ANALYSIS OF PESTICIDE RESIDUES IN SOFT DRINKS

INVESTIGATORS

Prof.(Dr.) H.B. MATHUR

Dr. SAPNA JOHNSON

Mr. AVINASH KUMAR

DATE

AUGUST 5, 2003

CENTRE FOR SCIENCE AND ENVIRONMENT

41, TUGHLAKABAD INSTITUTIONAL AREA, NEW DELHI 110 062

PH: 91-11-2995 6110/5124/6394/6399

FAX: 91-11-2995 5879 **E-MAIL:** CSE@CSEINDIA.ORG

WEBSITE: WWW.CSEINDIA.ORG

POLLUTION MONITORING LABORATORY

INDIA HABITAT CENTRE, CORE 6A, FOURTH FLOOR

LODHI ROAD, NEW DELHI - 110 003

PH: 91-11-2464 5334/5335

CONTENTS

1. ABOUT THE CSE LABORATORY

The Centre for Science and Environment (CSE), a non-governmental organisation based in New Delhi, has set up the Pollution Monitoring Laboratory (PML) to monitor environmental pollution. Its main aim is to undertake scientific studies to generate public awareness about food, water and air contamination. It is equipped with state-of-art equipments for monitoring and analysis of air, water and food contamination, including High Performance Liquid Chromatograph (HPLC), Gas Chromatograph (GC) with ECD, NPD, FID and other detectors, UV-VIS Spectrophotometer, Mercury Analyzer, Respirable Dust Sampler etc. It provides scientific services at nominal cost to communities that cannot obtain scientific evidence against polluters in their area. Given the state of scientific research in India --most of it being restricted to national defense and food security -- this is an effort to use science to achieve ecological security.

2. INTRODUCTION & ORIGIN OF THE STUDY

After the publication of the PML study on pesticides residues in bottled water which reported the presence of multiple pesticide residues in almost all the brands, a spate of letters were received enquiring about the presence of pesticide residues in soft drinks. In response to this public query it was decided to conduct a similar study in soft drinks.

A total of 12 brands-most popular and easily available -- were considered in this particular study. PML tested the samples with a widely and internationally used methodology based on United States Environment Protection Agency (USEPA) methodology for organochlorine pesticide and organophosphorus pesticide detection. Extraction was done as per the given methodology and analysis by Gas Chromatograph with Electron Capture Detector and with Nitrogen Phosphorus detector using a capillary column.

3. SOFT DRINK INDUSTRY AND REGULATIONS

3.1 Definition of soft drink

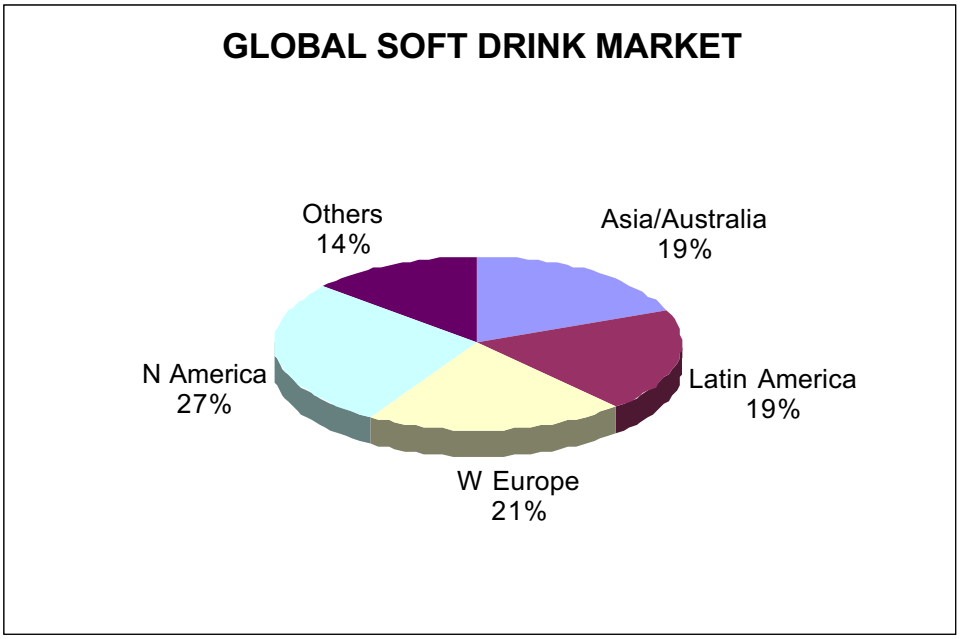
Soft drinks are non-alcoholic water-based flavored drinks that are optionally sweetened, acidulated, carbonated and which may contain fruit, fruit juice and/or salts; their flavor may derive from vegetable extracts or other aromatic substances. They constitute a defined and homogenous range, designated by a generic denomination and utilizing a single common list of additives. They include the beverages which comply with this definition, which utilize these additives and which do not claim to be part of adjacent categories such as fruit juices and nectars, dairy drinks, mineral waters, etc.

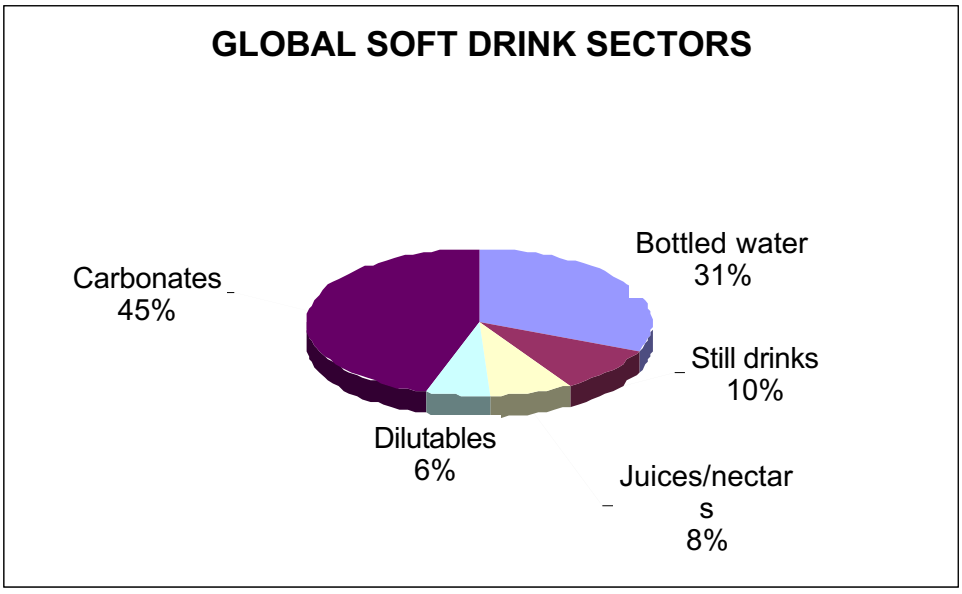
3.2. The Market

Global Scenario

Globally, carbonated soft drinks are third most consumed beverages. Per capita annual consumption of carbonated soft drinks is nearly four times the per capita consumption of fruit beverages. (Source: Data from the Beverage marketing Corporation, as reported by the Canadian Soft drink Association). Soft drink consumption is growing by around 5% a year, according to Global Soft drinks 2002. (Zenith International, 2002). Total volume reached 412,000 million litres in 2001, giving a global per capita consumption of around 67.5 litres per year.

GLOBAL SOFT DRINKS CONSUMPTION, 2001





Source: Zenith International, 2002 www.globaldrinks.com

viewed on BevNET.com (www.bevnet.com/news/2002/11.1.2002-Zenith.asp

North America is the largest soft drinks market with 27 per cent of total world soft drink sales and a consumption of 48 gallons per person per year (192 litres/ person / year). The European market accounts for 21 per cent, with a per capita consumption of 12.7 gallons per year (50.8 litres / person/ year). The fastest growth in soft drink consumption is in Asia and South America (www.beveragemarketing.com/news2p.htm). Carbonated drinks are the biggest soft drinks sector with 45% of global volume. The five fastest growing soft drink markets between 1996 and 2001 were from Asia, East Europe and the Middle East. The five fastest developing markets during 2001 and 2006 are all expected to come from Asia. Amongst them Pakistan is predicted to have the highest percentage growth rate while India is expected to make sizeable volume gains, as affluence spreads to more of its vast population. Indonesia, China and Vietnam complete the top five for future growth. The overall market should hit 523,000 million litres by 2006. A continuing 5% growth rate for soft drinks compares favourably with at best 1% for hot drinks, 2% for milk and 3% for alcohol. (Source: Zenith International, 2002).

Major Players-Global

The global soft drink industry is highly concentrated, being largely controlled by multinational companies. Among the major players are Coca-Cola Co. and PepsiCo. Coca–Cola leads the carbonated drink market in most countries in the world with 60% of the global cola market with its flagship Coca-Cola brand. Other notable players include Cadbury Schweppes.

Indian Scenario

Market

According to government estimates soft drinks marketed in India were 6540 million bottles in March 2001. The market growth rate, which was around 2-3% in '80s, increased to 5-6% in the early '90s and is presently 7-8% per annum. Most of the sales of soft drinks takes place during summers while just 5-6% of total sales take place in winters. In summers the high season lasts for 70-75 days, which contributes more than 50% of the total yearly sales. In terms of regional distribution cola drinks have main markets in metro cities and northern states of UP, Punjab, Haryana etc. Orange flavored drinks and sodas are popular in southern states. Western markets have preference towards mango-flavored drinks.

Major Players in India

The two global majors PepsiCo and Coca-Cola Co. dominate the soft drink market in India. Coca-Cola, which had winded up its India operations during the introduction of the FERA regime, re-entered India 16 years later in 1993. Coca-Cola bought local brands-Thumps Up, Limca and Gold Spot from Parle Beverages and soft drink brands Crush, Canada Dry and Sport Cola from Cadbury Schweppes in early 1999. Pepsi started a couple of years before Coca Cola in 1991 has bought over Mumbai based Duke's range of soft drink brands.

There are conflicting figures about their market share. According to one estimate (ORG)- Coca-Cola had 57% of soft drink market and Pepsi had 41 %. While another estimate (IMRB) figures were Pepsi- 49% and Coca- Cola- 48% during the same period January to May 2000. The soft drinks segment, dominated by MNCs, accounted for Rs 6,247 crore in sales in 2002. Thums Up, the brand taken over by Coca-Cola, is estimated at Rs 1,350 crore, bigger than Coca-Cola itself in India. Thums Up and Limca, two key brands that Coke acquired from Parle, account for no less than Rs 1,950 crore in sales for Coke. The Coca Cola Company in India has Rs 3,757 crore of sales of soft drinks, while PepsiCo's three main brands have sales of Rs 2,490 crore. (Source: Economic Times presentation, Retail Biz, 25th March 2003)

Consumption

According to NCAER survey 91% of the total consumption of soft drinks in the country is by lower, lower middle and upper middle class people. Per capita consumption in India is among the lowest in the world at 6 bottles per annum compared to 80 bottles in Thailand and 800 bottles in USA. Delhi market has highest per capita consumption in the country with 50 bottles per annum. (India Info line Sector Report, 2002).

Types

Non-alcoholic soft drink beverage market can be divided into fruit drinks and soft drinks. Soft drinks available in glass bottles, aluminum cans, PET bottles or disposable containers can be divided into carbonated and non-carbonated drinks. Cola, lemon and oranges are carbonated drinks and non-carbonated drinks include mango drinks. Soft drinks can also be divided into cola products and non-cola products. Cola products like Pepsi, Coca- Cola, Thumps Up, and Diet Coke, Diet Pepsi etc. account for nearly 61-62% of the total soft drinks market. Non-Cola products constitutes 36%, and based on the types of flavors available can be divided into Orange, Cloudy Lime, Clear Lime and Mango. (India Infoline Sector Report, 2002).

3.3 Soft drinks ingredients

The major ingredients of soft drinks include the following:

Water

The major ingredient of soft drinks is ***water*** and it accounts for 86%-90% of the soft drink composition.

Aromatic substances

Aromatic substances are added to soft drinks to give a pleasant taste and better stability to the taste. These could be natural aromatic substances like ***caffeine*** obtainable from a variety of leaves, seeds and fruits. Identical aromatic substance can be obtained more simply and cheaply, in purer forms from raw materials other than plant raw materials and have characteristics which correspond exactly with their natural equivalents.

Sweeteners

There are many different types of sweeteners like ***sugar*** (sacharose), another major ingredient in soft drinks as it is highly nutritious and is the invaluable carrier of the fruit aromas. It is made from sugar-beet or sugar-cane or sweeteners found naturally in many fruits and vegetables. Two simple types of sugar are found in fruits - fructose (fruit-sugar) and glucose (grape-sugar). There are also low-calorie artificial sweeteners like saccharin and aspartame (nutra-sweet). ***Saccharin***, is a non-nutritious sweetener which is extremely sweet, stable gives no energy (no calories). ***Aspartame*** is a nutrient-sweetener built up of two amino-acids, asparagin acid and phenylalanine (200 times sweeter than saccharin).

Carbon dioxide

Carbon dioxide is another important ingredient added to the soft-drinks in liquid form. It makes the drink more refreshing through its stimulation of the mouth's mucous membranes adding a sensation that the soft drink is colder than it actually is. The carbon dioxide also brings out the aroma since the carbon dioxide bubbles 'drag with them' the aromatic components. It also checks microbiological growth.

Acids

The most common acids used in soft drinks are ***citric acid, phosphoric acid*** and ***malic acid***. The function of acidity in the drink is to balance the sweetness, make the drink fresh and thirst-quenching.

Colouring matter

Colour is added to soft drinks to make them presentable and appetizing. Brown drinks are colored with ***caramel*** (when sugar is heated, its colour changes to brown, it becomes less sweet and acquires a burnt taste) or betakarotin, which is also the dominant colouring agent in carrots and oranges.

Preservatives

Preservatives like ***natrium benzoate*** and ***potassium sorbate*** are added to increase the life of the product. Sulphur dioxide can also be used as a preservative.

Antioxidants

Antioxidants are substances, which prevent reactions that destroy aromatic substances in soft drinks. The most common antioxidant used is ***ascorbic acid***, i.e. Vitamin C

Other additives

Emulsifying agents, stabilizing agents, and thickening agents are also added so that the contents of the drinks remain evenly distributed. Examples of stabilizing agents and thickening agents are ***pectin***, which is obtained from citrus fruits or apples, and ***alginates*** and ***carraghen***, which is obtained from algae. (www.unesda-cisda.org/public/framehealth.htm)

3.4 Manufacturing process

The production of soft drinks begins by making a syrup of sugar and water and an aromatic mixture (soft drink extract) made of raw fruit-juice, other aromatic agents as well as an acid. Soft drinks are acidified either by the addition of fruit juice or by the inclusion of an acid such as that found naturally in fruits (malic or citric acid) or phosphoric acid which is generally used in cola drinks. The components are then mixed into a soft drink concentrate - a syrup.

The water used is treated to remove the oxygen to avoid reactions which destroy the aromatic substances (oxidation). In case of carbonated drinks, the water is 'carbonated' with carbon dioxide under high pressure. All the air is removed to prevent froth formation when the package is opened. The syrup and the carbonated water are mixed in the correct proportions. The drink is then bottled, canned or put into other packaging for retail sale. (www.unesda-cisda.org/public/framehealth.htm)

3.5. Regulations

In ***India*** the soft drinks Industry is virtually unregulated. Rule 65 of the Prevention of Food Adulteration Act 1954, regulates the presence of insecticides and pesticides in food but “food” is so defined in Rule 65 as to exclude “beverages”. This Rule does not apply to soft drinks. Subsection A.01.01 in appendix B of PFA defines standards of quality for non-alcoholic beverages but makes no mention of pesticide residues.

There are specifications for “sweetened aerated water with no fruit juice or pulp or containing less than 10% fruit juice or fruit pulp” in part II (D) of the Food Products Order (FPO), 1955. It regulates the general characteristics of a beverage. On the quality of basic raw materiel-water it merely says “ water used in the manufacture shall be potable and if required by the licensing officer shall be got examined chemically and bacteriologically by any recognized laboratory”. The order however does not define what is potable nor does it provide any scope to regulate pesticide residues.

The Bureau of Indian Standards (BIS) has laid down specifications IS :2346:1992 for “carbonated beverages”. In the foreword to this document water is mentioned as ingredient in carbonated beverages ”the quality of carbonated beverage depends on the quality of *various ingredients* that goes into its manufacture -

water, acidulants, sweetening agents, emulsifiers, and stabilizers, flavor, color and carbon dioxide being the important ones"(emphasis added). The document than prescribes the requirements and methods by which the quality of carbonated beverages may be gauged. As part of the process, it lists the various ingredients that can be used to make carbonated beverages. In this list there is no mention of water. This BIS standard is voluntary in nature (unlike mandatory certification for bottled water).

The BIS has another standard- IS :4251-1967 (reaffirmed 1977) which prescribes standards for "Quality tolerances for water for processed food Industry". In its foreword it says: "In processed food Industry, water is used for a number of purposes such as processing, washing, flushing and general usage and also for boiler feed and cooling". It does not mention its use in making soft drinks. In any case it does not mention anything about pesticide residues.

The soft drink industry remains not only unregulated but it is also exempted from the provisions of Industrial licensing under the Industries (Development and Regulations) Act, 1951.It gets a one time license to operate from the ministry of food processing Industries, which includes a no objection certificate from the local government and a water analysis report from a public health laboratory. It also requires a no objection certificate from the concerned State Pollution Control Board. There is no mandatory requirement for Environmental Impact Assessment or siting regulations for the Industry. Its use of water- largely unpriced ground water is not regulated.

In contrast in ***United States*** , regulations provide that the standard of water used to make soft drinks must be the same as that used to make bottled water. "Raw water " used to make bottled water falls under the purview of US Food and Drug Administration (FDA). According to the rules water consumed in this form is a " food" therefore water used as an ingredient in beverages must meet the same standards as bottled water.

In addition to the US Safe Drinking Water Act a federal legislation under the Environmental Protection Agency **(EPA)** stipulates drinking water standards to protect public health. Its primary standards are legally enforceable nation wide. In the state of Massachusetts regulations stipulate that the source water used for bottled water and carbonated drinks must meet the Federal EPA National Primary Drinking water standards.

In ***Europe,*** the European Economic Council Directive 80/778/EEC and 98/83/EEC lay down the standards for quality of drinking water intended for human consumption. Such water it clearly specifies, shall include water used in any food production undertaking for the manufacture, processing, preservation or marketing of products or substances intended for human consumption, or effecting the wholesomeness of the food stuff in its finished form".

4. STANDARD FOLLOWED

Different International Agencies -- World Health Organization (WHO), Food and Agriculture Organization (FAO), the US Environment Protection Agency (USEPA)/ Food and Drug Administration Act(FDA) stipulate different limits for different pesticides. The European Economic Community's Directive (80/778/EEC) on "quality of water intended for human consumption" regulated at European level sets maximum admissible concentration for individual pesticides and related products in drinking water at 0.1 µg/L (0.0001mg/L) to ensure that the concentration is low enough to be toxic to human beings. This Directive shall be replaced and repealed by Directive 98/83/EEC on "quality of water intended for human consumption" with effect from December 25, 2003. The limit for pesticides is the same as in Directive 80/778/EEC except for aldrin, dieldin and heptachlor and heptachlor epoxide for which the parametric value has been made even more stringent at 0.030 µg/L (0.00003 mg / L).

In the present work, PML used European norms to compare the results of pesticide residue analysis in soft-drinks as norms for multiple residues do not exist in most regulations. These norms – for single and multiple residues -- have been recently gazetted by the Ministry of Health and will be applicable to the packaged drinking water in India.

5. LITERATURE REVIEW

The major ingredients found in soft drinks like water, aromatic substances, sweeteners carbon dioxide, coloring matter, acids, preservatives, antioxidants and other additives are present in different composition in different soft drinks. There is a growing concern in the medical and scientific communities about the harmful effects associated with the consumption of carbonated soft drinks.

Studies have shown that soft drinks can lead to numerous health problems. Some of these health problems are obesity, diabetes, tooth decay, osteoporosis, nutritional deficiencies, heart disease, and many neurological disorders. Various ingredients used in soft drinks can have adverse effects.

A common problem that is associated with consumption of a large number of soft drinks is the increased acid levels (citric, malic and phosphoric acid) throughout the body causing gastronomic distress due to the inflammation of the stomach and erosion of the stomach lining leading to painful stomachache as the stomach which maintains a very delicate acid-alkaline balance can be set out of balance by the consumption to a large number of soft drinks, which can create a constant acid state leading to indigestion and gassiness.

Carbon dioxide emitted from soft drinks is a waste product that humans excrete and can be harmful when ingressed. Large amounts of sugar, bubbles caused by carbon dioxide, and phosphoric acid that are found in soft drinks remove nutritious minerals from bones allowing the bones to become weak and increasing the risk for them to break. This is done by the phosphoric acid disrupting the calcium-phosphorous ratio, which dissolves calcium from the bones (Source: http://www.kauhawaii.com/softdrinks.html).

It has been reported that enamel, which is composed of cementum and dentin, naturally protects teeth. Acidic drinks increase dentin permeability by opening dentinal tubules leaving a dentin surface completely uncovered and removing the smear layer. (Prati C *et al,* 2003) . Dental cavities are often associated with consumption of carbonated beverage because the amount of sugars that are consumed is important in forming caries caused by the bacteria *mutans streptocci,* which is a part of dental plaque. *Lactobacillus* and *Actinomyces viscosus* are two other kinds of bacteria that adversely affect teeth and survive well in very acidic environments, produce high amounts of acid from sugars and other types of acid.

Comparison of the effects of aspartame-sweetened and sucrose sweetened soft drinks on food intake and appetite ratings of female restrained eaters suggested that substitution of sucrose-sweetened drinks for diet drinks does not reduce energy intake and may even result in higher intake during the subsequent day. (Lavin J H *et al*, 1997)

Caffeine- a methylated xanthine acts as a mild central nervous system stimulant present in carbonated beverages. Large amounts of caffeine consumption can cause diseases and disorders such as insomnia, nervousness, anxiety, irritability, and deviations from the normal heart rate. A major concern about caffeine is that it increases the excretion of calcium in urine, which increases the risk for osteoporosis in heavy caffeine consumers. Some epidemiological studies describe exposure to caffeine during pregnancy as well as the occurrence of congenital malformations, fetal growth retardation, miscarriages (spontaneous abortions), behavioural effects and maternal fertility problems. (Christian WS *et al* , 2001)

Many soft drinks contain caramel which is added to give dark appearance. The caramel coloring used in soft drinks may be a carcinogen. (Source: http://members.aol.com/profchm/Soft.html)

Mineral waters and soft drinks were investigated for erosive potential of human teeth and showed that mineral waters offer a safe alternative to more acidic beverages and their complex mineral ion compositions may positively influence any dissolution process at the tooth surface.(Parry J *et al,* 2001) .

A study was conducted on concentrations of aluminum in drinking waters, fruit juices and soft showed that aluminum ranged from 4.2 to 165.3 μg/l in drinking water, 49.3 to 1,144.6 μg/l in fruit juices and from 44.6 to 1053.3 μg/L in soft drinks. Aluminum in high quantities is known to have adverse effects. (Lopez FF *et al*, 2002)

According to a study conducted on soft drinks, mineral waters and juices available in the Danish market, dissolution of enamel increased logarithmically inversely with the pH of the drink. Orange juice pH 4.0 exhibited high buffering effect, supplemented with 40 mmol/l calcium and 30 mmol/l phosphate did not erode the enamel as the calcium and phosphate saturated the drink with respect to apatite. The capability of a soft drink or a juice to erode dental enamel depends not only on pH of the drink but also on the buffering effect. The latter is the ability of the drink to resist a change in pH and may add to the effect of change in pH. (Larsen MJ *et al*, 1999)

According to Food Safety Information Bulletin, 98, July 1998, Benzene, a hydrocarbon was detected in some carbonated drinks in UK. Investigations revealed that benzene entered into the soft drinks through the contaminated carbon dioxide used for carbonation. Benzene is a genotoxic agent.

6. MATERIALS AND METHODS

6.1. Sampling methodology

Soft drink bottles of different brands and flavors were purchased from various markets in Delhi during the month of May 2003. Extraction and pesticide residue analysis was carried out at the PML during the same month. Three samples of each of the 12 different brands (thirty six samples) were analyzed for 16 organochlorines, 12 organophosphorus pesticides and 4 pyrethroides. The 16 organochlorines, which were, tested cover wide spectrum of chlorinated pesticides. The organophosphorus and pyrethroides pesticides were tested are the most commonly used organophosphorus pesticides in India. Details of the samples purchased from India are in Annexure IA and Annexure IB gives the details of the soft drink samples procured from USA and tested in PML.

6.2. Equipments

Gas Chromatographs used for pesticide residue analysis were Thermoquest-Trace GC with the ^{63}Ni selective Electron-Capture Detectors with advanced software (Chromcard-32 bit Ver 1.06 October 98) and Nucon –GC- 5765 series equipped with Nitogen Phosphorus detector. GC column employed were capillary column, DB-17, J & W make and DB-5, J & W make (for cross verification). Rotatory evaporator (Buchi type) and a 10-μl syringe from Hamilton Co. were employed.

6.3. Solvents

All the solvents acetone, methylene chloride, hexane (HPLC) grade used for the analysis were purchased from E-Merck.

6.4. Chemicals

Pesticide reference standards were obtained from Sigma Chemicals USA. Weighing appropriate amounts of active ingredients in a 40 ml brown bottle with a Teflon-lined screw cap and dissolving the weighed standard with HPLC grade hexane prepared individual stock solutions. The resulting concentration was corrected for the stated purity. Appropriate aliquots of the obtained solutions were subsequently mixed

into a 50 ml volumetric flask, which was completed to volume with hexane. Anhydrous sodium sulfate, sodium chloride were purchased from s. d. Fine Chem Ltd.

6.5.Sample extraction and Clean up

The samples were analysed for organochlorines by using EPA method 8081A for organochlorines by Gas chromatography and EPA Method 8141A for organophosphorus compounds by gas chromatography: Capillary column technique.

Extraction: Soft drink samples were shaken well and filtered through Whatman filter paper no.1.pH of the samples were checked and it was found to be acidic, pH of all the samples was neutralised by using 0.1 N NaOH. After filtration, 500 ml of sample was taken in a 1 L capacity separatory funnel and 20 ml of saturated sodium chloride solution was added. The water sample was partitioned with 100 ml of methylene chloride (thrice) by shaking the separatory funnel vigorously for 2-3 min releasing the pressure intermittently. The layers were allowed to separate. The three extracts of methylene chloride layers were combined and passed through anhydrous sodium sulfate and concentrated to about 1-2 ml using rotary vacuum evaporator. Again 10 ml methylene chloride was added for adsorption chromatography

Clean up: Cleanup was done by EPA Method 3620B- Florisil clean up by column chromatography. Florisil was activated at 130^0 C overnight and cooled in a dessicator before use. Weight of florisil taken was predetermined by calibration using lauric acid. 20g florisil was packed in the 20 mm ID chromatographic column. And anhydrous sodium sulfate was added to the top of the florisil column(1-2 cm) and the column was pre-eluted with hexane. The methylene chloride extract was added to the top of the column and then eluted with Hexane (100ml), 30% methylene chloride in hexane (100ml) and finally with methylene chloride (100ml). Eluent was collected and concentrated to dryness. Final samples were prepared in Hexane (HPLC grade) and analyzed by GC-ECD for organochlorines and synthetic pyrethroides and GC equipped with NPD for organophosphorus pesticides.

6.6. Sample Analysis

For Organochlorines and synthetic pyrethroides

Organochlorine and synthetic pyrethroides were analysed by Gas Chromatograph (Thermoquest-Trace GC) with the ^{63} Ni selective electron-capture detector. This detector allows the detection of contaminants at trace level concentrations in the lower ppb range in the presence of a multitude of compounds extracted from the matrix to which the detector does not respond. The capillary column used was DB- 17- coated with 50% phenyl, 50% methyl polysiloxane (length 30m, ID 0.25 mm and film 0.25μm). The carrier gas and the makeup gas was nitrogen with a 0.4 ml/min and 60 ml/min-flow rate respectively employing the split less mode. 2.0μl of the final extract was injected at a temperature of 270^0 C. The oven temperature was kept at 120^0C with a hold time of 1 minute, then from 120^0C to 205 ^{0} C at a rate of 25^0 C/minute with a hold time of 1 minute then finally from 205 to 290^0C at a rate of 2^0 C / minute with a hold time of 12 min. The total run length was 59.9 minutes. The detector was maintained at 320 ^{0}C. Peak identification was performed by the GC software (Chromcard-32 bit Ver 1.06 October 98) calibration table set up with a relative retention time window of 0.65%.

For Organophosphorus

Organophosphorus pesticides were analysed by Gas Chromatograpph (Nucon –5765 series equipped with Nitogen Phosphorus detector). The capillary column used was another GLC capillary column – DB- 17-coated with 50% phenyl, 50% methyl polysiloxane (length 30m, ID 0.25 mm and film 0.25μm). The carrier

gas and the makeup gas was nitrogen with a 1.3 ml/min and 25-ml/min-flow rate respectively, Hydrogen at 8ml/min and Air at 80ml/min respectively employing the split less mode. 2.0µl of the final extract was injected at a temperature of 270^0 C. The oven temperature was kept at 120^0C with a hold time of 1 minute, then from 120^0C to 205 ^{0} C at a rate of 25^0 C/minute with a hold time of 1 minute then finally from 205 to 270^0C at a rate of 2^0 C / minute with a hold time of 1 min. The total run length was 38.90 minutes. The detector was maintained at 300 ^{0}C.

The samples were calibrated (retention time, area count) against standard mixture of known concentration of 12 organophosphorus pesticides. Each peak was characterized by comparing relative retention time with those of standards. Analysis also included blank sample and blank samples (8) fortified with standard solution of known concentration (low and high level) and were analysed as mentioned above.

6.7 Calculations

All calculations were done as described in USEPA method and the amount of residues in samples were obtained**.** When applicable, analyte concentrations were corrected for any amount of pesticides incurred in the blank (non-fortified samples)**.**

Recovery:

Recovery experiment was done as mentioned in extraction methodology, a known amount of standard pesticides was injected in the sample before extraction, to check how much were recovered after complete exercise. Generally with the ten set of extraction one recovery experiment was performed. Recovery was 90% for OCs, 85% for OPs and 88% for synthetic pyrethroides.

The reproducibility of results for all the pesticides was 95 percent and above for all the samples. However, the mean average reading of a individual sample analyzed in triplicate, has been reported in the results.

6.8 Confirmation and Quantification

Spiking

Identifications were confirmed by spiking the sample with known standard only to confirm the unknown Thin layer chromatography of the pooled extract was also performed. Solvent systems used were hexane: benzene (4:1, v/v). The spots corresponding to the position of standards were scraped, extracted and analysed by GLC.

Dual column

The identifications were crosschecked with another column - DB-5 coated with 5% diphenyl and 95% dimethylpolysiloxane length of different polarity. Elution pattern was different from the elution pattern in DB-17.

7 RESULTS AND DISCUSSION

A total of 36 soft drinks samples of 12 brands (3 each- different batches) were tested for 16 organochlorine pesticides, 12 organophosphorus and 4 pyrethroides pesticides most commonly used in India.

Organochlorines:

Amongst organochlorines - **γ– HCH (Lindane)** was detected in 100% of the 36 samples analysed. The range of concentration in the 12 brands varied from 0.0008-0.0042 mg/L. Minimum

concentration was detected in Diet Pepsi- 0.0008 mg/L (8 times the EEC limit) and maximum concentration was detected in Mirinda lemon- 0.0042 mg/L which is 42 times higher than EEC limit for drinking water i.e. 0.0001mg/L limit for individual pesticide as per the Drinking Water Directive 80/778/EEC. **Average concentration of lindane detected in all the samples was 0.0021 mg/L, which is 21 times higher than the EEC limit.** (ANNEXURE:II)

Hexachlorcyclohexane, previously called BHC (benzene hexachloride), is a mixture of eight isomers of which five are found in the crude product (α, β, γ, δ, ε). Only γ isomer of HCH was detected in soft drink samples and other isomers of HCH (α, β and δ) were within the European norms, which might be because γ-HCH is more resistant to biological and chemical degradation under aerobic conditions (El beit *et al*, 1981) and is most commonly used. γ-HCH (Lindane) appears in the list of pesticides for restricted use (the use of lindane formulations generating smoke for indoor use is prohibited; it can be used for the control of insect pests of field crops) and has powerful insecticidal properties. and is very effective against a wide variety of insects, including domestic insects and mosquitoes.

DDT along with its metabolites (DDD+DDE) was detected in 81% of the samples i.e 29 out of 36. The range of concentration in the 12 brands varied from 0.0001 to 0.0042 mg/L. Minimum concentration was detected in Blue-Pepsi. Maximum concentration was detected in Mirinda lemon -- 0.0042 mg/L which is 42 times higher than the maximum EEC limit. **Average concentration of total DDT in all the samples was 0.0015 mg/L, which is 15 times higher than the EEC limit.**

Use of DDT is banned in agriculture. However its restricted use is allowed in public health sector (10,000 MT per annum). DDT was detected in most of the samples perhaps due to its persistent nature. Since DDT is known to undergo metabolic conversion and dehydrochlorination, presence of metabolites of DDT i. e DDD and DDE encountered in this study might be due to such metabolic processes.

Heptachlor (banned with effect from September 20, 1996), aldrin (banned with effect from September 20, 1996), dieldrin (banned with effect from May, 1990), endosulfan, methoxychlor and chlordane were not detected in any of the samples.

Organophosphorus

Amongst organophosphorus pesticides - **Chlorpyrifos** was detected in 100% of the 36 samples analysed . The range of concentration in the 12 brands varied from 0.0015-0.0072 mg/L. Minimum concentration of 0.0015 mg/L was detected in Sprite, a Coke product and maximum was detected in Mirinda lemon flavor, a Pepsico product, which is 72 times higher than the EEC limit of 0.0001 mg/L for individual pesticides. **Average concentration of 0.0042 mg/L of chlorpyrifos was detected in all the samples that is 42 times higher than the EEC limit.**

Chlorpyrifos is a moderately persistent insecticide effective against mosquito and fly larvae, cabbage root fly, aphids. Chlorpyrifos has become one of the most widely applied insecticides in homes restaurants against cockroaches, termites.

Malathion was present in 97% of the 36 samples analysed. The range of concentration in the 12 brands varied from 0.0013 to 0.0196 mg/L. Minimum concentration of 0.0013 mg/L was detected in Sprite and maximum concentration was 0.0196 mg/L which is 196 times higher than the EEC limit. The **average concentration of malathion (0.0087 mg/L) was 87 times higher than the EEC limit.** Malathion was present in all samples except one sample of Sprite (BN 787).

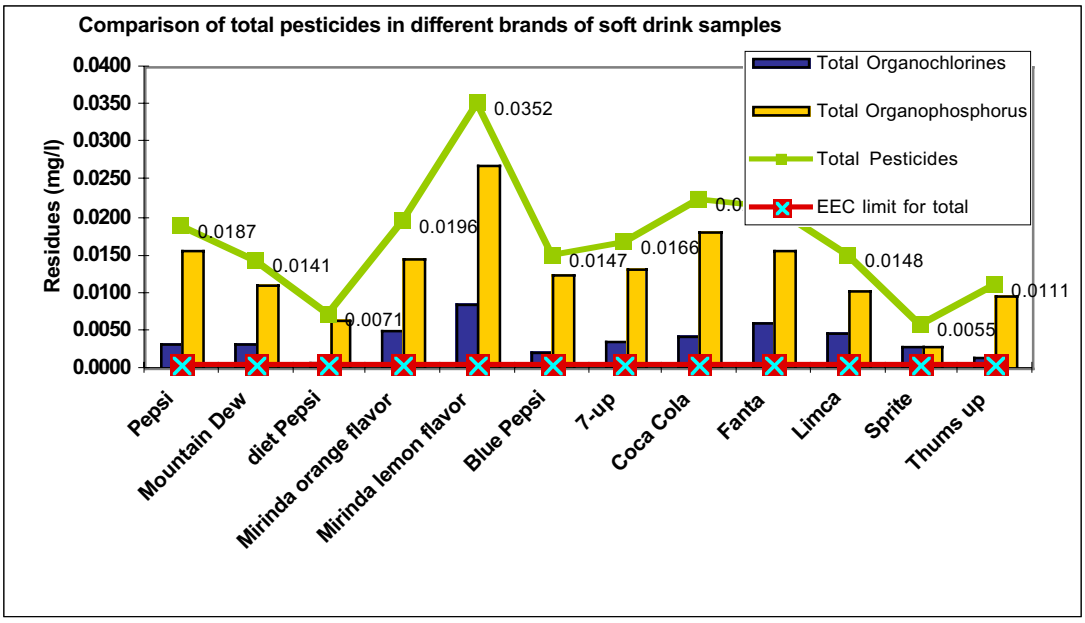
Malathion is an important and widely used contact insecticide and acaricide for the control of aphids, red spider mites, leaf hoppers and thrips on a wide range of vegetable and other crops. It is also

used to control insect vectors like mosquitoes. It is rapidly absorbed by practically all routes including the gastrointestinal tract, skin, mucous membranes, and lungs. Malathion requires conversion to malaoxon to become an active anticholinesterase agent. Most of the occupational evidence indicates a low chronic toxicity for malathion.

The organophosphorus pesticides are less persistent in water, soil, food and feed for animals than the organochorine pesticides; however they are relatively soluble in water and are highly toxic. They break down into nontoxic metabolites. (ANNEXURE:III)

Synthetic Pyrethroides

Out of 4 synthetic pyrethroides - permethrin, detamethrin, cypermethrin and fenavalerate, none was detected in any of the soft drink samples.



Total pesticides residues

The range of concentration **of total organochlorines** in the 12 brands varied from 0.0008-0.0084 mg/L. Minimum concentration of 0.0008 mg/L was detected in Diet Pepsi and maximum concentration was detected in Mirinda lemon- 0.0084 mg/L. **Average concentration of total organochlorines was 0.0038 mg/L in all the 36 samples.**

The range of concentration of **total organophosphorus** in the 12 brands varied from 0.0028-0.0268 mg/L. Minimum concentration of 0.0028 mg/L was detected in Sprite and maximum concentration of 0.0268 mg/L was detected in Mirinda Lemon. **Average concentration was 0.0129 mg/L in the 36 samples analysed.**

The range of concentration **of total pesticides** (organochlorines and organophosphorus) pesticides in the 12 brands varied from 0.0055-0.0352 mg/L. Minimum value of 0.0055 mg/L was detected in Sprite which is 11 times higher than the EEC limit and maximum value was detected in Mirinda lemon-0.0352 mg/ which is 75 times higher than the total EEC limit of 0.0005 mg/L. **Average concentration of total pesticides detected was 0.0168, which is 34 times higher than the total EEC limit**. (ANNEXURE:IV and Table 2)

The variation in different brands could be due to the different ingredients present in different brands, composition and pH.

Table: 2 Average residues in different brands of soft drink samples

S. No.	Brand	Total Organochlorines (mg/L)	Total Organophosphorus (mg/L)	Total Pesticides (mg/L)	EEC limit for total pesticides -0.0005 mg/L	Deviation from EEC limit (No. of times)
1	Pepsi	0.0032	0.0155	0.0187	0.0005	37
2	Mountain Dew	0.0033	0.0108	0.0141	0.0005	28
3	Diet Pepsi	0.0008	0.0063	0.0071	0.0005	14
4	Mirinda orange	0.0050	0.0146	0.0196	0.0005	39
5	Mirinda lemon	0.0084	0.0268	0.0352	0.0005	**70**
6	Blue Pepsi	0.0022	0.0125	0.0147	0.0005	29
7	7-up	0.0036	0.0130	0.0166	0.0005	33
8	Coca Cola	0.0044	0.0179	0.0223	0.0005	45
9	Fanta	0.0060	0.0154	0.0214	0.0005	43
10	Limca	0.0047	0.0101	0.0148	0.0005	30
11	Sprite	0.0027	0.0028	0.0055	0.0005	**11**
12	Thums up	0.0015	0.0096	0.0111	0.0005	22

Note: Average of 3 samples .

Total pesticide residues in the 36 soft drink samples manufactured in India was **0.0168 mg/l** which is **34 times the** EEC limit for total pesticides. Total pesticide residues in all brands of PepsiCo products (India) were 0.0180 mg/L, which is 36 times the total pesticide limit and in all brands of Coca - Cola Co (India) were 0.0150mg/L which is 30 times the total EEC limit for pesticides.

Comparison of Indian soft drink samples with US samples

The Indian Coca-Cola had pesticide residues 45 times higher than the EEC norms, while no pesticides were detected in the same product procured from the US. Similarly, the Indian Pepsi had pesticide residues 37 times higher than EEC norms while its US counterpart was without any pesticide residues.

8. HEALTH IMPACTS OF PESTICIDES

The four commonly found pesticides detected in the soft drink samples were – Lindane, DDT and its metabolites, Malathion and chlorpyrifos. Health impacts of these pesticides are as follows:

LINDANE

All isomers of HCH are stored in fats; the gamma isomer of HCH (Lindane) which was also found in soft dink samples analysed is stored at much larger rates than the other isomers, which are more readily metabolized and eliminated. Lindane is absorbed through respiratory, digestive or cutaneous routes and

accumulates in fat tissues. It damages human liver, kidney neural and immune systems and induces birth defects cancer and death. Chronic administration results in endocrine disruption in birds as well as in mammals.(Pages N *et al*, 2002)

Treatment with 1-40 mg of lindane/kg of body weight disrupts testicular morphology, decreases spermatogenesis, inhibits testicular steroidogenesis, reduces plasma androgen concentrations and may adversely affect reproductive performance in males. In females lindane disrupts the estrous cycle, reduces serum estrogen and progesterone levels decreases sexual receptivity

There has been a link of Lindane to immune system. Lindane is a ***potent carcinogen***. Rats exposed to gamma HCH showed evidence of liver cancer. (ATSDR, 1989). Chronic exposure to Lindane has been liked to increase in the risk of cancer of the aerodigestive tract and strong ***genotoxic*** effects on human tonsillar epithelium.(Source: J. Soc Biol, 2002 196(4):339-48)

Lindane was found to be ***estrogenic*** to female rats and mice, and also caused the testes of male rats to become atrophied. Seminiferous tubules and Leydig cells (important for production of sperms) were completely degenerated at dozes of 8 mg/Kg/day over a 10-day period (Gallo MA and Lawryk NJ, 1991).

The absorption of high doses of gamma - HCH is particularly toxic for the central nervous system and for the female and male reproduction apparatus in mammals where lindane is considered as an endocrine disruptor. Lindane is ***highly lipophilic*** and incorporates into biological membranes according to the following sequence: mitochondria >sarcoplasmic reticulum >myelin >brain microsomes >erythrocytes. Lindane exerts a stimulating action on synaptic transmission and inhibits the chloride current activated by gamma-amino butyric acid (GABA) of many muscular and nervous preparations by interacting with the receptors GABA - chloride channel complex. It seems to affect calcium homeostasis of many tissues. Lindane affects the excitable membranes and the cardio circulatory system. These alterations (may) represent a potential risk for human health. (Sauviat M *et al,* 2002).

DDT and its metabolites

DDT (dichlorodiphenyltrichloroethane) and its metabolites were detected in 81% of the soft drink samples. They have been linked to altered sexual development in various species, to a decrease semen quality and to increased risk of breast cancer in women. (Sharpe RM, *et al* 1993; Carlsen E *et al*, 1992; Stone R *et al*, 1994). DDT and its metabolites have also been shown to mimic ***estrogen***, binding to and activating the estrogen receptors (ER's) thereby often producing estrogen like effects (Jaga K, 2000) They may alter a number of harmful estrogen-regulated health effects in humans such as breast cancer (Coceo P *et al*, 2002) , spontaneous abortion (Korrick SA, *et al*, 2001), reduced bone mineral density (Beard J *et al*, 2000). DDT and its metabolites because of their ***lipophilicity*** and long half lives accumulate in the food chain. Their weak oestrogenic effects may result from altered metabolism and competition for binding to cytosolic and nuclear receptors of steroid hormones. (Levine R, *et al* 1991)

DDT reportedly induces cancer in animals, mimics estrogen activity, induces antiandrogen effects, and impairs Natural Killer (NK) cells and T lymphocyte responses. Occupational exposure to insecticides resulted in frequent infections and ***immunological abnormalities*** DDT, dichlorodiphenyldichloroethylene (DDE), and dichlorodiphenyldichloroethane (DDD) in blood levels have been associated with several immune parameters in patients occupationally exposed to insecticides. The majority of 49 patients who worked as farmers or farmhands in the former German Democratic Republic, were contaminated with more than 1 chemical-- most commonly DDE, PCBs, and HCB and 80% of them had been exposed for more than 20 years. (Daniel V *et al*, 2002).

Comparison of blood levels of HCB and total DDT in 159 women with breast cancer and 250 presumably healthy controls showed that mean levels of total DDT and HCB were significantly higher for

breast cancer patients than for controls. No differences in serum levels of total DDT or HCB were found between estrogen receptor positive and estrogen receptor negative patients with ***breast cancer*** which implies that persistent pollutants may occur in higher concentrations in blood samples from breast cancer patients from controls. (Charlier C *et al*, 2003)

There are mixture effects even when each mixture component is present at concentrations that individually produces insignificant effects. Lifetime treatment of mice with DDT induced liver tumours in a doze related manner and the tumors included overtly metastasizing hepatoblastomas (Hoyer AP *et al.*, 1998). Main metabolites of DDT (pp' DDE and pp' DDD) are both ***carcinogenic***. Exposure to DDE resulted in high incidence of liver tumors in both male and female mice. The combined exposure to DDE and DDD resulted in a marked increase and early appearance of liver tumors in both sexes (Turosov VS *et al* ; 1973).

Mixture of 4 organochlorines (op' DDT, pp' DDE, β-BHC and pp' DDT) acted together to produce proliferative effects in MCF-7 human breast cancer cells and the combined effect was additive (Gertrudis C *et al* 2001). A study suggests that exposure to a mixture of DDT, HCH and endosulfan and decreased fertility in males, an increase in birth defects and in neonatal deaths (Rupa DS, 1991). Detoxification processes both in humans and animals involve conversion of DDT to less toxic acetate; little is known about variations from person to person in these detoxification mechanisms, and even less about intermediate metabolism concerned. Regardless of detoxification mechanisms, DDT is stored cumulatively in body fat and excretion is extremely slow even after intake ceases. (Smith MI, 1946)

CHLORPYRIFOS

Chlorpyrifos, one of the most widely used organophosphorus pesticide has been reported to be a developmental neyurotoxicant specifically targeting the immature brain. (Barone *et al* 2000; Pope 1999). Fetal and childhood exposure to chlorpyrifos has raised concerns about developmental neurotoxicity. Exposure to chlorpyrifos resulted in adverse effects on brain cell development and cholinergic biomarkers. Neonatal rats were found to be more sensitive to chlorpyrifos than the fetal rats and animals exposed prenatally developed behavioral deficits in adolescence and adulthood. (Qio D *et al*, 2003). Developmental neurotoxicity of chlorpyrifos is thought to involve both neurons and glia, increasing the vulnerability of the developing brain. The vulnerability increases from the gestational exposure through later periods of development which glial neuronal interactions influence brain architectural, circuitary and function. Exposures occurring during childhood are as important as those occurring prenatally.

Chlorpyrifos is a suspected ***neuroterratogen***. Recent findings suggest that chlorpyrifos has a shifting cellular target, initially impairing the development of neurons and subsequently affecting the glia, which develop much later.(Garcia *et al* 2001, 2002). It was evaluated for potential developmental toxicity in rats and was found to show ***fetotoxic and terratogenic effects*** at maternal doze of 25 mg/kg per day, a doze that also produced maternal toxicity. Fetal weight and viability were decreased and fetal death and early resorption increased at this doze. (Farag AT *et al*, 2003). Studies carried out to evaluate potentiel toxicological effects of chlorpyrifos in rats showed that repeated exposure to subthreshold dozes of chlorpyrifos may lead to growth retardation, behavioural abnormalities and muscle weakness. (Tery AV Jr. *et al*, 2003)

Chronic exposure to chlorpyrifos has been shown to cause ***immunological change***. Comparison of chronic health complaints of twenty-nine individuals exposed to chlorpyrifos with respect to peripheral lymphocyte phenotypes; autoantibodies (nucleic acids and nucleoproteins, parietal cell, brush border, mitochondria, smooth muscle, thyroid gland, and central nervous system/peripheral nervous system myelin); mitogenesis to phytohemagglutinin and concanavillin and compared with 3 control groups (i.e., 1 positive 2 negative) showed an increase in CD 26 expression, a decrease in percentage of CD5 phenotype, decreased mitogenesis in response to phytohemagglutinin and concanavillin, and an increased frequency of

autoantibodies. The alterations in these peripheral blood markers were unaffected by medication, age, sex, or season. (Thrasher JD *et al*, 2002)

Chlorpyrifos toxicity becomes acute if transformed to ***chlorpyrifos oxon***, which is a potent anticholine esterase, 1000 times more toxic than chlorpyrifos. Active chlorine dispersed in water causes rapid abiotic transformation of chlorpyrifos to chlorpyrifos oxon. Chlorination is commonly used for treatment of domestic water supplies which is a new concern about the safety of domestic use of chlorpyrifos products. (Wu J *et al*, 2003. The effects of chlorpyrifos and its major metabolite chlorpyrifos oxon have been studied in two in vitro models, neuronotypic and gliotypic C6 cells. Chlorpyrifos inhibited DNA synthesis in both cell lines, but had greater effect on gliotypic cells. Chlopyrifos oxon, the active metabolite that **inhibits cholinesterase**, also decreased DNA synthesis in PC-12 and C-6 cells with a preferential effect on the latter. Diazinon also inhibits DNA synthesis with preference towards C-6 cells but is less effective than chlorpyrifos (Qiao D *et al* , 2001).

MALATHION

Malathion, a known ***cholinesterase inhibito*** *r*, responsible for the hydrolysis of body choline esters, including acetylcholine at cholinergic receptors. Primary site of action in insects is nervous system. It was found to ***induce progression of malignant transformation*** in epithelial cells in the rat mammary glands. It has been shown to induce changes in the epithelium of rat mammary glands, influencing the process of ***carcinogenesis;*** such alterations occur at the level of nervous system by increasing the cholinergic stimulation (Vladimir T *et al* , 2002). Malathion induces alterations in actin cytoskeleton and in cell adhesion of cultured breast carcinoma cells. (Cabello G *et al* 2003). It has been reported to induce a slight increse in the incidence of ***chromosomal aberrations in bone marrow cells*** of rats exposed in vivo. (Kawachi T *et al* , 1980). Malathion caused a significant increase in sister chromatid exchange in human foetal lung fibroplasts after a single doze of 40 μg/l or a double doze of 20 μg/l. (Nicholas AH *et al* , 1979). (Cabello G,2003).

In contrast to potent carcinogens, which induce mammary carcinomas in 100 per cent of intact females, parathion and malathion induced 14.3 and 24.3 per cent of mammary carcinomas. Type of tumors induced had papillary adenmatous patterns and ductal carcinomas with cribiform pattern (Willings SR *et al* , 1975). Malathion incorporated through epithelium of skin, mouth and respiratory tract, are activated in the liver by enzymatic processes producing malaoxon. (Silman I, and Futerman A., 1987). It also has been shown to cause ***birth defects*** in a variety of wildlife and at levels lower than other pesticides. When administered to adult animals, malathion and related thiophosphonates stimulate, and subsequently inhibit, the nicotinic sites in skeletal muscle, resulting in muscle weakness and paralysis. Neonates (newborn babies) are far more sensitive to these agents than adults, mainly because of a slower rate of detoxification of the metabolite (the metabolite in this case would be the liver breakdown product of malathion – malaoxon which has been shown to be far more toxic than malathion itself. (*Source:* Teratology, 36:7-9, 1987)

It was found to cause DNA abnormalities at all doses (0.02, 0.2, 2 and 20 ug/ml when added to human blood cells drawn from three healthy non-smoking men, aged 23, 24 and 25. It causes a dose-dependent increase in chromosomal aberrations as well as sister chromatid exchanges in human leukocyte cultures. A dose dependent decrease in mitotic index was also observed which suggests that malathion is a ***mild mutagen*** and at higher concentrations it might cause ***genotoxicity*** in humans. (Source*:* Mutation Research, 301:13-17, 1993).

9. CONCLUSIONS

From analysis of 36 samples of 12 different brands of soft drink samples it can be concluded that:

- Out of the 16 organochlorines, 12 organophosphorus and 4 synthetic pyrethroides analysed soft drink samples, Lindane, DDT and its metabolites, Malathion and Chlorpyrifos were most commonly found in 36 soft drink samples tested.
- Lindane (γ -Hexachlorocyclohexane), a potent carcinogen was detected in 100% of the samples analysed. The average concentration detected in all the samples was 0.0021 mg/L, which is 21 times higher than the EEC limit for individual pesticides. Lindane is the most toxic of all the isomers of HCH and has powerful insecticidal properties and is used for the control of insects of field crops and pests in houses.
- DDT (dichlorodiphenyltrichloroethane) was detected in 81 % of the samples analysed. The average concentration of total DDT (DDT+DDD+DDE) in all the samples was 0.0015 mg/L, which is 15 times higher than the EEC limit.
- Chlorpyrifos, a suspected neuroterratogen was detected in 100% of the 36 samples analysed with an average concentration of 0.0042 mg/L of chlorpyrifos which is 42 times higher than the prescribed EEC limit.
- Malathion was present in 97% of the samples analysed with an average concentration of malathion (0.0087 mg/L) which is 87 times higher than the EEC limit. Malathion was present in all samples except one sample of Sprite (BN 787).
- *Synthetic Pyrethroides* Out of 4 synthetic pesticides-cypermethrin, deltamethrin, fenvalerate and permethrin analysed, none was detected in any of the samples.
- The average concentration of total organochlorines was 0.0038 mg/L, that of total organophosphorus was 0.0129 mg/L and the level of total pesticides detected was 0.0168 mg/L, which is 34 times higher than the total EEC limit. The variation in different brands could be due to the different ingredients present in different brands, composition and pH.
- **No pesticide** residues were detected in the Coca-Cola and Pepsi samples from USA manufactured by the same multinationals.

10. REFERENCES

Agency for Toxic Substances and Disease Registry (ATSDR) (1989) Public Health Statement for Hexachlorocyclohexane. Atlanta, GA: US Department of Health and Human Services.

Barone S, Das KP, Lassiter TL, White LD (2000) Vulnerable process of nervous system development. A review of markers and methods. *Neurotoxicology* **21**: 15-36.

Beard J *et al* (2000) Reduced Bone Mineral Density and Decreased Sperm Counts. *Arch Environ Health* **55**, 177-180.

Cabello G , Galaz S, Botella L, Calaf G, Pacheco M, Stockert JC, Villanueva A, Canete M, Juarranz A (2003). The pesticide malathion induces alterations in actin cytoskeleton and in cell adhesion of cultured breast carcinoma cells. *Int J Oncol.* Sep, **23** (3) : 697-704.

Carlsen E, Giwercman A, Keiding N, Skakkebaek NE (1992) Evidence for decreasing quality of semen during past 50 years. *British Medical Journal* **305**:609-13.

Christian WS and Brent RL (2001) Terratogen Update: Evaluation of the reproductive and development risks of caffeine.*Terratology* **64**:51-78.

Coceo Petal (2002) Cancer mortality and environmental exposure to DDE in United States. *Environ Health Perspect ive* **108**: 1-4.

Curtis KM (1999) The effect of pesticide exposure on time to pregnancy. *Epidemiology* **10** (2) : 112-117.

Daniel V, *et al* (2002) Associations of dichlorodiphenyltrichloroethane (DDT) 4.4 and dischlorodiphenyldichloroethylene (DDE) 4.4 blood levels with plasma IL-4. *Arch Environ Health* Nov-Dec; **57**(6):541-7

El Beit IOD, Wheelock JV, Cotton DE (1981) Factors affecting soil residues of dieldrin, endosulfan, gamma HCH, dimethoate. *Ecotoxicol and Environ Saf* **5 :** 135-60

Farag AT, El Okazy AM, El Aswed AF(2003) Developmental toxicity study of chlorpyrifos in rats. *Reprod Toxicol* March-April;**17**(2):203-8

Gallo M A and Lawryk NJ (1991) Organophosphorus Pesticides. *In* Handbook of Pesticide Toxicology. Hayes, W. J., Jr. and Laws, E. R., Jr., Eds. Academic Press, New York, NY pp 3-5

Garcia SJ, Seidler FJ, Crumpton TL, Slotkin TA. (2000) Does the developmental toxicity of chlorpyrifos involve glial targets? Macromolecule synthesis, adenylyl cyclase signaling, nuclear transcription factors, and formation of reactive oxygen in C6 glioma cells *Brain Research* **891**: 54-68

Garcia SJ, Seidler FJ, Qiao D, Slotkin TA (2002) Chlorpyrifos targets developing glia: effects on glial fibrillary acidic protein. *Dev Brain Res.* 133: 151-161*J. Submicrosc Cytol Pathol* Jan; **35** (1) :1-9

Gertrudis C, Mario V, Arnaldo V, Viviana D, Isolde R, Nicolas H and Gloria C (2001) A rat mammary tumour model induced by the Organophosphorus pesticides Parathion and Malathion, possibly through Acetyl cholinesterase inhibition. *Env. Health Perspectives* **109** (5) : 211-214.

Hoyer AP et al (1998) Organochlorine exposure and risk of breast cancer *Lancet*, **352**:1816-1820.

Jaga K, (2000). What are the Implications of the interaction between DDT and Estrogen Receptors in the body . *Med Hypothesis* **54**: 18-25

Kawachi T, Yahagi T, Kada T, Tazima Y, Ishadate M, Sasaki M and Sugiyama T(1980) Cooperative program on short term assays for carcinogenecity in Japan. *IARC Sci. Publi* **27**:323

Korrick SA, etal (2001) Association of DDT with Spontaneous Abortion : a Case Control Study *Avn Epidemiol* **11** : 491-496

Lavin JH, French SJ and Read NW (1997). The effect of sucrose- and aspartame- sweetened drinks on energy intake, hunger and food choice of female, moderately restrained eaters. *International Journal of Obesity* **21**: 37-42.

Lopez FF, Carbrera C, Lorenzo ML amd Lopez MC (2002). Aluminum content of drinking waters, fruit juices and soft drinks: contribution to dietary intake. *The Science of Total Environment*, **23:** 205-213

Levine R. Recognized and possible effects of pesticides in humans. In: Hayes WJ Jr, Laws ER Jr, eds. Handbook of pesticide toxicology. Vol 1. General principles . San Diego: Academic Press, 1991:275-360

Nicholas AHY, Vienne M and Van den Berghe H (1979) Induction of sister chromatid exchanges in cultural human cells by an organophosphorus insecticide : malathion. *Mutat Res,* **67**: 167

Pages N, Sauvet MP, Bouvet S, Goudey-Perriere F(2002) Reproductive toxicity of Lindane, *J Soc Biol*. 196(4):325-38.

Parry J, Shaw L, Arnaud MJ and Smith AJ (2001) Investigation of mineral waters and soft drinks in relation to dental erosion. *Journal of Oral Rehabitilation* **28**: 766-772

Pope CN (1999) Organophosphorus pesticides: Do they all have the same mechanism of toxicity? *J. Toxicol Environ* Health **2**: 161-181.

Prati C et al (2003). Permeability and Morphology of dentin after erosion induced by acidic drinks. *J Periodontol* **74** (4) : 428-436.

Qiao D, Seidler FJ and Slokin TAA (2001) Developmental neurotoxicity of chlorpyrifos modeled in vitro: Comparative effects of metabolites and other cholinesterase inhibitors in DNA synthesis in PC-12 and C-6 cells. *Env. Health Perspectives* **109** (9): 909-913

Qio D, Seidler FJ, Tate CA, Cousins MM, Slotkin TA(2003) Fetal chlorpyrifos exposure: adverse effects on brain cell development and cholinergic biomarkers emerge postnatally and continue into adolescence and adulthood, April **111**(4):536-44).

Rupa DS, Reddy PP and Redii OS (1991) Reproductive performance in population exposed to pesticides in cotton fields in India. *Environment Research*, 55:123-128

Sauviat MP, Pages N (2002) Cardiotoxicity of lindane, a gamma isomer of hexachlorocyclohexane. *J Soc Biol* 2002 **196**(4):339-48

Charlier C, et al (2003) Breast cancer and serum organochlorines residues *Occup Environ Med*, May; **60** (5):348-51]

Sharpe RM, Skakkeback NE(1993) Are oestrogens involved in falling sperm counts and disorders of the male reproductive tract? *Lancet* , **341**:1392-5.

Silman I Futerman A (1987) Modes of Attachment of Acetylcholine esterase to the surface membrane *Eur J. Biochemistry* **170**: 11-20

Skakkebaek NE, *et al* (1998) Germ Cell Cancer and disorders of Spermatogenesis: An Environmental Connection. *Apmis* **106**: 3-11.

Smith MI (1946) Accidental Ingestion of DDT , with a note on its metabolism in Man. J.AM.A, **131**:519-520

Stone R. (1994) Environmental estrogens stir debate. *Science* **265** :308-10.

Terry AV Jr, Stone JD et al (2003) Repeated exposures to subthreshold dozes of chlorpyrifos in rats: hippocampal damage, impaired axonal transport, and deficits in spatial learning. *J. Pharmacol Exp Ther*,, Apr;**305** (1):375-84.

Thrasher JD, Heuser G, Broughton A (2002) Immunological abnormalities in humans chronically exposed to chlorpoyrifos. *Arch Environ Health*, May-Jun, **57**(3):181-7

Turosov VS, Day NE, Tomatis L, Gati E, Charles RT (1973) Tumors in CFI mice exposed for six consecutive generations to DDT. *J. Natl. Cancer Inst* **51**: 983-997

US Environment Protection Agency. Method 8081A Organochlorine compounds by Gas Chromatography. at http://www.epa.gov/epaoswer/hazwaste/test/pdfs/8081a.pdf

US Environment Protection Agency - 1994 Method 8141A Organophosphorus compounds by Gas Chromatography: Capillary Column Technique. Revision 1 September-1994 available at http://www.epa.gov/epaoswer/hazwaste/test/pdfs/8141a.pdf

US Environment Protection Agency. Method 3620B Florisil Clean Up available at http://www.epa.gov/SW-846/pdfs/3620b.pdf

Vladimir T, Valery R and Lorenzo T (2002) Dichlorodiphenyl trichloroethane (DDT): Ubiquity, persistence and risks. *Env. Health Perspectives* **110** (2): 125-128.

Willings SR, Jensens HM, Marcum RG (1975) An Atlas of subgross pathology of human breast with special reference to possible precancerous lesions. *J Natl Cancer Inst.* **55:** 231-273

Wu J, Laird DA.(2003). Abiotic transformation of chlorpyrifos to chlorpyrifos –oxon in chlorinated wate., *Environ Toxicol Chem* , Feb;**22**(2):261-4