors (such as fluorescence and diode-array detector) and later also making use of full-scan GC that has ion trap detector (GC–ITD). The basic extraction solvent employed by this method is acetone and as partitioning solvents, dichloromethane, and petroleum ether, accompanied by sodium sulphate. 30 mL of acetone was used to extract a 15 g sample and separation with 30:30 mL of dichloromethane–petroleum ether (15 g Na_2SO_4). The modified mini-Luke method (NL method) employs a solvent which is further decreased to 20 mL of acetone and 10:20 mL for dichloromethane–petroleum ether. The NL method employs a rapid, straight, and efficient (two times 30 s) Ultra-Turrax (IKA) homogenization and mixing, resulting in optimum extractability of incurred pesticide residues, which can also be applied for the most difficult matrices. A test portion of sample (15 g), when applied with Ultra-Turrax mixing, is a better representative in giving a lower sample processing error when compared with the 10-g test portion as employed in the QuEChERS extraction method (Ana Lozano et.al., 2016). Another difference seen with the QuEChERS method is employing an evaporation step in the NL method's extraction step. Though additional time is required for this step, it allows employing an ideal injection solvent for both GC (isooctane–toluene) and LC (methanol). In particular, this offers a significant important benefit for GC-injection-labile compounds such as captafol, captain, dicofol, folpet and chlorothalonil, when storing extracts for GC analysis. A clean-up step is not required for the NL method, while an additional dispersive solid phase extraction (SPE) clean-up step is required for QuEChERS, which requires additional time. When compared to the NL method and other conventional methods, the key benefit provided by QuEChERS is excluding chlorinated solvents like hazardous waste and DCM. Overall, very similar performance characteristics were displayed by both extraction methods during validation studies as

well as in proficiency tests, where each method had its own pros and cons (Ana Lozano et.al., 2016). QuEChERS is gradually gaining popularity amongst researchers to perform pesticides residues analysis in vegetables, fruits, and cereals as well as low-fat samples in general.

The sample extraction step, which takes about two-thirds of the total analysis time, was still found to be the weakest link and, at the same time, a time-determining step for the entire analytical procedure. It was also found to be responsible of causing errors and inconsistency amongst laboratories. However, in the recent past, advances in the sample preparation techniques encompassing diverse environmental samples have resulted in revision of the existing methods as well as development of new techniques, in order to decrease the quantities of chemicals used and save time, thereby enhancing the analytical process's overall performance. Thus, for extraction purposes, various rapid, low-cost, environment friendly and readily automated methods are now available. In addition, these new methods have been altered to achieve a better balance amongst selectivity, cost and sensitivity. Due to the associated complexity with matrices, the extraction procedures normally follow very specific clean-up procedures to get precise sample quantification. Reduced solvent methods, including solid phase extraction (SPE), supercritical fluid extraction (SFE), solid phase micro extraction (SPME), accelerated solvent extraction (ASE), microwave assisted extraction (MAE), modified Dutch mini-Luke method (NL method) and matrix solid phase dispersion (MSPD), and the quick, cheap, easy, rugged, effective, and safe (QuEChERS) method, have grown in terms of their maturity.

The QuEChERS method, initially introduced in 2003, is the most general multiresidue methodology for isolating a wide spectrum of pesticides. This approach guarantees great extract clean-up, high analyte recovery, and adaptability to deal with a

wide range of matrixes such as fruits and vegetables. 375 pesticides were determined in fruit and vegetables by Benerjee et al., (2012). Anagnostopoulos et al., (2013) reported 32 pesticides in olive and olive oil using QuEChERS acetate buffered method (AOAC), GC-MS.MS and LC-MS.MS with good recoveries ranging from 70-120%. 199 multiclass pesticides were quantified in fruits and vegetables in comparative study carried out by Lopez et al., (2014) using AOAC and citrate buffering (EN) QuEChERS methods and liquid chromatography- quadrupole-time of flight-mass spectrometry with automated detection (UPLC-QTOF—MS). Screening detection limits (SDLs) were 10 and 50 ng/g for 57% and 79% of the tested compounds, respectively. A simple multiresidue pesticide analysis method was developed and validated by He et al., (2015) for monitoring of 219 pesticides in cereals using AOAC and EN QuEChERS versions in a comparative study using GC-MS.MS analysis. Recoveries ranging from 70 to 120% with RSDs< 20% were achieved indicating acceptable accuracy of the method. Pano-Farias et al., 2016 developed a multiresidue method for analysis of carbamates (carbaryl & carbofuran), triazines (atrazine & ametrine) and organophosphates (methyl parathion) in papaya and avocado using QuEChERS (AOAC acetate buffering and CEN, citrate-buffering versions) and GC-MS analysis. Limit of detection (LOD) and limit of quantification (LOQ) were as the following; LODs: 0.03–0.35 ng /g (papaya), 0.14–0.28 ng /g (avocado) LOQs: 0.06–0.75 ng /g, (papaya) 0.22–0.40 ng /g (avocado). Arnnok et al., (2017) reported developing of multiresidue using d-SPE polyaniline-modified zeolite- NaY and HPLC-PDA analysis for multiclass pesticides (carbamates organophosphates, pyrethroids, neonicotinoids and sulfonylureas) from samples of (cabbages, cucumbers, tomatoes and strawberries). The method achieved LODs ranging from 0.001–1.00 µg/ mL and LOQs ranging from 0.005−2.50 µg/ mL. 76 representative compounds (multiclass-various) from green tea were reported by Chen et al., (2017)

using pressurized liquid extraction and solid phase extraction column (PLE / SPE). Graphitized carbon and primary secondary amine were used as sorbents for clean-up (GCB/PSA) and ultra-performance liquid chromatography -mass spectroscopy (UPLC-MS/MS) analysis. LOQs in samples were 0.4–36.4 ng/g. Farajzadeh et al., (2017), developed a method for determination Aryloxy pesticides (metalaxyl, haloxyfop–r–methyl, clodinafop–propargyl, dicofol–methyl, and bromopropylate) in vegetables (potato, tomato, onion, garlic, celery, radish, beet and carrot) using combination of DSPE and DLLME and GC-FID. Lozano et al., (2016) presented a modification and validation for Dutch mini-Luke using acetone /Na_2SO_4 and DCM/PEt for 175 pesticides in fruits and vegetables using GC-MS. Consumption of DCM was reduced by a factor of 3 and the recoveries were in the range 70-120% with RSDs < 20%. Modified QuEChERS dispersive solid phase extraction (d-SPE) based on a mixture of PSA and multi-walled carbon nanotubes (MWCNTs) for pesticides analysis in fruits and vegetables were developed and validated by Zhao et al., (2018) using LC-MS.MS. In most cases, the percentage recovery ranged from 71 % to 123 %, with an intra-day precision of less than 13% and an inter-day precision of less than 20 %. The proposed method was used successfully for the quantitative determination of pesticide in commercial berry sample in all areas of analytical sample preparation, future developments are expected to be directed towards application-driven processes in a bid to improve recovery, decrease consumption of organic solvent, get higher sample throughput and provide accurate results. Table 2.1 shows the extraction, clean up and analysis techniques used for determination of pesticides in fruit and vegetables. Quick, easy, cheap, effective, rugged, and safe (QuEChERS) extraction method is flexible extraction and clean-up method so, it was optimized regarding the pH to improve the compounds stability and recovery in case of acid base sensitive pesticides, this led to

developing of two buffered methods: European committee for standardization (EN) using citrate buffer for extraction and AOAC method which known as acetate buffered method.

Abdallah et al., (2018) reported developing and validation of QuEChERS method for determination of 42 pesticides in 200 date fruits samples collected from different markets in Al-Qassim region in Saudi Arabia using LC-MS.MS. Pesticides were detected in 18 % of the tested samples of which 7.5 % (15 samples) exceeded the maximum residue levels. Acceptable recoveries ranging from70-120 % and RSD values ≤ 20 % were achieved at three different concentrations :10, 50 and 100 µg/kg. The measure drawback of that study that the general QuEChERS method was applied without any modification and optimization to make it suitable for high sugar matrix such as date fruits. In addition, cleanup step was not applied to minimize the matrix effect. The dilution was used to minimize the matrix effect, and this may have two drawbacks; First, with dilution the extract, the concentration of some of pesticides may become lower than the LOD and give negative falls result. Secondly, as both pesticides and the co-extracts diluted by the same ration, the interferences of the co-extracts with pesticides still can cause suppression or enhancement for pesticides signal and lead to falls results. To avoid these problems, QuEChERS method should be modified and optimized for pesticide extraction and analysis in date fruit matrix and matrix effect should be taken in the account.

2.1.1 Method A (Original QuEChERS Procedure)

The original procedure for QuEChERS includes sample extraction with 10 mL of acetonitrile (MeCN) via vortex. To the sample, through mixing, gram quantities of salts (2 g of NaCl and 4 g of anhydrous magnesium sulphate ($MgSO_4$) are added to improve

separation of analytes between the solvent and the aqueous residue. After performing vortex mixing and centrifugation, the residual water is cleaned-up and removed through a d-SPE procedure (to the sample extract, a mixture of PSA adsorbent and anhydrous $MgSO_4$ is added). This step consumes lesser time when compared with the traditional SPE, and residual water and various polar matrix components, such as some polar pigments, organic acids, and sugars, are simultaneously removed. As a polar solvent, miscible with water, which possesses reasonable hydrophobic properties to perform efficient extraction of both non-polar and polar pesticides, MeCN is selected as the QuEChERS solvent. In the QuEChERS method, employing MeCN as a solvent has demonstrated successful extraction of various pesticides classes with different matrices (Rejczak & Tuzimski, 2015).

2.1.2 Method B (AOAC: Acetate Buffered QuEChERS)

For extraction step, 15.0 mL of 1 % (v/v) acetic acid in acetonitrile are added to 15 g homogenized rehydrated sample in 50 mL centrifuge tube. The tubes shaken for 1 min. Next, anhydrous $MgSO_4$ (6.0 g) and CH_3COONa (1.5 g) is added and Vortex for 10 min. Immediately centrifuge for 5 min at 3500 rpm (Rejczak & Tuzimski, 2015).

2.1.3 Method C (EN QuEChERS: Citrate Buffered QuEChERS)

For extraction step 10.0 mL of acetonitrile added to 10 g homogenized rehydrated sample in 50 mL centrifuge tube. The tubes were shaken for 1 min. Then, anhydrous $MgSO_4$ (4.0 g), NaCl (1.0 g), tri-sodium citrate dihydrate (1.0 g) and di-sodium hydrogen citrate sesquihydrate (0.5 g) were added and vortexed for 10 min. Centrifuge for 5 minutes at 3500 rpm right away.

Clean-up 1: 2 mL of the supernatant is put into another 15 ml centrifuge tube containing 50 mg of PSA and 150 mg of anhydrous $MgSO_4$, 50 g GCB. The mixture is vortexed and then centrifuged for 5 minutes at 4200 rpm. Finally, 0.5 to 1.0 mL of the supernatant is transferred to the GC-MS vail for analysis.

Clean-up 2: 1–2 mL of the supernatant is put into another 15-ml centrifuge tube containing 50 mg of PSA, 50 mg of C18, and 150 mg of anhydrous MgSO4. The mixture was vortexed and then centrifuged for 5 minutes at 4200. Finally, 0.5 to 1.0 mL of the supernatant is transferred to HPLC, or GC-MS vail for analysis (Rejczak & Tuzimski, 2015).

2.1.4 Some Common Pesticides in Date Fruits

2.1.4.1 Dimethoate

Dimethoate is a systemic and contact organophosphorus insecticide that was approved for use in the United States in 1962. It is used on a variety of field-grown agricultural commodities (including leaf greens, citrus, and melons), as well as tree crops and ornamentals. Dimethoate is used on alfalfa, wheat, cotton, and corn crops, accounting for more than 60% of total dimethoate use in the United States. Dimethoate is nonvolatile, water soluble, and inert in soil, where it dissolves over a period of typically 2-4 days, depending on soil conditions (U.S. EPA, 2006).

Omethoate arises naturally in plants and the environment within a few days of dimethoate application. As with dimethoate, omethoate degrades within two weeks in the environment. Omethoate appears to be the poisonous component of dimethoate in insects and mammals (IPCS, 1989). Both dimethoate and omethoate are very poisonous to honeybees, birds, and freshwater invertebrates (U.S. EPA, 2006).

Dimethoate is absorbed through the GI tract, the lungs, and the skin. Consumption of dimethoate-treated foods or drinking water can expose the public to dimethoate or omethoate. Average daily intakes have been estimated to be below safe levels (Gunderson, 1995).

Dimethoate, omethoate, and other organophosphate pesticides disrupt the nervous system's acetylcholinesterase enzymes, resulting in an overabundance of acetylcholine at nerve terminals. Nausea, vomiting, weakness, paralysis, and convulsions are all examples of acute cholinergic symptoms. Omethoate is approximately tenfold more hazardous than dimethoate and is a more effective cholinesterase inhibitor (Hassan, et al., 1969).

Dimethoate metabolizes to omethoate in insects, while mammals appear to have lower quantities of the enzyme responsible for this metabolic conversion. In animal experiments, it appeared as though inhibition of erythrocyte cholinesterase, but not plasma cholinesterase, was the most sensitive indicator of dimethoate exposure and toxicity (Sanderson and Edson, 1964). El- said et al., (2010) reported dimethoate residue in date fruits with concentration of 0.24 mg/kg which exceeding the MRL (0.01 mg/kg) using SFE and GC-MS techniques.

2.1.4.2 Carbendazim

Carbendazim (methyl 1H-benzimidazol-2-ylcarbamate) is a broad-spectrum systemic fungicide with the chemical formula $C_9H_9N_3O_2$ and a molecular weight of 191.19 g. It is also produced after the breakdown of thiophanate-methyl and benomyl fungicides (Fang et al., 2010; Mazellier et al., 2003). Annually, fungal pathogens inflict severe harm to food crops, resulting in low yields, insufficient nutrient content, and enormous economic loss.

As a result, the use of fungicides gained popularity as a means of averting these losses (Karlsson et al., 2014; Patel et al., 2015). Carbendazim is frequently used pre- and postharvest to manage fungal diseases caused by Fungi imperfecta, Ascomycetes, and Basidiomycetes on a variety of fruits, vegetables, medicinal plants, and food products (Tortella et al., 2013; Devi et al., 2015). Additionally, carbendazim and mancozeb exhibited efficient regulation of some plant problems such as chilli rots, leaf damage of sunflower, and mango common fungal diseases (Devi et al., 2015). Additionally, it is utilized in some industries such as fabric, paper, and leather industries (Selmanoglu et al., 2001).

Carbendazim is listed by World Health Organization as dangerous chemicals. Both carbendazim and carbonyl are known carcinogens (Goodson et al., 2015). It has been added to the list of substances known to cause endocrine disruption as a priority (Ferreira et al., 2008). Carbendazim has been forbidden in most of the European Union countries, the United States of America and Australia, due to its toxicity and perseverance (Zhang et al., 2013; Huan et al., 2016). But it is still employed in developing countries including Brazil, India, and China, in a variety of forms. Continuous use of carbendazim result in environmental contamination with ongoing effects on soil, animals and human health. The acceptable daily intake (ADI) of carbendazim is 0.03 mg/kg/day (Sharma, 2007). Residual carbendazim has a half-life of around 3 days to 12 months depending on nature of the soil, (Pourreza et al., 2015). The carbendazim's environmental durability is due to its benzimidazolic ring, which is resistant to degradation (Barnett et al., 1996). The highly toxic component, 2-amino-benzimidazole resulting from carbendazim degradation, binds to the spindle microtubules to prevent nuclear division (Yenjerla et al., 2009). Carbendazim is known

to be embryotoxic, teratogenesis, apoptosis, infertility, and developmental toxicity in various mammalian varieties (Rama et al., 2014; Singh et al., 2016).

By working with b-tubulin producing microtubular disruption, carbendazim suppresses microtubular polymerization in living cells resulting to altered chromosome segregation during cell division (2012; Rama et al., 2014). EL-Saeid et al., (2010) reported carbendazim detection in the three cultivars Khalas, Sukkari, and Nabout Seif (0.05, 0.05, and 0.03 mg/kg), respectively by (SFE). Carbendazim was also reported in date fruit from Al-Qassim (KSA) by Abdallah et al., (2018) with a concentration range of 0.010–0.760 mg/kg and exceeding the MRL (0.1 mg/kg) using HPLC-MS.

2.1.4.3 Chlorpyrifos

Chlorpyrifos is non-volatile organophosphorus pesticide derived from thiophosphoric acid, chemically known as O, O-diethyl O-3,5,6-trichloro-2-pyridyl phospho-rothioate. Commercially chlorpyrifos is accessible under a variety of brand names, including Dursban, Lorsban, Empire20, Equity, and Whitmire PT270 (Prasad et al., 2006; Rathod & Garg, 2016).

Chlorpyrifos is pesticide with a broad spectrum of activity that is used as an insecticide and acaricide (Christensen et al., 2009). Chlorpyrifos is available in a variety of formulations, including sprayable material, wettable granular material, powder, dry flowable solution, dust, microencapsulated material, pellets, and soluble concentrate (Prasad et al., 2006). Chlorpyrifos is a water-insoluble compound but is soluble in the majority of organic solvents (Smegal, 2000; Rathod & Garg, 2017). Chlorpyrifos is stable in both neutral and acidic aqueous solutions, however its stability declines as the pH increases. The chlorine group on chlorpyrifos enhances the compound's lipid solubility and half-life in the body, resulting in a more gradual and permanent decrease

in cholinesterase (ChE) levels when compared to other organophosphorus (Christensen et al., 2009). Chlorpyrifos is commonly applied to protect the agricultural crops due to its effect on a wide variety of insect species. However, as it is cost-effective, does not impact seed activity and having low residue levels in food grains. In non-target organisms like humans, chlorpyrifos often cause poisoning. Research performed across the U.S. to assess mortality among pesticide applicators, revealed that the effects of chlorpyrifos on contributors' health depends on the level, period, frequency of exposure, and an individual's health and some environmental factors. Chlorpyrifos is quickly cleared from the body after acute human exposure. Small quantities of chlorpyrifos metabolites can typically be sensed in the blood or urine after a single exposure of 48 h (Rathod & Garg, 2017).

Chlorpyrifos toxicity is comparable in both target and non-target species. Direct ingestion, oral administration during suicide attempts, or accidental inhalation, skin absorption during spraying of chlorpyrifos all result in toxicity in individuals and toxic effects on the central nervous system, respiratory system and cardiovascular system of the individual (Al-Meqbali et al., 1998). It creates three forms of action in body mode of mammals which are enzyme acetyl cholinesterase (AChE) inhibition, oxidative stress and endocrine disturbance (Schulze et al., 2012).

Chlorpyrifos is converted into chlorpyrifos-oxone in the liver, more potent than chlorpyrifos and more effectively works to serene ester hydrolases, such as AChE (Rathod & Garg, 2017). Chlorpyrifos-oxone is respiratory poison and 1000 times more potent than chlorpyrifos to neurons and 65 % more toxic to glia (Srivastava et al.,2011)

Chlorpyrifos-oxon is converted largely to TCP (3,5,6-trichloro-2-pyridinol), but also to diethyl phosphate (DEP) and diethyl thiophosphate (DETP). In addition, TCP glucuronide and sulfate conjugates have been detected in human urine. Chlorpyrifos-

oxon is the sole chlorpyrifos metabolite that inhibits ChE, while the other metabolites are regarded less hazardous (Al-Meqbali et al., 1998). The kidneys are responsible for chlorpyrifos removal; it is excreted in human urine as TCP, DEP, and DETP; the half-life of chlorpyrifos elimination in urine has been estimated to be 27 hours following oral or cutaneous contact. The detoxification of chlorpyrifos is mainly curried out through the hydrolysis of the chlorpyrifos oxon by A-esterase (Rathod & Garg, 2017).

According to the result of a research carried out on a human volunteer, the half-life of urinary dialkyl phosphates after oral administration was 15.5 hours and 30 hours following cutaneous administration. On average, 93 % of the oral dose and 1 % of the cutaneous dose were recovered as urine metabolites (Chatterjee & Sarma, 2003). For chlorpyrifos, a human exposure limit has been established (Schulze et al., 2012). Daily Acceptable Intake (ADI) 0.01 mg/kg/day.

The chlorpyrifos orally administered is partially defecated unchanged through the excrement, the part absorbed in the blood, is rapidly metabolized into less harmful compounds in the liver and easily defecated through urine (Mishra et al., 2014). 84 % of chlorpyrifos are mainly excreted in urine and about (5%) excreted in excrement within 72 h of exposure (Pawar et al., 2015). The orally taken chlorpyrifos is mainly defecated unchanged through the faeces, the part absorbed in the blood, is swiftly converted into less toxic compounds in the liver and readily defecated by urine (Mishra et al., 2014). Eighty-four percent of chlorpyrifos are largely eliminated in urine and around 5 percent excreted in faeces within 72 h of exposure (Pawar et al., 2015).

EL-Saeid et al., (2010) reported some pesticide's residue levels in three cultivars of date fruits viz., Khalas, Sukkari, Nabout Seif and their seeds using SFE method. The concentrations of chlorpyrifos were 0.16, 0.18, and 0.15 mg/kg, respectively. The KSA-

MRL for chlorpyrifos is 0.02 mg/kg, and the chlorpyrifos residue level was 6 times higher than the KSA-MRL.

2.2 Household Methods for Elimination of Pesticides Residues from Date Fruits

Most of the pesticide's residues appear to be found on the surface of vegetables and fruits where they can be removed by the activity of rinsing (Krol, 2000). There are many studies performed on the effects of cleaning agricultural produce to decrease or remove pesticide deposits as a stage in industrial crop treatment. These investigations are of little functional use to the customers. Nevertheless, decontamination procedure before use has also been studied for consumers e.g., warm water was found to be more effectual in eliminating pesticide remains in comparison to tap water (Uno et. al., 1984). Other material such as alkali and detergent solution (Awasthi, 1986), brine mixture (Jadhar, 1987), Palmolive® solution (Krol, 2000), hydrogen peroxide, peroxyacetic acid, ozone and chlorine dioxide (Hwang et al., 2001) have also been discovered to be more effective in eliminating the pesticide from farming commodities. Lee et al. (1991) noticed 60 % decrease of chlorpyriphos when rice grain was cleansed with water. Hwang et al. (2001) found out a significant reduction from 56% to 97 % of pesticides remains when soiled apple was processed with ozone. Tahir, et al., (2014) examined the effects of water on the elimination of residual pesticides found on the surface of vegetables. Filtered flour, tamarind juice, vinegar, water and soda salt solutions were studied for cleaning chlorpyrifos residues from cauliflower by Nowowi et al., (2016) and positive results were obtained. Tianxi et al., (2017) reporting that using baking soda, $NaHCO_3$ 10 mg/mL as washing solution for 12 -15 min is completely remove thiabendazole and phosmet surface residues from apples.

There are a few studies carried out on removing of pesticides from date fruits. Distilled and ozonated water were used as wash treatments after harvesting for 15–60 min in order to test the dipping times to remove amitraz and dicofol from different types of date fruits (Osman, 2015). Osman et al., 2014 reported removal of chlorpyrifos from date fruits using running water (H_2O), acetic acid, citric acid, hydrogen peroxide (H_2O_2), potassium permanganate ($KMnO_4$) and running water (H_2O). The study used relativity long washing time ranging from 5 to 60 min which may seriously damage the nutritive value of date fruits under uncontrolled temperature. Temperature and washing duration are the most important factors which should be optimized during washing of date fruits for pesticides residue removal.

Table 2.1: Basic Principles the Modern Extraction, Clean Up and Determination Techniques Applied for Pesticides Residue Determination in Fruits and Vegetable Samples

Analyte	Sample	Extraction /clean up	Clean-up procedure	Determination	Reference
349 pesticides (multiclass-various)	Fruit and vegetable samples (grape, pomegranate, okra, tomato, and onion	QuEChERS (Acetone)	DSPE with primary secondary amine (PSA) and/or graphitized carbon black / bonded silica	GC–EI-MS/MS	Banerjee et al., 2012
Insecticides, fungicides and herbicides of 32 different chemical groups	Olive oil and olives	QuEChERS (Acetonitrile)	i-Freezing out ii-AOAC official method 2007.01	GC-MS/MS LC-MS.MS	Anagnostopoulos et al.,2013
199 pesticides (multiclass-various)	Fruit and vegetable commodities (apple, broccoli, celery, leek, melon, nectarine, onion, pear, pepper, tomato, and grapes.	Acetonitrile 1% acetic acid	QuEChERS (EN, citrate-buffering versions)	UPLC– QTOF–MS	Lopez et al., 2014
219 pesticides	Cereals	Acetonitrile	QuEChERS (AOAC; acetate buffering and CEN, citrate-buffering versions)	GC-MS.MS	He et al., 2015
Carbamates (carbofuran & carbaryl), triazines (atrazine & ametryn), and organophosphates (methyl parathion	Papaya and avocado	Acetonitrile	QuEChERS (AOAC; acetate buffering and CEN, citrate-buffering versions	GC-MS	Pano-Farias et al., 2016
20 organochlorines	Romanian graps	Methanol	Stir bar sportive extraction (SBSE)-thermal desorption	GC-ECD	Ioan Simon et al., 2016
Multi-class pesticides (including carbamates, organophosphates, sulfonylureas, pyrethroids and neonicotinoids)	Fruit and vegetable samples (cabbages, cucumbers, tomatoes and strawberries)	Acidified Acetonitrile	DSPE using polyaniline-modified zeolite NaY	HPLC-PDA	Arnnok et al., 2017

Table 2.1, continued

Analyte	Sample	Extraction /clean up	Clean-up procedure	Determination	Reference
76 representative compounds (multiclass-various)	Green tea	Dichloromethane	PLE/SPE column (GCB/PSA	UPLC-MS/MS	Chen et al., 2017
Aryloxy pesticides (metalaxyl, haloxyfopr–methyl, clodinafop–propargyl, diclofop–methyl, and bromopropylate	Vegetables (potato, tomato, onion, garlic, celery, radish, beet and carrot)	Acetonitrile (as elution solvent)	Combination of DSPE and DLLME (sorbent (C8) is mixed with a dispersive solvent (iso–propanol) and then dispersed into an aqueous solution placed into a narrow–bore tube.	GC-FID	Farajzadeh et al., 2017
Pesticides	Date fruits	Supercritical fluid extraction SFE\CO2 and Microwave extraction MSE	No clean up needed	GC-/ECD/NAD	EL-saeid, et at., 2010
14 Organochlorines	Brazilian fruit pulps	Acetonitrile/dispersive solid-phase extraction d-SPE (QuEChERS) EN (citrate buffered version	clean up sorbent. (A); MgSO4, PSA, C18 and GCB (B); MgSO4, PSA, C18	GC-ECD	Mario Paza. et al., 2016
172 pesticides	Fruits and vegetables	i-Acetone /Na2SO4 (extraction step)	ii-DCM /PEt (modified NL method NL) partitioning.	GC-MS.MS	Ana Lozano et al., 2016
42 pesticides	Date fruits	Acetified acetonitrile and citrate buffer salts were used for extraction	No clean-up needed; the upper layer was filtered with 0.22µm PTFE syringe filter into 1.8 vials for direct chromatographic analysis.	LC-MS.MS	Abdallah et al., 2018
Pesticides	Strawberry, raspberry, blueberry and blackberry	Acetonitrile	Modified-QuEChERS dispersive solid phase extraction (d-SPE) based on a mixture of PSA and multi-walled carbon nanotubes (MWCNTs)	GC-MSW.MS LC-MS.MS	Zhao et al., 2018

2.3 Methods of Sample Preparation and Pre-treatment for Heavy Metals Analysis

Heavy metals analysis in organic or inorganic matrices involves sample preparation methods for partial or whole dissolution of the sample matrix before instrumental analysis. These include digestion of the matrix, extraction, and preparation of the analytes for the analysis. Therefore, digestion methods are the most frequently applied approaches for sample preparation of inorganic contaminants. Digestion methods such as dry or wet decomposition in open or closed systems, using thermal, ultrasonic, or radiant (infrared, ultraviolet, and microwaves) can be effectively applied for inorganic compound analysis to all types of samples (soil, plant, biological, and water samples) and they signify an essential step in various analytical methods for the analysis of heavy metals and other inorganic compounds. sample digestion has been developed over the last decades including dry ashing, wet digestion, microwave digestion, ultraviolet irradiation, ultrasound, and slurry sample preparation methods that are improved for all types of samples (plant materials, biological samples, soil, and water), with the purpose of transfer the elements into solution, prior to quantifying them using suitable analytical tools (Kailasa & Wu, 2012).

Most samples containing inorganic compounds also contain organic substances that require pretreatment before analysis. Generally, pretreatment (filtration and acidification) is required for liquified inorganic compounds (metals), which can then be analyzed directly. Digestion of the sample involves the complete destruction and dissolution of the sample matrix to release inorganic species from the matrix.

In metal analysis, combustion is one of the simplest and most convenient digestion methods. Figure 2.2a shows various tools that have been used in combustion methods for sample ashing. Ashing is the dissolution of the inorganic compounds in

organic materials by decomposition or complete oxidation. In the last decades, digestion procedures such as dry ashing, wet ashing, and microwave digestion methods have been widely used for the decomposition of organic materials from environmental and biological samples (Marshall, 2010).

2.3.1 Dry Ashing

Dry ashing is simple and has been widely used for the digestion of inorganic compounds in various samples. this technique, involved the decomposition of organic substances with thermal treatment at 500–600°C, followed by dissolution of the residues in acids to release the inorganic elements. Usually, several types of muffle furnaces (110 V to 208 or 240 V) are employed for dry ashing. Currently, microwave furnaces with high heating efficiency are available. They reach extremely high temperatures in a short period of time. Electronically excited oxygen plasma is utilized for low-temperature ashing and it is very useful for volatile elements (Nemati et al., 2010).

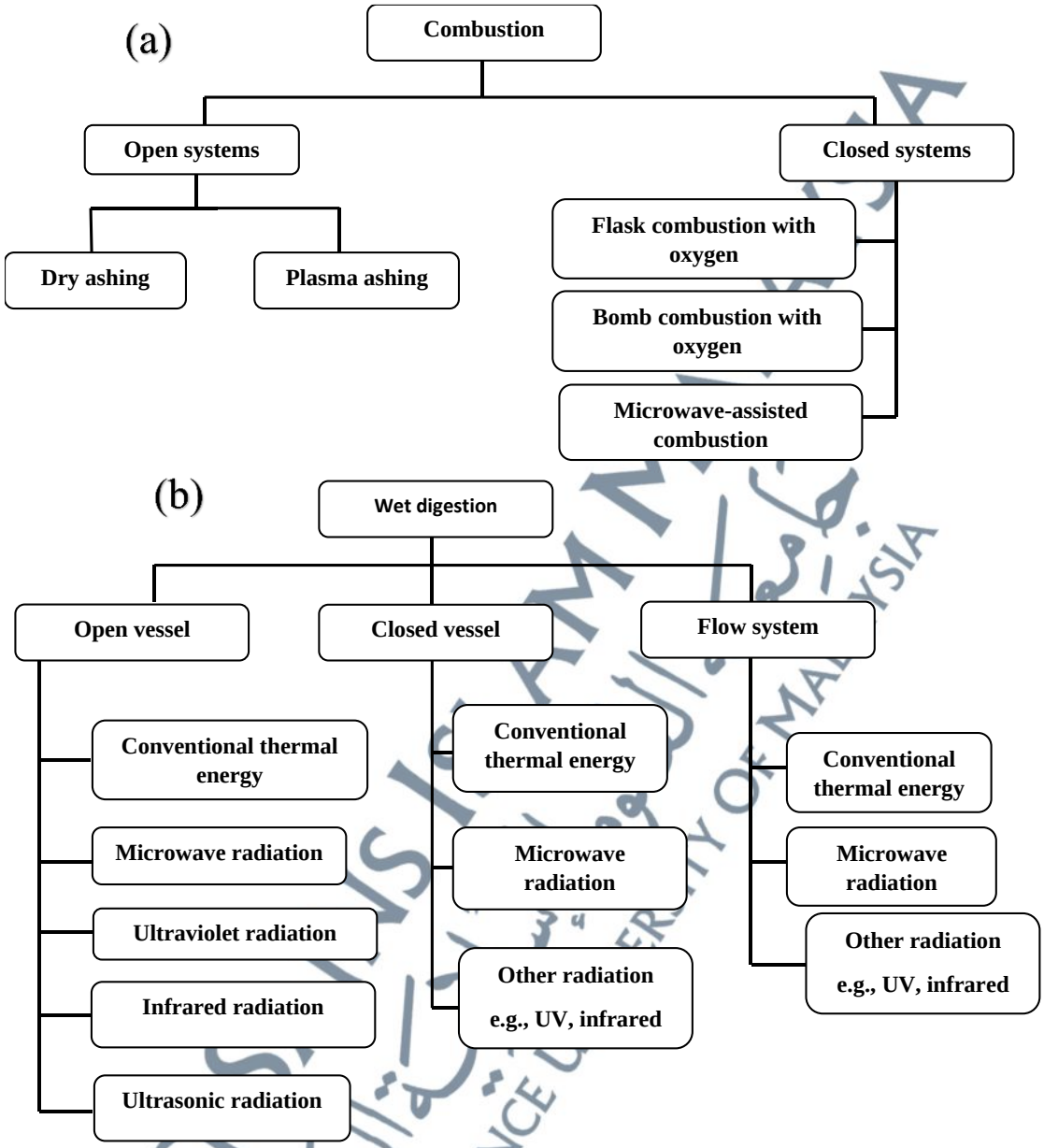
Crucible type is critical in the ashing process because the types of crucibles used must be appropriate for the samples. There are many types of crucibles used for the ashing of inorganic samples including quartz, porcelain, Pyrex, steel, and platinum. The selection of a suitable crucible is dependent on the sample and the furnace temperature. because of low cost, high-temperature stability (<1200) ease of cleaning, and resistance to acids, Porcelain crucibles are extensively used for dry ashing. A number of dry ashing methods have been developed and officially recognized for the determination of ashing content and inorganic contaminants in various samples (Horwitz & Latimer, 2005).

The advantages of the dry ashing method are the ability to digest a large number of samples, and the remains can be liquified in small volumes of acid. It does not require

any expensive reagents. Elements present at low levels in the samples can be analyzed effectively. That advantage is not possible with other digestion methods, such as wet digestion methods. Drawbacks to this method are that it is time-consuming and needs more electrical power. In addition, it is not suitable for the mineralization of volatile compounds of Hg, Se, Zn, V, Cr, Pb, Cu, Cd, Ni, and B (Hoenig, 2003).

2.3.2 Wet Digestion

Wet digestion depends on the dissolution of inorganic components by acid or mixtures of acids that are carried out in open vessels, in tubes, on a hotplate, or in closed vessels at elevated pressure with thermal or microwave heating. Various methods have been used for elemental analysis that involves the conversion of the sample into a solution. Different acid treatment procedures are used for efficient extraction of elements from various geological, biological, and environmental samples with specially designed apparatus (Stoeppler, 2012). Inorganic species can be produced by adding strong acid mixtures and applying heat by (thermal, microwave, UV, or infrared resources). Nitric acid is widely used for the primary oxidation of organic matrices in various samples; however, a single acid used in wet digestion does not give complete and rapid oxidation of the matrix. Thus, a mixture of acids is often used. The common physical properties of acids and vessels to be used have been discussed for the wet digestion of samples (Kailasa & Wu, 2012). Figure 2.2 (b) shows the classification of wet digestion methods using different energy devices and systems.



Sources: Kailasa & Wu (2012)

Figure 2.2: Classification of Sample Decomposition Methods for Inorganic Species(a) Combustion, and (b) Wet digestion

2.3.3 Conventional Wet Digestion Methods for Heavy Metals Analysis

In conventional wet digestion procedures, samples are firstly digested in a mixture of strong acids or oxidizing agents, such as nitric acid (HNO_3), perchloric acid ($HClO_4$), sulfuric acid (H_2SO_4), hydrochloric acid (HCl), and hydrogen peroxide (H_2O_2). The

digested samples are then heated to near-dryness cooled and re-dissolved in a suitable solvent (usually 1 % HNO_3). These digestion methods successfully decompose the organic materials in biological samples and lessen the dissolution substances in the final solution. Therefore, the matrix effect and spectral interferences are removed or minimized. Direct wet digestion methods are frequently carried out in concentrated HNO_3 or in a mixture of HNO_3 and H_2O_2. Studies that used this dissolution technique reported good recoveries for certain heavy metals in various biological matrixes analyzed by GFAAS and ICP. (De Brätteret al.,1995; Tüzen, 2002). Compared with other acids such as $HClO_4$, HCl, and H_2SO_4, nitric acid (HNO_3) showed the least background spectral interference (Matusiewicz, 2003) and has become the most preferred sample preservative and dissolution reagent for elemental analysis. With H_2O and reactive oxygen species as decomposition products, H_2O_2 has become the preferred additional oxidizing reagent used in conjunction with HNO_3 to facilitate wet digestion of biological samples. This dissolution method is simple, safe, rapid, and subject to less potential contamination and loss of volatile elements (Aldjian et al., 2011).

2.3.4 Analytical Tools for Determination of Heavy Metals in Food and Environmental Samples

Several analytical techniques have been employed for the determination of trace metals in biological and environmental samples such as atomic absorption spectrometry (AAS) including both techniques: flame atomic absorption spectrometry (FAAS) and graphite furnace atomic absorption spectrometry (GFAAS), inductively coupled plasma-atomic emission spectrometry (ICP-AES),inductively coupled plasma-mass spectrometry (ICP-MS), neutron activation analysis (NAA), energy dispersive X-ray fluorescence (EDXRF) ,differential pulse anodic stripping voltammetry (DPASV)

(Zhong et al., 2016). GFAAS will be used for quantifying the heavy metal contents in date fruits samples in the present work because of its high selectivity, accuracy, sensitivity, effectiveness, and low cost in addition to the availability of the instrument.

2.3.5 Heavy Metals in Date Fruits

There are many studies on investigating the heavy metals in date fruits; determination of some trace elements as pollutants in date fruits and agricultural soils at Zilfi province (Sudan) was carried out by Suleman (2014). The concentrations of the elements in soil were found as follows in ppm: Ag (0.7-42), Al (1628.7-6264.5), As (40-59.8), Bi (0.02-0.1), Cd (0.08-4.2), Co (1.1-11.5), Cr (19.2-126.2 (ppm), while, the concentrations of the examined heavy metals in date fruits were found as the following in μg/g: Ag (0.3-1.2), Al (24.6-152.9), As (4.3-6.5), Bi (0.004-0.008), Cd (0.03-0.2), Co (0.03-1.6), and Cr (4.1-15.5). Washed date samples showed lower concentrations of heavy metals than unwashed ones. In the study carried out by Taha et al., (2015) on date fruits from the species Sugai (KSA), washed and unwashed samples were analyzed for metal contents using inductivity coupled plasma and atomic absorption spectrophotometer. The results of the study showed that the dates have considerable contents of Fe and Mn. While the poisonous elements such as arsenic (As), cadmium (Cd), nickel (Ni) and bismuth (Bi) was found to be less in washed samples compared to unwashed samples. The study conducted by Salama et al., (2019) revealed that toxic heavy metals such as, arsenic (As), lead (Pb) and cadmium (Cd) are exceeding the maximum allowable levels (MAL) and the estimated daily intake (EDI). AS and Pb were found higher than maximum tolerable daily intake in samples from some locations of KSA. The calculated hazard risk index predicted that both AS and Pb may have a possibility of health risk on consumer's health. Therefore, the heavy metals

content and risk assessment should be investigated for the imported date fruits from Middle East. However, a suitable, low-cost digestion method for determination of heavy metals in date fruits matrix should be investigated.

2.4 Natural Antioxidants

In food, naturally occurring inhibitors typically derive from plant materials. phenolics and polyphenolics, like tocopherols are the active components of secondary plant metabolites derived from phenylalanine initially, and from tyrosine in some plants. The resulting poly-phenylpropanoids can then be subjected to additional transformation to yield benzoic acid derivatives, flavonoids, isoflavones, and some complex polyphenols. Consequently, natural food phenolics are present as a complex combination of molecules that combine to generate a cocktail of many active components in free or bound forms (Shahidi & Naczk, 1995). Hence the effectiveness of preparations is governed by their chemical structures and regulated by the concentration and system-dependent hydrophilic-lipophilic balance (HLB) of contributing molecules. Thus, the source material, and the potential existence of synergistic and antagonists, are determining the mode of action of natural antioxidants which comprise several mechanisms.

2.4.1 Assessment of Antioxidant Activity

The antioxidant activity can be assessed by quantifying primary or secondary lipid autoxidation products or by tracking other variables. Usually, the interruption of the formation of hydroperoxide or the development of secondary autoxidation products by chemical or sensory methods can be used as a determining factor. These procedures can be applied to either complete foods, their extracts or to model systems. It is

possible to conduct studies on foods under regular storage conditions as well as under accelerated oxidation conditions using methods such as the active oxygen method (AOM), the Schaal oven test, oxygen uptake/absorption, oxygen bomb calorimetry, or by using a fully automated rancimate apparatus (Kochhar & Rossell, 1990). The increase of the induction duration by adding an antioxidant is correlated with antioxidant effectiveness, often expressed as an antioxidant index or protective factor. The most frequently used tests for evaluating antioxidant activity are listed in the Table 2.2.

Table 2.2: Assays Used to Evaluate the Antioxidant Activity

Assay	Reference
Ferric thiocyanate method (FTC)	Osawa & Namiki (1981); Zin et al. (2002), Pastor et al., 2020
Tiobarbituric acid test (TBA)	Ottolenghi (1959); Kikuzaki & Nakatani (1993), Karagöz et al., (2015).
Oil stability index (OSI) assay	Chen & Ho. (1997); Yu et al., (2002)
Free radical scavenging capacity (2,2-diphenyl-1-I-picrylhydrazyl radical (DPPH) assay	Shimada et al., (1992); Oktay et al., (2003). Foti, M.C. 2015
Chelating activity (2,2' -bipyridyl competition assay)	Yamaguchi et al., (2000), Li et al., 2011
Antioxidant capacity (B- carotene -linoleic acid assay)	Pratt (1992); Dimitris & John. (2001), Mraihi et al., 2013
Superoxide anions scavenging assay (xanthine -xanthine oxidase system & Phenazine methosulphate-NADH System (non-enzymic method)	Jadwiga & Ryszard (1987), Bhatia et al. (2021)

2.5 Analysis and Quantification of Phenolic

There are many spectrophotometric methods to determine the phenolic contents in plant materials. These tests are based on various concepts and are employed to determine various structural classes of phenolics. It is possible to quantify all extractable phenolics together using spectrophotometric methods, but this is not always the case.

(Swain & Hillis, 1959; Price & Butler, 1977). There are some developed methods for determining individual phenolic chemicals, such as sinapine (Tzagoloff, (1963); Flourat, et al., 2019 and Quinn, et al., 2017), while others were developed to determine a specific class of phenolic substances, such as phenolic acids (Price et al., 1978; Brune et al., 1991). The modified vanillin test (Price et al., 1978), Folin-Denis assay (Swain & Hillis, 1959), Prussian blue test (Price & Bulter, 1977) and Folin-Ciocalteu assay (Hoff & Singleton, 1977; Maxson & Rooney, 1972; Deshpande & Cheryan, 1987; Aryal, et al., 2019) are most commonly used spectrophotometric methods. Table 2.3 shows various assays used to assess total phenolic contents.

Table 2.3: Summary of Assays Used to Evaluate Total Phenolic Content

Assay	Uses	Reference
Vanillin assay (Condensation of vanillin with proanthocyanins in acidic solutions)	Quantification of Proanthocyanidin in fruits	Goldstein & Swain (1963); Burns (1971). Shahidi et al., (1991)
Prussian blue assay (Reduction of ferric to ferrous ion-by polyphenolic)	Quantification of polyphenolic	Price & Butler (1977); Deshpande and Cheryan (1987); Shahidi, & Zhong, (2015)
Folin-Denis's assay (Reduction of phosphomolybdic-phosphotungstic acid to blue-colored complex by phenolics)	Quantification of total phenolics in plant material and beverages	Folin & Denis (1912) Swain & Hillis (1959) Blainski et al., 2013
Folin-Ciocalteu assay (blue complex between reagents and phenolics)	Determine the total content phenolic of food phenolics.	Hoff & Singleton (1977); Maxson & Rooney (1972) Aryal, et al .,2019
Reinecke salt assay (formation of water-insoluble complex between Reinecke salt and Quaternary 'nitrogen base)	Determination of sinapine in hot methanol extracts	Calndinin (1961); Austin & Wolff (1968); Flourat, et al., 2019
Titanium tetrachloride assay (Formation of tetrachloride titanium complexes with sinapine in presence of HCl)	Determination of sinapine in acetone water (60:40) extracts	Eskin (1980); Quinn, et al., 2017

2.6 Analysis and Quantification of Total Sugars

The colorimetric methods for determination of total saccharides that based on the reaction between a hydrolysed carbohydrate solution and a colouring reagent produces a colour that is observable in the visible electromagnetic spectrum, is one of the most flexible, reasonably simple and cheap methods for determining concentration of

carbohydrates. Reagents widely used for colour production include phenol (C_6H_5OH) (DuBois et al., 1956), alkaline ferricyanide ($2K_4$ Fe $(CN)_6$) (Englis & Becker, 1943) and anthrone (C_{14} H_{10} O) (Dreywood, 1946).

The Phenol – Sulfuric acid method of DuBois et al. (1956) has been the most reliable method and widely used in several areas among colorimetric methods for analysis of carbohydrates. The dehydration of hydrolysed saccharide into the furfural derivatives during the reaction with concentrated sulfuric acid depends on the Phenol-Sulfuric Acid process (Albalasmeh et al., 2013). Further furfural derivatives with phenol forms coloured complexes that have maximum absorbance at 490 nm (DuBois et al., 1956; Rao & Pattabiraman, 1989).

While it is simpler to use than many of the available methods, there are some significant disadvantages in the Phenol Sulfuric Acid process. First, Phenol and its vapours are corrosive to skin, eye and breathing system. Thus, repeated skin contact can lead to dermatitis or burns of second and third degree. Similarly, prolonged, or chronic phenol vapor inhalation causes pulmonary edoema. Long-term exposure to phenol also has a significant effect on the central nervous system, kidneys, and liver (Michalowicz & Duda, 2007, Albalasmeh et al., 2013). Second, in glucose-equivalent concentrations, the product of the conventional Phenol – Sulfuric Acid process is presented. This representation may have possible drawbacks in the treatment of complex carbohydrates that are not simple glucose polymers. Finally, the chemical reactivity of carbohydrate derivatives (sulfuric acid) depends heavily on whether the carbohydrates are neutral or anionic. Thus, the coefficients of molar absorption will differ greatly depending on the charge of the analysed carbohydrates (Mecozzi, 2005).

However, Albalasmeh et al., (2013) introduced the Sulfuric Acid–UV method as a method for determining sugar concentrations and carbon content of aqueous solutions

based on the UV absorbance of furfural derivates formed by reaction with concentrated sulfuric acid. The suggested Sulfuric Acid–UV approach employs aqueous solutions of neutral (glucose, fructose, sucrose, starch, dextran, and actigum) and anionic (polygalacturonic acid (PGA) and xanthan) carbohydrates often utilized in environmental research and industrial applications.