18

In-Vivo and In-Vitro Methods for Evaluation of Pesticides on DNA Structure

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1. Introduction

It is well known that the growth of the population and convenience of human's life depends on industry and agriculture. In this context, the pesticides have contributed greatly to the increase of yields in agriculture by controlling pests and diseases. The pesticides currently in use include a wide variety of compounds belonging to different chemical classes. More than 800 chemicals marketed as multiple formulations are used in the European Union as insecticides, herbicides, fungicides. There is a large range of positive outcomes or benefits from the pesticides. Reduced crop loss resulting from spraying fungicides is an obvious benefit. The benefits of pesticides can be classified in three stages such as: immediate effects, primary, and secondary benefits. The outcomes of pesticides use is immediate, and the main effects of pesticides can be divided as follows: (1) controlling agricultural pests (including diseases and weeds) and vectors of plant disease; (2) controlling human and livestock disease vectors and nuisance organisms; (3) preventing or controlling organisms that harm other human activities and structures.

The secondary benefits are the consequences of the pesticides, effects. From the three main effects listed above, 26 primary benefits have been identified ranging from protection of recreational turf to saved human lives. The secondary benefits are less immediate, less intuitively obvious or longer term consequences. It follows that for secondary benefits, it is more difficult to establish causes and effects nevertheless, and they can be powerful justifications for pesticide use. These classifications are summarized in Fig. 1.

However, the effectiveness of pest management is the wide application of pesticides throughout the world. Unfortunately, the result of these widespread uses is the contamination of soils, surface and specially ground waters. In fact, due to the macropore structure of soils and rapid and not uniform leaching via preferential flow paths, a fraction of the pesticides percolates into ground water before it can degrade or be adsorbed by the soil. Although this leaching through the vadose zone to ground water is a complex process and controlled by a variety of processes such as soil water flow, solute transport, heat transport, pesticide sorption, transformation and degradation, volatilization, crop uptake, and surface run off (Fig. 2), the result is the wide contamination of drinking waters and food chain (Abhilash, & Singh,2009).

The Food and Agricultural Organization (FAO, 1988) has been concerned about various reports of ill health arises of pesticides. The World Health Organization had estimated that

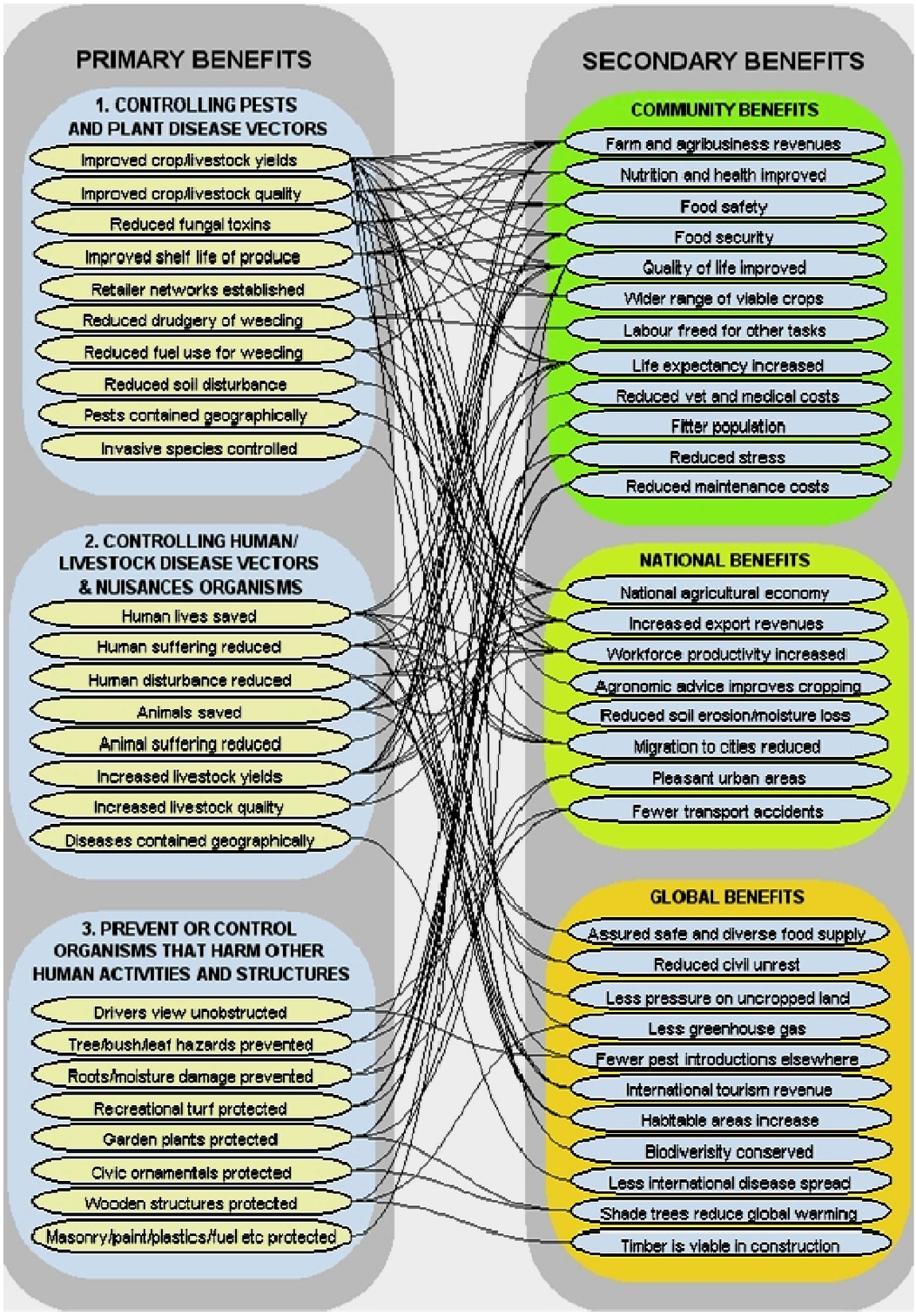


Fig. 1. The benefits of pesticides that classified in three stages such as: immediate effects, primary, and secondary. The linkages are not easy to follow, but serve to illustrate the complexity of the interactions between them. With permission (Cooper,. & Dobson, 2007).

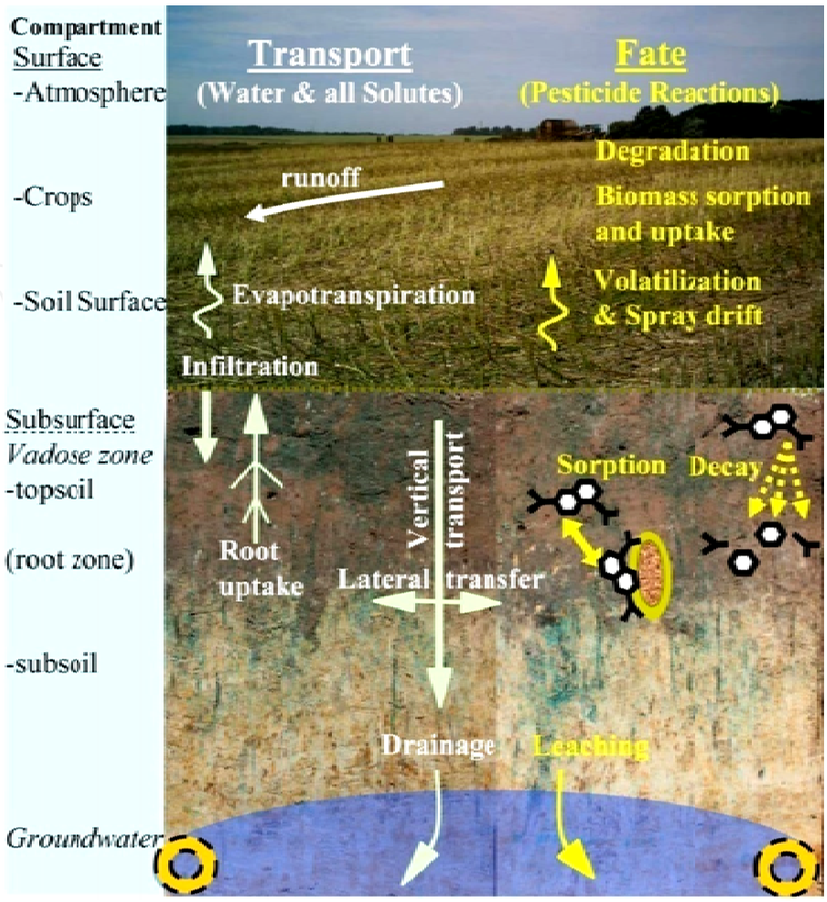


Fig. 2. Principal processes governing pesticide transport and fate in agricultural structured soil systems. With permission (Köhne, Köhne, & Simůnek, 2009).

several millions people were being poisoned via pesticides (WHO, 1986). Pesticides have been considered potential chemical mutagens. Experimental data revealed that various agrochemical ingredients possess mutagenic properties inducing gene mutation and chromosomal alteration or DNA damage (Bolognesi, & Morasso, 2000). Increasing incidence of cancer, chronic kidney diseases, suppression of the immune system, sterility among males and females, endocrine disorders, neurological and behavioral disorders, especially among children, have been attributed to chronic pesticide poisoning. Human health hazards vary with the extent of exposure. Moderate human health hazards from the exposure to pesticides include mild headaches, flu, skin rashes, blurred vision and other neurological disorders while rare, but severe human health hazards include paralysis, blindness and even death. Pesticide pollution to the local environment also affects the lives of birds, wildlife, domestic animals, fish and livestock. Although it is documented that, the pesticides are toxic for living organisms with variable specificity for targeted species, the chronic toxicity in humans such as neurotoxicity, endocrine disruption, immunotoxicity or carcinogenicity, are still to be explored (Hodgson, & Levi, 1996; Carpy, Kobel, & Doe, 2001). The risk for cancer is frequently discussed since some of these compounds have demonstrated carcinogenic potential in-vivo, more particularly in rodents (Cueto Jr., 1980). In some epidemiological studies conducted on farmers, it is thought that occupational

exposure to pesticides contributes to an increased incidence of various cancers at specific sites. These concern especially tumors of the lip, skin, prostate or brain, Hodgkin disease and non-Hodgkin lymphoma (NHL) (Blair, & Zahm, 1991; Georgellis, and et al., 1999). Several chemical classes of pesticides, like organochlorine insecticides (Leary and et al., 2004) or phenoxyacetic acid herbicides have been more specifically incriminated (Zahm, & Blair, 1992). Cancer is the third leading cause of death in adults and is the second cause in the children, with heart failure being the most frequent one. Leukemia is the group of childhood malignancies with by far the highest incidence, representing close to a third of all cases; childhood brain tumors (CBT) and lymphomas are the second and third most frequent groups (Rull, 2009). Knowledge about the causes of childhood cancer is scant, with ionizing radiation and some therapeutic drugs being the only well-established causal factors. Several other factors, however, have been suggested, comprising natural factors such as infections, and man-made factors such as electromagnetic fields, traffic exhaust, and pesticides. The existing epidemiological studies on associations between childhood cancer and either parental or child exposure to pesticides have been recently reviewed (Daniels,

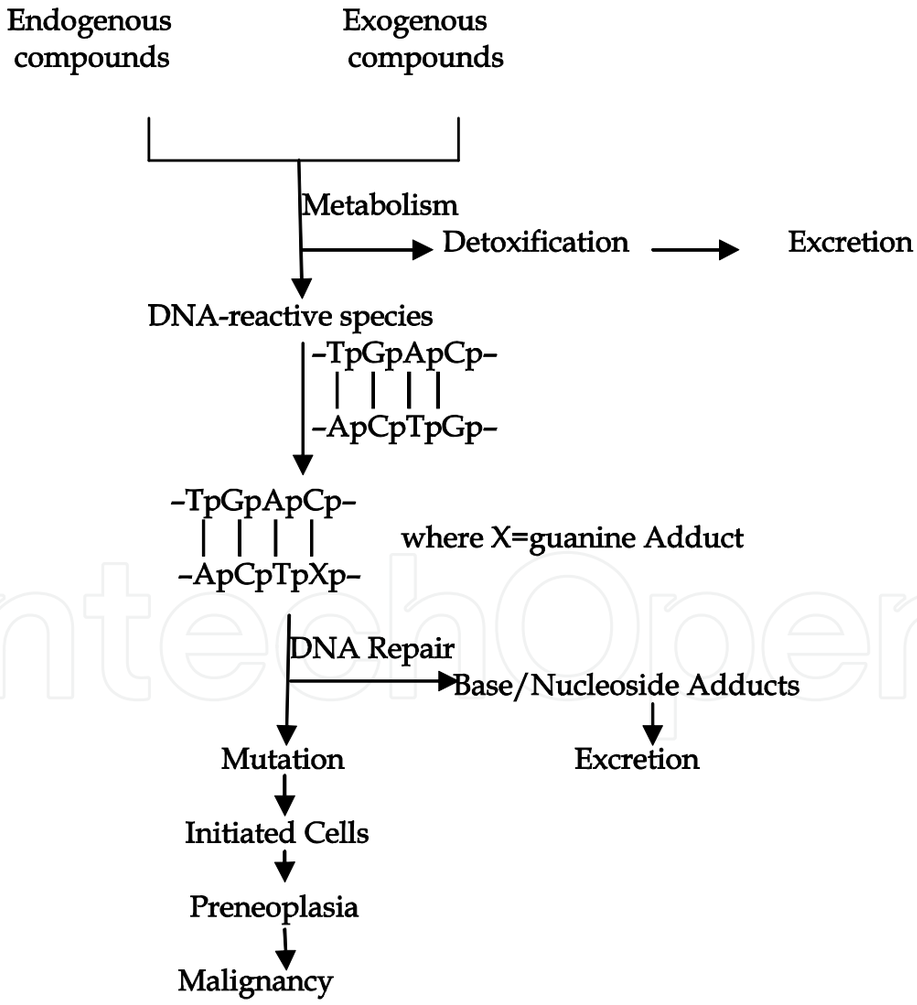


Fig. 3. The role of DNA adducts in mutation and cancer.

1997; Zahm, & Ward, 1998; Nasterlack, 2006; Nasterlack, 2007). Nonetheless, there are a variety of mechanisms by which pesticides may potentially initiate or promote cancer, including mutagenicity, tumor promotion immunotoxicity and hormonal disruption. It is completely clear that when DNA interacts with carcinogenic and/or mutagenic compounds it can play a key role in the advent of cancers, unusual proliferation and metastasis of malignant cells. For example aromatic amines, nitro aromatic amines, nitrosamines, hydrazine, aflatoxins, pesticides, and halogenated hydrocarbons can be activated by P-450 to form compounds capable of covalent binding to DNA. Alkylation of DNA is believed to be the first step in the initiation of chemically induced carcinogenesis and cancer (Prakash and et al., 1998). DNA adducts, if not repaired before the onset of DNA replication or misreported, are capable of inducing gene mutations and putatively initiate the conversion of exposed natural cells to irreversibly altered preneoplastic states. Figure 3 depicts sequence of the main events that are considered crucial in chemically induced carcinogenesis.

However, to discover the mutagenic and carcinogenic effects of pesticides, the development of accurate, reliable and rapid methods is of interest for evaluating effects of these compounds and analyzing their mechanisms of action especially on DNA.

2. DNA conformations and modes of interaction

DNA is found in cells, and usually is as a right-handed double helix. The two chains (strands) of the double helix have complementary sequences of nucleic bases. The structure and numbering of nucleic bases are shown in Fig. 4.

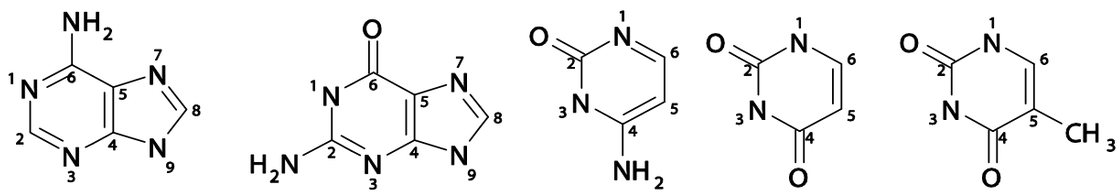


Fig. 4. Structure and numbering of the bases adenine, guanine (purines) and cytosine, uracil, thymine (pyrimidines). Hydrogen's are not shown.

The double helix of DNA is of great length and short diameter. The minor groove is narrow and shallow, only about 10 Å in width. The major groove is deeper and wider, approximately 24 Å in width. The grooves form because the patterns of hydrogen bonding between complementary bases of DNA cause sugar groups to stick out at 120˚ angles from each other instead of 180˚. It has been known from early X-ray diffraction studies on fibers that DNA can exist in more than one conformation depending on the fiber salt concentration, the degree of hydration, the metal ion, etc. It was found from X-ray or spectroscopic data that in A-DNA and also RNA the sugar pucker exists in C3'-*endo* conformation, while in B-DNA the conformation is converted to C2'-*endo* (Fig. 5).

It is known that in solutions there is an equilibrium between the C2'-*endo* and C3'-*endo* pucker in the deoxyribose. Since nucleic acids are polyanions, they require counter ions in order to neutralize the negatively charged phosphate groups. DNA can be interacting with other compounds through a variety of modes and each may be exploited to stabilize the bound ligand at a DNA site. For example the metal complexes are known to bind to DNA

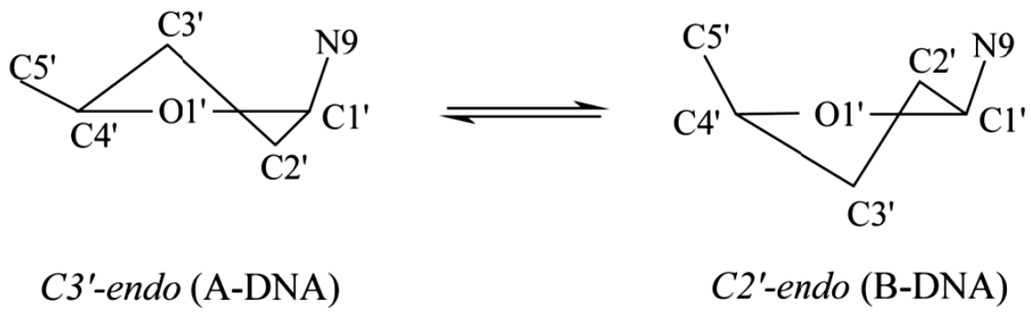


Fig. 5. The numbering of the pentose ring and C2'-*endo*, C3'-*endo* sugar puckering in DNA.

through a series of interactions, such as Z-stacking interaction associated with intercalation of aromatic heterocyclic groups between the base pairs. Intercalators are small molecules that contain a planar aromatic heterocyclic functionality which can insert and stack between the base pairs of double helical DNA, while the groove binders bind DNA within either grooves of the double helix. In general, the binding of an intercalator to DNA is driven entirely by a large favorable enthalpy reduction but with an unfavorable entropy decrease, and the binding of a groove binder to DNA is driven by a large favorable increase in entropy. Once a pesticide binds DNA within either grooves of the double helix, a variety of non-covalent molecular interactions, such as hydrophobic interactions between the bound pesticide and either groove floor/walls, specific hydrogen bonds and van der Waals contacts between the pesticide and DNA, may occur, and the apparent enthalpy increase should be the summation of all of these molecular interactions. In principle, there are six modes for reversible binding of molecules with double-helical DNA: (i) electrostatic attractions with the anionic sugar-phosphate backbone of DNA, (ii) interactions with the DNA major groove, (iii) interactions with the DNA minor groove, (iv) intercalation between base pairs via the DNA major groove, (v) intercalation between base pairs via the DNA minor groove, and (vi) a threading intercalation mode. Depending on structural features of both the molecule and DNA, many molecules show more than a single interaction mode with DNA. Notwithstanding, the strong interactions have binding modes which fall into two categories: intercalation and specific hydrogen-bonding interactions in the DNA grooves. The development of high throughput screening techniques for study of molecular interactions and biological activities of pesticides with DNA has been an increasingly popular area of research. Two most approaches to study pesticides/DNA interactions are widely used *in-vivo* and *in-vitro* strategies. The following sections we will review and introduce the basic procedures that are especially used for in-vivo study of DNA interactions such as: ^{32}P postlabeling analysis of DNA-adducts, alkaline single cell gel electrophoresis (Comet assay) and alkaline elution (or unwinding) techniques, and techniques that are used for in-vitro study of DNA interactions such as: spectroscopy of Uv/Vis, quenching fluorescence, competitive fluorescence and circular dichroism(CD); electrochemistry containing the linear sweep voltammetry (LSV), cyclic voltammetry (CV) and differential pulse voltammetry (DPV).

3. In-vivo methods for studying DNA-pesticides interactions

Some pesticides are genotoxic/carcinogenic or are converted into genotoxicin during metabolism. Thus DNA damage has the potential as a biomarker and can act as endogenous

or exogenous compounds. The actual damage and alterations to DNA when interact with pesticides occur in two basic forms, the detection of which employs specific techniques:

1. Pesticides bind covalently to DNA to form "DNA adducts". The DNA adducts are usually detected by the non-specific ^{32}P postlabeling techniques.
2. Strand breaks can be measured by two methods, the alkaline single cell gel electrophoresis (Comet assay) and alkaline elution (or unwinding).

3.1 ^{32}P postlabeling analysis of DNA-adducts

The sites susceptible to adduct formation include nucleic acid bases, as well as the sugar-phosphate backbone. For example, there are 17 potential sites for reaction in DNA treated with N-nitroso alkylating agents, and of these sites the phosphate and ribose moieties each has one nucleophilic site. The exocyclic oxygen and amino groups of nuclei acid bases, N1, N3, and N7 positions of purines, and N3 position of pyrimidines are frequently involved in adduct formation. The N3 and N7 positions of guanine are most susceptible to electrophilic attack, compared with the exocyclic oxygen. The DNA adducts, if not repaired, have the

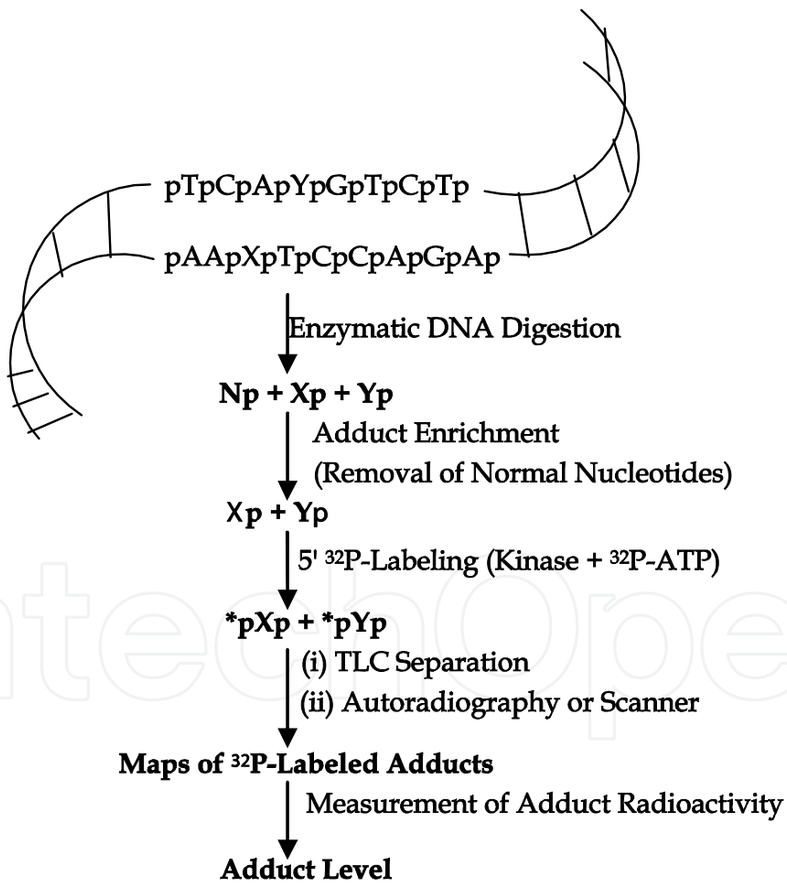


Fig. 6. The ^{32}P-postlabeling assay for DNA adducts detection. The assay involves six steps: digestion of DNA to 3'-mononucleotides of normal (Np) and adducted (Xp, Yp) deoxyribonucleotides; enrichment of adducts; ^{32}P-labeling of adducts; separation, detection, and quantization of adducts. Asterisks indicate the position of the ^{32}P label (Phillips, & Arlt, 2007).

potential to interfere with normal DNA replication, which leads to base substitution/frame shift mutations and/or cell killing. DNA-adducts in the cells are effective molecular dosimeters of genotoxic contaminant exposure, and the ^{32}P-postlabeling assay has been used to measure covalent DNA-pesticides adducts. Figure 6 gives an outline of various steps involved in the analysis of DNA adducts by the ^{32}P-postlabeling assay.

In the ^{32}P-postlabeling method, DNA is enzynmatically digested to 3´-monophosphates of normal deoxynucleosides (Np) and adducts (Xp, Yp). Adducted nucleosides are enriched by extracting them with butanol or converting Np to N by 3´-dephosphorylation with nuclease P1 treatment. These two adduct enrichment procedures are applicable to the enrichment of bulky or aromatic DNA adducts, but not to the most simple alkylated adducts like methyl- and ethyl-substituted products. Simple alkylation of purines at the N7 position creates a positive charge and such adducts can be enriched as a group or class by an anion-exchange column chromatography. The adducted nucleotides are subsequently labeled enzymatically with polynucleotide kinase and [γ-^{32}P]ATP at the 5´-end (Fig. 7), separated by thin-layer chromatography (TLC), detected by exposure to X-ray films or scanning by electronic autoradiography, and quantified by measuring the ^{32}P incorporation.

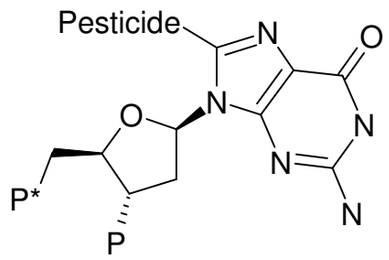


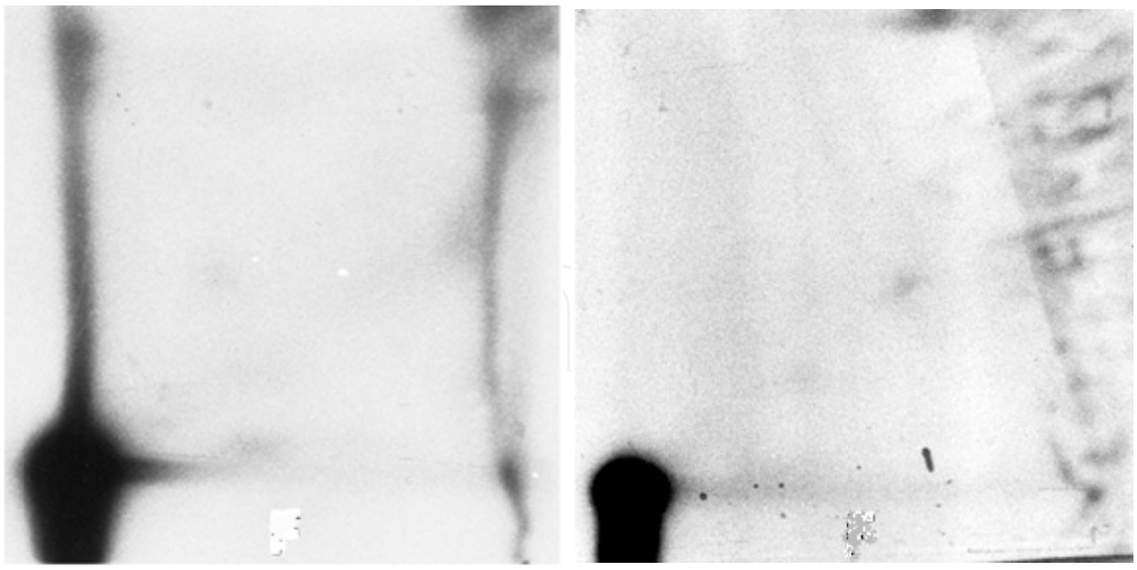
Fig. 7. Structures of the ^{32}P-labeled DNA adducts formed by interaction with pesticide.

As it was said, two procedures are used for adduct enrichment: butanol extraction and nuclease P1. The butanol method allows the extraction of aromatic adducts into 1-butanol in the presence of a phase-transfer agent (tetrabutylammonium chloride). The nuclease P1 method is based on the finding that certain adducted nucleotides are resistant to 3'-dephosphorylation and remain as substrates for kinase, while normal nucleotides are hydrolyzed to nucleosides which are not substrates for kinase due to the lack of a phosphate at the 3'-end. Both methods have been shown to be suitable for the enrichment of most aromatic adducts derived from chemicals containing one or more aromatic rings, such as polycyclic aromatic hydrocarbons, aromatic amines, and pesticides. Some aromatic amine adducts, however, are sensitive to nuclease P1 activity, resulting in their poor recovery and weak detection. On the other hand, some adducts are not extractable by butanol, but they are resistant to nuclease P1-catalyzed 3'-dephosphorylation. Because of the difference in adduct recovery by the two methods, it is recommended that both methods be used for the enrichment of unknown adducts. In addition to these two procedures, an anion-exchange chromatography method should be considered for the purification of adducts containing a positive charge, such as N7-alkylpurines, N1 adenine, and N3 cytosine. The purification on an anion-exchange column is based on the positive charge on the adducts, because these are less retained and readily eluted in a low salt buffer, while unmodified nucleotides are strongly retained on the column under these conditions. For separation of ^{32}P adducts the thin layer chromatography (PEI-(polyethyleneimine) cellulose) is a suitable method. Based on the polarity of adducts, different TLC techniques such as two dimentional and

multidirectional TLC procedure (MD-TLC) are used. The ^{32}P postlabeling method has an advantage over the others in that prior structural knowledge is not required for adduct detection. Furthermore, this technique could be used for detecting adducts formed by chemicals of diverse structures, such as alkylating agents, polycyclic aromatic hydrocarbons, aromatic amines, and pesticides. The ^{32}P-postlabeling assay is very sensitive and can detect a single adduct in 10^9–10^{10} nucleotides from 1 to 10 μg of DNA. Potential pitfall of the postlabeling technique includes the lack of structural identification of the adduct. The assay may give false-negative results due to either adduct loss or lower sensitivity for some adducts. The loss of adducts could occur because of the instability of adducts or the inefficiency of certain steps in the assay. In addition, some small adducts are difficult to analytically resolve from unmodified nucleotides, making the assay less sensitive for the detection of these adducts than bulky aromatic adducts. A false-positive response could be due to the phosphorylation of nonnucleic acid components, such as the metabolites containing hydroxyl groups, if they are present as contaminants in DNA preparations at high levels. It is, therefore, important to purify the DNA well, particularly that from *in vitro* exposures, where the test article is generally investigated at higher concentrations than in *in vivo* studies. It appears that a standard DNA isolation procedure using solvent extraction methods is adequate to purify DNA from contaminants. Another concern about the ^{32}P-postlabeling assay is the detection of I-compounds or endogenous adducts on the TLC plates. These adducts, if present, can contribute to background spots and interfere with the detection of exposure-related adducts. Since I-compounds increase with aging, it is recommended that younger animals be used to reduce background activity to the extent it is permissible in the experimental design. Also, different TLC solvents or different versions of the ^{32}P-postlabeling assay may be explored to resolve adducts of interest from I-compounds.

3.1.1 Sample preparation

For in-vivo study of Pesticides-DNA adduct formation by ^{32}P-postlabeling assay, the DNA is commonly isolate from the rat tissues and liver rats, calf thymus, and the tissues of fish or plants. In general, the tissues are homogenized by a solvent and the cells are lyses. After that, the nuclei are isolated by centrifugation. The DNA is extracted with a chloroform/isoamyl alcohol mixture, and then treated with RNAses A and T1 before final extraction by chloroform/isoamyl alcohol mixture. The isolated DNA is digested to mononucleotides with micrococcal nuclease and spleen phosphodiesterase. The adducted nucleotides of digested sample are concentrated by either 1-butanol extraction or nuclease P1. Adducted nucleotides are preferentially extracted over the normal bases into the organic phase during the butanol extraction, while nuclease P1 is able to cleave the 3'phosphate group from the normal bases due to the resistant bulk of adducted bases. The pervious studies conducted on Chlordane (CLD) as an organochlorin pesticide demonstrated that the CLD increases liver tumor incidences in rodents and is a tumor promoter. However, Whysner and coworker by using the ^{32}P postlabeling assay on male and female $B6C3F_1$ mice, concluded that, the mode of action for tumorigenesis by CLD is not based on adduct formation (Whysner and et al., 1998). Results showing typical TLC separations for liver DNA of mice exposed to CLD for 2 weeks are illustrated in Fig. 8a and 8b. As it is observed no spots were identified by either the 1-butanol extraction or nuclease P1 enrichment methods. Furthermore, the pathology findings demonstrated no association between liver cancer and CLD exposure (International Agency for Research on Cancer, 1991).



(a) (b)

Fig. 8. ^{32}P-Postlabeling results using **(a)** 1-butanol extraction **(b)** nuclease P1 showing typical TLC plates of $B6C3F_1$ mouse liver DNA for CLD-DNA adduct formation after 2-week.

Shah and coworkers used the ^{32}P postlabeling assay in order to study adduct formation between the Guthion (azinphos methyl), Sencor (metribuzin), Lorox (linuron), Reglone (diquat), Daconil (chlorothalonil) and Admire (imidacloprid) pesticides with calf thymus DNA (Shah and et al., 1997). The pesticides were reacted with calf thymus DNA. In addition the metabolites of the pesticides were obtained enzymatically using arochlor induced rat liver S9 fraction, in an NADPH generating system. The resulting metabolites were reacted with calf thymus DNA and the DNA was analyzed for the presence of adducts by either the nuclease P1 or butanol enrichment. Nuclease P1 enrichment resulted in adducts for all the pesticides. Compared to the level of adducts in control DNA, the levels in pesticide-treated DNA were higher for all the pesticides, except Daconil. The increase in adduct numbers for pesticide-treated DNAs ranged from 4.9–12.4 times the control-DNA indicating pesticide genotoxicity in this *in vitro* system. Enrichment using butanol extraction gave three adducts unique to Sencor-DNA. These adducts were different from those obtained with nuclease P1 enrichment of the same. B(a)P was the positive control for the *in vitro* metabolism, and two adduct enrichment procedures were nuclease P1 digestion and butanol extraction (Fig. 9).

3.2 Alkaline single cell gel electrophoresis (comet assay)

One of the other procedures that enable us to assess the DNA damage is the alkaline single cell gel electrophoresis or comet assay. In general, a small number of cells suspended in a thin agarose gel on a microscope slide are lyses, electrophoreses and stained with a fluorescent DNA finding dye. The technique is based on the fact that broken DNA migrates more easily in an electric field than intact molecules. When the slide is visualized with a fluorescence microscope, the observed objects resemble comets with a head region containing undamaged DNA and a tail containing the broken DNA (Fig. 10). The amount of DNA able to migrate and the distance of migration indicate the number of strand breaks present in that cell. Greater migration of the chromosomal DNA from the nucleus is an indication of higher level of DNA damage. Two versions of the Comet assay are currently in use; one introduced by Singh et al. (Singh, and et al., 1988), who used alkaline

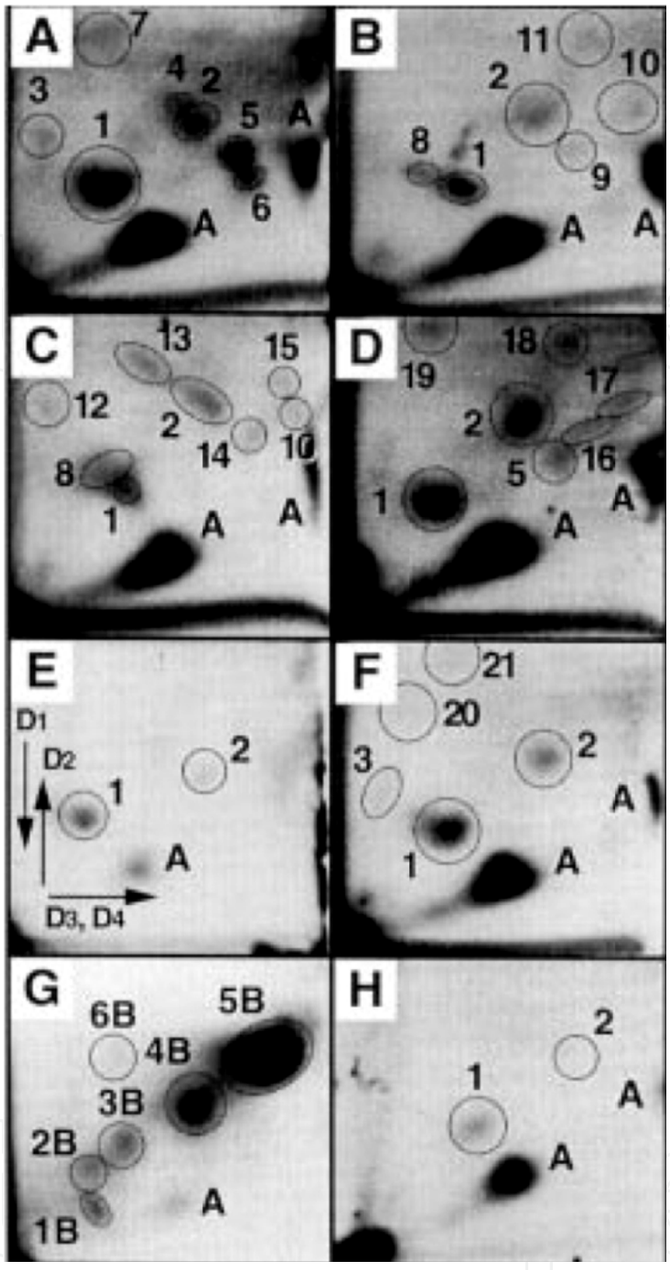


Fig. 9. Autoradiograph of PEI cellulose TLC maps of nuclease P1 enriched and postlabeled DNA, modified *in vitro* with metabolites of (A) Guthion, (B) Lorox, (C) Sencor, (D) Reglone, (E) Daconil, (F) Admire, (G) B(α)P and (H) negative control. Calf thymus DNA was reacted with the S9 metabolites of the above mentioned compounds. The adducts were enriched with nuclease P1 and labeled. The labeled adducts were separated on PEI cellulose plates using solvents for bulky adducts. Adducts are highlighted by encircling and numbered based on their position on the plate. All the autoradiograms were exposed for 18 h at –80°C with intensifying screens except for B(α)P which was exposed for 3 h. 'A' represents spots arising from ATP. The arrows represent the direction of migration with the solvents D1, D2, D3 and D4.

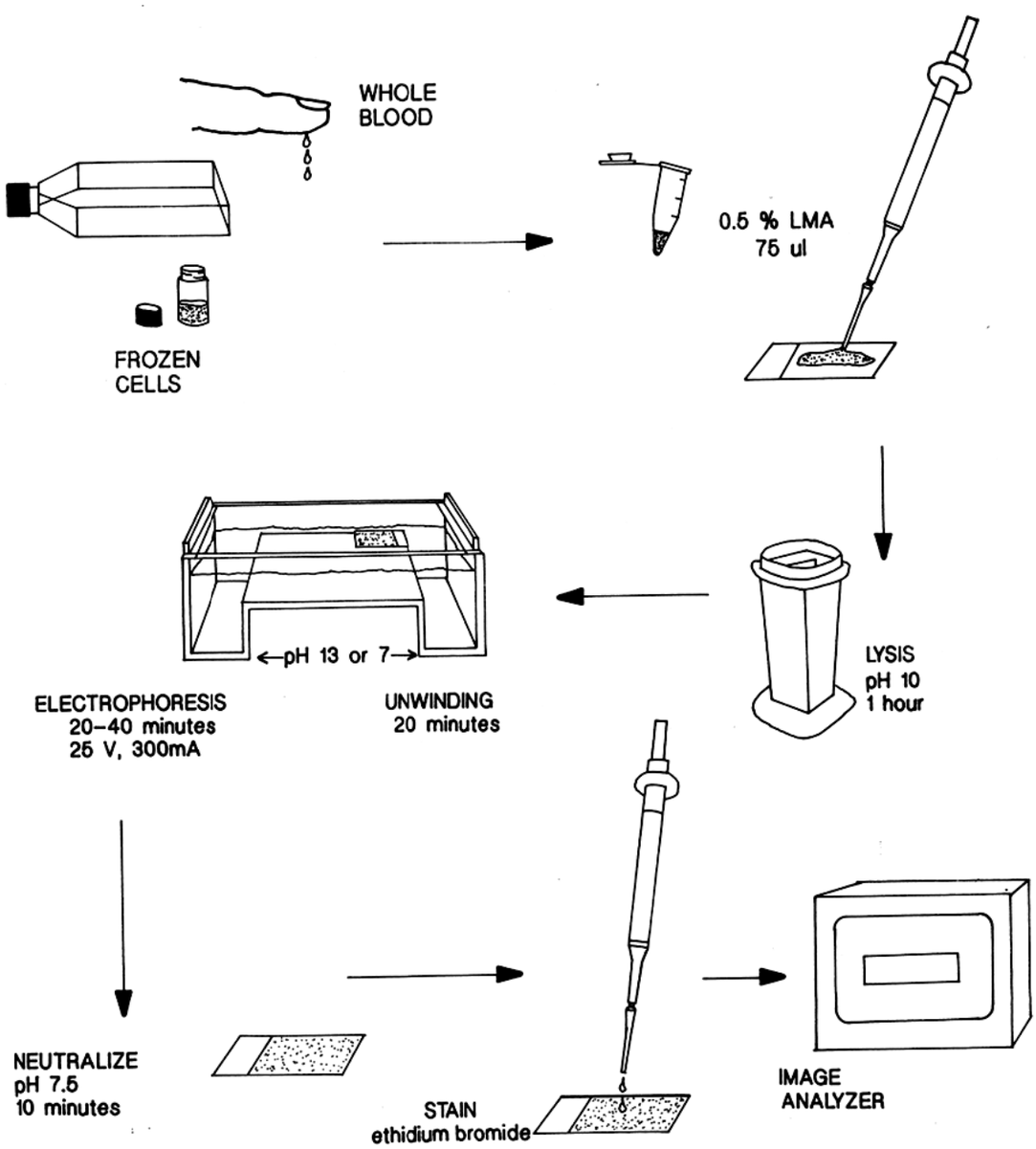


Fig. 10. SCGE Protocol.

electrophoresis (pH>13) to analyze DNA damage after treatment with X-rays or H_2O_2, which is capable of detecting DNA single-strand breaks and alkali labile sites in individual cells. This version is known as the ''*single cell gel electrophoresis (SCGE) technique*'', although for historical reasons many investigators refer to this method as the ''*Comet assay*''. Subsequently, Olive and co-workers developed versions of the neutral technique of Ostling and Johanson, which involved lyses in alkali treatment followed by electrophoresis at either neutral ([a] Olive, and et al., 1990) or mild alkaline (pH 12.3) conditions ([b] Olive and et al., 1990) to detect single strand breaks. The Singh and Olive methods are identical in principle and similar in practice, but the Singh method appears to be at least one- or two-orders of magnitude more sensitive. In the Singh version of the assay, a single cell suspension of the mammalian cell culture or tissue under study is embedded in low melting-point agarose in an agar gel sandwich on a microscope slide, lysed by detergents and high salt concentration at pH>10 and then electrophoresed for a short time under alkaline conditions. Lyses remove the cell contents except for the nuclear material. DNA remains highly super coiled in the presence of a small amount of non-histone protein but when placed in alkali, it starts to unwind from sites of strand breakage. Cells with increased DNA damage display increased migration of the DNA from the nucleus towards the anode under an electrical current, giving the appearance of a ''comet tail''. Depending on pH conditions for lyses and electrophoresis, the sensitivity of the technique can change. Employing neutral conditions for both variables allows us to detect DNA double strand breaks, but the pH 12.3 detects single strand breaks and delays DNA repair sites, while at pH>13 the sensitivity allows the evaluation of alkali labile sites, single strand breaks and delay repair sites of DNA. Therefore it is important to know the purpose of the study.

3.2.1 Slide preparation

Generally, the cells are mixed with 0.5% low-melting agarose at 37˚C and then placed on a microscope slide coated with 0.5% normal agarose. When the agarose is solidified, an additional layer of agarose is added. After the preparation of the three layers of this material (Fig. 11), the cells are lysed in a solution containing 2.5mM NaCl, 100mM Na_2-EDTA, 10mM Tris, pH 10, with 10% DMSO and 1% Triton X-100 for 1-24 h, and then the slides are put into an alkaline (300mM NaOH, 1mM Na_2-EDTA, and 0.2% DMSO, pH 13.5) or neutral buffer for double and single strand breaks detection, respectively, in a electrophoresis chamber, allowing the DNA unwinding. The electrophoresis is carried out, resulting in the migration of small pieces from the core of DNA toward the electric field. After electrophoresis, the slides are rinsed with neutralization buffer such as Tris-Hcl or PBS, and cells are stained with a fluorochrome dye such as Ethidium Bromide (5µg ml^{-1}).
A wide range of pesticides were studied by Comet assay for determination of their ability to induce in vivo genotoxic effects on DNA (see Table 1).

3.2.2 How to evaluate DNA damage by comet assay

For the evaluation of DNA damage by comet assay, the basic method is the image analysis of individual cells using the commercial software programs (such as SPSS) for collecting data. The parameters selected from the images for the quantification of DNA damage are length of DNA migration, tail length, tail moment, tail DNA% and head DNA%.
The length of DNA migration, usually presented in mm. Migration length, is related directly to fragment size and would be expected to be proportional to the extent of DNA damage. The migration length could be measured using different approaches; with a micrometer in

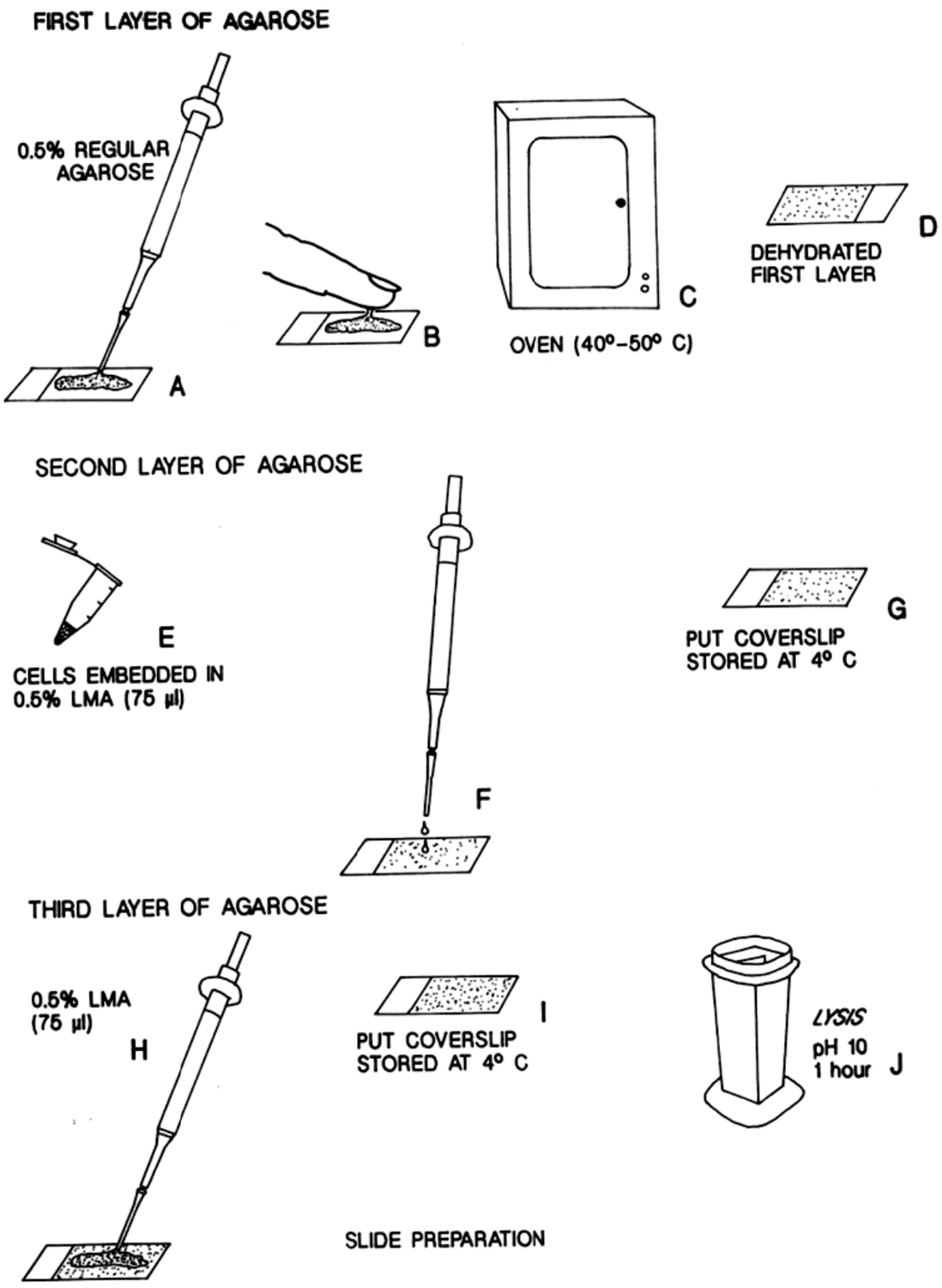
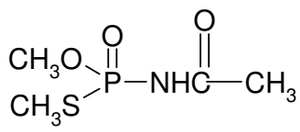
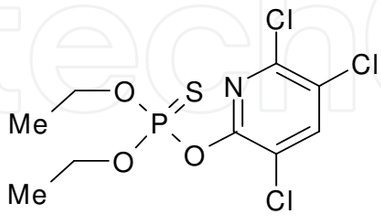
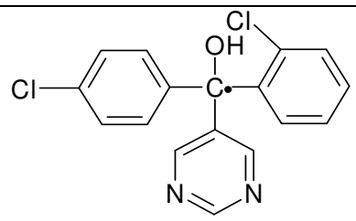
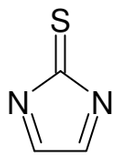
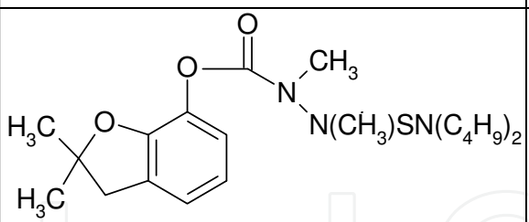
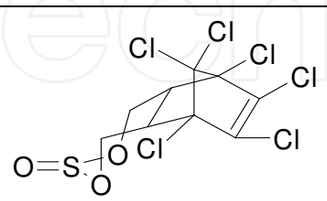
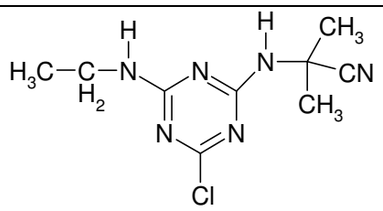
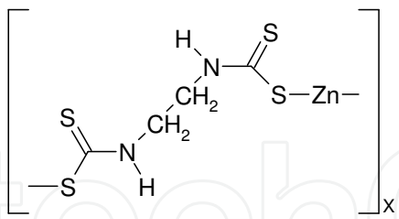
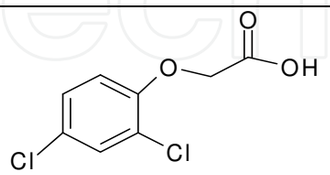
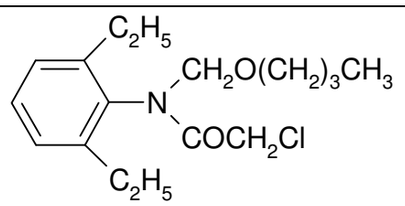
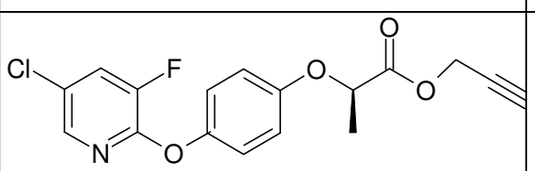
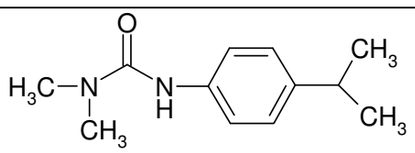
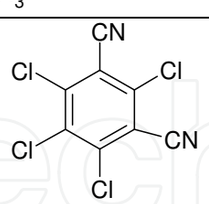
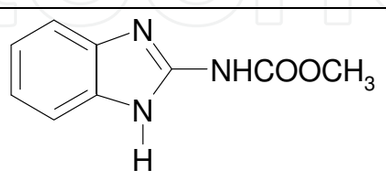
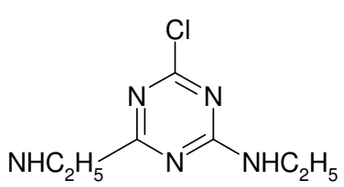


Fig. 11. Protocol for permanent slide preparation.

Reagent Name	Structure Formula	Type of Cells	Ref.
Acephate		Leucocytes of Swiss albino mice	(Rahman & et al., 2002)
Chloropyriphos		Leucocytes of Swiss albino mice and Lymphocyte and gill cells of *Channa punctatus* fish	Rahman & et al., 2002; Ali & et al., 2008)
Fenarimol		Leukocytes of two different rodent species (rat and mouse)	(Poli & et al., 2003)
2-Imidazolidinethione		Liver, lung, spleen, kidney, bone marrow from mouse	(Sasaki & et. al., 1997)
Carbosulfan		Erythrocyte and gill cells of *Channa punctatus* fish	(Nwani & et. al., 2010)
Endosulfan		Gill and kidney tissues of *Channa punctatus* fish	(Pandey , & et a., 2006)
Cyanazine		Leukocytes of mice	(Tennant & et al. 2001)

Reagent Name	Structure Formula	Type of Cells	Ref.
Zineb		Chinese hamster ovary (CHO) cells	(González , & et al., 2003)
2,4-D		Erythrocytes of *Clarias batrachus* catfish	(Ateeq & et al., 2005)
Butachlor		Erythrocytes of *Clarias batrachus* catfish	(Ateeq & et al., 2005)
Clodinafoppropargyl		Hemocytes of *Bombyx mori* silkworm	(Yin , & et al., 2008)
Isoproturon		Chinese Hamster Ovary (CHO) cells	(Vigreux & et al., 1998)
Chlorothalonil		Chinese Hamster Ovary (CHO) cells	(Vigreux & et al., 1998)
Carbendazim		Chinese Hamster Ovary (CHO) cells	(Vigreux & et al., 1998)
Atrazine		Human blood samples	(Garaj-Vrhovac, & Zeljezic, 2000)

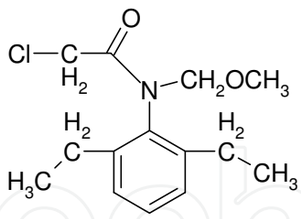
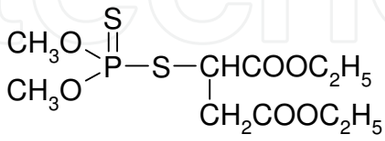
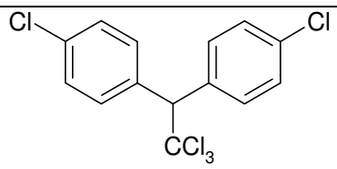
Reagent Name	Structure Formula	Type of Cells	Ref.
Alachlor		Human blood samples	(Garaj-Vrhovac, & Zeljezic, 2000)
Malathion		Cerebral tissue and peripheral blood of rats	(Reus, & et al., 2008)
P,P′-DDT		Human peripheral blood mononuclear cells (PBMC)	(Yanez & et al., 2004)

Table 1. The DNA damage by pesticides using the SCGE or Comet assay

the microscope eyepiece, a rule on photographic negatives / positives of cell images or in the camera monitor, and by using the image analyzer. Currently, the criteria used to identify the trailing and leading edge of the migrating DNA seem to depend on the investigator and/or software program. Furthermore, some investigators use the term ''DNA migration'' to describe total image length while others apply the term to migrated DNA only. A variant of this parameter is to present the ratio of length /width or width/ length, with cells exhibiting no damage having a ratio of approximately 1. (Olive, & Durand, 1992) discounted the utility of DNA migration as a parameter for DNA damage in the neutral or pH 12.3 alkaline assays, based on the observation that the length of DNA migration reached a plateau while the percentage of migrated DNA continued to increase. However, this limitation in migration length is not a characteristic of the pH.13 alkaline assay, where length has been reported to be the best parameter for this version of the assay. The computerized image analysis system to collect SCGE/Comet data, favors the evaluation of relative amount of migrated DNA, presented either as the percentage of migrated DNA or as the ratio of DNA in the tail to DNA in the head. This parameter assumes signal linearity in quantifying the amount of DNA ranging over multiple orders of magnitude and that the staining efficiency of the fluorescent dye is identical for migrated and non-migrated DNA. The concept of tail moment (tail length×tail intensity or percentage migrated DNA) as a parameter for DNA migration was introduced by Olive ([b]Olive, & et al., 1990). In a study conducted on damaging effects of pesticides on CHOK1 cells by Vigreux and co workers((Vigreux & et al., 1998) using SCGE, twenty-five randomly selected cells per slide were visually analyzed and submitted to image analysis (Fig. 12). Cells were eye-graded into three categories depending on DNA migration level: intact cells (IC) and slightly damaged cells (SDC), damaged cells (DC), and highly damaged cells (HDC) . These HDC, characterized by a small head and exceeding DNA fragmentation, may represent apoptotic cells.

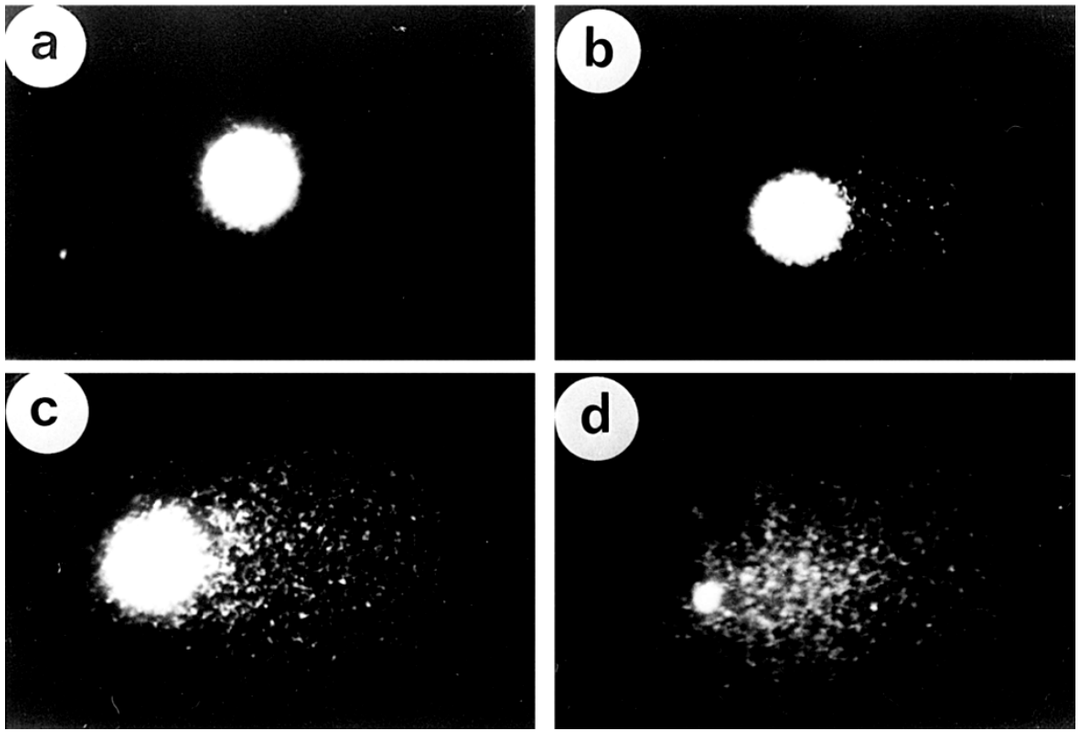


Fig. 12. Individual CHOK1 cells with various degrees of DNA damage: **(a, b)** intact cell (IC) and slightly damage cells (SCD) (tail moment 0–5) ; **(c)** damage cell (DC) (tail moment 5–60) and **(d)** highly damage cell HDC tail moment was not calculated with permission (Vigreux & et al., 1998).

Yin and et al., to evaluate DNA damage by pesticides and score each cell, used the relative intensity of the head and tail fluorescence of images (from undamaged DNA, stage 0, to maximally damaged DNA, stage D; Fig. 13a) (Yin , & et al., 2008). The visual analysis revealed that the exposure of hemocytes of the fourth instars silkworm, *B. mori*, to clodinafop-propargyl induced significantly elevated levels of DNA damage, and by increasing clodinafop-propargyl concentration the percentage of damaged DNA increased. Analysis of comet percentage of hemocytes in the second and fifth instars larvae of silkworm was skipped. The representative images (Fig. 13) illustrate how the damaged DNA was increasingly allowed to migrate to form a larger comet tail with the increase in the dose. The comet tail length of the hemocytes was 10.14, 9.5 and 6.6 times, and the tail moment was 86.8, 40.17 and 22.5 times compared with that of the control at the same dosages, respectively.

3.3 Alkaline elution or unwinding procedure

The DNA alkaline elution technique in combination with fluorometric measurement of the eluted DNA is a sensitive and suitable method for measuring single and double-strand DNA breaks, alkali-labile sites, and with minor modifications, protein associated strand breaks, DNA–protein and DNA–DNA cross links induced by physical, chemical and enzymatic processes in a mammalian test system. The alkaline elution method was developed by Kohn (Kohn, & Ewig, 1973), and the fluorometric method was developed by Cesarone.

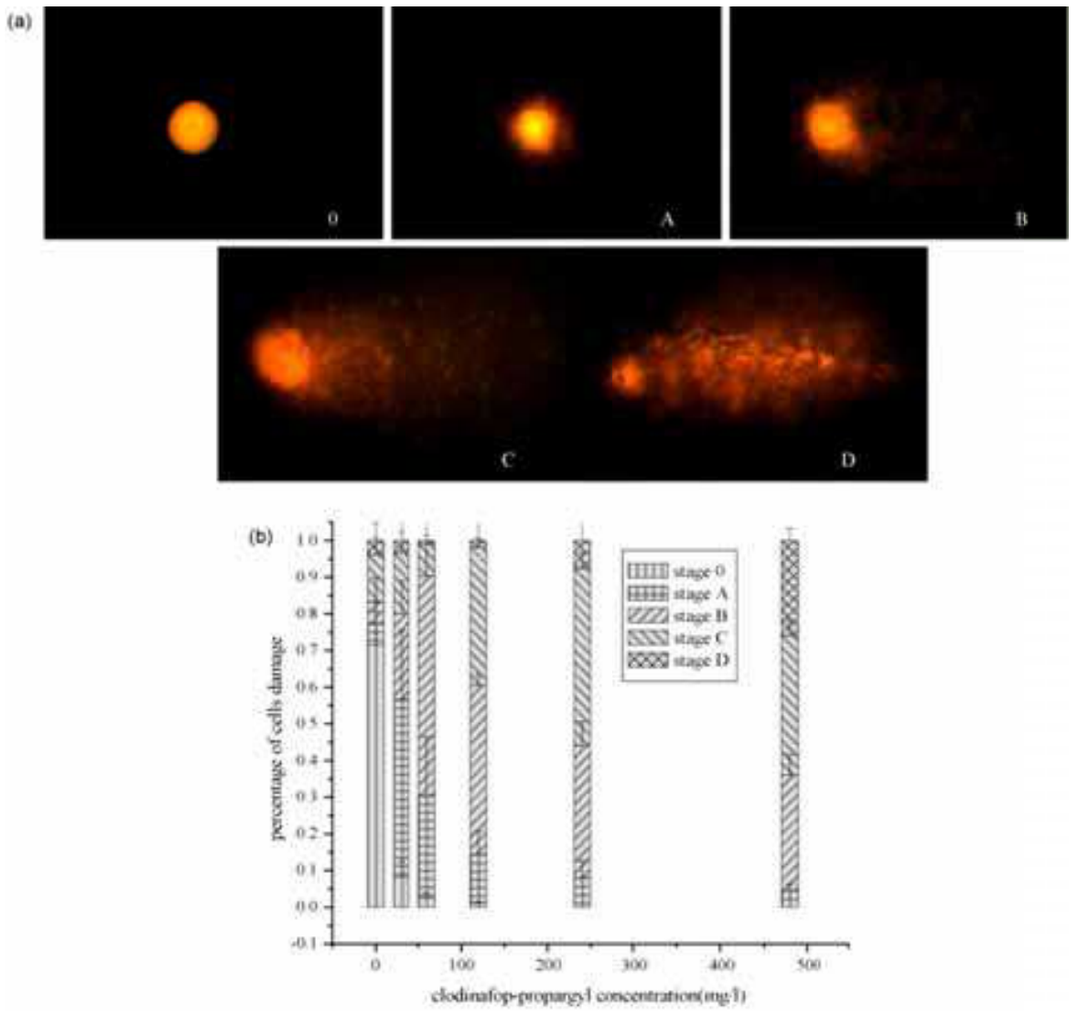


Fig. 13. **(a)** The different cell damage stages (0–D) used for scoring the SCGE by visual approach. **(b)** The percentage of cells damage stages (0–D) in the fourth instars larvae of silkworm, *B. mori* exposed to clodinafop-propargyl by visual approach. *Y* bars represent percentage of cells damage. *X* bars represent concentration of clodinafop-propargyl (Yin , & et al., 2008).

According to this method, cells are lysed on membrane filter and large nonelutable double strand-DNA is released. The filter is then eluted with an alkaline solution; single strand-DNA is released and slowly pumped through while fractions are collected to determine the elution kinetics. The elution rate of the single strand-DNA was proven to be size dependent and used as a measure of the DNA single strand breaks. Initially, for quantification of the DNA amount eluted during the alkaline elution procedure, prelabeling of cells with radioactive probes was used. Soon after, DNA quantification using fluorometric methods was considered preferable to avoid the use of radioactive materials. In original procedure, after extraction of crude nuclei from the tissues and centrifuge, the pelleted nuclei are resuspended in saline solution (EDTA, pH 7.4), an appropriate of solution containing a minimum of 1×10^6 cells or nuclei is placed in the membrane filter (25 mm diameter, 2.5 - 5 μm pore size). Then the nuclei or cells are lysed (2-5 mL solution containing 2% SDS

(Biorad), 0.025 M EDTA, 0.1 M glycine and 0.5 mg mL^{-1} proteinase K (BRL), pH 10) on the surface of the filter and single-stranded DNA fractions are elute with a solution containing EDTA and 10% tetraethylammonium hydroxide, pH 12.3, at low flow rate (0.1-0.3 mL min^{-1}). In a period of intervals, the fractions are collected. The fluorometric determination of DNA is performed with Hoechst 33258 reagent. This reagent, when complexed with DNA, enhanced the fluorescence yield.

The assay can be completed in 2.5 days, and for the diaminobenzoic acid dihydrochloride (DABA) method, it is labor-intensive for measuring the DNA in elution fractions. In DABA method, DNA cannot be assayed directly from the elution fractions and must be recovered by precipitation; also in order to get a sufficient amount of DNA, a minimum of 1×10^6 cells are required. In DABA method, it must be notified that the elution filters must have sufficient surface area so that they will not clog, and the fraction volumes must be large enough to obtain a sufficient amount of DNA per fraction. To extract the DNA from the eluting solution, a standard ethanol precipitation method is used which requires increasing the fraction volume 3-fold and incubating the samples at −20 ◦C for several hours. Therefore, wide attempts are made to minimize and automate the procedure along with elevation the efficiency (Gealy & et al., 2007). However, by this assay the elution rate constant, *K*, can be calculated from the equation (1):

$$K(mL^{-1}) = \frac{-Ln\ fraction\ of\ DNA\ retained\ on\ the\ filter}{eluted\ volume} \tag{1}$$

Bolognesi and coworkers (Bolognesi, & et al., 1997) evaluated the genotoxicity effects of Glyphosate (*N*-phosphonomethylglycine) and its technical formulation (Roundup) on liver and kidney of Swiss CD1 mice by alkaline elution assay. Figure 14 shows the results

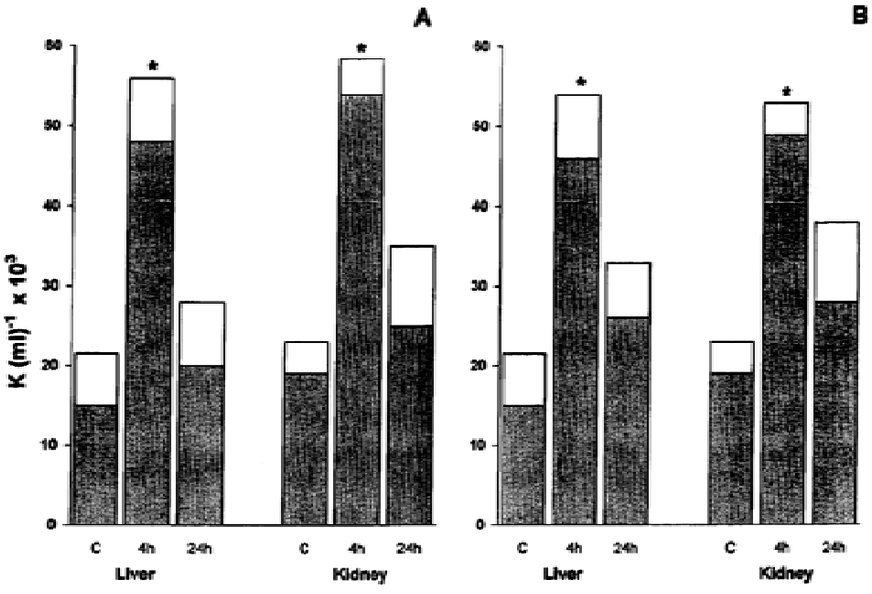


Fig. 14. Induction of DNA single-strand breaks and alkali labile sites by glyphosate (A) and Roundup (B). Dose was 300 mg/kg expressed as glyphosate. Mean data with SD of at least four independent experiments (8 animals) in treated animals and at least six experiments in controls (12 animals) are shown. * indicates $p < 0.05$.

obtained with the alkaline elution assay in treated liver and kidney with glyphosate or Roundup. A significant increase in the elution rate constant was observed 4 h after treatment with the technical formulation or its active ingredient, glyphosate. The increase in the elution rate was consistently higher in kidney of mice treated with glyphosate or Roundup. After 24 h, the elution rate constant returned to control values (data not shown). This transient effect could be attributed to the rapid elimination of this compound from the body and/or to repair of the DNA damage. No significant difference was observed in the extent of damage for the two compounds.
Pino (Pino & et al., 1988) reported that atrazine given orally to rats caused DNA damage in the stomach, kidney, and liver as measured by an alkaline elution assay. Thus, there is agreement that atrazine can cause DNA damage as measured by the alkaline elution-type DNA damage assays, but more than likely, the small amount of DNA damage that occurs is repaired.

4. In-vitro procedures for study of DNA-pesticides interactions

The in-vivo procedures described above are applicable for detection of Adduct-DNA complexes and dose not covers the non-covalent interactions. Therefore, recently the in-vitro methods have been developed. The Ultraviolet-visible spectrophotometry (Uv-vis), Fluorescence, circular dichroism (CD), and voltammetry are common procedures that are usable for analysis of non-covalent complexes including for DNA or RNA-targeting specially drugs. These techniques have now found important applications as a screening tool in drug discovery. Recently in our laboratory for the first time, we developed the in-vitro procedures for assessment of pesticides-DNA interactions to achieve a model of interaction for evaluation of genotoxicity effects of pesticides. Therefore, in this part we will discuss the procedure and the interpretation of data for assessing the mode and damaging effect of pesticides on DNA conformations. The data interpretations and how to use these procedures make the large body of this section.

4.1 The preparation of DNA solution and measuring concentration

Generally, the highly polymerized calf thymus-DNA (CT-DNA) is used for evaluation of damaging effect of pesticides. The stock solution of DNA is prepared by dissolving appropriate amount of DNA in a suitable buffer such as HEPES, phosphat, Tris–HCl and/or 10 mM NaCl solutions at pH 7.2 and dialyzing exhaustively against the same buffers for 24 h. The purity of the DNA is checked by monitoring the absorption ratio at 260/280 nm (A_{260}/A_{280}). The ratio must be more than 1.8, which indicate that DNA is fully free of protein. DNA concentration (Molar per nucleotide) of the stock solution is determined by UV spectrophotometery using the molar absorption coefficient $7000M^{-1}\ cm^{-1}$ at 258 nm (Ahmadi, & et al., 2010).

4.2 Uv-vis titration

Electronic absorption spectroscopy is very suitable for interaction studies related to DNA. DNA has an absorption peak at 260nm, which arises due to the π–π* transitions of DNA bases. However, when DNA and pesticides are interacting, a clear change in DNA spectrum absorbance is observed. In other words, the change of stacking pattern, disruption of the hydrogen bonds between complementary strands, covalent bonding of DNA bases and intercalation reactions caused the hypochromism and/or hyperchromism, red and/or blue

shifts of this band. Hypochromism is due to the contraction of DNA in the helix axis and changes in the conformation of DNA, while hyperchromism results from the damage of the DNA double helix structure. Several studies revealed that interaction of foreign molecules with DNA by an intercalation mode causes significant elevation in absorbance (>0.07 Abs.) and a high red shift in wavelength (>6 nm), and the extent of hypochromism is commonly consistent with the strength of the intercalative interaction. In a study by author and Bakhshandeh conducted on the in-vitro study of damaging effects of 2,4-Dichlorophenoxyacetic Acid on DNA structure, the Uv/vis spectrum shows a weak hyperchromic and red shift (Fig.15a) (Ahmadi, & Bakhshandeh, 2009). We concluded that 2,4-D neither covalent nor classical intercalate binding with DNA bases and these Uv/vis results may be due to the conformation changes of DNA structure via the groove binding interaction with 2,4-D. In another study on effect of clodinafop-propargyl herbicide on DNA structure by author and co workers, the Uv/vis titration shows a significant increase in absorbance (>0.1 Abs.) and red shift (~7nm) (Fig. 15b). The author concludes that the clodinafop-propargyl herbicide interacts with calf thymus DNA by an intercalative mode of binding (Askari, Ahmadi & et al., 2008).

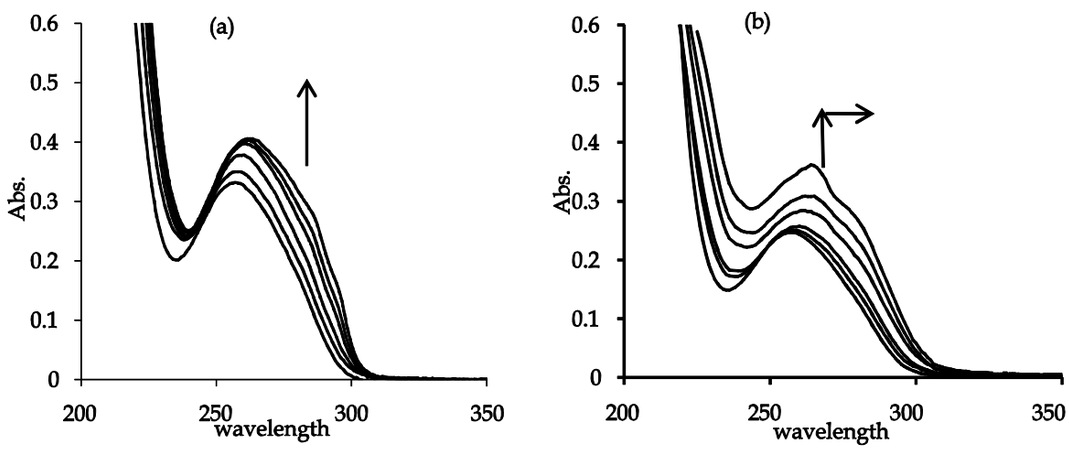


Fig. 15. Change in Uv/Vis absorption spectra of 5.0×10^{-5} M of DNA in the presence of **(a)** 2,4-D; **(b)** clodinafop-propargyl.

From the Uv/vis titration spectra, the bonding constant of pesticide-DNA can be calculate as follows:

Generally a solution of DNA (5.0×10^{-5} M, 2 ml) is place in a Uv cell, thermo state at a given temperature, and the spectrum of the solution measuring. Then the pesticide (1.0×10^{-2} M) can be transfer step-by-step to the titration cell using a pre-calibrated micropipette, and the spectrum of the solution is measure after each transfer. Addition of the pesticide solution is continue until the desired r_i=[pesticide]/[DNA]=1.0 is achieved. To calculate the Pesticide-DNA bonding constant, the data can be treated according to the following equations:

$$\text{DNA} + \text{pesticide} \leftrightarrow \text{DNA} - \text{pesticide complex} \tag{2}$$

$$K = \frac{[\text{DNA} - \text{pesticide complex}]}{[\text{DNA}]_{\text{uncomplexed}}[\text{pesticide}]_{\text{uncomplexed}}} \tag{3}$$

For weak binding affinities, the data can treat using linear reciprocal plots based on the equation (4):

$$\frac{1}{A - A_0} = \frac{1}{A_\infty - A_0} + \frac{1}{K(A_\infty - A_0)} \cdot \frac{1}{[\text{pesticide}]} \tag{4}$$

Where, A_0 is the absorbance of DNA at 260nm in the absence of pesticide, A_∞ is the final absorbance of the pesticide-DNA complex and A is the recorded absorbance at different pesticide concentrations. The double reciprocal plot of 1/ (A – A_0) versus 1/[pesticide] is linear and the bonding constant (K) can be estimated from the ratio of the intercept to the slope. The author calculated the bonding constant of several pesticides such as Chloridazon, Fenitrothion and 2-Imidazolidinethione with DNA. If any pesticide has an obvious and sharp absorbance at higher wavelength of 260 nm, the K_b can be calculated from the spectrophotometric titration by keeping the concentration of pesticide constant and varying the DNA concentration. Based on the variations in the absorbance spectra of pesticide upon binding to DNA, the binding constant, K_b, can be calculated according to the equation (5):

$$\frac{A_0}{A - A_0} = \frac{\varepsilon_P}{\varepsilon_{P-D} - \varepsilon_P} + \frac{\varepsilon_P}{\varepsilon_{P-D} - \varepsilon_P} \frac{1}{K[DNA]} \tag{5}$$

Where A_0 and A are the absorbance of pesticide in the absence and presence of DNA, ε_P and ε_{H-P} are the absorption coefficients of pesticide and its complex with DNA, respectively. The plot of $A_0/(A-A_0)$ versus 1/[DNA] is linear and the bonding constant (K) can be estimated from the ratio of the intercept to the slope

4.3 Competitive fluorescence measurements

To estimate further the binding affinity of the pesticides with DNA, the variation of quantum yield of DNA fluorescence with pesticides can be monitored by stern-Volmer quenching constant. Fluorescence quenching refers to any process which decreased the fluorescence intensity of a sample. A variety of molecular interactions can result in quenching, including excited-state reactions, molecular rearrangement, energy transfer, ground-state complex formation, and collision quenching. In fact, two quenching processes are known: static and dynamic quenching. Both of them require molecular contact between the fluorophore and the quencher. Static quenching refers to formation of a nonfluorescent fluorophore–quencher complex. Dynamic quenching refers to the quencher diffusion to the fluorophore during the lifetime of the excited state and upon contact, the fluorophore returns to the ground state, without emission of a photon. Both static and dynamic processes are described by the Stern–Volmer equation (6):

$$\frac{F_0}{F} = 1 + K_{SV}[Q] \tag{6}$$

Where F_0 and F are the steady-state fluorescence intensities in the absence and presence of quencher (Pesticides), respectively, K_{SV} is the stern-Volmer quenching constant and [Q] is the concentration of quencher. It is well known that the fluorescence intensity of DNA itself is very weak; therefore, direct use of DNA fluorescence emission properties to monitor the interaction of pesticides with DNA is not possible. The standard method for fluorescence

enhancement of DNA is based on Ethidium bromide (EB) usage. EB is one of the sensitive fluorescence probe that with plane structure can bind to DNA. The interaction pattern of EB with DNA belongs to the intercalation model with the binding affinity in the order of 10^7. However, when EB intercalated into DNA, its emission intensity and excited-state lifetime dramatically increased. An experimental strategy for determining the quenching constant for the pesticide molecule interacting with DNA based on the quenching of EB fluorescence via a competition for binding sites in DNA has become a standard method in nucleic acid chemistry. Recently, due to carcinogenic properties of EB, the methylene blue (MB) was replaced and has become a safe reagent in nucleic acid chemistry. Methylene blue (MB) is one of phenothiazine dyes with a planar structure. The interaction of MB with DNA has been studied with various methods. Most studies indicated that at a low ionic strength buffer and a low concentration of DNA, the major binding mode of MB with DNA is through intercalation; otherwise, MB interacts with DNA by nonintercalative binding. However, two modes of interactions were postulated for MB with DNA: intercalate, and nonintercalate or groove binding.

In our laboratory we found that the addition of CT-DNA to an MB solution caused significant quenches of fluorescence of MB, and in [DNA]=[MB] ratio of 1 it reaches a minimum, and the spectrum is constant even when adding more DNA. By the addition of pesticide to the DNA–MB solution, the fluorescence of MB should increase. If the formation of pesticide– DNA complex is complete, the probe fluorescence intensity should be sufficiently close to the corresponding pure MB fluorescence intensity. This enhancement in fluorescence is due to the release of bonded MB molecules from DNA molecule. The formation constant of pesticides with DNA can be calculated based on the recorded fluorescence data using the modified Benesi–Hildebrand equation (7):

$$\frac{1}{(F - F_0)} = \frac{1}{K(LQ[MB - DNA]_0[Pesticide]_0)} + \frac{1}{(LQ[MB - DNA]_0)} \quad (7)$$

Where F_0 and F represent the fluorescence signals of MB–DNA in the absence and presence of pesticide, $[MB\text{-}DNA]_0$, and $[pesticide]_0$ represents the initial concentration of MB–DNA complex and pesticide, L is the instrumented constant, K is the formation constant of the pesticide–DNA complex, and Q is the quantum yield for the pesticide–DNA complex. By plotting $1/(F\text{-}F_0)$ versus $1/[pesticide]_0$, K can be obtained from the ratio of the intercept to the slope of the resulted curve. The interaction of DNA with Fenitrothion, Diazinon, has been studied by quenching competitive fluorescence, (see Fig16a, b).

Figures 16a and 16b shows the fluorescence emission spectrum of EB with DNA and the effect of the addition of Fenitrothion and Diazinon to EB bound DNA. As it is observed, by addition of the Fenitrothion and/or Diazinon a significant quenching of EB-DNA fluorescence occurred, reflecting an intercalative mode of binding between DNA and Fenitrothion or Diazinon.

Also, the interaction of DNA with clodinafop-propargyl have been studied by enhancement competitive fluorescence (see Fig17). As it is observed by addition of clodinafop-propargyl to the DNA–MB solution, the fluorescence of MB increased. The increase in the fluorescence intensity should be due to a greater amount of free MB molecules; in other words, the MB molecules were released after the addition of clodinafop-propargyl and the fluorescence of solution increased.

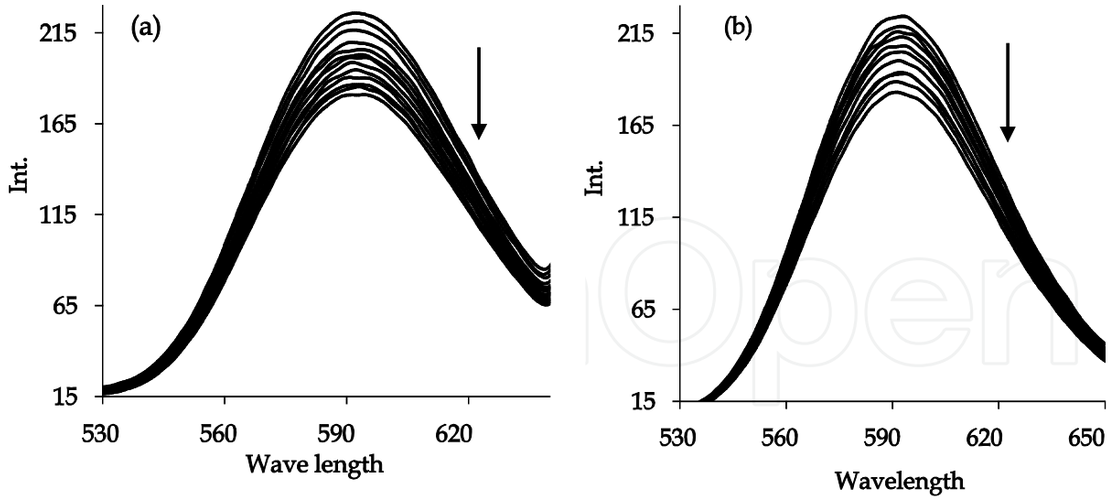


Fig. 16. Fluorescence quenching of DNA (5.0×10^{-5} M) with **(a)** Fenitrothion, **(b)** Diazinon with varying concentration (0.0, 0.492, 0.984, 1.476, 1.968, 2.46, 3.444, 4.428, 5.421, and 6.396×10^{-5} M) at 25°C. Fluorescence was excited at 320 nm, and the monitoring wavelength was 590 nm.

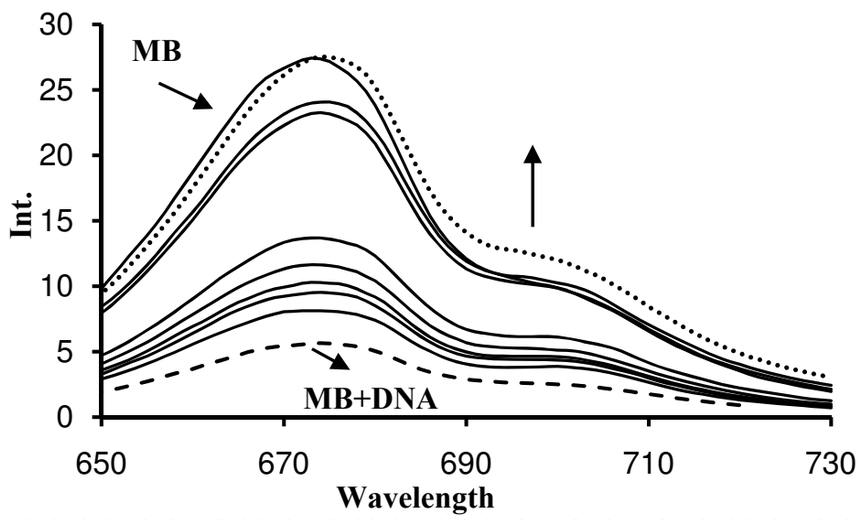


Fig. 17. Emission spectra of the MB–DNA complexes in the presence of the increasing clodinafop-propargyl (CP) concentrations in 0.01 M HEPES at room temperature. r_i=[CP]/[DNA+MB]=0.0(MB+DNA), 0.01, 0.02, 0.06, 0.2, 0.6, 1.0, 1.4, and 2.

4.4 Circular Dichroism (CD) spectroscopy

Circular dichroism (CD) spectroscopy CD has been extensively used in analysis of DNA conformation. In fact the CD provided essential information about the conformational properties of nucleic acids in solution. In the past decade, most DNA conformation studies included CD spectral data because they are informative, specific for various conformations of DNA and relatively easy and inexpensive to measure. The pervious studies revealed that CD resolved an apparent contradiction regarding the A-like conformation of (G-C)-rich

molecules of DNA, which was not solved by NMR spectroscopy, Raman spectroscopy, or other methods that are informative about the details of local DNA conformation. Also CD can distinguish between groove binders and intercalators but cannot identify individual binding sites. CD spectroscopy is complementary because it reflects the global properties of base stacking in DNA in a surprisingly realistic and sensitive way. The CD spectrum of free CT DNA is conservative and consists of a negative band at 245 nm and a positive one at 275 nm. The negative spectrum corresponds to the helical structure of DNA (helicity), and the positive spectrum corresponds to stacking of the base-pair that is characteristic of DNA in the right handed B-form. The effects of several pesticides such as 2,4-D, Chloridazon, fenitrtion, Diazinon, Clodinafop-propargyl, permetrin, 2-Imidazolidinethione, on DNA conformation have been reported by CD techniques. Depending on the structure formula of pesticides, the effects on DNA conformation were different.

For example, the CD spectra of Diazinon-DAN were measured at various ratios of Diazinon to DNA. In Figure 18, the changes in the CD spectra of DNA in the presence of increasing concentrations of Diazinon are depicted. The positive band (at 275 nm) in the CD spectrum has a very small change with addition of Diazinon. The negative band (at 245 nm) in the CD spectrum of DNA has an increase in the ellipticity to negative values with no shift in the band maximum.

These observations suggest that Diazinon binding to DNA induces certain conformational changes, such as condensation of DNA molecule.

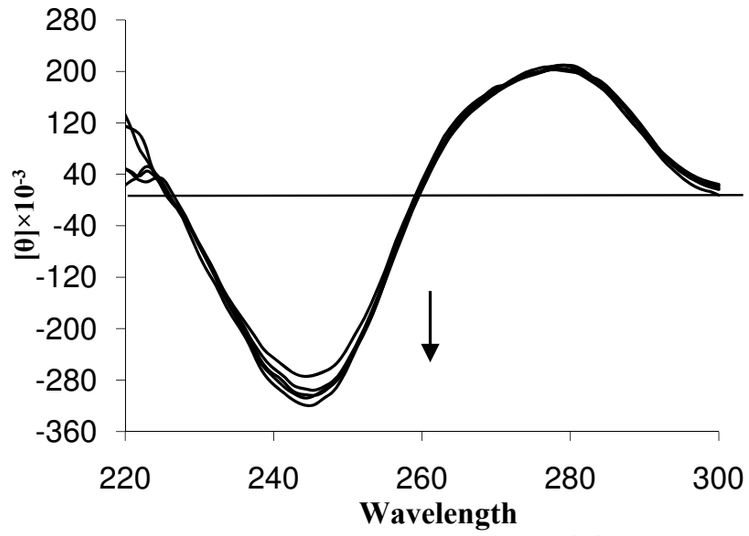


Fig. 18. CD spectra of the DNA in the presence of different amount of Diazinon (r¼0.0, 0.05, 0.2, 0.5, and 0.1) in 0.01M HEPES buffer (pH 7.3).

Also the bonding constant of DNA with pesticides can be calculated using the equation (8):

$$\frac{1}{\Delta A} = \frac{1}{(\varepsilon_b - \varepsilon_f)L_T} + \frac{1}{(\varepsilon_b - \varepsilon_f)L_T K_a} \times \frac{1}{M} \tag{8}$$

Where ε is the extinction coefficient; the subscripts b, f, and T denote bound, free, and total; and L is the DNA concentration. M is the concentration of pesticide and ΔA is the change of the CD response at a particular wavelength.

4.6 Voltammetric techniques in biomolecules

Recently, the electrochemical techniques were used as rapid and inexpensive methods for the study of DNA interactions with different compounds. The advantages of this technique are low cost, ease of use, high sensitivity, and a wide dynamic range of operations. Today the voltammetric methods enable us to estimate and predict the conformational changes of DNA by pesticides and evaluation of carcinogenic and mutagenic effects of pesticides or other molecules on DNA. The changes of electrochemical signals provide a very interesting evidence for elucidation of interaction mechanism. The common voltammetric techniques that usable for study effects of pesticides on DNA are linear sweep voltammetry (LSV), differential pulse voltammetry (DPV), cyclic voltammetry (CV), and alternative current voltammetry (ACV). In general, under specific conditions such as DNA concentration and ionic strength of background electrolyte, DNA can produce transmetic response around -1.2 and -1.4V versus SCE; however, the pure DNA is electrochemically inactive in potential range of 0.0 to -1.2 V at Hanging Mercury Drop Electrode (HMDE), Pt, Au and Glassy Carbon (GC) versus Ag/AgCl at pH=5-9. Therefore all voltammetric studies are based on considerable diminution of reduction/oxidation peak current and positive or negative shift in peak potential of electroactive pesticides when DNA is added to the voltammetric cell. This decrease in current is due to the decrease of diffusion of pesticide–DNA complex, not due to the increased viscosity of the solution or the blockage of the electrode surface by DNA adsorption. In this section we will discuses the application of these procedures in evaluating of in-vitro pesticides-DNA interactions and the basic of mesurment of interaction parameters such as bonding constant, site size bonding, number of redox electrons, and diffusion coefficient of molecules. The basic theory of voltammetric procedures are presented in literature (Bard, & Faulkner, 2001).

4.6.1 Linear Sweep Voltammetry (LSV)

To determine the composition of the supramolecular complex and the equilibrium constant of the binding reaction, the linear sweep voltammetry is a suitable procedure. Linear sweep voltammetry is sometimes abbreviated to LSV. In this method, a static indicator electrode (A cm^2 in area) is used and its potential is scanned at constant rate v ($V\ s^{-1}$) from an initial value (E_i) in the positive or negative direction. After reaching a peak, the current decreases again. The current decrease after the peak occurs, because the thickness of the diffusion layer increases with time. Even if the potential scan is stopped after the peak, the current continues to decrease with time in the same way (Fig. 19).

In order to improve the sensitivity of detection, minimizing the reading error the second order derivative linear sweep voltammetric (DLSV) peaks is used. In this manner it should be assumed that pesticide molecules interacted with DNA only to form a single complex of DNA-n Pesticide. The binding number and the equilibrium constant of the binding reaction can be deduced as follows:

$$\text{DNA} + \text{n Pesticide} \leftrightarrow \text{DNA} - \text{n Pesticide} \tag{9}$$

The equilibrium constant is:

$$\beta_n = \frac{[\text{DNA} - \text{n Pesticide}]}{[\text{DNA}][\text{Pesticide}]^n} \tag{10}$$

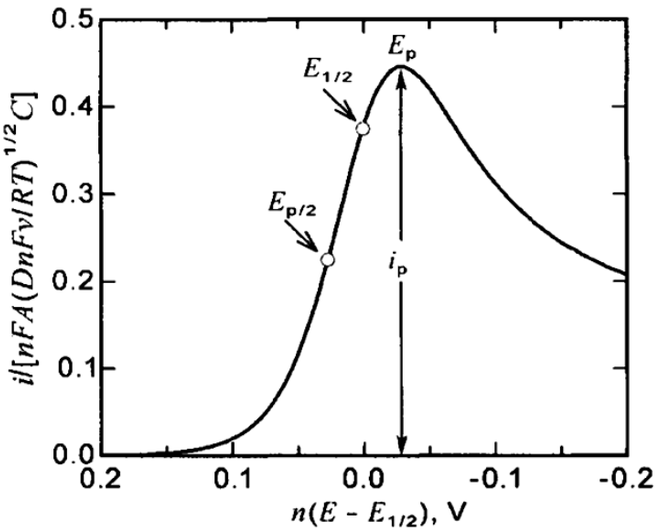


Fig. 19. Linear sweep voltammogram for a reversible process. E_p, peak potential; $E_{p/2}$, half-peak potential; $E_{1/2}$, half wave potential; i_p, peak current.

And the following can be deduced:

$$\Delta I_{max} = KC_{DNA} \tag{11}$$

$$\Delta I = K[DNA - n\ Pesticide] \tag{12}$$

$$[DNA] + [DNA - n\ Pesticide] = C_{DNA} \tag{13}$$

Therefore:

$$\Delta I_{max} - \Delta I = K(C_{DNA} - [DNA - n\ Pesticide]) = K[DNA] \tag{14}$$

Introducing Eqs. (10), (11), (13), and (14) gives:

$$\frac{1}{\Delta I} = \frac{1}{\Delta I_{max}} + \left(\frac{1}{\beta \Delta I_{max}}\right)\left(\frac{1}{[Pesticide]^n}\right) \tag{15}$$

or

$$Log\left[\frac{\Delta I}{(\Delta I_{max} - \Delta I)}\right] = \log \beta_n + n \log[Pesticide] \tag{16}$$

Where ΔI is the difference of peak current of reduction or oxidation of pesticide in the presence and absence of DNA and ΔI_{max} correspond to the obtain value when the concentration of pesticide is extremely higher than that of DNA. The C_{DNA}, [DNA], and [DNA-n Pesticide] correspond to the total, free, and bound concentrations of DNA in the solution, respectively. From the linear relationship between $\log[\Delta I/(\Delta I_{max}-\Delta I)]$ versus log[Pesticide], the stoichimetry and log β_n can be calculate from intercept and slope, respectively. The stoichiometry and log β_n of diazinon with DNA were reported using the

LSV. In Fig. 20 curve a was the relationship of Ip with the concentration of Diazinon, curve b represented the change of peak current after the addition of 1.0 ×10^{-5} M on varying the concentration of Diazinon, and curve c showed the differences between curve a and curve b, which represented the relationship between ΔIp (Ip_a-Ip_b.) and the concentration of Diazinon. From Eq. (16) the relation of log[ΔI/($ΔI_{max}$ - ΔI)] with log[Diazinon] was calculated and from the intercept and slope the n=1.798 and log $β_2$=8.42 were deduced, which indicated that a stable 1 : 2 complex of DNA-2Diazinon was formed under the selected conditions.

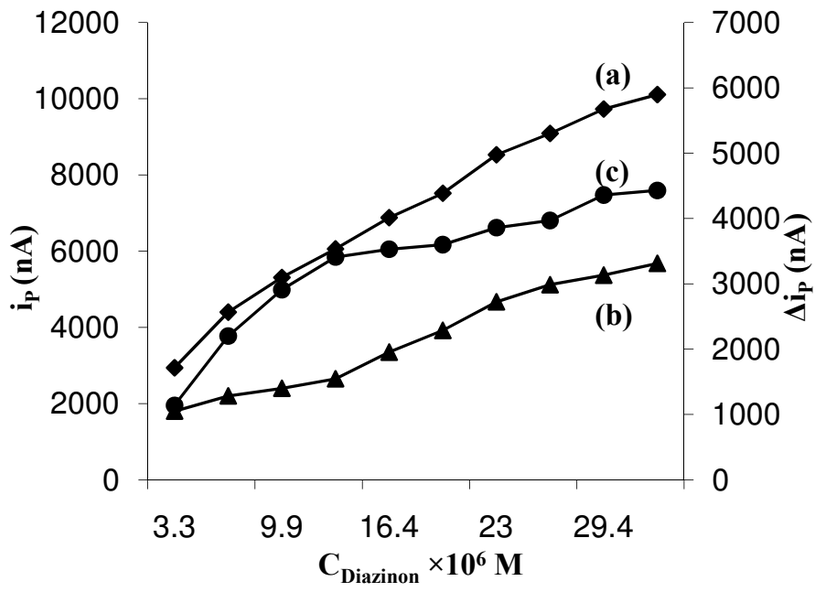


Fig. 20. Relationship between Ip and $C_{Diazinon}$ (a, b), ΔIp and $C_{Diazinon}$(c); (a) C_{DNA}= 0; (b) C_{DNA}= 1.0 ×10^{-5} M; (c) ΔIp= Ip_a-Ip_b (Ahmadi & et al.,2008).

4.6.2 Cyclic Voltammetry (CV)

In CV, the potential is linearly scanned forward from E_1 to E_2 and then backward from E_2 to E_1, giving a triangular potential cycle. Figure 21 shows some examples of cyclic voltammograms for the process, Ox + ne ↔ Red where only Ox is in the solution. Curve 1 is when the process is reversible. In the forward scan, a cathodic peak is obtained by the reduction of Ox to Red, as in LSV. In the backward scan, an anodic peak appears, due to the re-oxidation of the Red, which was generated during the forward scan. For a reversible process, the cathodic and anodic peak currents are equal in magnitude ($|i_{pc}| = |i_{pa}|$) and the cathodic peak potential (E_{pc}) is ($58/n$) mV more negative than the anodic peak potential (E_{pa}). These are important criteria for reversibility. Moreover, the half-wave potential, which is used to obtain the formal redox potential, is obtained by $E_{1/2}$=(E_{pc}+E_{pa})/2.

By decreasing the reversibility, the difference between the two peak potentials increases. Curve 2 is for a process that is considerably irreversible. Compared with curve 1, the cathodic peak appears at much more negative potential, the anodic peak at much more positive potential. If the process is completely irreversible, the anodic peak does not appear in the measurable potential region. From the irreversible CV curve, we can obtain kinetic parameters (rate constant and transfer coefficient) for the electrode reaction, the bonding formation and bonding site size usually by a simulation method. Curve 3 is for the case in

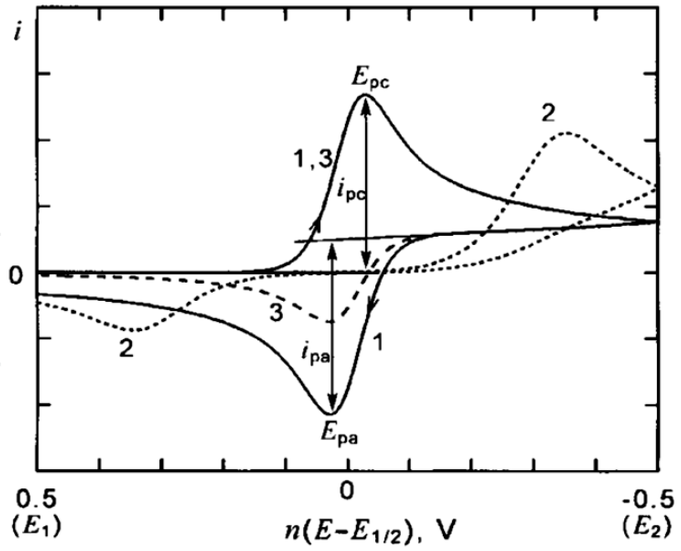


Fig. 21. Cyclic voltammograms for the electrode reaction Ox+ne ↔Red, which is reversible (curve 1), irreversible but α=0.5 (curve 2), and reversible but accompanied by a conversion of Red to an electroinactive species (curve 3). $E_{1/2}$ is for reversible process.

which $Ox \leftrightarrow Red \underset{\sim}{k} A$ i.e. Red can be reversibly re-oxidized to Ox but, before the re-oxidation, some part of the Red is converted to non-electroactive species A. In CV, the voltage scan rate can be varied over a wide range, 0.001 to 2 V s^{-1} or more.

The application of cyclic voltammetry for interaction study of electroactive pesticides with DNA provides valuable information's. In voltammetric studies before all the electrochemical behavior of electroactive species at the surface of electrode should be assessment. Generally the CV is a common technique that widely used. From the cyclic voltammogrames the reversibility and/or irreversibility of electrochemical reaction is observable. Also, from the changes of the peak current (i_p) and peak potential (E_p) at the electrode surface versus scan rate (ν), $\nu^{1/2}$ and $ln\ \nu$ the absence and presence of an excess of DNA the type of mass transport of species to the surface (adsorption or diffusion), $(\alpha n)_f$ and $(\alpha n)_b$, K_s and E^0 can be calculated. The symmetry of the energy barrier (α) and all electrons (n), can be calculate, using the Anson equation (17):

$$E_p = E^0 - \frac{RT}{\alpha nF}\left[ln\left(\frac{K_s}{D^{\frac{1}{2}}}\right) + 0.5ln\left(\frac{\alpha nF\nu}{RT}\right) + 0.78\right] \quad (17)$$

The α is obtained from the slope of the linear between E_p and $ln\nu$. As the $n\Delta E_p$ should be less than 200mV, the Laviron equation (18) also is usable for calculation of K_s and E^0.

$$E_p = E^\circ + \left(\frac{RT}{\alpha nF}\right)\left[ln\left(\frac{RTK_s}{\alpha nF}\right) - ln\,\nu\right] \quad (18)$$

Where E^0 (V) is the formal potential, R is the universal gas constant (8.314 J K^{-1} mol^{-1}), T (K) is the Kelvin temperature, K_s (s^{-1}) is the electrochemical rate constant and F is the Faraday constant (96,487 C mol^{-1}). According to Laviron equation the plot of E_p vs. $ln\nu$ is linear with a

slope that allows *an* being determined, and an intercept from which K_s can be calculated if the value of E^0 (V) is known. The value of E^0 in Laviron equation can be obtained from the intercept of the E_p vs. ν curve by extrapolation to the vertical axis at ν=0.

4.6.3 Cyclic voltammetric titration of pesticides with DNA

The electrochemical titration is more valuable to quantify the interaction parameters of an electroactive molecule with DNA than other methods. By addition of different amounts of DNA to the voltammetric cell containing an electroactive pesticide (5×10^{-5} M), the cathodic or oxidative peak current pesticide begin to decrease and the formal potential shifts to more negative or positive values which suggest the interaction of the pesticide with DNA. The addition of DNA to voltammetric cell continue untile the $R = \frac{[DNA]}{[Pesticide]} \geq 10$, and the CV currents of pesticide decrease and stabled. If an electroactive molecule (*E*) nonspecifically reacts with a DNA duplex at a binding site, which is composed of base pairs (S), a DNA-electroactive molecule complex (E-S) is produced as follows:

$$E + S \leftrightarrow E\text{-}S \tag{19}$$

As the pesticides non-specifically binds to the DNA duplex and covers S consecutive base pairs (i.e. one binding site), the binding constant, *K*, can be given by the equation (20):

$$K = \frac{C_b}{C_f C_s} \tag{20}$$

Where C_b, C_f, and C_s represent the equilibrium concentrations of the DNA-Pesticide complex, free Pesticide and free binding site, respectively. The total concentration of pesticide, C_t, is:

$$C_t = C_b + C_f \tag{21}$$

The average of number of binding sites (x) along a DNA duplex molecule with an average total number of base pairs *L* can be described by the following form: $x = \frac{L}{s}$, where *s* is the binding site size of the electroactive molecule interacting with DNA. It means that, the number of DNA base pairs is occupied (or covered) by a binding molecule. Thus, the total concentration of binding sites (xC_{DNA}) can be expressed as follows:

$$xC_{DNA} = C_b + C_s \tag{22}$$

where

$$C_{DNA} = \frac{C_{NP}}{2L} \tag{23}$$

C_{NP} represents the concentration of nucleotide phosphate, which is determined by the UV absorption at 260 nm. The total concentration of binding sites can also be expressed as follows:

$$\frac{C_{NP}}{2s} = C_b + C_s \tag{24}$$

The ratio of the NP concentration and the total concentration of electroactive molecules can be defined as *R*:

$$R = \frac{C_{NP}}{C_t} \tag{25}$$

For an irreversible reaction in CV, the total cathodic current (I_{pc}) under the fixed potential with any R can be calculated:

$$i_{pc} = B\left[(\alpha n)_f^{\frac{1}{2}} D_f^{\frac{1}{2}} C_f + (\alpha n)_b^{\frac{1}{2}} D_b^{\frac{1}{2}} C_b \right] \tag{26}$$

In fact B represents the appropriate, concentration-independent terms in the voltammetric expression. A Nernstain reaction in CV at 25 ^{o}C is shown as follows:

$$B = 2.99 \times 10^5 nA\nu^{\frac{1}{2}} \tag{27}$$

Where n is the number of electron transferred, A the electrode area, v the scan rate, D_f and D_b are the diffusion coefficients for free and bound molecules, α_f and α_b are the electron transfer coefficients for free and bound molecules, and C_f and C_b are the bulk concentrations of the free and bound irreversibly electroactive species.

Based on Carter et al., the binding constant, K, can be expressed as the following form:

$$K = \frac{C_b}{C_f\left(\frac{[NP]}{2s} - C_b\right)} \tag{28}$$

Where s is the size of binding site in terms of base pairs. Making appropriate substitutions and eliminating C_b and C_f from Eq. (26), a new equation was obtained:

$$i_{pc} = B\left\{ (\alpha n)_f^{\frac{1}{2}} D_f^{\frac{1}{2}} C_t + \left[(\alpha n)_b^{\frac{1}{2}} D_b^{\frac{1}{2}} - (\alpha n)_f^{\frac{1}{2}} D_f^{\frac{1}{2}} \right] \times \left[\frac{b - \left(b^2 - \frac{2K^2 C_t^2 R}{s} \right)^{\frac{1}{2}}}{2K} \right] \right\} \tag{29}$$

Where $b = 1 + KC_t + \frac{KRC_t}{2s}$.

Since i_{pc}, C_t and [NP] are experimentally measurable and $(\alpha n)_f$, $(\alpha n)_b$, have already been acquired as mentioned above, the binding constant (K) and binding site size (s) of the pesticide-DNA, D_f and D_b can be obtained from a nonlinear regression analysis of the experimental data (i_{pc} versus [R] plot) according to Eq. (29).

The in-vitro interaction of Fenitrothion with DNA by CV technique and nonlinear fit analysis using the equation (29) (see Fig. 22) yielded K =1.03×10^4, s =1.204, $D_f = 5.2\times10^{-2}$ and $D_b=1.72\times10^{-5}$.

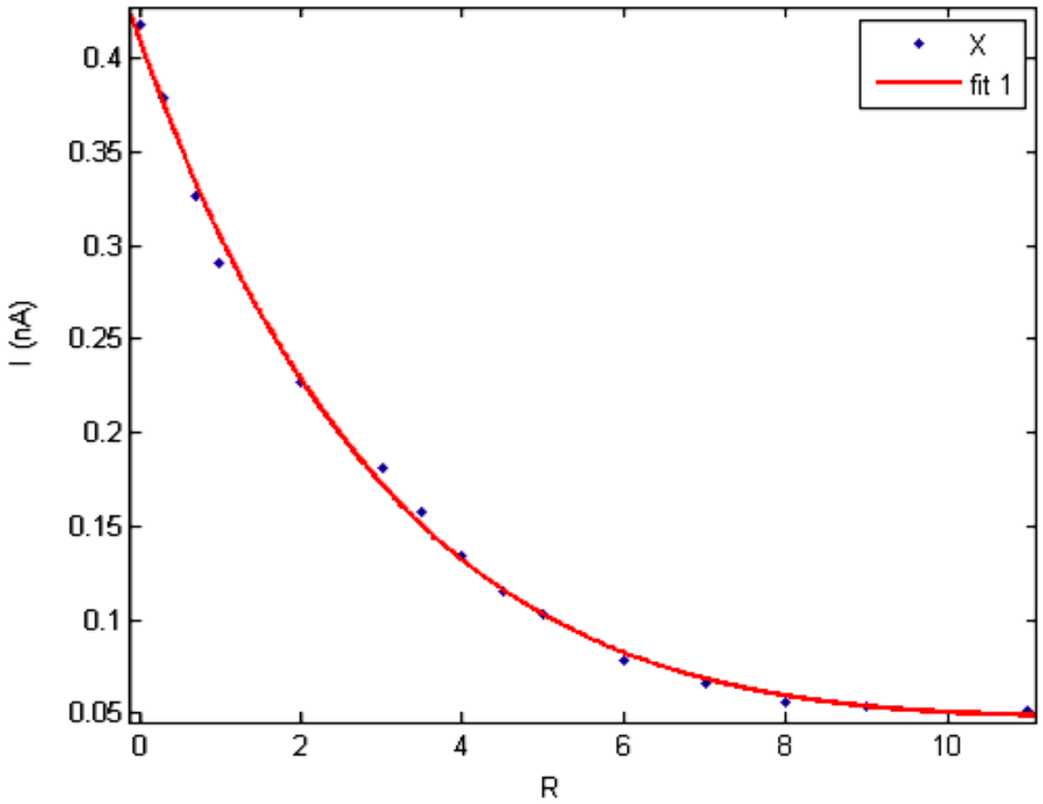


Fig. 22. Dependence of i_p of 5×10^{-5} M Fenitrothion on the concentration of added DNA by cyclic voltammetry(blue dots), and nonlinear fiting (solid Red line) using the equation (29).

4.6.4 Differential Pulse Voltammetry (DPV)

Differential pulse voltammetric titration is another valuable technique for monitoring of in-vitro DNA interactions. It is performed by keeping the concentration of electroactive species constant while varying the concentrations of DNA. The current titration equation can be described by equation (30):

$$\frac{1}{C_{DNA}} = K_f \frac{(1-A)}{1-\frac{i}{i_0}} - K_f \tag{30}$$

Where C_{DNA} is the concentration of DNA, K_f is the bonding constant of Pesticide-DNA, i_0 and i are the peak currents of electroactive species without and with DNA, respectively, and A is the proportional constant. The condition of using this Eq. (30) is that a 1:1 association complex is formed and C_{DNA} is much larger than the total concentration of electroactive pesticide in solution.

The interaction of 2-Imidazolidinethione (ETU) with guanine in binary mixtures of water-acetonitril were studied by DPV (Fig. 23, Table). In this study the hydrogen bonding were proved between DNA and ETU by electrochemical procedur.

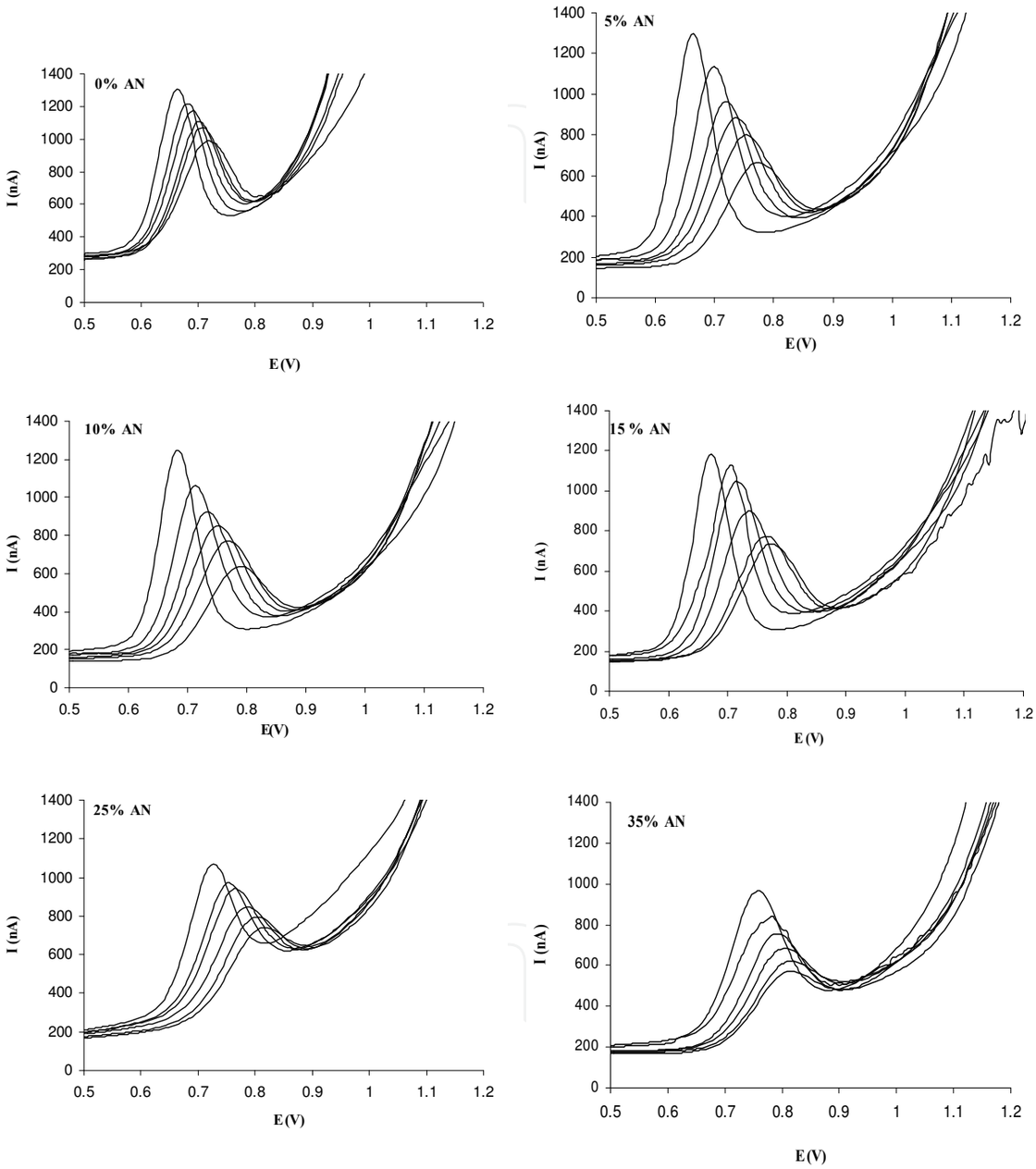


Fig. 23. Differential pulse voltammograms of 4×10^{-5} M of GU in the addition of different amount of Im (0.0, 2.0, 4.0, 6.0, 8.0, 10.0 × 10^{-5} M) in Tris-HCl buffer and binary mixture of AN- buffer with pH=7.2.

%AN	Linear Equation [58]	R^2	Log K_f
0	$\frac{1}{C_{Im}} = \frac{2.23\times10^3}{(1-\frac{i}{i_0})} - 7.48\times10^2$	0.9982	2.87
5	$\frac{1}{C_{Im}} = \frac{5.07\times10^3}{(1-\frac{i}{i_0})} - 1.27\times10^3$	0.9971	3.1
10	$\frac{1}{C_{Im}} = \frac{5.12\times10^3}{(1-\frac{i}{i_0})} - 2.24\times10^3$	0.9968	3.35
15	$\frac{1}{C_{Im}} = \frac{4.27\times10^3}{(1-\frac{i}{i_0})} - 3.13\times10^3$	0.9988	3.49
25	$\frac{1}{C_{Im}} = \frac{5.45\times10^3}{(1-\frac{i}{i_0})} - 6.92\times10^3$	0.9962	3.84
35	$\frac{1}{C_{Im}} = \frac{8.84\times10^3}{(1-\frac{i}{i_0})} - 7.33\times10^3$	0.9986	3.87

Table 2. The linear equation and log K_f of Im-GU complex calculated by DPV in different binary mixture of buffer-AN.

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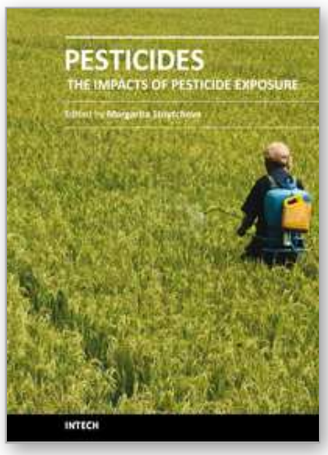

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Pesticides are supposed to complete their intended function without â€œany unreasonable risk to man or the environmentâ€. Pesticides approval and registration are performed â€œtaking into account the economic, social and environmental costs and benefits of the use of any pesticideâ€. The present book documents the various adverse impacts of pesticides usage: pollution, dietary intake and health effects such as birth defects, neurological disorders, cancer and hormone disruption. Risk assessment methods and the involvement of molecular modeling to the knowledge of pesticides are highlighted, too. The volume summarizes the expertise of leading specialists from all over the world.

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