CHAPTER 1

1. Introduction

1.1 General

Nowadays, it is a common trend for most countries to encourage organic farming, integrated pest management and biotechnology because the use of herbicides, insecticides, fungicides, growth regulators and rodenticides, collectively called agrochemicals or pesticides are found to be endangering the environment. Although the above trend is welcomed, the world demand for pesticides will increase 4.4% annually to value over US$34 billion in 1998, according to Luci Young of The Freedonia Group, Inc. (Cleveland, Ohio) (1). Much of the anticipated growth will occur in the less developed countries in Asia and the Pacific, Africa and in South and Central America, whilst a constant demand is being experienced in many of the more developed areas of Europe, North America, Australia and Japan where factors such as food surpluses and land set-aside schemes are already beginning to influence the pesticide market. Table 1 shows the world pesticide consumption (1) from 1983 - 1998.

				% Annual Growth	
	1983	**1993**	**1998**	**93/83**	**98/93**
North America	4.0	7.4	9.0	6.3	4.0
South America	1.3	2.3	3.0	6.3	5.4
Western Europe	5.8	7.2	9.0	2.1	4.6
Eastern Europe	2.9	2.6	3.2	- 1.2	4.4
Africa/Mideast	0.9	1.3	1.6	2.9	5.1
Asia/Oceania	5.6	6.8	8.4	2.0	4.2
Total Consumption	20.5	27.6	34.2	3.0	4.4

Table 1 -World Pesticide Consumption (billion US dollars)

Generally speaking, the pesticide market is affected by changes and trends in agriculture, climatic variables (e.g. rainfall, temperature) and various government policies. At present, the adoption of more sophisticated farming techniques in developing nations has encouraged the use of chemical pest control agents. Western Europe's agricultural reforms have hindered demand by restricting the area planted. Both regions, however, face mounting regulatory and environmental pressures to improve the safety of pesticides and limit the production and export of potentially dangerous compounds.

It was reported that the USA was one of the world's largest users of pesticides. The market for pesticides is enjoying gains as product mixes shift to more sophisticated chemistry and are generally more expensive. A recent industry analysis (2), prepared by The Freedonia Group, Inc. estimated that demand in USA will increase by 4% per annum to reach US $ 7.5 billion by end of this century. Conventional pesticides - herbicides, insecticides and fungicides will continue to dominate the market with over 90% of aggregate industry consumption. Biopesticides will gain importance, as evidenced by the commitment of large, established firms - including Ciba and DuPont to research and development of these products. Table 2 shows the USA demand for pesticides from 1985 - 2000.

				% Annual Growth	
	1985	**1995**	**2000**	**95/85**	**00/95**
Pesticide demand	4.198	6.166	7.500	3.9	4.0
Herbicides	2.640	3.458	3.990	2.7	2.9
Insecticides	1.004	1.576	1.950	4.6	4.4
Fungicides	0.312	0.592	0.755	6.6	5.0
Biopesticides	0.008	0.080	0.200	25.9	20.1
Others	0.234	0.460	0.605	7.0	5.6

Table 2 - The USA demand for pesticides (billion US dollars)

Pesticides will continue to be needed to help produce sufficient food, fibre and other essential commodities like rubber and oil palm to meet the demands of a rapidly expanding global population which is expected to exceed 6 billion by the turn of the century.

In Malaysia, agriculture accounts for 30% to 32% of the GNP (3) and contributes significantly to export earnings. Rubber and oil palm are the main export crops, accounting for 29% of total exports. Cocoa, rice, vegetables, and fruits are the other major crops. Table 3 shows the major crops and their planted acreage in Malaysia.

CROPS	PLANTED AREA (Ha)
Rice	645,000
Coconut	216,000
Oil palm	3,050,000
Cocoa	209,000
Coffee	9,470
Sugarcane	23,500
Pepper	11,400
Rubber	1,713,000
Tea	3,200
Tobacco	12,100
Durian	140,800
Pineapple	10,000
Other Fruits	90,000
Flowers/Ornamentals	830
Vegetables	38,600
Total areas	6,172,900

Table 3 - The major crops in Malaysia and their planted acreage (4)

In the Malaysian Agricultural Chemicals Association (MACA) Annual Report 1997/98 (4), it was reported that the Malaysian crop protection chemicals industry has reached "maturity". The market in 1997, at end-users price levels grew by about 8.3% to an estimated RM326 million. The overall performance of this sector is very much dependent on the performance of the different commodities, the change in crops cultivated, the stages of growth and the incidence of pests or disease outbreaks. Herbicides account for 75% of the total Malaysia pesticide market, insecticides for 16%, fungicides for 5.0% and rodenticides for the remainder. Fig. 1 shows the Malaysia pesticide market in 1997.

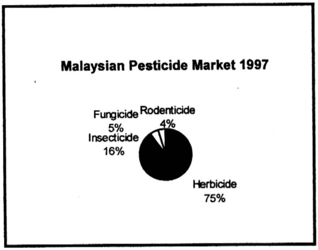

Fig. (1)-The Malaysian Pesticide Market in 1997 (4)

The Malaysian crop protection chemicals market as measured in ringgit (RM) terms from 1991 -1997 are shown in Table 4.

Crop Protection chemicals	1991	1992	1993	1994	1995	1996	1997
Herbicides	230	210	200	210	220	227	245.0
Insecticides	40	41	39	41	43	47	52.0
Fungicides	13	13	13	14	15	16	17.5
Rodenticides	10	12	10	11	11	11	11.5
Total	293	276	262	276	289	301	326.0

Table 4 : The Malaysian Crop Protection Chemical Market from 1991 - 1997 (RM million) (4)

In Malaysia, about 90% of the pesticides are consumed in the cultivation of rubber, oil palm and rice. Approximately 75% are herbicides which are applied for weed control in the rubber and oil palm plantations. Table 5 shows the major crops, and the common pests and diseases associated with their cultivation.

CROPS	PESTS AND DISEASES
oil palm & rubber	bagworm(*Metisa plana,Cremastopsyche pendula*), nettle caterpillars(*Setora nitens,Ploneta diducta*) seedling leaf diseases; oil palm-(*Botryodiplodia sp., Glomerella sp.,Melanconium sp., Curvularia sp., Drechslera sp., Cochliobolus sp,Helminthosporium sp., & Pestalotiopsis sp.*), rubber-(*Cylindrocladium quinqueseptatum, Colletotrichum sp., Pestalotiopsis versicolor, Drechslera heveae, Colletotrichum derridis, & Ascochyta heveae*), pink disease(*Corticium salmonicolor*) and broad leaves and other weeds
cocoa	pod borers(*Conopomorpha cramerella*), cockchafers(*Apogonia sp., & Lepadoretus compressus*), black pod(*Phytophthora palmivora*), canker(*Phytophthora palmivora*) vascular streak dieback(*Oncobasidium theobromae*).
Rice	stemborers(*Chilo suppressalis, Chilo polychrysus, Scirpophaga incertulas, Scirpophaga innotata,Sesamia inferens*), brown planthopper(*Nilaparvata lugens*), whitebacked planthopper(*Sogatella furcifera*), green leaf hopper(*Nephotettix sp.*), & rice bugs(*Leptocorisa acuta*).
Vegetables	diamond back moth(*Plutella xylostella*), fruitpod borers(*Maruca testulalis*), aphid(*Aphis sp.*), Red mites(*Tetranychus truncatus*), thrips(*Thrips sp.*), downy mildew(*Pseudoperenospora*), late blight(*Phytophthora infestans*), & anthracnose(*Colletotrichum sp.*)

Table 5 : Some major crops and their common pests and diseases

With continued emphasis on the development of plantation crops, it is expected that herbicide use in Malaysia will continue to expand, while that of insecticides will remain constant.

1.2 Key Pesticides

The key pesticides used in Malaysia are paraquat, dalapon, diuron, 2,4-D amines, methamidophos, chlorpyrifos, cypermethrin, malathion and carbaryl. Paraquat, dalapon, 2,4-D amines and glyphosate are commonly used for weed control in plantations. To a lesser extent, diuron, sodium chlorate, DSMA (Namate) and various herbicide mixtures are employed to control special weed problem such as lalang '*Imperata cylindrica*'. Approximately 30% of the total herbicides used are applied to lalang. Insecticides are more commonly used on vegetables, rice and tobacco. The important products used are chlorpyrifos, methamidophos, carbofuran, cypermethrine and BPMC (Osbac). Vegetables grown in Malaysia yield an average of 5 crops per year and spraying intervals vary from 1 week to 10 days. Insecticide applications on rice are seasonal, while those on tobacco vary with yield. Insecticides are used on oil palm only during sporadic infections of nettle caterpillars and bagworms. The use of fungicides in Malaysia is minimal, primarily in vegetables and tobacco farms. Small quantities are used in rubber plantations for control of pink disease (*Corticium salmonicolor*). The major fungicide used is mancozeb. Rodenticides are used to control rats in oil palm plantations and to a lesser extent in the rice fields.

1.3 Pesticide Industry Profile

The supply and production of pesticides in Malaysia is carried out by the private sector. 50% of the pesticide registrants belong to the Malaysian Agricultural Chemicals Association (MACA) and 85% of pesticide products are sold by MACA members. The pesticides are supplied directly to large estates or through a dealer network to farmers. Approximately 55% of all pesticides in Malaysia are imported, the remainder (mainly herbicides) are locally formulated or manufactured.

1.4 Vegetable Farms in Malaysia

Fresh air, some rain and sunshine are not the only ingredients to raising a healthy crop of vegetables. Commercially grown vegetables need some protection to achieve the shiny, fresh and crunchy look that sells at wet markets or supermarkets. Usually when a basket of fresh looking vegetables arrives at the wholesale market, the grower gets a better price because the consumers will be more attracted to the produce. To achieve the fresh-look, the farmers who have to battle the elements and pests that can wipe out their farms in one stroke, apply frequent pesticide spraying and use a cocktail of different chemicals for more potency. By this method, the use of pesticides provides unquestionable benefits in increasing agricultural production. However, it has the drawback of pesticide residues which remain on the vegetables, constituting a potential health risk to consumers.

1.5 Other Problems Related to the use of Pesticides

Application of pesticides not only kills pests but also their predators and parasites, thereby disturbing the natural regulating mechanisms. In addition, pollinating insects which are vital for plant propagation, especially for many tropical crops, may be affected by pesticides. Another problem which is encountered in chemical control is the increasing development of resistance in insects towards specific pesticides which leads to a vicious circle of repeated applications with higher dosages leading to more resistant species. It has been reported that arthropods, plant pathogens, and rodents have all developed resistance to pesticides (5). In north eastern Mexico, for example, multiple resistant pests have led to the virtual replacement of cotton production by other crops.

The environmental fate of pesticides can extend beyond agricultural systems. During and after application, the pesticides may be transported by wind or water to the surrounding compartments resulting in hazardous concentrations in the surface water and in soils. Long-term effects may be expected when persistent pesticides that do not degrade easily or disappear by volatilisation or adsorption are used. These pesticides and their metabolites may even migrate to ground water systems and contaminate present and future drinking water resources.

In the past, the side effects of pesticides and their degradation products on birds and mammals were often related to the use of persistent organochlorine insecticides. However, less persistent pesticides may also cause deleterious side effects (6). Spraying of toxic compounds and their accidental release may cause acute mortality or long-term effects in populations of birds, fish and other aquatic animals. Secondary poisoning by feeding on prey containing pesticide residues may also affect many organisms. The effects on non-target organisms not only depend on the type of pesticides used, the dose rate and the use pattern, but also on local circumstances such as the condition of the soil and water, climate, disturbance cycles (e.g. drought, floods), geomorphology, ecosystem structures, presence of areas and species of recognised ecological value and the potential for recovery of affected species. Risk assessments of a pesticide therefore cannot be extrapolated from one location to another.

1.6 Prevention of Negative Effects of Pesticides

A chemical pesticide should be chosen not only for its effectiveness against the pest species but because of its low toxicity to non-target species organisms. The correct and safe use of pesticides (e.g. efficient and effective application) should be emphasised. Integrated Pest Management (IPM) is, therefore, concerned with combining a number of control techniques in a particular cropping system, for a whole range of pest species in order to provide an overall coherent strategy for pest management. According to Peter Kenmore (7) of the Food and Agriculture Organisation (FAO) of the United Nations, IPM is currently accepted as the

" farmer's best mix of control tactics in comparison with yields profits and safety of alternatives". Other experts have described IPM as " a set of practices that maintain pest problems at a level below that which causes economically significant losses". It emphasises minimal usage of pesticides through a combination with other pest control methods such as proper monitoring of pest outbreaks in relation to weather conditions, the use of insect traps, natural predators, pest resistant cultivation, mixed cropping and crop rotation. IPM is presently seen as essential to the development of sustainable agriculture. There are normally several options that can be used for the control of a particular pest, farmers selection of a control or a management technique is based on the relative merits of each practice in terms of the ability of each option to meet the farmers' particular constraints and goals in relation to expected profit and their perception of the risk. For successful adoption of IPM, the farmers must i) be able to identify the problems; ii) have access to appropriate control measures; iii) have the confidence to make appropriate choices. As side effects are often a result of the improper use or improper choice of pesticides in relation to the local conditions, it is important that proper education and training for farmers be carried out on the level of application. Education is very important for the successful implementation of IPM, and misapplication of pesticides is mainly caused by a lack of education in farm communities. If this problem persists, the IPM programme will not be a feasible alternative solution.

Many countries have now formulated legislation for the registration, trade and use of pesticides, including the testing of pesticide characteristics, packaging and labelling. Statistics on the impact and use, however, are often not available. Deficient infrastructure is often the cause of extensive and unsafe use of pesticides. Legislation, and control of the distribution, application, packaging, labelling and protective measures are also weak.

Various international guidelines and programmes have been developed to supply governments and users with information on hazards related to the use of pesticides. These have been developed either jointly or independently by bodies such as the following :-

- Food and Agriculture Organisation of the United Nations (FAO)
- World Health Organisation (WHO)
- Organisation for Economic Co-operation and Development (OECD)
- European Union (EU)
- United Nations Environment Programme (UNEP)
- UNEP/ International Register of Potentially Toxic Chemicals (IRPTC)
- UNEP/ WHO/ International Labour Organisation (ILO) International Programme on Chemical Safety (IPCS)

1.7 Maximum Residue Level (MRL) and Legislation

It was reported (8) that since mid 1950s, countries have begun to legislate for pesticide residues in food/feed, by the use of MRLs. However, problems arose with the development of a number of separate national MRL regulations. Usually a national MRL applies both to domestically produced food and to imported food commodities. In the country of origin more than 95% of food consignments studied would normally comply with the MRLs prevailing in the country. If however, an exporting country and an importing country have established MRLs at different levels, trade obstacles may occur. As the result, a joint meeting between the FAO Panel of Experts on the use of pesticides in agriculture and the WHO Expert Committee on pesticide residues was held in 1961. The purpose of this meeting was to consider a programme to study the principles for establishing MRLs for pesticide residues in food, bearing in mind the aspect of consumer safety. Since 1965, the joint sessions of the FAO and WHO Committee of the Joint Meeting on Pesticide Residues (JMPR) have been held on annual basis.

Parallel with the establishment of the JMPR, another development took place. This was the establishment of the Codex Alimentarius Commission in the early 1960s, when the government of Austria invited a number of experts from various European countries to a meeting in Vienna, to discuss the possibility of arriving at internationally acceptable standards for food.

The Codex Alimentarius Commission is responsible for establishing food standards . Its first meeting was held in Rome in 1963 and since then meetings have been held at regular intervals. The Commission comprises a great number of committees dealing with standards for individual food groups and for more general subjects related to food. The Netherlands has taken responsibility for the regular sessions of the Codex Committee on Pesticide Residues (CCPR). CCPR consists of delegates from about 40 member countries in their capacity as government representatives. The sessions are also attended by a number of observers from other international organisations and from the agro-chemical industry. The prime objective of the CCPR is to reach agreement on internationally acceptable maximum limits for pesticide residues in food and feed commodities in international trade. The MRL proposals in the CCPR sessions are those which have first been developed by the JMPR. In dealing with these proposals, the CCPR follows a step-wise procedure. Proposals for maximum residue limits which have reached the penultimate procedural step are regularly published by FAO and WHO in their series “Recommended international maximum limits for pesticide residues”.

1.8 Pesticide Residues in Food

Public concern over pesticide residues in food has been increasing during the past 15 - 20 years to the point where it has become a significant food safety concern. This leads on one hand to the establishment of legal directives to control their levels through the maximum residue levels (MRLs) and on the other to a continuous search for pesticides which are less persistent and less toxic for human beings. Thus this search has led to the extraordinary increase in the number of pesticides registered or recommended for use and the analytical difficulties encountered for their control. In Malaysia, the total number of registered pesticides for the periods 1993 -1995 and 1995 -1997 are 1810 and 1815 respectively (9). This situation has put pressure on regulatory agencies to increase the scope of analysis and number of samples analysed in their monitoring programs. Procedures were developed to detect as many pesticides as possible, reliably and rapidly in the most cost effective manner.

To monitor agricultural products that contain pesticide residue levels higher than the MRLs, each country has its own government agencies which can determine pesticide residues through two different but complementary approaches : (i) regulatory monitoring focused on raw agricultural commodities which measures the levels in individual lots for determining compliance with legal tolerances, and (ii) the total diet study. The methods of Storherr *et al* (10), Carson *et al* (11), Mills *et al* (12) and Luke *et al* (13-14) are used to determine organophosphorus pesticides (OPP) levels in the total diet study.

1.9 Analytical Procedures

Pesticides which are currently used on a worldwide scale include organophosphorus (OP), carbamates and the phenylurea herbicides. Since many of the organochlorine (OC) pesticides have been withdrawn because of evidence on their toxicity, persistence and bioaccumulation in the environment, the organophosphorus and carbamate compounds have become important groups replacing the organochlorine pesticides in many agricultural applications. Organophosphorus pesticides show persistence in soil for a maximum of 3 months, which is the lowest value for all the different groups of pesticides; about 8 months for phenoxy herbicides; 12 months for carbamates and more than 18 months for urea triazine herbicides.

The sequence of steps in a typical analysis are the extraction, clean-up and detection. Any analytical method used in residue monitoring programs should be such that it can detect the compound below the permissible level while providing methods that are selective, precise, and reproducible for qualitative and quantitative determinations. Although the present analytical capability is sufficient to meet these requirements, many residue isolation and purification techniques have not kept pace with advances in analytical capabilities and, therefore, they tend to limit the identification of pesticide residues that may be present in complex biological food matrices. Ideally, residue isolation should involve as few steps as possible which require minimal expendable materials, be easy and fast to perform, and result in extracts that have minimal interference when they are analysed. Official pesticide extraction techniques for complex matrices are tedious to perform and involve multiple steps, and can result in interference that can complicate the analysis. Sample throughput is greatly reduced in multistep procedures, and such methods cannot meet the present challenge of testing a wide variety of food samples for the many chemical residues that may be present.

There are numerous multiresidue methods (MRM) which test for a single class of compounds such as OP (15-18, 115,149), OC (19), and N-methyl carbamate (NMC)(20,21,71), but few can screen for these pesticides with a single sample extraction. The MRMs developed by Luke *et al.* (13,14) , Specht and Tilkes (22) and Steinwandter *et al.* (23) can be applied to foods of vegetable and animal origin. The MRM of Lee *et al.* (24) and Fillion *et al.* (127), were only applied on fruits and vegetables. These MRMs employ water miscible solvents such as acetone or acetonitrile, followed by the water removal step. This approach has the advantage of extracting a wide variety of pesticides but it also extracts a large amount of the polar matrix coextractives. Labour intensive steps, such as liquid-liquid partitioning (LLP), column chromatography, or salting out, are then required to remove the coextracted water.

MRMs are continuously being revised to reduce their disadvantages and to diminish the drawbacks (toxicity of solvent used and partition step). A volatile solvent system must be used because rapid evaporation of a large solvent volume would be required without loss of volatile compounds. The solvent system must also be sufficiently polar to extract the most polar pesticides and yet be immiscible with water. Leary *et al.*(25) reported the difficulties encountered in the development of an extraction procedure for polar compounds. Methamidophos is a very polar compound and in their preliminary work, Leary *et al.*(25) indicated the need for an extracting solvent which is at least as polar as ethyl acetate. Halogenated solvents such as dichloromethane were eliminated from consideration because of the hazards and disposal costs. Solvent mixtures with large percentages of water miscible solvent such as methanol, ethanol and acetone were not considered because of water miscibility. Toluene, propanol, larger alcohols, isooctane, and cyclohexane were eliminated because of insufficient volatility. Roos *et al.*(26) first reported the use of ethyl acetate and sodium sulphate in a multiresidue extraction procedure to eliminate the LLP step. Holstege *et al.*(86) modified the method by addition of acetone, methanol, or ethanol in the ethyl acetate to increase the polarity of the solvent system. It was found that the mixtures containing 5% methanol or ethanol in ethyl acetate gave the lowest matrix co-extractives (86).

The ethyl acetate methods are also called on-line extraction methods because they omit a separate LLP step. The theoretical principle of the on-line method is presented by the Gibbs triangle. Other solvents which can be used in these on-line methods are mixtures such as hexane-acetone mixture (8:2), and ethyl acetate-xylene (27,28). Previously, it was difficult to carry out microscale extractions with these conventional methods. However, the on-line methods can be miniaturised very easily, so that the solvent consumption is reduced to $\frac{1}{10}$ - $\frac{1}{100}$ of the original amount. This microtechnique has been validated by the analysis of OPPs in fruits and vegetables (29 - 32). To replace classical LLP, and to reduce analysis costs and pollution, an solid phase extraction (SPE) method has been developed. In this process, the compound is isolated from a liquid sample by differences in the relative solubility between a liquid mobile phase and a stationary phase.

Most MRMs include a clean-up step using an adsorption column, in particular Florisil, which is a mixture of aluminium oxide (alumina) and silica gel. Most adsorbent columns provide good clean-up only when they are eluted with solvent mixtures of low polarity, eluting less polar residues and leaving more polar co-extractives in the column. By increasing the polarity of the eluting solvent a greater portion of the interfering substances will be eluted and the less effective will be the clean-up. Florisil has gained the greatest attention of all the sorbents used in the pesticide residue analysis. As Florisil retains lipids (25g Florisil with 3% water retains 1g of fat), it is particularly well suited for the clean-up of fatty foods. When a Florisil column is eluted with solvent mixtures of low polarity, non-polar residues are recovered almost quantitatively. A major disadvantage is that its activity may vary from one batch to another. However, for the analysis of plant material, Forisil has been recommended by many authors (33,34,89,90) in the framework of multi-residue procedures for fruits and vegetables. Florisil can be replaced by alumina, particularly for the analysis of fatty foods. Basic alumina decomposes some organophosphates, and some more polar pesticides are not quantitatively eluted from neutral or acidic alumina columns. Alumina columns were used for clean-up by Ambrus *et al.*(35).

In general, silica gel is less efficient than alumina and does not adequately separate pesticides from plant co-extractives (36). Its importance in the analysis of plant pesticide residues in the fractionation of certain residues according to their polarity without appreciable losses. Many authors (34,37,38,89) have used silica gel columns or mini-columns for the clean-up of sample extracts. Lores *et al.* (39) used an improved silica gel clean-up method for organophosphorus pesticides. The method used employed a 3.5g silica column pre-loaded with 1% acetic acid. Recoveries ranging from 92% to 101% for the 11 organophosphorus compounds were reported.

Analytical instruments are needed to determine, quantify and confirm pesticide residues in vegetables for both research and regulatory purposes. Currently, the pesticides are generally analysed by spectrophotometry, thin layer chromatography (TLC), high performance liquid chromatography (HPLC) and gas chromatography (GC) or gas chromatography-mass spectrometry (GC-MS). Previous reports have noted that the spectrophotometric (40-42) and TLC (43-45) methods are inferior to the other methods in terms of detection sensitivity and specificity. HPLC (46-49) is a well known measurement technique that has good operability because it can be performed at room temperature. However, HPLC methods using retention time and fixed wavelength data via conventional detectors, such as ultraviolet absorption and fluorescence, might not be sufficiently specific and selective to perform certain identification of the target pesticides in complex matrices. UV absorbance has been the most commonly used detection in HPLC determination of NMCs pesticides (50-58), probably because of its wide applicability and consequent presence in HPLC systems. However, UV detection is subject to interference from sample co-extractives and also lacks sensitivity for some compounds which limit its usefulness for the analysis of environmental samples. Sparacino and Hines (59) carried out studies on the absorption maximum and extinction coefficients for 14 NMC pesticides and metabolites and found that with the exception of carbaryl (222 nm), methomyl (233 nm) and mobam (223 nm), the absorption maximum occurred at 202 nm or less. This is the region where plant co-extractives also commonly absorb strongly. In order to overcome these limitations some workers have employed derivatization of NMCs either before HPLC determination or on-line following chromatographic separation. Nelsen and Cook (60) improved the resolution of carbofuran from soil extracts which converted carbofuran to its phenolic moiety. The phenol was well resolved from co-extractives. Any phenol initially present as a metabolite was removed in the initial base wash. Lauren and Agnew (61) reacted the phenolic metabolites of carbofuran with FDNB to form 2,4-dinitrophenyl ether derivatives and achieved detection limits of 2-4 ng. Pietrogrande *et al.* (62) hydrolysed carbaryl and derivatized the resulting 1-naphthol with 4-aminoantipyrine. The derivative was determined by HPLC-UV at 460 nm, a wavelength at which co-extractive compounds are not likely to absorb. Fluorescence detection is not as widely employed as UV detection, since most Nmethylcarbamates (NMC) do not possess native fluorescence. However, for those which fluoresce, or can be made to fluoresce by derivatization, fluorescence detection offers a degree of selectivity and sensitivity which is often an order of magnitude or more prefer than that obtained by UV. Frei and co-workers (63) were the first to report the use of fluorescence detection for the determination of NMC pesticides. Fourteen NMCs were rendered fluorescent by derivatization with dansyl chloride prior to injection. Detection limits were between 1 ng to 10 ng. This approach was used to determine carbaryl (64,65) and carbofuran (66) residues in vegetables. A significant development occurred in 1977 when Moye *et al.* (67) introduced a post-column derivatization reaction for NMCs. Sodium hydroxide, introduced by a post-column reagent delivery pump, was used to hydrolise the NMC at 90°C and methylamine was released. This methylamine was subsequently reacted with a mixture of o-phthalaldehyde (OPA) and 2-mercaptoethanol, introduced by a second post-column pump, to form a highly fluorescent derivative identified as (1-hydroxyethylthio)-2-methylisoindole (68) The detection limit for methomyl was as low as 0.1 ng. In a series of studies, Krause refined the chromatographic and derivatization parameters (69), introduced a complex extraction and clean-up procedure for crop samples (70) and validated the method through collaborative studies (71,72). The method was rapidly adopted by a large number of workers for determination of various NMCs in a variety of substrates including water (73-75) and plant tissues (76-81).

Similarly, GC (58,82-88) is also an easy analytical method to use for compounds that are volatile and do not thermally decompose at high temperature. In recent years, the separation capability of this method has been improved remarkably by the use of the capillary column, and simultaneous measurement of many different types and classes of pesticides has become possible when equipped with selective and sensitive detection methods such as electron-capture detection (ECD)(89,90), nitrogen-phosphorus detection (NPD)(91,92), and flame-photometric detection (FPD)(93,94). In the past 30 years, the ECD has been the most used in pesticide residue analysis. It presents a very high sensitivity to polychlorinated hydrocarbons and other halogenated pesticides but its selectivity is rather poor because all kinds of electron attracting functional groups such as nitro groups and aromatic structures also give a response on this detector (95,96). However, ECD is normally used together with other element selective detectors in monitoring programs. Usually, the extracts obtained are injected into the various detectors mentioned above because their combined use makes better identification and detection of the pesticide residue possible. The most widely used combination of detectors is ECD, NPD and FPD (89,95,97,98). Other combinations employed are those formed by ECD, FPD and electrolytic conductivity detector (ELCD) (84,99,100), or ECD,NPD,FPD, ELCD and atomic emission detector (AED)(101). The detectors are sometimes connected in parallel, since it allows the results to be obtained with only one injection (84,97). Luke *et al.*(14) used GC-ECD to determine 77 pesticide residues simultaneously. NPD is an important detector used in pesticide residue analysis, because of Its selectivity for phosphorus and nitrogen containing compounds. The sensitivity of this detector is usually better for phosphorus than for nitrogen. OPPs, carbamates, triazines and their metabolites, and fungicides were determined by NPD from different fruits and vegetables (28,102-105). The FPD, in phosphorus mode, has frequently been the instrumental technique of choice for the analysis of OPPs (16,93,94,106-109). Interference in analytical determinations may occur when it is used when sulphur is present (27,93). A new detector introduced in 1989, the AED, is used for its selective detection of the elements such as fluorine, chlorine, bromine, iodine, phosphorus, sulphur and nitrogen. Its applicability was compared with other element selective detectors and showed higher selectivity in the determination of chlorine, fluorine and phosphorus containing pesticides than other detection methods for 12 agricultural products (101). However, problems arise when pesticides that are of low volatility (bipyridylium herbicides) or thermally unstable (benzoylurea or N-metylcarbamates) are to be analysed by GC (110). These compounds would normally require derivatization in order to continue with GC analysis or otherwise it needs careful attention, because the peaks which were detected in the GC traces, might not correspond to the compound itself but to a degradation product that is generally formed in the injection port. One such group is the carbamates, compounds are thermally unstable under conventional GC conditions. It has been reported that some of the compounds such as carbaryl and carbofuran determined by GC using cold column injection gave good recoveries (111,112). It is recommended that, if GC methods are still preferred, then the problems of decomposition of carbamates giving phenols and isocyanates should be overcome by prior derivatisation with appropriate reagents such as acetic anhydride. For phenylurea, Gregory and George (113) reported that the thermal instability of phenylurea herbicides such as diuron and monuron makes it difficult to analyse them under the normal GC conditions because of degradation to their respective isocyanates upon heating. To avoid these problems, derivatization with reagents such as heptafluorobutyric anhydride can be applied. In the analysis of highly polar compounds (114,115), derivatization reactions are required to convert the substances into less polar compounds. All these shortcomings require time consuming and complicated pre-treatment of the samples.

Gas chromatography - mass spectrometry (GC-MS) has been particularly important in environmental research and monitoring because it is a highly sensitive and specific technique suitable for confirmation or identification by full scan or single ion monitoring (SIM) of target pesticides in complex matrices (114,116-121). It has become the "staple" for identification and measurement of volatile and semivolatile compounds in air, foodstuffs, water, and wastes. The most widely used technique for pesticide residues analysis is GC-MSD with electron impact (EI) ionisation. Quantification is usually achieved by the technique of SIM. With this technique, the selectivity is also improved (93,94,96,97,122-126). Fillion *et al* (127) described a rapid and efficient multiresidue method for analysis of 189 pesticides in fruits and vegetables by GC-MS. However, it has the same problems encountered with GC analyses. It is obvious that the multiresidue methods using GC, HPLC and

GC-MS with specific detectors can only cover a limited range of pesticides (146-151) and therefore multiple injections of each sample into different instruments and dual column confirmation of positive analytes are required.

1.10 Liquid Chromatography - Mass Spectrometry (LC - MS)

Liquid chromatography coupled with mass spectrometry (LC-MS), a comparatively new analytical method has been developed to overcome the above problems of GC, HPLC and GC-MS. It would avoid the determination of false positives. Draisci *et al* (128) reported that confirmation methods for residues of veterinary drugs in foods should be quantitated and confirmed by mass spectrometry (MS), since it is sufficiently specific in itself to provide unambiguous identification and quantitation of a compound. This method is the technique of choice in confirmation analysis of thermally unstable, highly polar and non-volatile organic residues in food. Liquid chromatography (LC) coupled with MS has great potential for performing drug residue identification in complex biological matrices and has identified furazolidone (one of the nitrofurans) in swine serum and avian eggs through FIA-API-MS. Although the first coupling of LC and MS was reported about 20 years ago, its use for environmental monitoring is still in its infancy. This is so because of its complexity, lack of ruggedness, limited sensitivity and the technical difficulties in interfacing the solvent flow rate with the high vacuum of a mass spectrometer source. As a result, it limited the widespread use of LC-MS in method development during the early years. Subsequently the development of various types of ionisation interface such as moving belt (129,130), silicone membrane separator (131), direct liquid introduction (DLI)(132), thermospray (TSP)(133-135) and atmospheric pressure ionisation (API)(136-139) were utilised to introduce the effluent from the LC into the MS. Slobodnik *et al* (140) has published a review on the use of LC-MS technique for polar pesticides covering both the principles of ionisation and applications of LC-MS with TSP, particle beam (PB) and API interfaces.

1.10.1 Thermospray (TSP) Interface

TSP was originally developed by Vestal and co-workers (135) and it was an established LC-MS technique and widely used in the past. The TSP interface is schematically depicted in Fig. (2). The LC effluent entering the interface is heated and is passed through a small orifice to form an aerosol jet. The aerosol spray is directed perpendicular to the mass spectrometer entrance; the desolvation chamber and the mass spectrometer are separated by one or more skimmers and they are differentially pumped. Ions in the aerosol may be forced into the mass spectrometer by a repeller electrode, which is commonly placed opposite the skimmer. Some of the droplets in this spray are electrically charged because of the statistical distribution of ions in the micrometer-sized droplets. This results in the formation of ions by desorption ion evaporation. Ion evaporation is a solution phase ionisation technique applicable to most nonvolatile compounds. In ion evaporation, a volatile buffer typically 0.1 M ammonium acetate is added to the LC mobile phase which can cause static charging of the micrometer size droplets that form after thermal vaporisation. As the charged droplets desolvate, the field strength increases until sufficiently high surface fields (10^8 V cm^{-2}) are reached and evaporation of the ions from the droplets becomes as favourable as evaporation of the neutrals (desolvation). The extraction process allows as much as 2 ml min^{-1} of LC effluent to be introduced while maintaining an MS pressure of 10^{-5} torr.

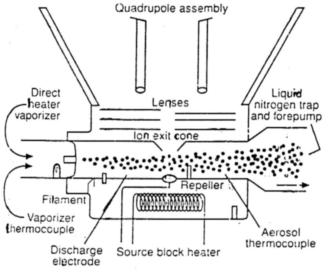

Fig. (2) - The schematic diagram of Thermospray Interface.

Typically, ionisation by evaporation which is a 'soft ionisation' process, produces ions with a minimum of internal energy and thus leads to a low abundance of ion fragmentation processes. The TSP mass spectra show abundant pseudo-molecular ion $[M+H]^+$ ions for samples of high proton affinity. Otherwise, $[M+NH_4]^+$ ions (NH_4^+ from ammonium acetate) are detected. In negative ion detection, $[M-H]^-$ or $[M+\text{buffer or solvent anion}]^-$ ions are observed. However, either mode of ionisation is soft and usually results in limited fragmentation.

The introduction of the TSP interface brought about a breakthrough in interfacing conventional-sized LC with MS. TSP mass spectra usually yield little structural information and they are, therefore, less useful for the identification of the unknown compounds. As the identification capability is often required in environmental analysis. TSP-MS is mainly used for target compound analysis.

1.10.2 Particle Beam (PB) interface

The introduction of the PB interface, originally known as the 'mono-disperse aerosol generating interface for chromatography'(141) enables the coupling of a wide range of LC separations to conventional electron ionisation (EI) and chemical ionisation (CI) MS procedures. A scheme of a PB interface is shown in Fig. (3).

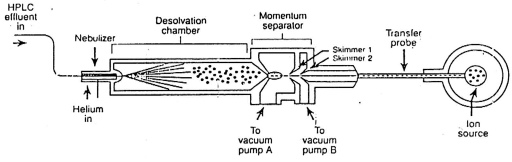

Fig. (3) - **The schematic diagram of Particle beam interface**

The EI spectra are more easily referenced to mass spectral data bases, which assist in identifying unknown components in the sample extracts. In addition, the fragmentation produced in EI offers valuable information for structure elucidation. CI spectra from the same interface offer the opportunity to obtain molecular weight information, which complements the structural information gained through EI analysis. The particle beam interface (142) involves four discrete steps: aerosol formation, desolvation, separation of solvent from analyte, and ionisation of the analyte for the acquisition of mass spectra. The initial step, aerosol formation, is accomplished by a co-axial helium nebulisation of the LC effluent. The resulting aerosol is desolvated in the heated desolvation chamber held near atmospheric pressure. Solute molecules in the desolvating droplets precipitate to form particles whose momentum enables them to transverse the two-stage momentum separator and transfer line to the MS source. The source's heat vaporises the particles into the gas phase for ionisation.

Particle beam LC-MS is nowadays mainly used for the identification of non-target compounds in real world matrices. The interface is commercially available under various names such as 'particle beam' (with concentric pneumatic nebuliser), 'thermabeam' (with thermally assisted pneumatic nebulisation), 'universal interface'(with thermally assisted pneumatic nebulisation and additional membrane separation). Of all LC-MS interfacing methods, LC-PB-MS comes closest to GC-MS. The flow rates that can be accommodated by the PB interface are generally compatible with conventional-size LC; they are between 0.1 and 1.0 ml min^{-1} (143). Two closely related drawbacks of LC-PB-MS are the low sensitivity and the non-linearity of the response. The low sensitivity is due to the low analyte transmission efficiency of the interface(144), typically between 0.5 and 1%. The non-linearity of the signal intensity in LC-PB-MS at low analyte concentrations was first reported by Bellar and co-workers (145) General acceptance of the PB interface in environmental analysis will require considerably improved detection limits; the present values, which typically are in the 10 - 500 ng range, are often not sufficient.

1.10.3 Atmospheric Pressure Ionisation(API)

API interface is currently the most widely used interface. In an API-MS system, the ion source region (located outside the mass spectrometer, at ambient pressure) is separated from the high vacuum mass analyser region by a small ion sampling orifice. This orifice must be large enough to permit the introduction of a significant proportion of the ions from the atmospheric pressure region into the high vacuum region. The LC column effluent is sprayed in the vicinity of the orifice, so that free jet expansion and concomitant adiabatic cooling can occur. The formation of a spray is effected by applying heat, a coaxial nebuliser gas stream, an electrostatic potential, with ultrasonic vibration of the capillary, or a combination of these. The free jet cooling contributes to the formation of large clusters of analyte and solvent molecules, which are bound by Van der Waals forces. Desolvation of the clusters is effected by collisions; the collision rate is enhanced either by leading the ions through a gas curtain or by accelerating them through an electric field gradient.

The development of LC-API-MS system offers new opportunities for the determination of thermolabile and polar compounds. API is a term covering different principles of ionisation (152-157) such as electrospray (ESP), IonSpray (ISP) and the heated nebuliser.

1.10.3.1 Electrospray (ESP)

ESP is the most recent LC-MS technique to win wide recognition. ESP is the softest ionisation technique available for LC-MS and has permitted, for the first time, large labile molecules to be studied intact. Proteins having molecular weights up to 150,000 Da have been successfully analysed by ESP. Fig. (4) shows the schematic diagram of the ESP interface.

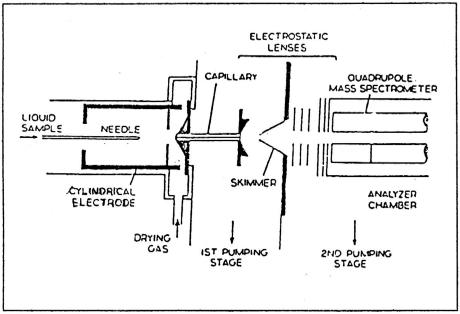

Fig (4) - The schematic diagram of electrospray interface

In ESP, sample molecules are simultaneously nebulised and ionised at atmospheric pressure. The sample solution is passed through a steel capillary tube situated a few millimeters from a hollow counter electrode. A potential difference of several thousand volts is maintained between the capillary and the counter electrode so that solvent emerging from the capillary forms an electrostatic spray towards the counter electrode. Gas phase ions formed by ion evaporation at atmospheric pressure are then sampled through a two stage momentum separator into the high vacuum of the mass analyser.

A raw ESP mass spectrum often contains a series of peaks representing ions of different charge states. Raw data of this type are usually processed to yield a single mass peak indicative of the true molecular weight. The major drawback of LC-ESP-MS is that the maximum allowable flow rate is in the order of 10 μL min^{-1}, with lower flow rates giving better performance. Regarding the compatibility with conventional size LC, the ESP interface is often quoted to be a 'concentration-sensitive' device (158). This would imply that splitting of the LC effluent flow will not cause a loss of sensitivity.

1.10.3.2 IonSpray (ISP)

The ISP interface was originally introduced to enhance the ion evaporation of the ESP. The main advantage of the ISP interface, developed by Bruins *et al.*(159), over the ESP interface is the tolerance of higher flow rates. In the ISP interface, the electrospraying process is assisted by coaxial pneumatic nebulisation of the LC column effluent. ISP has been coupled successfully to a benchtop-size mass spectrometer (160). Flow rates of 40-50 μLmin^{-1}, which are compatible with 1mm I.D. LC column, can be accommodated. ISP shows improved performance over

TSP with thermolabile ionic compounds, because ISP operates at room temperature, while heat has to be applied with TSP.

1.10.3.3 Heated Nebuliser

The heated nebuliser is the most common interface currently used on an API source system. It takes full advantage of the large gas and solvent vapour throughput tolerated by the API source and provides a wide range of reversed phase solvent compositions at flow rates between 0.2 - 2.0 ml/min. Pneumatic nebulisation of the total HPLC effluent into the atmospheric pressure ionisation interface eliminates the vacuum system problems generally associated with LC-MS interfacing. This interface uses a nebuliser and make-up gas flow (N_2) to ionise the HPLC effluent . The spray travels through a large diameter quartz tube which is heated sufficiently to dry the vapour. Ionisation of solvent molecules is initiated by a corona discharge needle. The solvent ions which are formed produce the analyte ions by atmospheric pressure chemical ionisation (APCI) of the analyte. These ions are focused and declustered through a dry nitrogen curtain gas and are then passed through the orifice into the high vacuum analyser region of the mass spectrometer where they are mass analysed. Fig.(5) shows the basic components of the heated nebuliser LC-MS interface and the atmospheric pressure chemical ionisation(APCI) source (140).

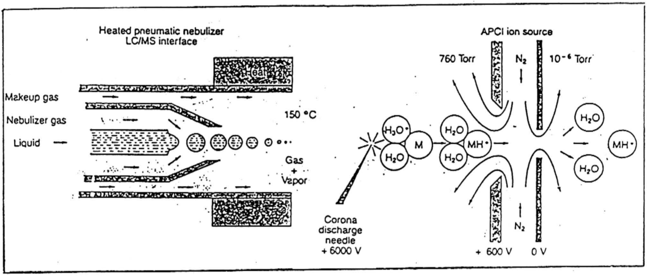

Fig. (5) - The basic components of the heated nebuliser LC-MS interface and the atmospheric pressure chemical ionisation (APCI) source.

The APCI process is a soft ionisation method typically generating only protonated molecular ions $(M+H)^+$ in PCI mode or deprotonated molecular ions $(M-H)^-$ in NCI mode, thus producing the molecular mass information which is considered the most important criterion for identification of the analyte. Huang *et al* (152) reported that lower detection limits are obtainable via selected ion monitoring (SIM) of only the protonated molecular ion of each compound which can provide low picogram detection of these compounds.

Generally, all API techniques provide soft ionisation in which little structural information is directly obtained. However, if structural information is desired for identification purposes, the problem can be overcome by the application of an appropriate voltage difference on two regions - the sample cone and the first skimmer of the API source generally varies the frequency and energy of collision of the analyte and drying gas molecules and thus induces fragmentation of the primarily formed ions, this mode of operation is termed pre-analyser collision induced dissociation (CID). A typical example of enhancement of structural information by changing the pre-analyser CID voltage is shown in Fig.(6). Giraud *et al* (161) reported that the selectivity of a single quadrupole MS can be considerably increased by monitoring two ions - the precursor ion derived from the intact molecule at low orifice voltage, the protonated molecular ion $(M+H)^+$ and its major fragment ion produced at high orifice voltage, thus allowing for the unambiguous pesticide identification and confirmation. Combination of retention time data, molecular mass information from the $[M+H]^+$ or $[M-H]^-$ ion and other characteristic fragment ions provide satisfactory information for the identification of target analyte without a large risk of false positive (140).

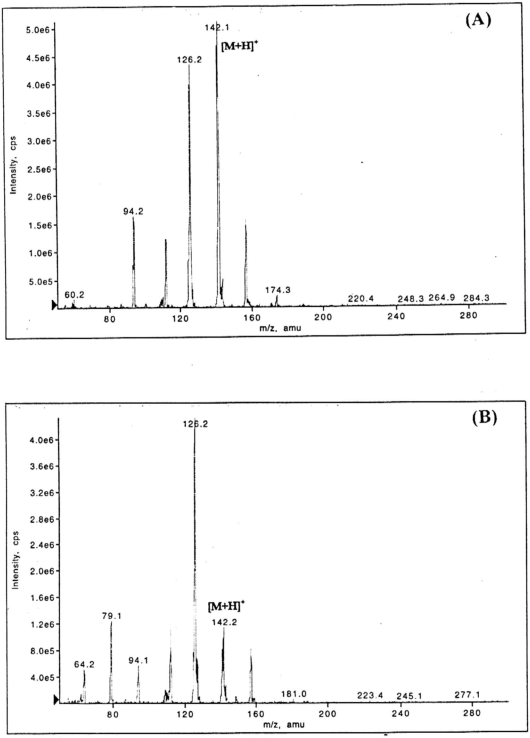

Fig.(6) -The mass spectra of methamidophos at low orifice V = 20 volts (A) and high orifice V = 60 volts (B)

Very few studies on the application of LC-MS with a heated nebuliser for pesticide analyses have been published. The potential of this technique for the analysis of some pesticides has been demonstrated (153) and its application for the quantitative determination of pesticides has been described by some workers (155,162,163). Pleasance *et al* (162) used it for the determination of residues of carbamates in green peppers, Kawasaki *et al* applied this technique for the determination of organophosphorus pesticides (163) and carbamates (155) in human serum. Silvia and Damia (164) used it for trace determination of organophosphorus pesticides in water samples. Doerge and Bajic (165) have determined several groups of pesticides in ground water using LC-API-MS. And Blanchflower *et al.*(166) applied it for the determination of 5 thyreostats 'anti-hormone compounds which increase the mass of the animals by enhancing the water retention in tissues' in urine and thyroid tissue.

1.11 Flow Injection Analysis (FIA) in Pesticide Formulations

In the commercial development of pesticides, one of the first steps that a manufacturer employs is to chemically produce the compound in a form which is called the "technical grade material". This technical grade material or toxicant may be sold in solid form such as crystals, powder, or in liquid or gas forms. Since it is not normally used in these forms for pest control, the technical grade material must be formulated. Formulation involves processing the technical grade material by any method that will provide its effectiveness, storage, handling, safety and ease of application.

The formulation process is usually accomplished by grinding the material to a powder or dissolving it in a petroleum solvent. The toxicant may then be diluted with other substances to make the desired formulation and is then known as the active ingredient, which is often abbreviated as "AI". The other substances which are added to the formulation are called adjuvants. An adjuvant is a substance which enhances the physical properties of an active ingredient, but by itself may not have any killing properties. Some examples are xylene, talc, flur, and bran.

The publication of Collaborative International Pesticide Analytical Council (CIPAC) Handbooks (167) provided producers and users of up-to-date methods in the study of pesticide formulations. All CIPAC methods which are used to analyse the active ingredient of the pesticides are based on HPLC-UV, GC, infrared and titrimetric methods. As the importance of economic and safety management increases in laboratories, the approach to pesticide formulation analysis has begun to change. Procedures were developed to determine pesticide formulations reliably and rapidly in the most cost effective manner. Bushway and co-workers(168) used an LC method to analyse simultaneously separate formulations of diazinon and chlorpyrifos. The combined analyses of different formulations that have different active ingredients can lead to cost reduction and increased safety without sacrificing the analytical integrity. The economic savings would come in the form of fewer columns, less solvent, and more efficient use of time. Safety would be enhanced because less laboratory waste would be generated. Furthermore, a decrease in waste would save money because a proper waste disposal is expensive.

Although flow injection analysis (FIA) has long been applied in many fields of natural sciences, most of the major detector systems used in conventional FIA are based on optical absorption or emission and electrochemical techniques (169-175). There are also many previous reports (176-178) regarding the applications of FIA-MS in pesticide analysis and to determine and characterise the pollutants from a complex matrix and the method has been recognised as an analytical method that has an excellent specificity and sensitivity.

The development of FIA-API-MS with a heated nebuliser offers new opportunities for the determination of non-volatile, thermolabile and polar compounds such as methamidophos, carbaryl and carbofuran. It can be developed into a rapid and accurate single residue method (SRM) for pesticide formulations. Both the pseudo-molecular ion of the pesticides are monitored under PCI/NCI using SIM conditions for identification and quantification(183).

1.12 OBJECTIVE

The objectives of this study is to develop rapid and accurate methods: (i) multiclass and multiresidue determination on different structural pesticides such as organohalogen, organophosphorus, N-methylcarbamate and phenylurea simultaneously in raw agricultural produce such as vegetables using liquid chromatography coupled with atmospheric pressure ionisation - mass spectrometry (LC-API-MS) and a heated nebuliser, and (ii) the FIA technique for the analysis of the active ingredients in some pesticide formulations. A simple and effective procedure for sample extraction adopted from that of the Queensland Health Scientific Services, Australia with some modifications (179) was used in this study.