DETECTION AND QUANTIFICATION OF PHTHALATES IN LIQUID FOOD PRODUCTS BY GC-MS

A dissertation submitted to

THE TAMILNADU Dr.M.G.R.MEDICAL UNIVERSITY
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in partial fulfilment of the requirements for the award of the degree of

MASTER OF PHARMACY
IN
PHARMACEUTICAL CHEMISTRY

Submitted by
Reg.No. 261415714

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CERTIFICATE

This is to certify that the dissertation entitled **"DETECTION AND QUANTIFICATION OF PHTHALATES IN LIQUID FOOD PRODUCTS BY GC-MS"** submitted by the candidate bearing the Register No:**261415714** in partial fulfilment of the requirements for the award of degree of **MASTER OF PHARMACY in PHARMACEUTICAL CHEMISTRY** by the Tamilnadu **Dr. M.G.R Medical University** is a bonafide work done by him during the academic year 2015-2016 in the **Department of Pharmaceutical Chemistry, College of Pharmacy, Madras Medical College, Chennai- 600 003**.

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LIST 0F SYMBOLS AND ABBREVIATIONS

GC-MS	Gas Chromatography-Mass Spectrometry
UV	Ultra violet
IR	Infrared
RH	Relative Humidity
Eh	Redox potential
Min	Minutes
PVC	Poly vinyl chloride
PAE	Phthalic acid esters
BPA	Bis phenol-A
DEP	Di ethyl phthalate
DIBP	Di isobutyl phthalate
DnBP	Di-n-butyl phthalate
BMOEP	Bis-2-methoxy ethyl phthalate
DIPP	Di isopentyl phthalate
IPPP	Isopentyl pentyl phthalate
DPP	Di-n-propyl phthalate
DHP	Di hexyl phthalate
BBP	Benzyl butyl phthalate
DEHP	Di-2-ethyl hexyl phthalate
DnOP	Di-n-octyl phthalate
MEP	Mono ethyl phthalate
MIBP	Mono isobutyl phthalate
MNBP	Mono-n-butyl phthalate
MMP	Mono methyl phthalate
MEHP	Mono-2-ethyl hexyl phthalate
Mg	Milligram
µg	Microgram

ɳg	Nanogram
Kg	Kilogram
SPE	Solid Phase Extraction
SPME	Soli Phase Micro- Extraction
HPLC	High Performance Liquid Chromatography
GPC	Gel Permeation Chromatography
GC	Gas Chromatography
LC	Liquid Chromatography
FID	Flame ionisation detector
ECD	Electron capture detector
SIM	Selected ion monitoring
API	Active Pharmaceutical Ingredient
NMR	Nuclear Magnetic Resonance
RSD	Relative standard deviation
ICH	International Conference of Harmonisation
L	Litre
PPM	Parts per million
PPB	Parts per billion
Ml	Millilitre
LC-MS	Liquid Chromatography- Mass Spectroscopy
DAP	Di alkyl phthalate
PCNA	Proliferating cell nuclear antigen
DOTP	Di octyl terephthalate

CHAPTER 1

INTRODUCTION

Packaging is the process by which the products are suitably placed so that they should retain their therapeutic effectiveness from the time of their packaging till they are consumed. “ Packaging may be defined as the art and science which involves preparing the articles for transport, storage, display and use”. A package is a manufactured product consisting of any material or material combination used to present, contain, protect, handle and distribute goods, from raw materials to finished products, in every phase of the distribution chain. Thus the basic packaging functions follow:

- To contain the product
- To present and identify the product
- To protect the products physical integrity
- To preserve the products properties and quality characteristics
- To prepare the product for handling during transportation and commercial distribution.
- To inform the consumer

Packages have changed with time in response to the following social factors: population growth, urbanization, necessity to avoid food detoriation and loss, internationalization of commerce, greater awareness about hygiene, consumption of more natural foods and environmental detoriation.[1,2]

1.1. PACKAGING MATERIALS

Nowadays a wide and diversified supply of packaging materials and designs is available to suit the specific requirements of industry and consumers. Packaging materials can be grouped as follows:

- Metallic materials (tinplate, electrolytic chromium -coated steel and aluminum)
- Glass
- Paper and paperboard
- Plastics
- Wood
- Other materials (ceramics, natural fibers etc.)
- Combinations of the above

Many types of containers have been developed with these materials in order to meet the specific requirements of diverse products commercialized in urban societies in large amounts. Nevertheless, the packages can be grouped into a few well –established container types: rigid containers (eg. Classic cans), metal and plastic drums, glass or plastic bottles and jars, cartons and wood baskets and flexible packages manufactured with paper, carton, plastic or complex structures (e.g. Pouches, bags, coatings, etc).[3,4]

With such variety, it is obvious that a single package type will not suit the requirements of all foodstuffs. The most adequate package must be selected for each specific use, taking into account diverse parameters, such as

- Product characteristics (nature, composition, sensitivity to atmospheric factors)
- Possible product/container interactions
- Transportation and commercial distribution
- Type of consumer
- Shelf life
- Cost
- Possible reuse of materials or recycling, and environmental impact.

1.2. FACTORS AFFECTING PACKAGED FOOD

For many foods, the product shelf life is limited by specific or key attributes that can be predicted at the time of product development. This is either on the basis of experience with similar products or observations of them, or from a consideration of

- The make-up of the product (intrinsic factors)
- The environment that it will encounter during its life (extrinsic factors)
- And the shelf life limiting processes that this combination of intrinsic and extrinsic factors is likely to result in.[5]

Intrinsic factors are the properties resulting from the make-up of the final product and include the following:

- Water activity (available water)
- pH/total acidity
- Natural microflora and surviving microbiological counts in final product
- Availability of oxygen
- Redox potential
- Natural biochemistry/chemistry of the product
- Added preservatives (e.g. salt, spices, antioxidants)
- Product formulation
- Packaging interactions (e.g. tin pickup, migration).

Extrinsic factors are a result of the environment that the product encounters during life and include the following:

- Time–temperature profile during processing

- Temperature control during storage and distribution
- Relative humidity (RH) during storage and distribution
- Exposure to light (UV and IR) during storage and distribution
- Composition of gas atmosphere within packaging
- Consumer handling.

1.2.1. Chemical and biochemical process

Many important deteriorative changes can occur as a result of reactions between components within the food, or between components of the food and the environment. Chemical reactions will proceed if reactants are available and if the activation energy threshold of the reaction is exceeded. The rate of reaction is dependent on the concentration of reactants and on the temperature and/or other energy, e.g. light induced reactions. A general assumption is that for every10^{0}C rise in temperature, the rate of reaction doubles.

Specialized proteins called enzymes catalyse biochemical reactions. They can be highly specific catalysts, lowering the activation threshold so that the rate of reaction is dramatically increased[6].

1.2.2. Oxidation

A number of chemical components of food react with oxygen affecting the colour, flavour, nutritional status and occasionally the physical characteristics of foods. Foods containing a high percentage of fats, particularly unsaturated fats, are susceptible to oxidative rancidity and changes in flavour. Saturated fatty acids oxidize slowly compared with unsaturated fatty acids. Antioxidants that occur naturally or are added, either slow the rate of, or increase the lag time to, the onset of rancidity.

Three different chemical routes can initiate the oxidation of fatty acids: the formation of free radicals in the presence of metal ion catalysts such as iron, or heat, or light; photo oxidation in which photo-sensitizers such as chlorophyll or myoglobin affect the energetic state of oxygen; or an enzymic route catalyzed by lipoxygenase. Once oxygen has been introduced into the unsaturated fatty acids to form hydro peroxides by any of these routes, the subsequent breakdown of these colourless, odourless intermediates, proceeds along similar routes regardless of how oxidation was initiated. It is the breakdown products of the hydro peroxides – the aldehydes, alcohols and ketones that are responsible for the characteristic stale, rancid and cardboard odours associated with lipid oxidation.

1.2.3. Microbiological process

Under suitable conditions, most microorganisms will grow or multiply. Bacteria multiply by dividing to produce two organisms from one, their numbers increasing exponentially. Under ideal conditions some bacteria may grow and divide every 20 min, so one bacterial cell may increase to 16 million cells in 8 hrs. Under adverse conditions this doubling or generation time is prevented or extended – a feature that is exploited when developing food products and processes to achieve the desired shelf life.[7]

The presence of food poisoning organisms (pathogens) is not necessarily evident from changes in the food, and may only be apparent from the effects they produce, ranging from mild sickness to death. With many human pathogens, the greater the number of cells consumed, the greater the chance of infection and the shorter the incubation period before the onset of disease. Therefore, destruction, inhibition or at least control of growth is essential. For some invasive pathogens e.g. viruses, Campylobacter, the infectious dose is low and growth in the food may not be necessary.

1.3. MIGRATION FROM PLASTIC PACKAGING

There is always a risk that migration of components from plastics (in particular) and other packaging materials can occur and although with most solid medicaments this risk is low, it must always be considered.The popularization of polymeric packaging materials has resulted in increased concerns over the migration of undesirable components into foods. This has the potential to affect product quality as well as safety. These concerns are generally focused on the levels of residual monomers and plastics additives, such as plasticizers and solvents, present in polymers intended for direct or close contact with food.

Additives are used to aid the production of polymers and to modify the physical properties of the finished material. For instance, plasticizers, added to give a plastic the desired flexibility, have been identified as a potential threat to health. The World Health Organization has published opinions on a number of commonly used plasticizers with comments on toxicity.[8,9]

The use of plasticized PVC as cling film has been targeted as a potential problem in terms of migration. Studies have been conducted on the migration of the plasticizer di-(2-ethylhexyl) adipate from PVC films into food during home-use and microwave cooking and in retail food packaging. The level of migration increased with both the length of contact time and temperature of exposure, with the highest levels observed where there was a direct contact between the film and food, and where the latter had a high fat content on the contact surface. Use of a thinner PVC film was suggested as means of reducing the migration of this plasticizer.[10,11]

1.4. PHTHALATES RELATED ISSUES IN INDIA

DAILY MAIL (MAR 2, 2016): Dermatologist warn that make-up can cause acne breakouts, painful skin rashes - and is also linked to breast cancer and tumors. Dr.Macrene Alexiades-Armenakas, a dermatologist and Associate Clinical Professor at Yale University's School of Medicine, has revealed that costume make-up can cause acne breakouts, allergic reactions - and even long-term deadly illnesses. The doctor, who is the director and founder of the Dermatology and Laser Surgery Center in New York, tells Daily Mail Online that the toxins found in many facial products 'have been associated with cancer.

Phthalates could be detectable in the blood of people who wear stage make up daily. Phthalates, especially diethyl phthalate and di butyl phthalate, which are the ones in cosmetic products, cause breast cancer. According to the Breast Cancer Fund, phthalates, which are found in many plastics and cosmetic products, disrupt the body's hormones. Phthalates have also been linked to birth defects, neurodevelopmental problems in newborns and fertility issues.

DAILY MAIL (MAR 02, 2016) reported that plastic food wrap harbours a host of toxins. Heat makes chemicals in plastic storage boxes and bottles leach into food and drink and lead to health problems connected to cancers, fertility and fetal development.

Experts at Johns Hopkins University in the US concur, warn that heating food covered with plastic can melt the plastic on to the food. Of particular concern is cling film made from PVC, which contains hormone-disrupting phthalates, a chemical that keeps plastic soft.

PHARMABIZ.COM (MAR 01, 2016) urged the Government to come out with study on safety of PET bottles use for packaging liquid. A Dehradun based NGO reported that medicines packed in PET bottles were laced with contaminants that included phthalates and heavy metals. The NGO claimed that use of PET bottles had severe adverse effects on human health due to presence of endocrine disruptors and leaching which takes place under varying storage and temperature conditions and the age of packaging.

Considering the harmful effects of phthalates, the current situation warrants the adoption of suitable analytical techniques that detect the presence of phthalates in packaged food samples.

THE HUFFINGTON POST (JAN 22, 2016): Healthy Child Healthy Pet: Safer Toys For Pets which are Phthalate Free

Healthy Child Healthy World provides parents with information that can prevent many childhood diseases. It provides cutting edge scientific research which links human health to exposure to chemicals in the environment. It states that although some phthalates are banned from use in children's products in the U.S., pregnant women are still exposed to phthalates used in consumer and personal care products.

THE TIMES OF INDIA(JAN 6, 2016) MYSURU reported studies conducted on mice which showed that a combination of environmental factors, including exposure to plastic and certain pesticides, disrupts the endocrine system. This could have implication in the light of increasing infertility, falling sperm count, rising cases of reproductive disorders and testicular cancer in young men. Expert pathologist Dianne Creasy said 15-20% of couples are infertile compared to 7-8% in the early 1960s. Along with declining sperm count, there is increasing incidences of reproductive disorders like cryptorchidism and hypospadias in newborn boys. Also, more men aged between 20 and 30 seem to have testicular cancer.

The chemicals that were suspected to have endocrine disrupting effect on unborn or newborns include pesticides, BPA (found in polycarbonate plastics used in water bottles) and plasticizers (material to make plastic more malleable).

INDIA TODAY (NOV 16, 2015) reported the harmful effects of phthalates in cosmetics which can cause damage to lungs, liver and kidneys. The phthalates present in these cosmetics seep into the body by inhaling and through skin contact.

CHROMATOGRAPHY TODAY (SEP 29, 2015) reported the health implications of phthalate exposure. A group of researchers from Peking University has reported on a possible link between phthalate levels in women’s urine and an increased risk of miscarriage in pregnant women.

Phthalates are most commonly used are as plasticizers or solvents for plastics and they can be found in products as diverse as pharmaceuticals, flooring, toys and food packaging. They are incorporated into plastic products during the manufacturing phase of the plastic by heating the plastic and phthalate together and then cooling.

THE ECONOMIC TIMES (SEP 03, 2015) reported that the chemicals in soaps & shampoos may cause miscarriage risk. Exposure to certain substances commonly found in personal care products such as soaps and shampoos and food packaging could be associated with increased risk of miscarriage.

The study of more than 300 women suggested that exposure to certain phthalates - substances commonly used in food packaging, personal-care and other everyday products - could be associated with pregnancy loss, mostly between 5 and 13 weeks of pregnancy.

CHEMICAL WATCH (AUG 26, 2015) reported three phthalates to the cause of miscarriage - DEP, DiBP and DnBP.

Chinese researchers have found that levels of three urinary phthalate metabolites in pregnant women are associated with a higher risk of miscarriage. The researchers also mention a Russian study which found an increased spontaneous abortion rate in female factory workers exposed to high levels of phthalates. Phthalates are also known to affect concentrations of the hormone progesterone, which is involved in the implantation of embryos.

Five major metabolites of phthalates found were

- Mono ethyl phthalate (MEP)
- Mono isobutyl phthalate (MiBP)
- Mono n-butyl phthalate (MnBP)
- Mono methyl phthalate (MMP) and
- Mono (2-ethylhexyl) phthalate (MEHP)

The scientists concluded that increased urine levels of MEP, MiBP and MnBP were associated with an increased risk of miscarriage. This implicates women’s exposure to diethyl phthalate (DEP), di-isobutyl phthalate (DiBP) and di-n-butyl phthalate (DnBP) respectively. DEP and DnBP are mainly used in cosmetics, personal care products and food packaging. DnBP and DEHP are also used in building products such as paints, flooring and PVC items.

1.5. PHTHALIC ACID ESTERS (PAE)

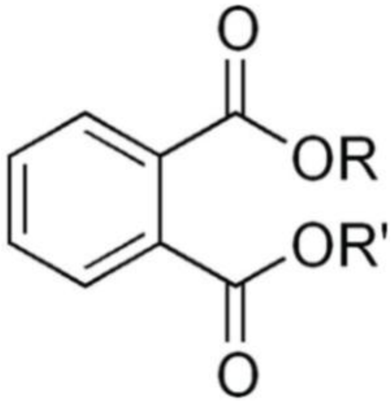
Phthalic acid esters (or phthalates) are di alkyl or aryl alkyl esters of 1,2- benzene di carboxylic (or phthalic) acid; a class of plasticizer used to impart flexibility and durability to polyvinyl chloride (PVC) and other plastics. Most of the phthalates, in the pure form, are colorless liquids that are poorly soluble in water but soluble in organic solvents or oils. Dimethyl phthalate (DMP), diethyl phthalate (DEP), di butyl phthalate (DBP), di-iso-butyl phthalate (DIBP), benzyl butyl phthalate (BzBP), di-(2-ethylhexyl) phthalate (DEHP) and di-n-octyl phthalate (DnOP) are the common phthalates available in commerce. Specifically, DEHP accounts for 50 % of total phthalate production. In addition to its use as a plasticizer, DMP is used as an insect repellent; DEP is used in shampoos, scents, soaps, lotions and cosmetics, and in industrial solvents and medications; DBP and DIBP are used in adhesives, cosmetics, industrial solvents and medications; DEHP is used in soft plastics, including tubing, toys, home products, food containers and food packaging and BzBP is used in vinyl flooring, adhesives, sealants and industrial solvents.

Phthalates are chemically inert, have high density, low to medium volatility, high solubility in organic solvents and are easily released to the environment during aging of polymer materials. Phthalates have been reported as functional solvents in the aromatic, essential oil, and even beverage industries. Phthalates are widely used as plasticizers in the manufacturing of plastics especially for food packaging.

Human beings have high possibility to be exposed to phthalate esters since these compounds are widely used in the production of agricultural, industrial and household detergents. Phthalate plasticizers migrate from plastic containers or closures into water, soft drinks and alcoholic beverages. Nowadays people are easily exposed to phthalates (phthalic acid esters, PAEs) particularly infant. Besides having carcinogenic and estrogenic effects to human, some of the phthalates are toxic and able to induce asthma.

Dibutyl phthalate (DBP) tends to impair androgen-dependent development for the male reproductive system and inhibits the production of testosterone. This phthalate can be rapidly absorbed into the systemic circulation and spread through the body in a short time. Mono-2-ethylhexyl phthalate, which is one of the urinary metabolites, was detected in the urine content of the workers, who were exposed to high concentration of diethyl phthalate (DEP), di butyl phthalate (DBP), and di (2-ethylhexyl) phthalate (DEHP) mono-2-ethylhexyl phthalate in several PVC production factories. This causes the workers have lower concentration of testosterone compared to the other normal individuals. The US Food and Drug Administration's Center for Devices and Radiological Health and Health Canada have reported the risk assessment of DEHP that migrated from PVC medical devices in hospitalized patients.[12]

1.5.1. Chemistry of Phthalates

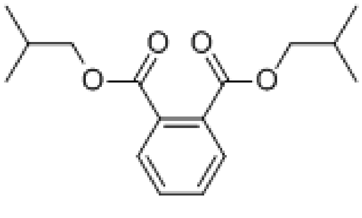


R^1, R =Alkyl Chain

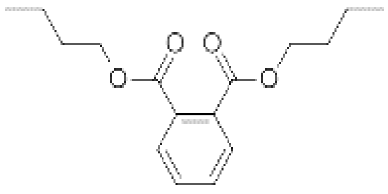
General structure of phthalates

The term ‘phthalate esters’ is restricted to the ortho form of benzene di carboxylic acid prepared by reaction of phthalic acid with a specific alcohol to form the desire ester. Most of the esters are colorless liquids, have low volatility, and are poorly soluble in water but soluble in organic solvents and oils. Two other isomeric forms of benzene di carboxylic acid esters are also available having important industrial applications-the -meta form (or isopthalate esters) and the para form (or terephthalate esters).

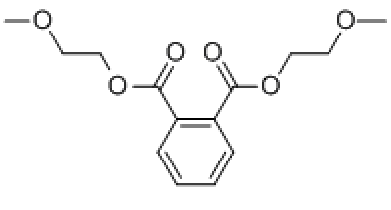
Structure of phthalates



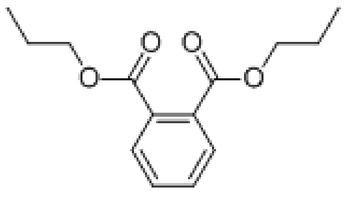
DIBP



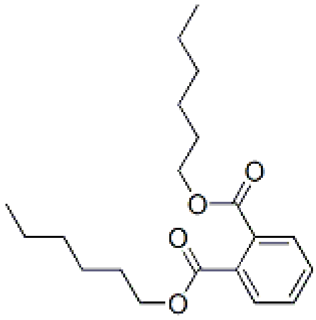
DBP



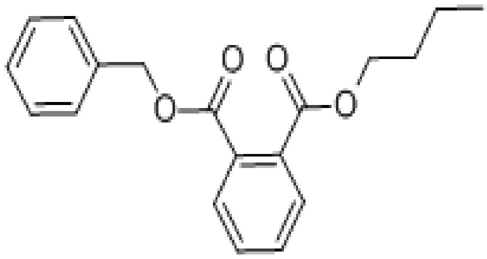
BMOEP



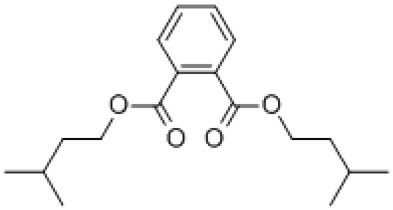
DPP



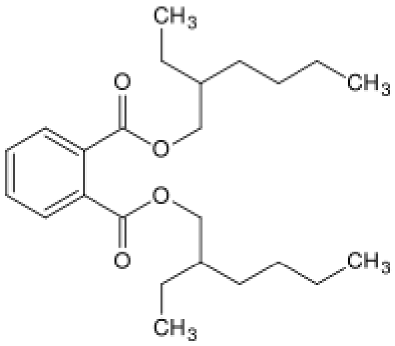
DHP



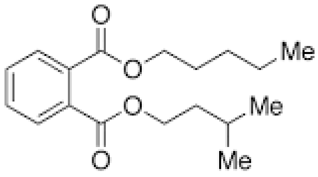
BBP



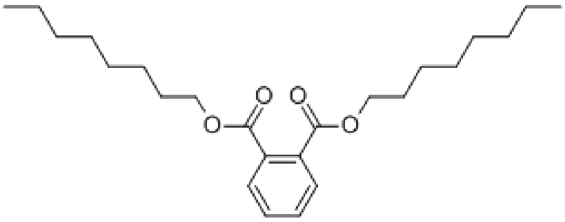
DIPP



DEHP



IPPP



DnOP

1.5.2. Physico-chemical properties of some common phthalates

Common Name	Acronym	Molecular weight	Boiling point	Water solubility (mg/l,25^0c)
Dimethyl phthalate	DMP	194.2	282	5220
Diethyl phthalate	DEP	222.2	295	591
Diiso butyl phthalate	DIBP	278.3	327	9.9
Dibutyl phthalate	DBP	278.3	340	9.9
Butyl benzyl phthalate	BzBP	312.4	370	3.8
Di-n- hexyl phthalate	DnHP	334.5	350	0.159
Di-2-ethyl hexyl phthalate	DEHP	390.6	384	0.0025
Di-n-octyl phthalate	DNOP	390.6	390	0.0025

Physical state

Phthalates are all in the liquid state at ambient temperatures and have melting points below the freezing point (< 0°C). The pure solutes boil between temperatures of 230°C (DMP) and 486°C (DiDP), but high molecular weight phthalate esters tend to decompose when exposed at elevated temperatures for longer times. The densities (20°C) are all very close to unity and differ only negligibly within the complete range of compounds.

Water solubility

Phthalate mono-esters are more polar than their parent di-esters and show higher water solubilities. Once ionized, the molecules tend to form micelles at basic conditions (pH > 9-11) and are dispersed well in the aqueous phase. The solubilities of hydrophobic compounds decrease with increasing temperatures.

Octanol-Water partition

The octanol-water partition coefficient (*Ko/w*) basically represents the equilibrium distribution of a solute between water and octanol and therefore is a physical constant that is linked to its hydrophobicity. Generally, the *Ko/w* or the hydrophobicity increases proportionately to the molecular weight of alkyl phthalates or the chain length of the alcohol moiety. High molecular-weight plasticizers (DEHP, DiNP, DiDP) show therefore very high affinity for apolar matrix substances like fat, bio-matrices or (suspended) solids in surface or waste water. Mono-alkyl phthalates are because of their free carboxylic acid group much more polar.

Vapour pressure

Phthalate esters can be considered as semi-volatile compounds and are present in the vapor phase at environmentally relevant temperatures. The mono-esters MEHP, MiNP and MiDP show analogously low vapor pressures.

1.5.3. General Applications of Phthalate Esters

- A very wide spectrum of uses has been found for the various phthalate esters. During the Second World War, dimethyl and di butyl phthalates were used in insect repellent formulations. By far, however, the largest market for these esters is as plasticizing agents for poly vinyl chloride, for example, the cellulosics and certain types of elastomers.

- These plasticizers give the polymer the desired flexibility and softness and may account for up to 40% of the final weight of the material. They are not chemically bound to the polymer, but dispersed in the matrix of the polymer chains to decrease the interaction forces of adjacent chains, lower the glass transition temperature of the polymer and causes chain mobility and material flexibility.

- Di-octyl phthalate (DOP) and its isomer, di-2-ethylhexvl phthalate (DEHP) are probably the most widely used plasticizers today, although other phthalates are used, sometimes in combinations, to give certain additional properties desired in the final material.

- A large number of industrial and consumer products of the poly (vinyl chloride) type will generally contain one of the phthalate esters as a plasticizer. These products include floor tiles, various types of furnishings for house-holds and transportation vehicles, food packaging systems, industrial tubings and conduits, medical tubings, catheters and blood containers, certain types of dental materials, coatings for drugs and numerous other products.[13]

- The phthalate esters are used as defoaming agents in the manufacture of paper, in cosmetic products as a vehicle (primarily di-ethyl phthalate for perfumes, in lubricating oils and in other industrial and consumer applications in which the esters add specific desired properties.

- There is also some evidence suggesting that the esters are present in certain plants and organisms as normal metabolites. Several of the phthalate esters have been approved by the FDA for food-packaging applications, and thus they have been accepted as generally safe under the conditions of use.

1.5.4. Pharmaceutical applications of phthalates

- Phthalates are used as functional excipients in a large number of oral pharmaceutical formulations. They are most commonly used as plasticizing agents in enteric film-coating materials or as a matrix binder for tablets, capsules, beads and granules.

- Due to their plasticizer properties, phthalates can be included in soft gelatin capsule formulations.

- They may also be used to control the viscosity of certain liquid formulations.

- Phthalates confer the following properties to tablets and capsules:

- Resistance to degradation of the tablet/capsule coating in the acidic environment of the stomach during transit to the site of absorption in the intestine. The solubility of the phthalates at neutral and high pH and insolubility at low pHs protects the tablet during prolonged contact with acidic gastric juices and ensures its dissolution in the neutral environment of the intestines.

- Maintenance of flexibility of solid dosage forms (e.g. tablet/capsules) for quality purposes (e.g. to prevent cracking) and to enhance oral administration (e.g. increased ease of swallowing).

- Viscosity modification during production of pharmaceutical formulations to control characteristics such as thinness of the sealing coat whilst maintaining adequate barrier to moisture.

- Control of drug-release characteristics of modified-release preparations.

- Increase of the palatability of bitter tasting formulations by effectively sealing off the underlying drug formulation (the phthalates are tasteless and odourless).

1.5.5. Human exposure to phthalates

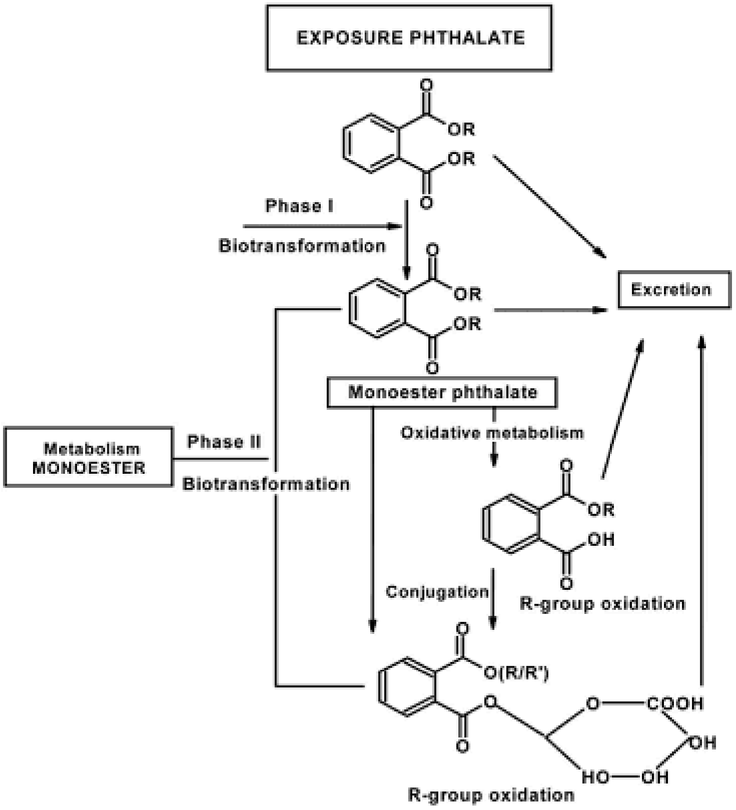
People can be exposed to phthalates by:

- Using consumer products that contain phthalates.
- Breathing household dust contaminated with phthalates.
- Eating foods contaminated with phthalate compounds that have leached from plastic containers into foods.
- Having a medical treatment, such as a blood transfusion or dialysis, that uses equipment made of plastics.
- Living near a manufacturing facility that makes products containing phthalates.

The exposure mainly due to the characteristic of phthalates, which is not chemically bound to the polymeric material, therefore, can leach to the environment as a pollutant or into the body by transfer from storage materials to products such as food, infant formulas, or pharmaceutical products.

Infants have the highest tendency to be exposed to phthalates because they tend to put their toys or pacifiers, which may contain phthalates into their mouth. Phthalates in toys which are specifically designed to be chewed by infant may leach into their saliva and then ingested into their stomach. Although the leached phthalates in the saliva are low, the amount is high enough to cause adverse effects to the infant such as liver, reproductive tract and kidney disorders. Cancer is the adverse effect for more serious cases. [14]

Due to these effects, many countries in Europe such as Austria, Denmark, Finnish, Greece and Danish had banned the use of phthalates additives in various soft PVC toys and childcare products for children under three years old at the end of the 1990s. Other countries such as Finnish and Danish had stipulated a permissible limit for each relevant phthalates in these products, which should not more than 0.05%.

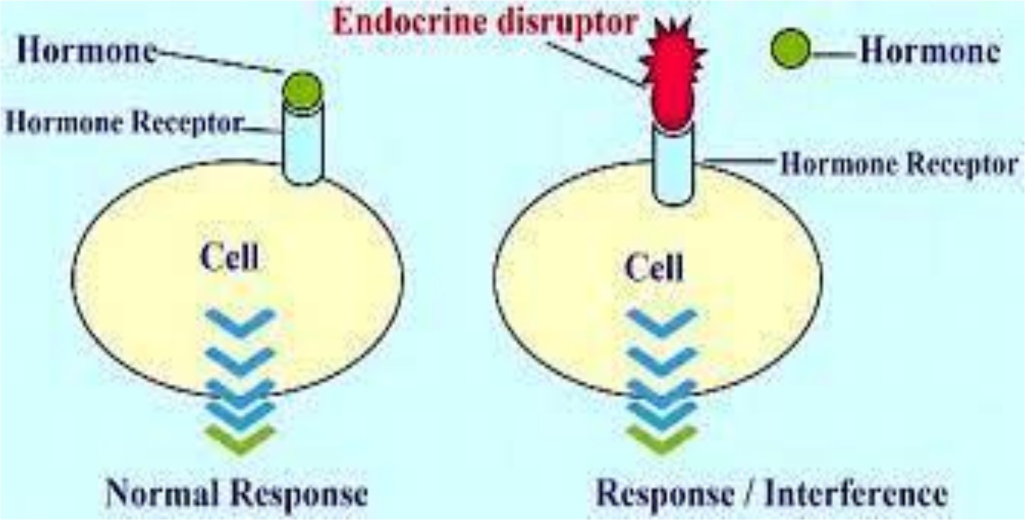


Phthalate esters can be rapidly metabolized to their corresponding monoesters within hours in the human body. The half-life of phthalate esters in the human body is in the range 6–20 h. Phthalates with a short alkyl chain (i.e., DMP, DEP, DIBP, DBP and BzBP) are metabolized mainly to monoesters, whereas long-alkyl-chain phthalates (i.e., DEHP, DINP, and DIDP) are metabolized, first, to the corresponding monoesters which are then further metabolized to more oxidized secondary metabolites. Both monoesters and secondary metabolites can be conjugated with glucuronic acid in phase II metabolism. All free and glucuronidated metabolites of phthalates are excreted in urine and feces. Urinary phthalate metabolite concentrations are widely used as a measure of recent human exposure to phthalate esters. As plasticizers, phthalates are not chemically bound to products and can, therefore, easily leach from the products into the environment. Consumer products containing phthalates can be a direct source of human exposure.

Phthalates are lipophilic and therefore tend to concentrate in the lipid phase of foodstuffs. Since dairy products like cream, butter and cheese are high-fat foods, they are expected to be more contaminated with phthalates than low-fat foods .Transport in the atmosphere is an important pathway for distribution of phthalates in the environment Since phthalates are typically present at trace levels (ng to μg/m³ range), direct analysis of air samples is not possible and enrichment techniques are required.

Health threats

PAEs in the environment and food chain can act as hormones, simulate the body's natural endocrine responses, interfere with the normal role of hormones, and affect the body's most basic physiological control mechanisms. Phthalates are reported to cause carcinogenic, teratogenic and mutagenic effects and constitute a health hazard to humans.



Phthalates are potentially hazardous to human health-especially to children's health-due to their classification as endocrine disruptors. This has resulted in regulations regarding the types and levels of phthalates allowable in plastic toys, water containers, textiles and foods.

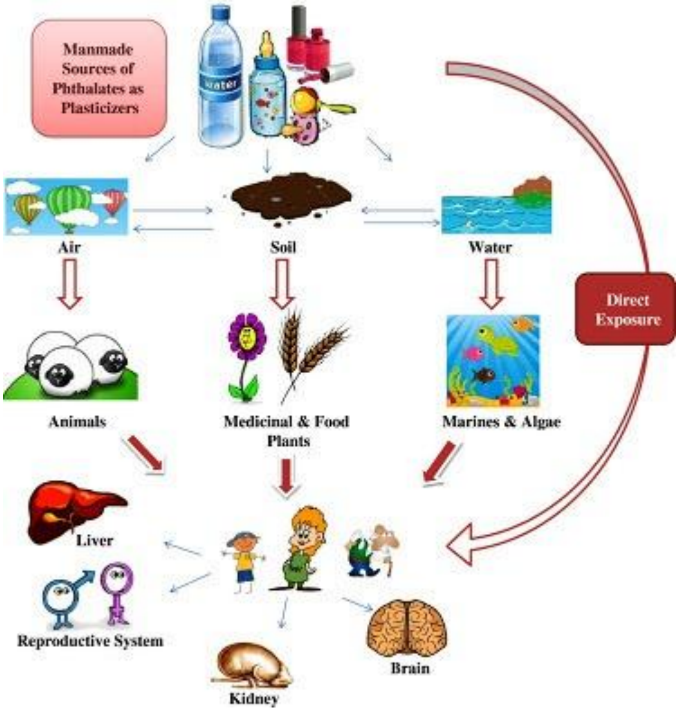
Recent studies have revealed a negative relationship between environmental phthalate exposure and intelligence in children. A study investigated the association between urinary concentrations of phthalate metabolites and biomarkers of oxidative stress and inflammation. The results indicated that concentrations of DEHP, DBP, and BzBP metabolites were associated with increased oxidative stress and inflammation.

Major health hazards of phthalates

- Premature birth
- Birth defects of male sex organs
- Reduced fertility
- Prostate and testicular cancer
- Learning disabilities
- Behaviour problems
- Asthma and allergies
- Early puberty in girls
- Breast growths in boys
- Obesity and diabetes

In 2009, eight phthalate esters, including DBP, DIBP, BzBP, DEHP, DNOP, diisodecyl phthalate (DIDP), di-n-pentyl phthalate (DnPP) and di isononyl phthalate (DINP) were added to the list of chemicals of concern by the US Environmental.

Human studies



Pediatric exposure

Phthalates have been found to cross the placenta and pass into breast milk so prenatal exposure and exposure from breast feeding may occur in humans. Infant exposure to lotion, powder, and shampoo were significantly associated with increased urinary concentrations of mono ethyl phthalate, mono methyl phthalate and mono isobutyl phthalate. There exists an association between low birth weight and DBP levels in maternal blood samples, as well as umbilical cord blood. DEHP and MEHP levels were associated with reduced birth length.[15-17]

Respiratory effects

Exposure to phthalates -BBP, DEHP and DBP via house dust, has been related to asthma and allergic symptoms in children. High levels of phthalates from PVC products can modulate the murine immune response to a co-allergen. Heated PVC fumes possibly contribute to development of asthma in adults.[18,19]

Hepatic effects

DEHP is a well known hepatotoxin in rodents. Large amounts of specific phthalates fed to rodents have been shown to damage their liver and testes and initial rodent studies also indicated hepato carcinogenity.[20,21]

Male reproductive health

Recent studies have reported effects of phthalates on male reproductive health such as reduced sperm production in laboratory animals. Phthalates may induce antiandrogenic or estrogenic effects in humans. Recently, it has been suggested that *o*-DAP's may contribute to the occurrence of the "testicular dysgenesis syndrome" in humans, that is, testicular germ cell cancer, cryptorchidism, hypospadia and low sperm count.[22-24]

Female Reproductive health

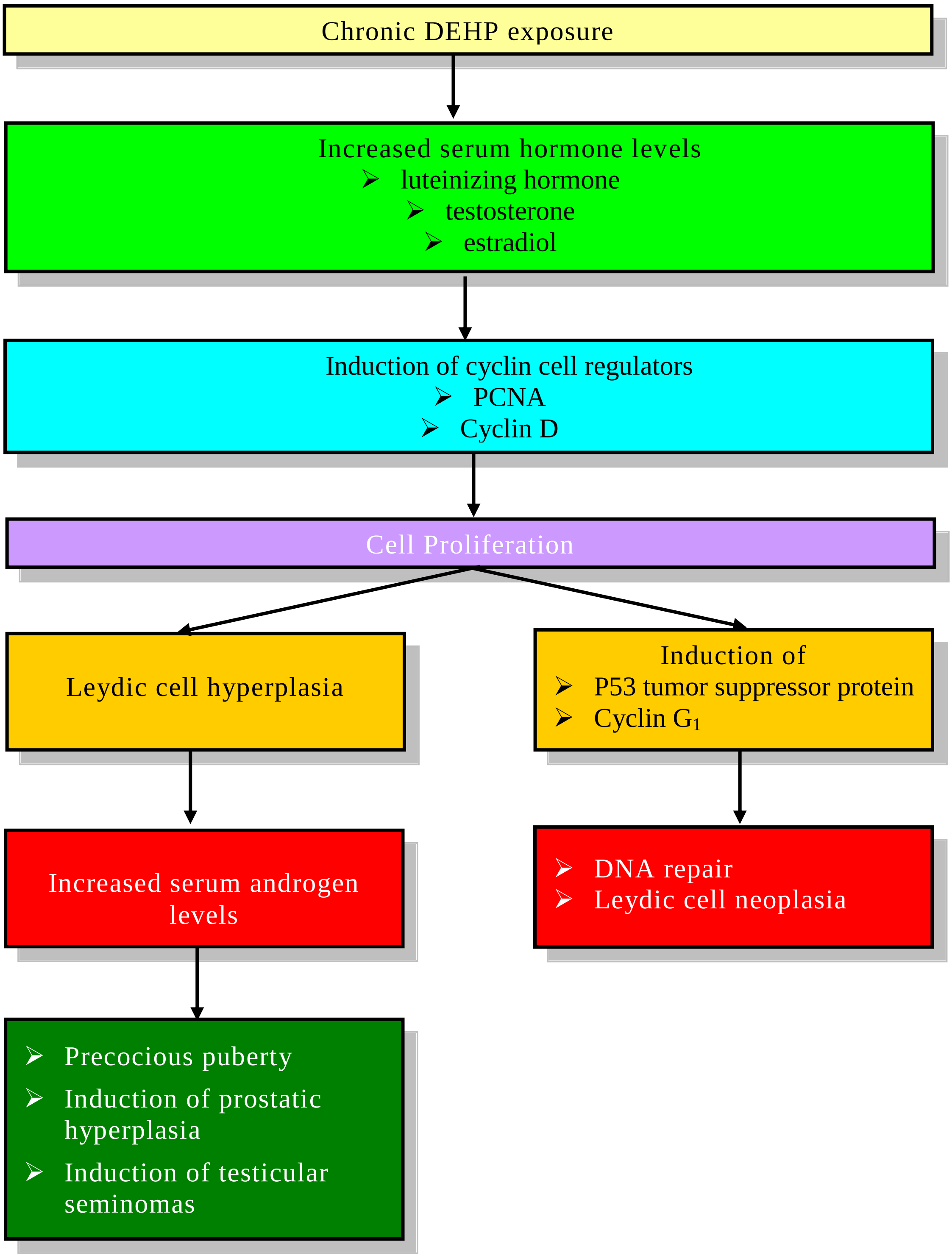
There is a possible association between plasticizers with known estrogenic and antiandrogenic activity and the cause of premature breast development in human female population with exposure to DEHP.[25,26]

Potential health hazards of selected phthalates

S.NO	Health hazard	Phthalate associated
1.	Pediatric exposure:	
	Preterm birth (shorter pregnancy duration)	DEHP
	Prenatal toxicity (antiandrogenic in fetal life)	DINP
2.	Respiratory effects:	
	Asthma and allergy in children	BBP and DEHP
	Asthma, allergy, persistent wheezing, persistent cough, persistent phlegm, nasal congestion and respiratory infections.	DEHP
3.	Hepatic effects:	
	Increased liver weights, elevated liver enzyme levels, histological changes and in some cases tumours.	DEHP and DINP
	Peroxisomal proliferation (a process related to metabolism of cholesterol and fatty acids).	DEHP
4.	Developmental and reproductive effects:	
	Toxic to reproduction, causing changes to both male and female reproductive systems in mammals e.g.	
	Development of testis in early life	BBP,DEP and DEHP
	Reduce testosterone	DEHP
	Poor semen quality	DMP, DEP, DBP,
	Genital defects e.g.	BBP, DEHP and DnOP
	Ano genital defects in male infants	BBP (rats),DBP (rats)
	Premature breast development in females	DEP, DEHP and DBP

Mechanism of DEHP in human body

Chronic exposure of DEHP interrupts with the hormonal changes in the human body as depicted below:



Acceptable daily intake

PHTHALATE	ACCEPTABLE DAILY INTAKE (µg/kg/day)
Di ethyl phthalate (DEP)	ND
Di-n-butyl phthalate (DBP)	200
Butyl benzyl phthalate (BBP)	1000
Di cyclohexyl phthalate (DCHP)	ND
Di-(2-ethyl hexyl) phthalate (DEHP)	5.8
Di-n-octyl phthalate (DnOP)	370
Diisononyl phthalate (DINP)	120

Considering the potential health effects of selected phthalates, specifically related to children, the European Union and the U.S. has banned the use of six phthalates in the production of children's toys: DINP, DEHP, DBP, BBP, DIDP and DnOP. Today, non-phthalate plasticizers are growing three times faster than the rest of the plasticizer market and represent more than 10% of the entire global market.

1.6. ALTERNATIVE PLASTICIZERS

Bio plasticizers

- Grind sted soft –n-safe (COMGHA)
- Polysorb ID 37 (isosorbide diesters)

Chemical alternatives

- 1,2-Cyclohexane dicarboxyllic acid diisononyl ester

- Dioctyl terephthalate (DOTP OR DEHT) - DOTP is a general purpose plasticizer that is considered safer than ortho-phthalate plasticizers due to its excellent toxicological profile. The terephthalates exhibit none of the peroxisome proliferation of liver enzymes that some ortho-phthalates have shown in several studies

- Citric acid - Because it is one of the stronger edible acids, the dominant use of citric acid is as a flavoring and preservative in food and beverages, especially soft drinks.

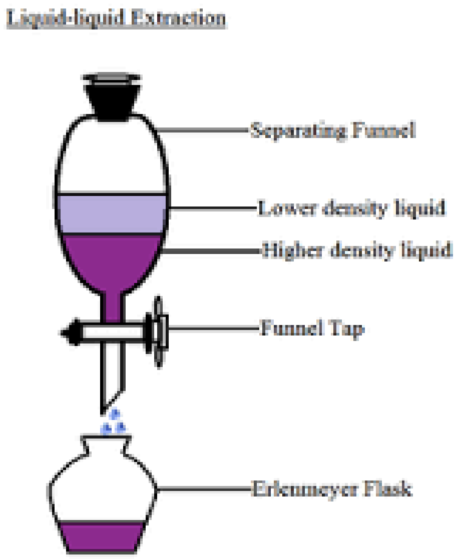
1.7. ANALYSIS OF PHTHALATES

The procedure for analysis of phthalate esters in food samples is similar to that for many organic contaminants and involves extraction, purification, separation, and instrumental detection. Because types of foodstuff vary widely, no one procedure can be used for all foods.

1.7.1. Extraction methods

Methods reported for liquid samples that do not contain lipids (for example water, juices, and other beverages), liquid–liquid (L–L) extraction with organic solvents, solid-phase extraction (SPE) with cartridges and solid-phase micro-extraction (SPME) with commercially available fibers have been used in the extraction of phthalates. SPME enables rapid, solvent-free extraction. The SPE method has been used for determination of phthalate esters in bottled water and wine. Selection of SPE cartridges with appropriate sorbents is important for efficient and selective extraction of phthalate esters from liquid foods.

Liquid-liquid extraction is one of the most widely employed and useful techniques in pharmaceutical sample preparation. This is due to a number of characteristics, including simplicity, rapid method development, and reasonable selectivity. Liquid-liquid extraction is usually performed with non-polar organic solvents, for example hexane, dichloromethane, or isooctane, followed by phase-separation by centrifugation and removal of moisture by treatment with anhydrous sodium sulfate.[27]



Liquid-liquid extraction involves adding a solvent to the sample that is immiscible, followed by selective partitioning of analytes versus contaminants between the two phases. In the interest of extraction completeness, it is necessary to use an adequate amount of extracting solvent to capture all of the analytes from the original samples. This extracting solvent is added to the sample, then the two phases are agitated, by vortexing or shaking, to bring about substantial physical mixing. After agitation, the phases are allowed to separate.

For liquid foods that contain lipids, for example milk (milk powder) and oil (including cooking oil), phthalate analysis requires several additional steps. The L–L extraction method would co-extract lipids and organic compounds with the phthalates (because of the lipophilic properties of phthalates). Removal of lipids from the extracts by gravity column chromatography or SPE can be cumbersome, because phthalates and lipids are of similar polarity.

Headspace SPME has been for determination of phthalates in milk samples. Lipids from the extracts can be separated and removed by two techniques that have been commonly used in food analysis: gel-permeation chromatography (GPC) and L–L extraction.

Dichloromethane–cyclohexane (1:1, v/v), cyclohexane ethyl acetate (1:1) or dichloromethane–hexane (1:1) have been commonly used as the mobile phases in the GPC separation of lipids. Alternatively, L–L extraction can be used for removal of lipids from food extracts. L–L extraction with acetonitrile and hexane can separate lipids from phthalates.

In comparison with the GPC method, L–L extraction consumes less solvent and therefore reduces background levels of phthalate contamination in the analytical procedure.

The choice of method depends on the kind of sample and co-extractives. Liquid-liquid extraction method is used in this analysis to extract the phthalates from the liquid food samples

1.7.2. ANALYTICAL TECHNIQUES

Chromatographic techniques

There are many different phthalate species, and different phthalates are thought to have different health impacts. Chromatography is one of the key techniques used in testing phthalates. The most widely used separation technique is gas chromatography with capillary columns. Liquid chromatography such as HPLC is used less, one reason being the much better chromatographic resolution of single compounds with capillary columns compared to HPLC. Another reason is the common use of mass selective detectors in GC while these detectors are not used as frequently in HPLC. Typically used are columns with a 5% phenyl methyl siloxan film, but resolution of certain pairs of phthalates is not satisfactory (e.g. dihexyl phthalate and BBzP, DEHP and DCHP. [28,29]

Detection techniques

Mass specific detection in conjunction with selected ion monitoring seems to be the method of choice after chromatographic separation either by GC or LC. As all phthalates except DMP form the ion with m/e 149 the esters are easy to identify. Traditionally, flame ionization detectors (FID) and electron-capture detectors (ECD) were used for detection of phthalates in foods.[30]

GC-MS

Gas chromatography (GC) coupled with different types of detector, preferably mass spectrometric (MS) detection, is used in the separation, detection, and quantification of phthalate esters in food samples. Flame ionization detectors (FID) and electron-capture detectors (ECD) were used for detection of phthalates in foods but are now replaced with more selective MS detectors.

MS detection enables addition of isotopically-labeled phthalate internal standards before sample extraction; this internal standard enables more accurate quantification of phthalates in food samples. The isotopically labeled and native phthalate esters can be discriminated from each other by the use of an MS detector, which eliminates potential interferences from the analysis. In the MS analysis, molecular and fragment ions of phthalates can be targeted by use of selected ion monitoring (SIM) mode, and the relative ratios among the ion fragments can be used to confirm the detection of phthalates.

In this study gas chromatography with mass spectroscopy followed by selected ion monitoring (SIM) mode is used for the detection and quantification of phthalates.

1.8. ANALYTICAL METHOD DEVELOPMENT

Quality assurance and control of pharmaceutical formulations is essential for ensuring the availability of safe and effective drug formulations to consumers. Hence analysis of pure drug substances and their pharmaceutical dosage forms occupies a pivotal role in assessing the suitability to use in patients. The quality of the analytical data depends on the quality of the methods employed in generation of the data. Hence, development of rugged and robust analytical methods is very important for statutory certification of drugs and their formulations with the regulatory authorities.

A well-developed method should be easy to validate. A method should be developed with the goal to rapidly test preclinical samples, formulation prototypes and commercial samples. There are five common types of analytical methods, each with its own set of validation requirements:

- Identification tests
- Potency assays
- Quantitative tests for impurities
- Limit test for the control of impurities
- Specific tests

The first four tests are universal tests, but the specific tests such as particle size analysis and X-ray diffraction are used to control specific properties of the active pharmaceutical ingredient (API) or the drug product. The most widely used methods for quantitative determination of drugs and metabolites in biological matrices such as blood, serum, plasma or urine includes Gas chromatography (GC), High-performance liquid chromatography (HPLC), Thin layer chromatography (TLC), combined GC and LC mass spectrometric (MS) procedures such as LC-MS, LC-MS-MS, GC-MS and GC-MS-MS. Techniques like NMR, IR and Mass spectrometry are used for structure identification.

Safety and efficacy of pharmaceuticals are two fundamental issues of importance in drug therapy. The safety of a drug is determined by its pharmacological-toxicological profile as well as the adverse effects caused by the impurities in bulk and dosage forms. The impurities in drugs often possess unwanted pharmacological or toxicological effects by which any benefit from their administration may be outweighed. Therefore, it is quite obvious that the products intended for human consumption must be characterized as completely as possible. The quality and safety of a drug is generally assured by monitoring and controlling the impurities effectively. Thus, the analytical activities concerning impurities in drugs are among the most important issues in modern pharmaceutical analysis.

The goal of any chemical analysis is to separate a sample into its individual components in order to evaluate each component free from interference from the other components. Chromatography is a general technique that separates a mixture into its individual components. Those components are referred to as analytes - the chemical compound of interest to the analyst.

1.9. METHOD VALIDATION

An analytical method is a laboratory procedure that measures an attribute of a raw material, drug substance or a drug product. Analytical method validation is the process of demonstrating that an analytical method is reliable and adequate for its intended purpose. Any method that is utilized to determine results during drug substance and formulation development will have to be validated. Reliable data for release of clinical supplies, stability and setting shelf life can only be generated with appropriate validated methods. The validation of analytical procedures, i.e., the proof of its suitability for the intended purpose, is an important part of the registration application for a new drug. Additional peak tailing, peak resolution and analyte recoveries are important in case of chromatographic methods. The ICH has harmonized the validation requirements in two guidelines. The first one summarizes and defines the validation characteristics needed for various types of test procedures; the second one extends the previous text to include the experimental data required and some statistical interpretation. These guidelines serve as a basis worldwide both for regulatory authorities and industry and bring the importance of a proper validation to the attention of all those involved in the process of submission of drug master files. The analytical research and development units in the pharmaceutical industry are responsible for preparation and validation of test methods.

1.9.1 Validation Parameters

Accuracy

Accuracy is the measure of how close the experimental value is to the true value. Accuracy studies for drug substance and drug product are recommended to be performed at 80, 100 and 120% levels of label claim. At each recommended level studied, replicate samples are evaluated. The RSD of the replicates will provide the analysis variation or how precise the test method is. The mean of the replicates, expressed as % label claim, indicates how accurate the test method is. Recovery data, at least in triplicate, at each level (80, 100 and 120% of label claim) is recommended. The mean is an estimate of accuracy and the RSD is an estimate of sample analysis precision.

Detection Limit and Quantitation Limit

These limits are normally applied to related substances in the drug substance or drug product. Detection limit is the lowest concentration of analyte in a sample that can be detected, but not necessarily quantitated, under the stated experimental conditions. Quantitation limit is the lowest concentration of analyte in a sample that can be determined with acceptable precision and accuracy under the stated experimental conditions.

Linearity and Range

The linearity of an analytical procedure is its ability (within a given range) to obtain test results which are directly proportional to the concentration of analyte in the sample.

The range of an analytical procedure is the interval between the upper and lower concentration of analyte in the sample for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy and linearity. Range is the interval between the high and low levels of analyte studied.

The linear range of detectability that obeys Beer's Law is dependent on the compound analyzed and detector used. The working sample concentration and samples tested for accuracy should be in the linear range.

Precision

Precision of an analytical procedure expresses the closeness of agreement between a series of measurements obtained from multiple sampling of the same homogenous sample under the prescribed conditions. Precision is the measure of how close the data values are to each other for a number of measurements under the same analytical conditions. ICH has defined precision to contain three components: repeatability, intermediate precision and reproducibility. The precision of an analytical procedure is usually expressed as the variance, standard deviation or coefficient of variation of a series of measurements.

(a) Repeatability

Repeatability expresses the precision under the same operating conditions over a short interval of time. Repeatability is also termed intra-assay precision.

(b) Intermediate precision

Intermediate precision expresses within laboratory variations like different days, different analysts, different equipments, etc. intermediate precision was previously known as part of ruggedness. The attribute evaluates the reliability of the method in a different environment other than that used during development of the method. The objective is to ensure that the method will provide the same results when similar samples are analyzed once the method development phase is over. Depending on time and resources, the method can be tested on multiple days, analysts, instruments, etc.

(c) Reproducibility

As defined by ICH, reproducibility expresses the precision between laboratories. Multiple laboratories are desirable but not always attainable because of the size of the firm.

Robustness

ICH defines robustness as a measure of the method's capability to remain unaffected by small, but deliberate variations in method parameters. Robustness can be partly assured by good system suitability specifications. Testing by varying some or all conditions, e.g., age of columns, column type, column temperature, pH of buffer in mobile phase, reagents, is normally performed.

Specificity/Selectivity

The analyte should have no interference from other extraneous components and be well resolved from them. A representative chromatogram or profile should be generated and submitted to show that the extraneous peaks either by addition of known compounds or samples from stress testing are baseline resolved from the parent analyte.

Recent development in pharmaceutical and biotechnological field generates a cumulative demand for analytical methods. Rapid and accurate quantification of the substrate and drug product is important in process development. It is very important to develop efficient and accurately validated analytical methods to develop safe and effective drugs.

CHAPTER 2

AIM AND OBJECTIVE

Analytical instrumentation plays an important role in production and evaluation of new product. Analytical technique is a method that is used to determine the concentration of a chemical compound or chemical element. There are a wide variety of techniques used for analysis, from simple weighing to titration to advanced techniques using highly specialized instruments.

Analytical techniques play a crucial role in new drug development. An appropriate analytical method is required to study the pharmacokinetic, therapeutic and toxicological effects of new drug entities. The present work is focused on developing new analytical technique for determination and quantification of phthalates contamination in liquid food products.

In the present scenario, the market is flaked with consumables containing phthalates induced food samples and consumers are taken to ride. No studies have been carried out for determination of phthalates in food products in INDIA. The world we live in today is directly influenced by the progression of science. From ancient to modern times, the role of science has always been to further the development of society. This project addresses aspects concerning the well-being of the individuals and families in the society.

Plan of work

In this study an attempt has been made to identify, detect and quantify the phthalates in packaged liquid food samples including water, the primary objective of this work being:

- Liquid-liquid extraction of the phthalates from the samples for analysis.

- Detection and identification of the phthalates in the samples by Gas chromatography and Mass spectroscopy.
- Quantification of the phthalates in the samples.
- To create awareness about phthalates and its toxic nature.

CHAPTER 3

REVIEW OF LITERATURE

1. Zitko et al. (1972) reported the determination, toxicity and environmental levels of phthalate plasticizers, phthalates extracted from biological samples with hexane and quantitated by gas chromatography. The Di-2-ethyl hexyl phthalate in commercial fish food was found to be 8-9µg/g of lipid [31].

2. John et al. (1973) studied the toxicity and health threats of phthalate esters. Long term feeding studies in several species of animals indicated that a no effect dose may range from approximately 0.1 to 0.2 g/kg per day [32].

3. Charles et al. (1997) reported the environmental fate of phthalate esters and the bio concentration factor for DEHP in Algae and Fish to be 3173±3149 and 280±230 respectively [33].

4. Robert et al. (2002) studied the risks to human reproduction on phthalate exposure. They reported that DBP is a testicular toxicant in three species of young adult laboratory animals in high dose of 1000 mg/kg bw/day.[34]

5. Bart et al. (2003) carried out the determination of phthalates in environmental, food and biomatrices by using capillary gas chromatography with mass spectrometry. Trace concentrations of phthalate mono esters (MBP,MEHP,MiNP and MiDP) in water can be readily derivatized to their corresponding ethyl alkyl ester in the presence of ethyl chloroformate, pyridine and ethanol. Typical concentrations between 0.1 and 2.5µg/ml MBP were found in waste water samples [35].

6. Kayako et al. (2003) carried out quantitative detection of nine phthalate metabolites in human serum using reversed- phase high-performance liquid chromatography –Electrospray ionization –tandem

mass spectrometry. The samples were subjected to an enzymatic deconjugation to hydrolyze the glucuronidated phthalate mono esters and solid –phase extraction to isolate the mono esters from other serum components. The limit of detection of all nine phthalate mono esters in serum was found to be 0.6 to 1.3ɳg/ml [36].

7. Feng at el. (2005) developed a headspace solid-phase micro extraction method combined with gas chromatography mass spectrometry for the determination of phthalate esters in cow milk. The Di-2- ethyl hexyl phthalate present in the raw cow milk was found to be 16.0 to 215ɳg/g .[37]

8. Jean et al. (2006) reported the analysis consumer cosmetic products phthalate esters by using high performance liquid chromatography with UV detector, average recoveries of the phthalate esters was found to be 90%. The DEP was detected most frequently at concentrations up to 38,663 ppm and DBP was found in fewer products, but at levels up to 59,815 ppm [38].

9. Sorensen et al. (2006) carried out the determination of phthalates in milk and milk products by liquid chromatography followed by tandem mass spectrometry. The analysis was carried out in a variety of samples like raw milk, infant formula, powdered, liquid and yoghurt with fruit. The DEHP present in the raw milk, infant formula and yoghurt was found to be 7-30, 10-23 and 15-37 ɳg/gm.[39]

10. Koch et al. (2007) reported the quantification of 22 phthalate metabolites in urine using fast high performance liquid chromatography with MS/MS. Analytical limits of quantification for all metabolites were in the range of 0.05 to 0.5 µg/ml, which is sufficiently low to determine environmental exposures to phthalates [40].

11. Carrillo et al. (2007) reported the determination of phthalates in wine by headspace solid-phase micro extraction followed by gas –chromatography mass spectrometry. The Di ethyl phthalate present in the wine sample was found to be 5.5 ɳg/ml .[41]

12. Del carlo et al. (2008) reported the determination of phthalate esters in wine using solid-phase extraction method followed by gas chromatography mass spectrometry. The DMP and DEP was not detected in this wine sample. The Di ethyl hexyl phthalate present in the sample was found to be 57 to 76ɳg/ml.[42]

13. Debajit et al. (2008) reported the validation and application of an HPLC method for determination of Di-2-ethyl hexyl phthalate and mono 2-ethyl hexyl phthalate in liver samples by revers phase gradient elution method. The developed method gave recovery of more than 95% at five different concentrations between 4.25 and 24.78µg/ml. Both DEHP and MEHP show a linear detection response with a coefficient of variation more than 0.999 [43].

14. Cao et al. (2008) determined phthalate and adipate in bottled water by using headspace solid –phase micro extraction and gas chromatography with mass spectrometry. The Di ethyl hexyl phthalate and DBP present in the bottled water sample was found to be 0.052 to 0.338 and 0.075 to 1.717 ɳg/ml.[44]

15. Thomas et al. (2009) developed methods for the determination of phthalates in food and the recovery of Di-ethyl hexyl phthalate and Di- ethyl phthalate present in the sample by using HP 5 MS column and gas chromatography with electron capture detector was found to be 90-100 and 50-60% respectively[45].

16. Kim et al. (2009) studied the determination of phthalates in raw bovine milk by using gas chromatography with time of flight anayser followed by mass spectrometry and dietary intakes. Di-2-ethyl hexyl phthalate was only determined and quantified in this sample. The amount of DEHP present in the raw bovine milk sample was found to be 57ɳg/ml .[46]

17. He et al. (2010) reported the extraction and quantification of di butyl phthalate ester from the soyabean milk by selective solid-phase

extraction using molecular imprinted polymers. The Di butyl phthalate present in the soyabean milk sample was found to be 8.8ɳg/ml .[47]

18. Guo et al. (2010) developed a method for analysis of phthalates in ham sausages by using solid-phase extraction and gas chromatography – mass spectroscopy. DEHP was only detected in this method, others phthalates were not detected. The amount of Di-2-ethyl hexyl phthalate present in the sausage sample was found to be 155ɳg/gm. [48]

19. The United States consumer product safety commission (2010) reported on toxicity of phthalates. The acceptable daily intake of DBP and DEHP was found to be 200 and 5.8 µg/kg/day[49].

20. Chong et al. (2011) determined six phthalates in polypropylene consumer products by sonication –assisted extraction and gas chromatography –mass spectroscopy method. These techniques are possible to detect phthalates at the level of 1-70 mg/kg. The overall recoveries were 79.2-91.1% .The percentage level of DEHP present in the food container was found to be 0.083[50].

21. Melissa et al. (2011) studied phthalates migration from toys using super critical fluid chromatography and single quadrupole LC-MS. The SFC/MS approach facilitated identification and quantification of mixed isomer of phthalates with good repeatability. The LOD and LOQ of DiNP present in the sample was found to be 12.5 and 50 ɳg/ml respectively [51].

22. Li et al. (2011) reported the simultaneous determination of nine types of phthalate residues in commercial milk products using high performance liquid chromatography with electron spray ionization followed by MS/MS. The Di butyl phthalate present in the commercial milk sample was found to be 120-131ɳg/g .[52]

23. Kanchana et al. (2012) reported the analysis of phthalate esters contamination in drinking water samples using solid phase extraction and gas chromatography, the DEHP concentration in three samples was found to be greater than the regulatory maximum admissible concentration. The DMP, DBP and DEHP present in the mineral water sample was found to be 0.16±0.01, 0.33±0.01 and 0.49±0.07 mg/l respectively [53].

24. Jimmy et al. (2012) developed a rapid, sensitive, and robust method of detection of phthalates in food using GC-MS or LC-MS. The GC-MS and LC-MS method can be run on both single and triple quadrupole mass spectrometers. The GC-MS methods can detect as little as 50 ppb of the phthalates, while the LC-MS methods are sensitive to as low as 1ppb,using triple quadrupole MS. The presence of DEHP, DINP and DNOP in beverage sample was found to be 72.5, 137 and 19.0 mg/l [54].

25. Ying et al. (2012) reported the challenges encountered in the analysis of phthalate esters in food stuffs and other biological matrices and they investigated a method of liquid –liquid extraction to remove lipids from oils than GPC method. The concentration of DMP, DEP, DBP and DEHP present in the oil sample was found to be 0.62±0.09, 0.18±0.32, 7.05±0.59 and 86.9±5.30 ɳg/g respectively [55].

26. Russo et al. (2012) developed an analytical method for determining phthalate esters in wine samples by solid-phase extraction and gas chromatography coupled with ion-trap mass spectrometer detector. The DEHP and DBP present in wine sample was found to be 2.4 to 16.0 and 7.3 to 23.0 ɳg/ml.[56]

27. Jianxia et al. (2012) reported the determination of phthalates in liquor beverages by single quadrupole GC-MS using simple liquid-liquid extraction and n-hexane as solvent. The DEHP present in the samples was found to be 0.006-0.236 mg/l[57].

28. Fierens et al. (2012) reported the transfer of eight phthalates through the milk chain, extraction using acetone: n-hexane (1:1) ratio. The instrumental analysis was performed by means of gas chromatography-low resolution – mass spectrometry with electron impact ionization. The presence of DMP, DEP and DCHP in pouch contained measurable levels were found to be 0.01, 0.06 and 0.1 ng/cm^2 [58].

29. Chen et al. (2013) reported the determination of phthalates in drinking water by using UHPLC with UV detection. The external standard method was used to establish the calibration curve and quantify the phthalates in drinking water samples. The linearity was observed from 0.05-10 µg/ml and recovery is 77-110% [59].

30. Anila et al. (2014) studied the determination of phthalate esters in soft drinks by using mass spectrometer provides high sensitivity for the detection of phthalate esters in selected ion monitoring(SIM) acquisition mode. The percentage recovery of DEHP at 1000 ɳg/ml is found to be 78% [60].

CHAPTER 4
MATERIALS AND METHODS

4.1. REFERENCE STANDARDS USED

S.No	Sample	Manufacturer
1.	DIBP	Sigma- Aldrich
2.	DBP	Sigma- Aldrich
3.	BMOEP	Sigma- Aldrich
4.	DIPP	Sigma- Aldrich
5.	IPPP	Sigma- Aldrich
6.	DPP	Sigma- Aldrich
7.	DHP	Sigma- Aldrich
8.	BBP	Sigma- Aldrich
9.	DEHP	Sigma- Aldrich
10.	DNOP	Sigma- Aldrich

4.2 REAGENTS AND INSTRUMENTS

Reagents

All the solvents used in spectrophotometric analysis were of AR grade and the solvents used for chromatographic studies were of HPLC grade.

Instrumentation

Equipment used: Agilent GC-7890A, MS-5975.

Table- 1 GC-MS Instrument Conditions

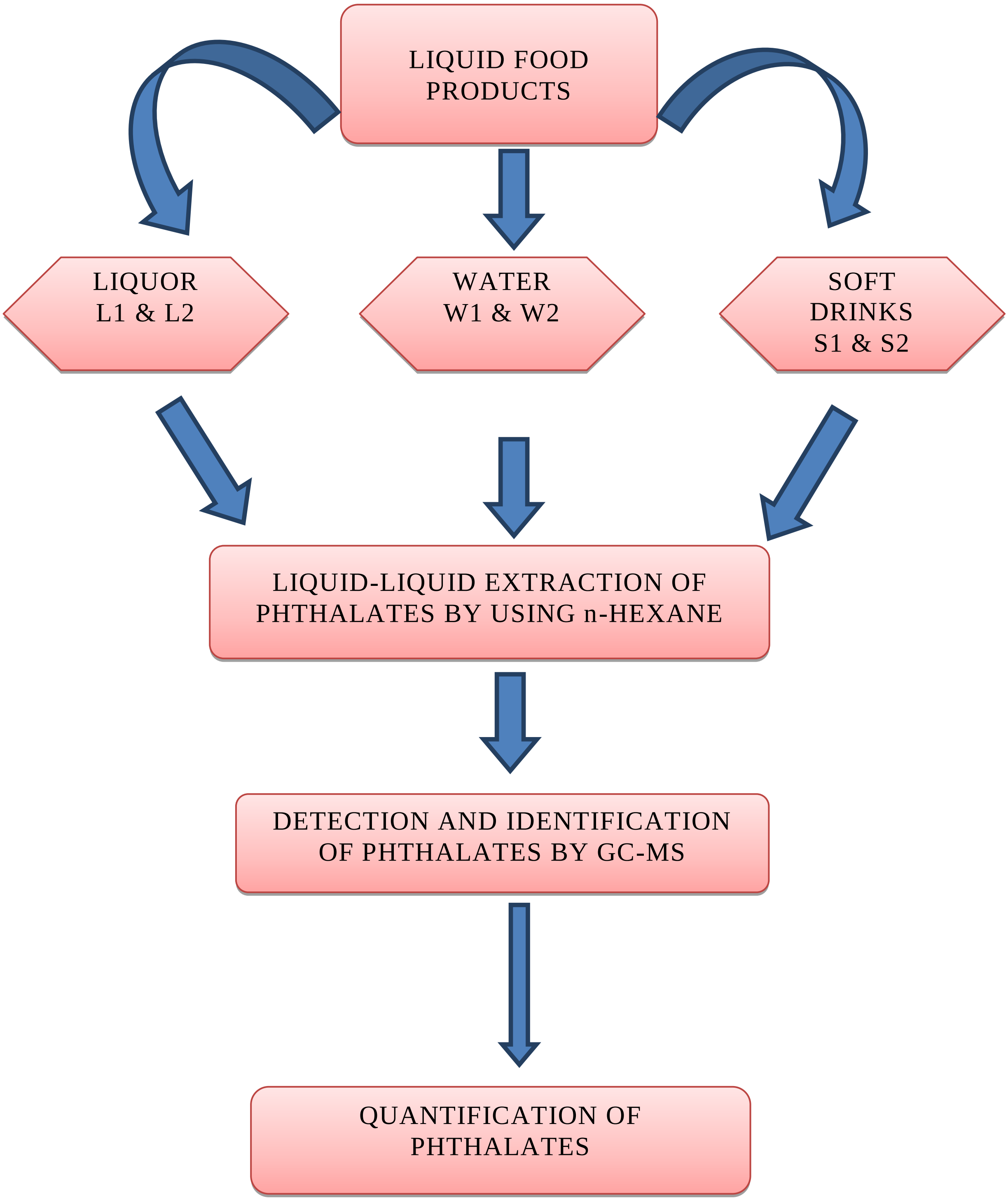
Instrument used	Agilent GC-7890A, MS-5975C or equivalent
Column	DB-5 ms,20m×0.25µm× 0.25µ
Acquisition mode	SIM/scan
Injection mode	Pulse split
Split ratio	1:05
Liner & liner volume	Single tapered with wool,~1ml
Injection volume	1µl
Injection temperature	280^0C
Interface temperature	310^0C
Ion source temperature	230^0C
Flow rate	1ml/min
Carrier gas	Helium
Temperature program	Initial temperature =150^0C, hold for 10 min, raise at 20^0C/min to 250^0C for 2 min; 30^0C/min to 300^0C hold for 12 min.

Samples used

Six samples – 2 samples each of liquor (L1 and L2), water (W1 and W2) and soft drinks (S1 and S2) were used in the present study. The samples were procured from the local market for the purpose of developing analytical method.

Extraction method: Liquid – Liquid extraction

SCHEME OF WORK



Preparation of standard solution

The stock solution having concentration of 1000mg/ml of the phthalate esters were prepared separately by dissolving accurately weighed quantities of the phthalate standards in hexane/acetone (8:2 v/v). Further dilution of standard stock solutions were made with hexane to get working standard solution of 5000, 2000, 1000 and 500 µg/ml.

Calibration curve of standard

The calibration curve was constructed by taking various concentrations of the standards in the concentration range of 0.5 to 5 mg/l. The peak area was measured for the various dilutions. The linearity curve was constructed between concentration and peak area.

Analysis of marketed formulation

Liquor sample

Liquid-liquid extraction method was used to extract the phthalate residues present in the liquor sample. Prior to extraction the liquor sample was subjected to evaporation for removal of ethanol. To the residue 10ml of n-hexane was added and shaken well for 5 minutes. Then finally the supernatant was transferred for analysis.

Water & Soft drinks

10ml of each of water and soft drinks sample were mixed with 10ml of n-hexane and shaken for 5 minutes. After extraction the supernatant liquid was transferred for the analysis.

4.3 METHOD VALIDATION

Linearity

For the linearity studies, from the working standard solution, further dilutions were made to get concentration of the phthalates in the range of 0.5 to 5 mg/l. The calibration curve of peak area against concentration was plotted. Correlation coefficient and regression line equations for the standards were calculated.

Precision

The precision was determined with standard samples of the phthalates prepared in triplicates at three different concentration levels covering the entire linearity range. The precision was calculated and reported as % RSD.

Accuracy

To study the accuracy of the proposed methods, recovery studies were carried out by standard addition method. A known amount of standard was added to pre-analyzed sample and percentage recoveries were calculated.

Specificity

The specificity of an analytical method is assessed by its ability to measure accurately an analyte in presence of interferences like synthetic precursor, excipients, degradants or matrix component.

CHAPTER 5

RESULTS AND DISCUSSION

The present work deals with the development of a new analytical method for the detection of phthalates in liquid food samples by GC-MS method. The developed method was applied to quantify the phthalates in the selected market samples.

All the developed methods were validated as per ICH guidelines to prove the reliability of the methods. Only with proper validation, the data obtained can be reliable and trustworthy. The parameters which were validated are linearity, accuracy, precision, limit of detection, limit of quantification, robustness, selectivity and specificity. The validation acceptance criteria was met for the developed method.

The linearity of an analytical method is based on its ability to elicit test results, that are directly or by a well-defined mathematical transformation proportional to the concentration of the analyte present in the sample within a given range. The developed method was found to be linear in the concentration range reported. The regression analysis was carried out, to check the correlation coefficient, intercept and slope of the regression line, which estimates the degree of linearity.

Accuracy of the proposed method was confirmed by recovery studies which indicate that, the co-formulated substances do not interfere in the determination. The low values of standard deviation in the recovery data indicate the reproducibility of the proposed method. The percent relative standard deviation determined in the developed methods was below 2% indicating the precision of the method.

The proposed method was successfully applied to the determination of the phthalates in the marketed samples. Statistical analysis was carried out and the results were found to be satisfactory. The regression analysis of the plot using the method of least square was made to evaluate the intercept, slope, regression coefficient and standard deviation of slope and intercept. The high value of the regression coefficient (close to unity) of the regression equation and the negligible value of the intercept depict the linearity of the calibration plot in all the methods. System suitability parameters demonstrated the suitability of the developed chromatographic conditions for the analysis.

The chromatogram of the mixed standards in various concentrations is shown in figure 1-4. The retention time of the standards is furnished in Table-3.

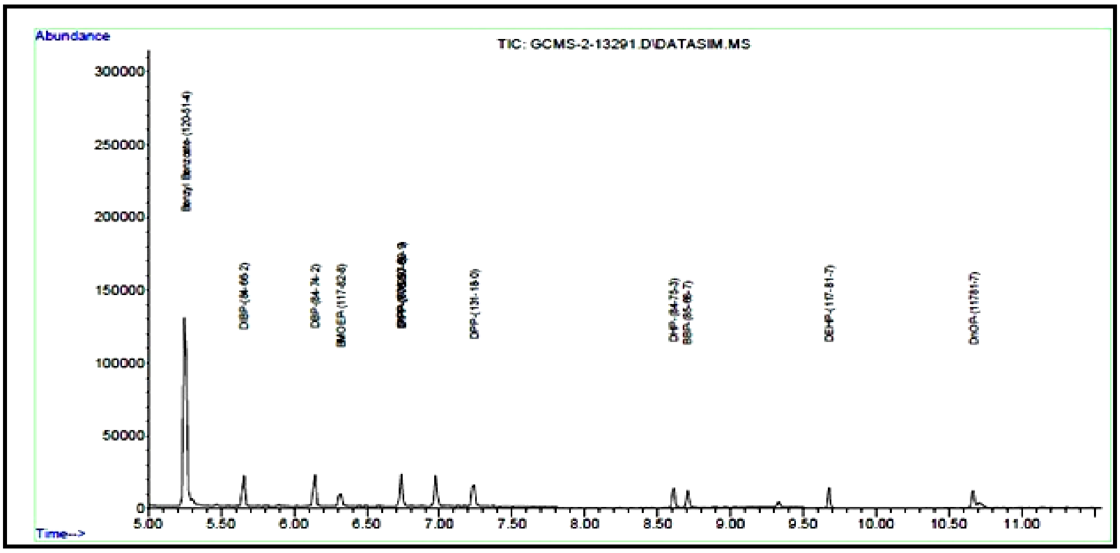


Figure -1 Chromatogram of mixed standard at 0.5 ppm

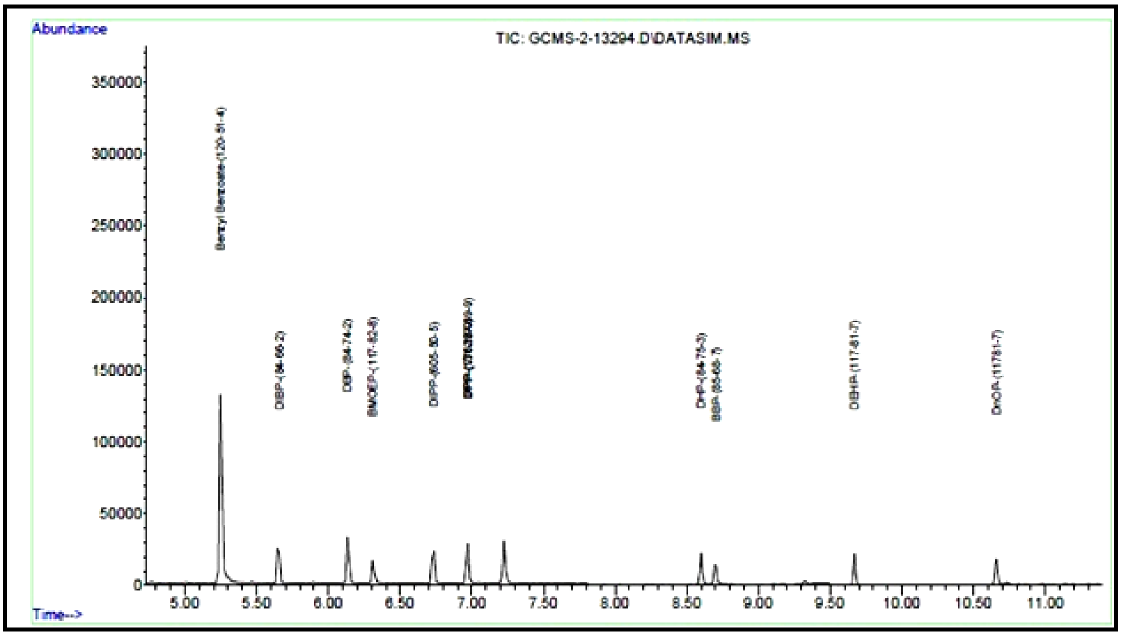


Figure -2 Chromatogram of mixed standard at 1ppm

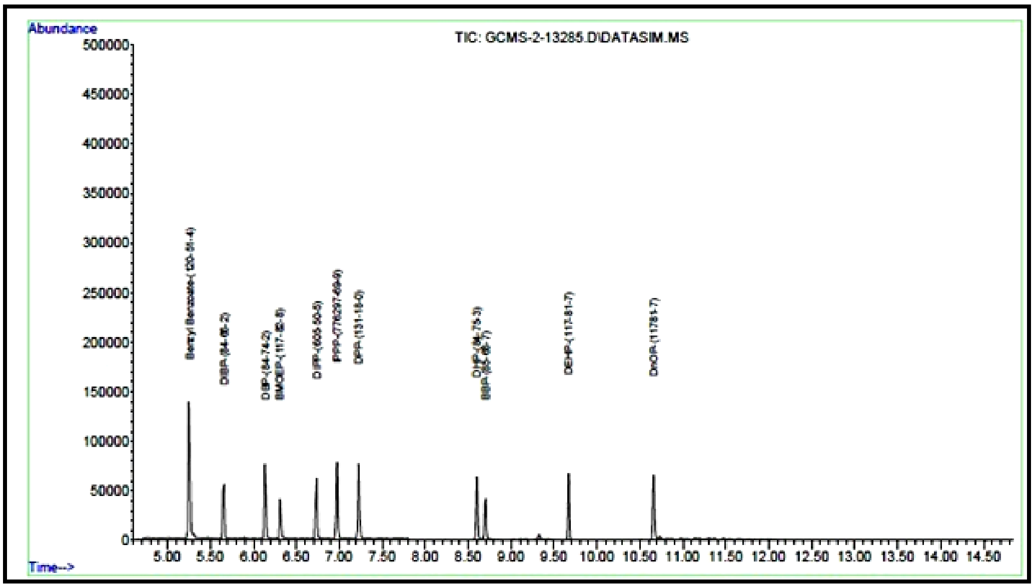


Figure -3 Chromatogram of mixed standard at 2ppm

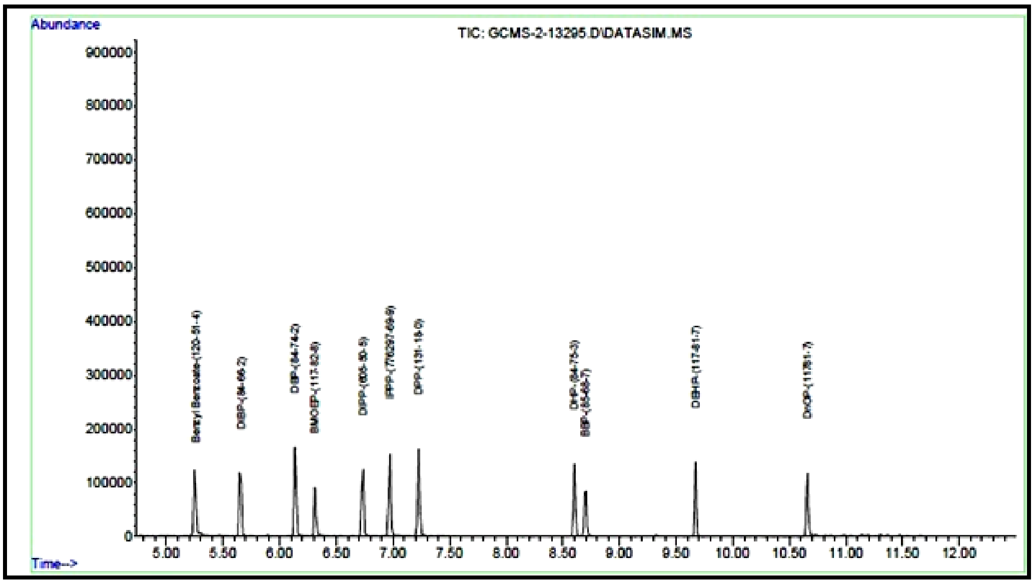


Figure-4 Chromatogram of mixed standard at 5ppm

Method Validation:

Linearity:

Linearity was observed at concentrations ranging from 0.5 to 5 mg/L for all the phthalates. The calibration curves of the standards are shown in figures 5 to 14. The peak areas were calculated for the standard curve with linear regression of very good precision with an average r^2 value of 0.999. The calibration data is given in Table-2. The low values of the standard error of slope and intercept of ordinate showed that, the calibration plot does not deviate from linearity.

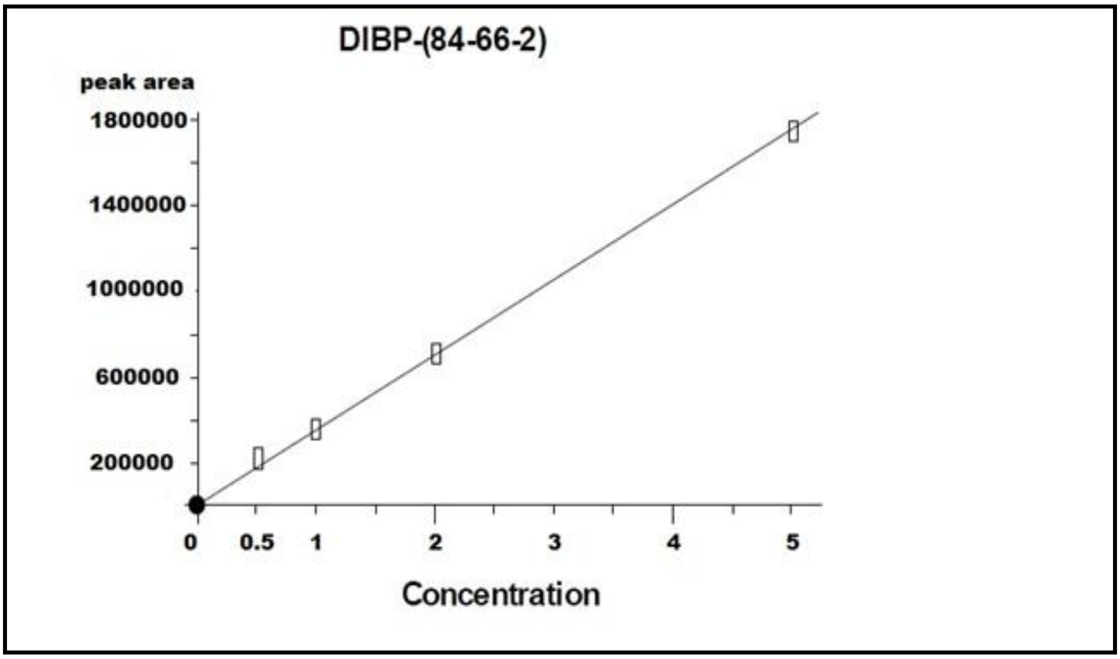


Figure -5 Linearity graph of DIBP

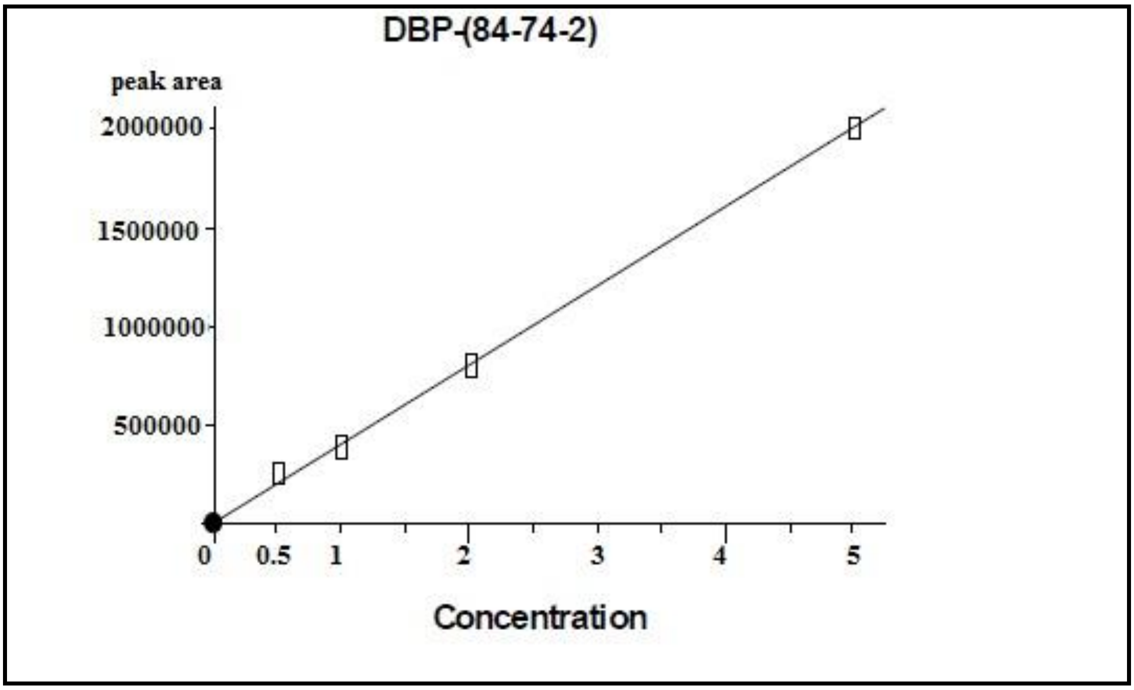


Figure-6 Linearity graph of DBP

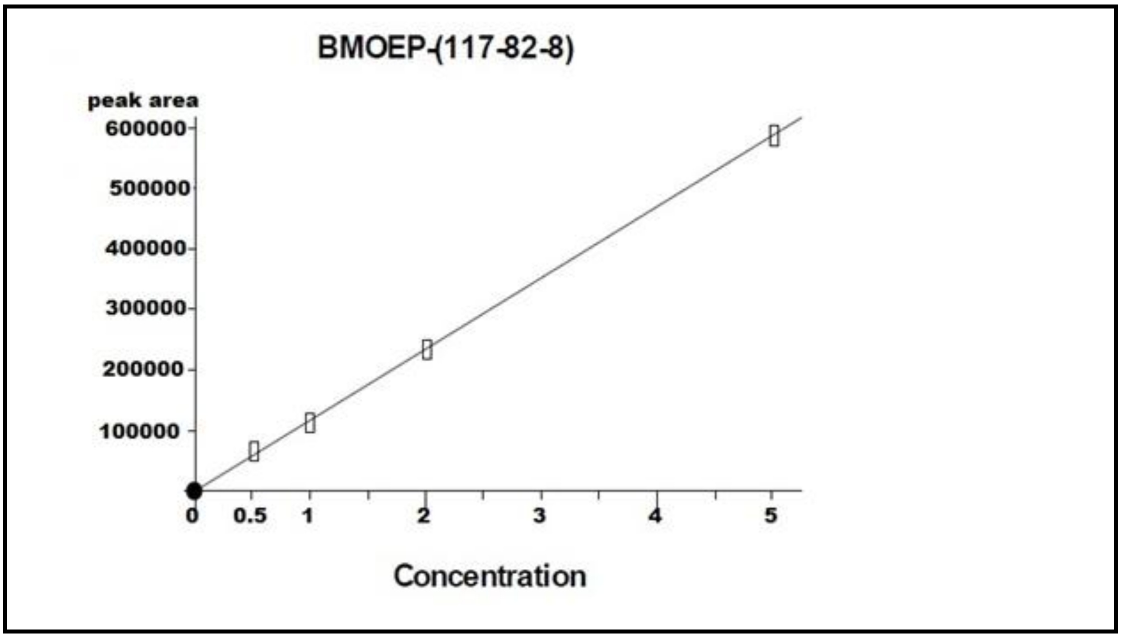


Figure-7 Linearity graph of BMOEP

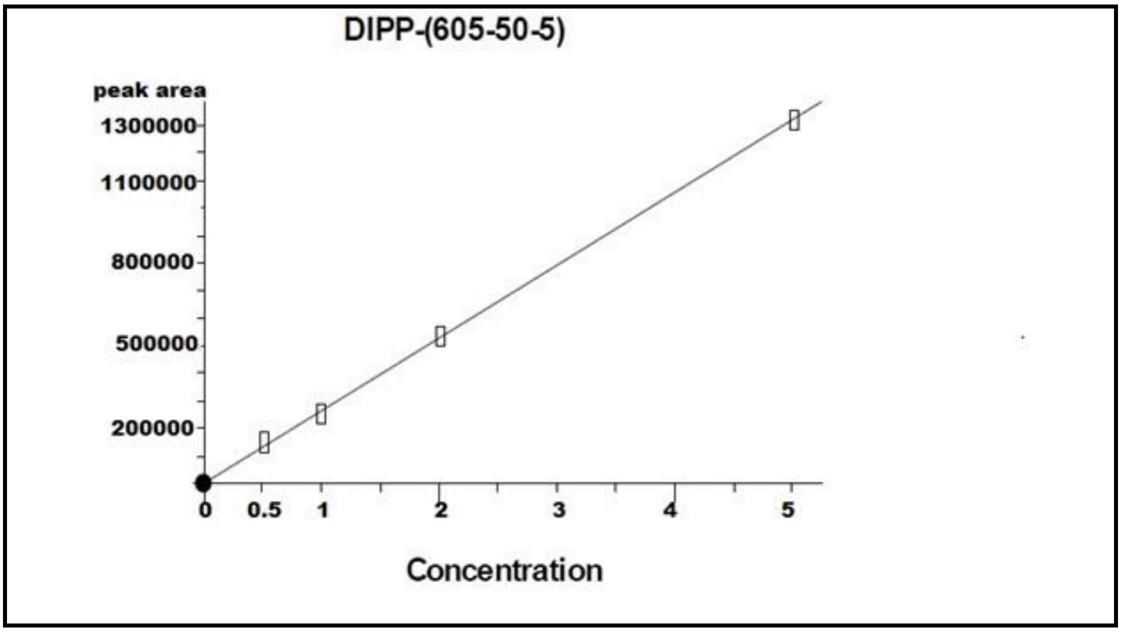


Figure-8 Linearity graph of DIPP

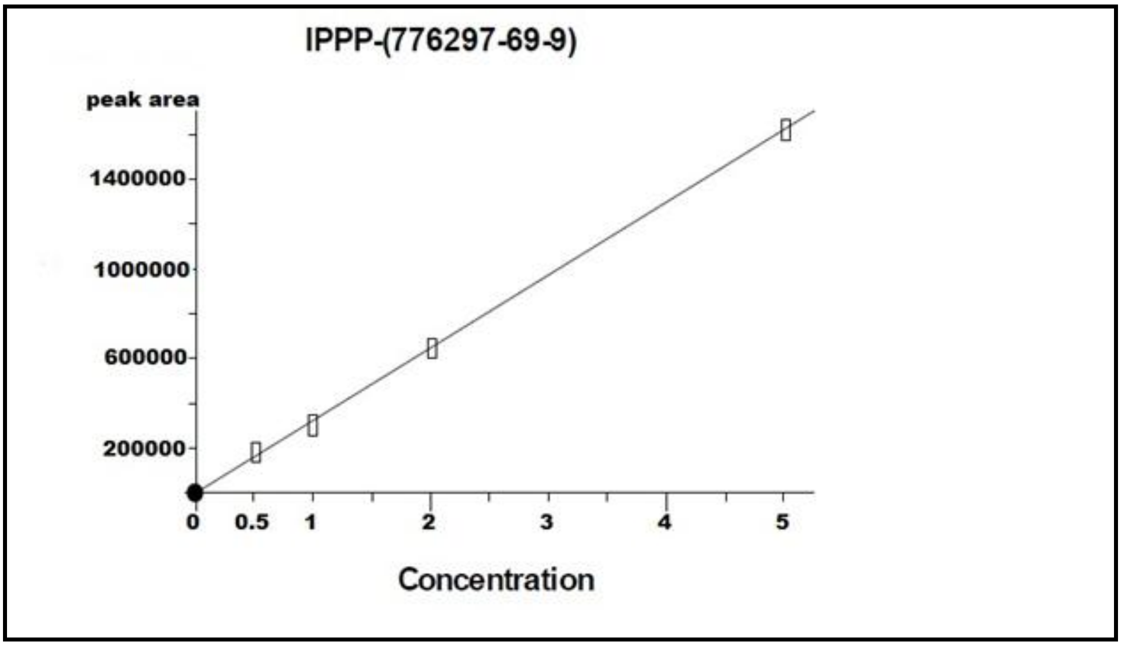


Figure-9 Linearity graph of IPPP

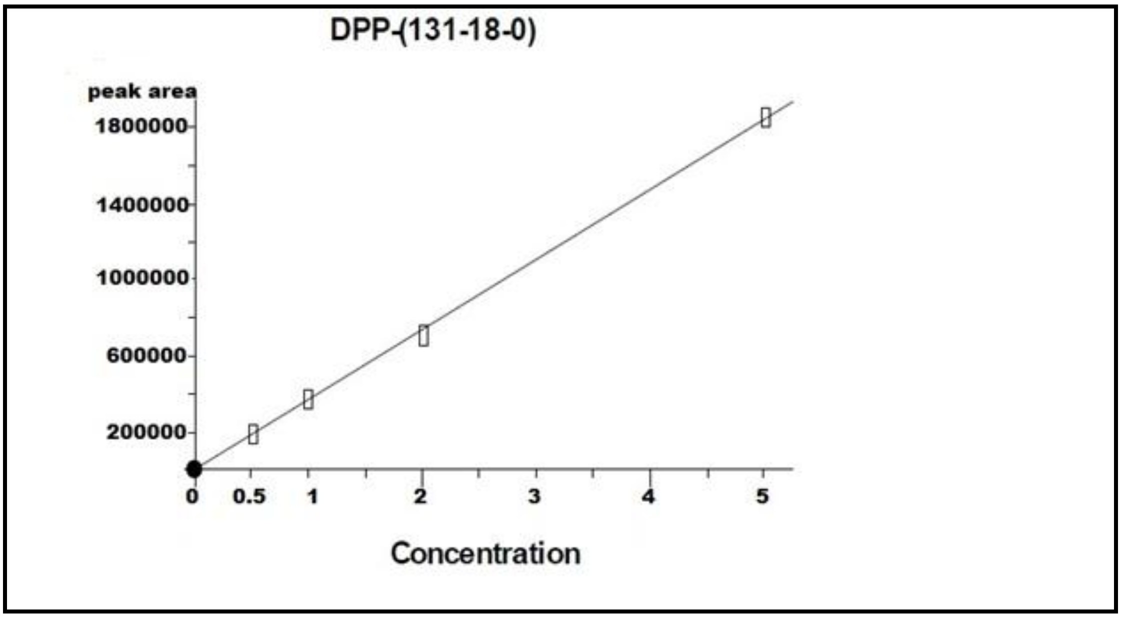


Figure-10 Linearity graph of DPP

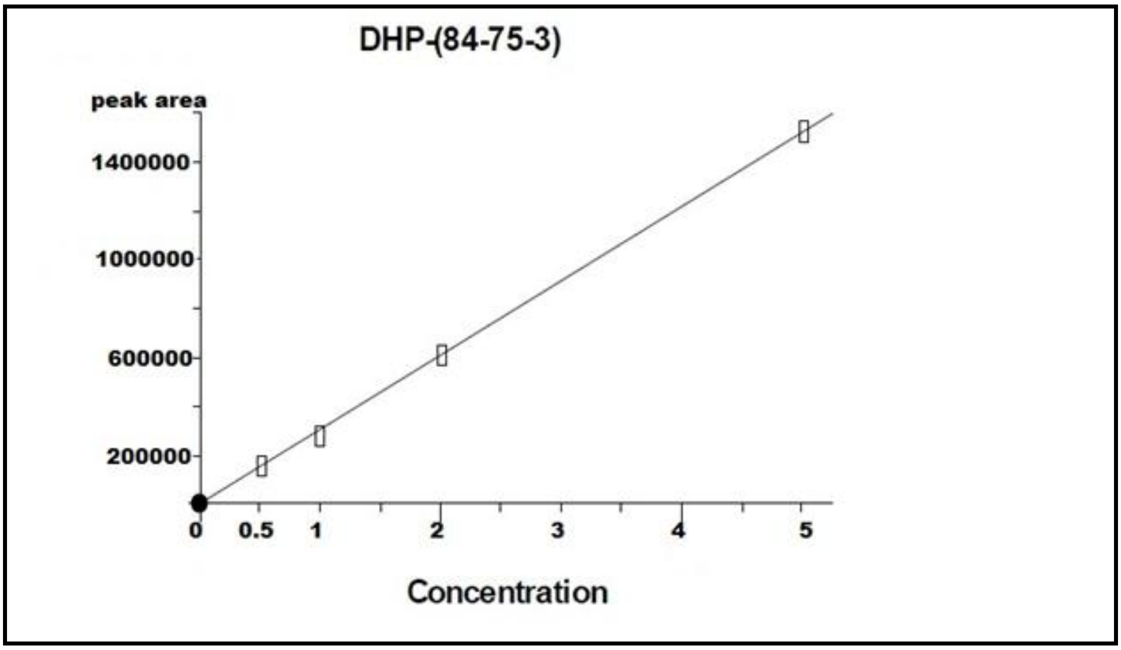


Figure-11 Linearity graph of DHP

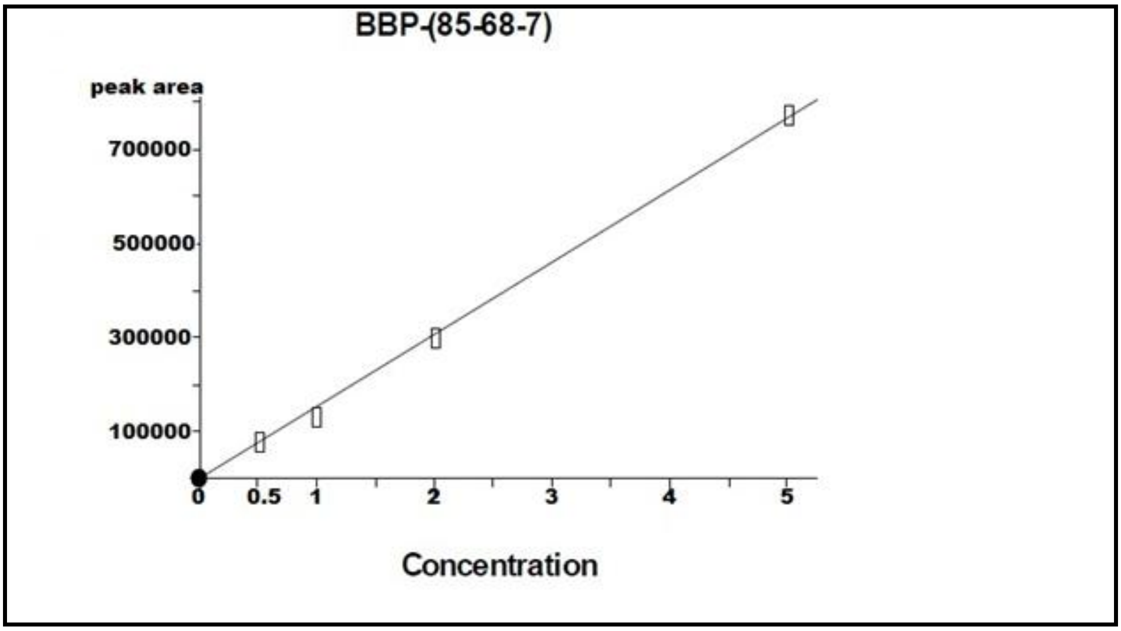


Figure-12 Linearity graph of BBP

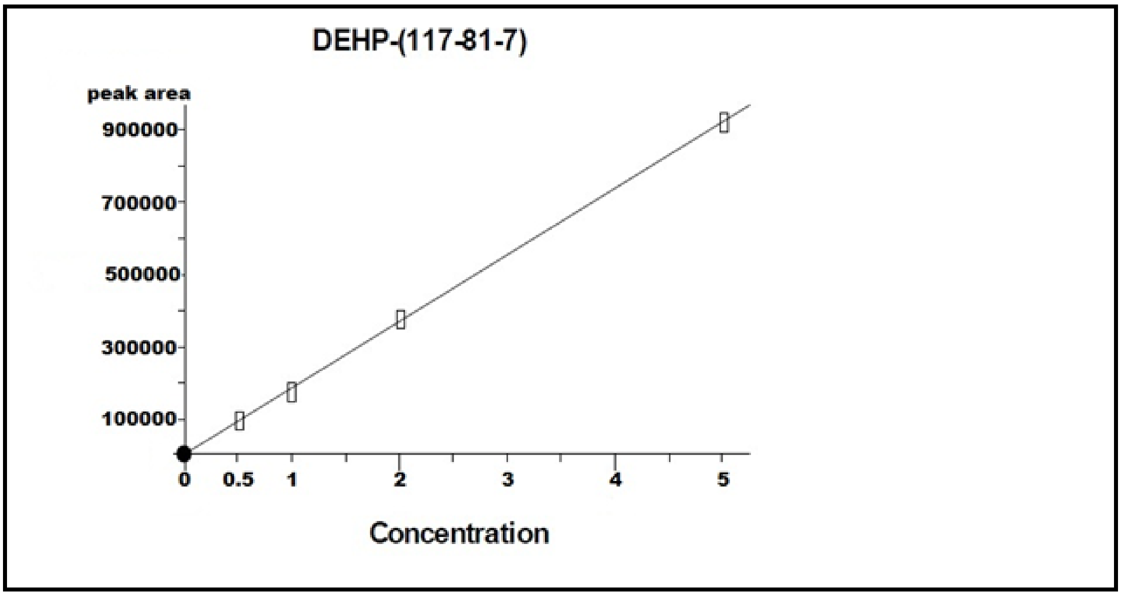


Figure-13 Linearity graph of DEHP

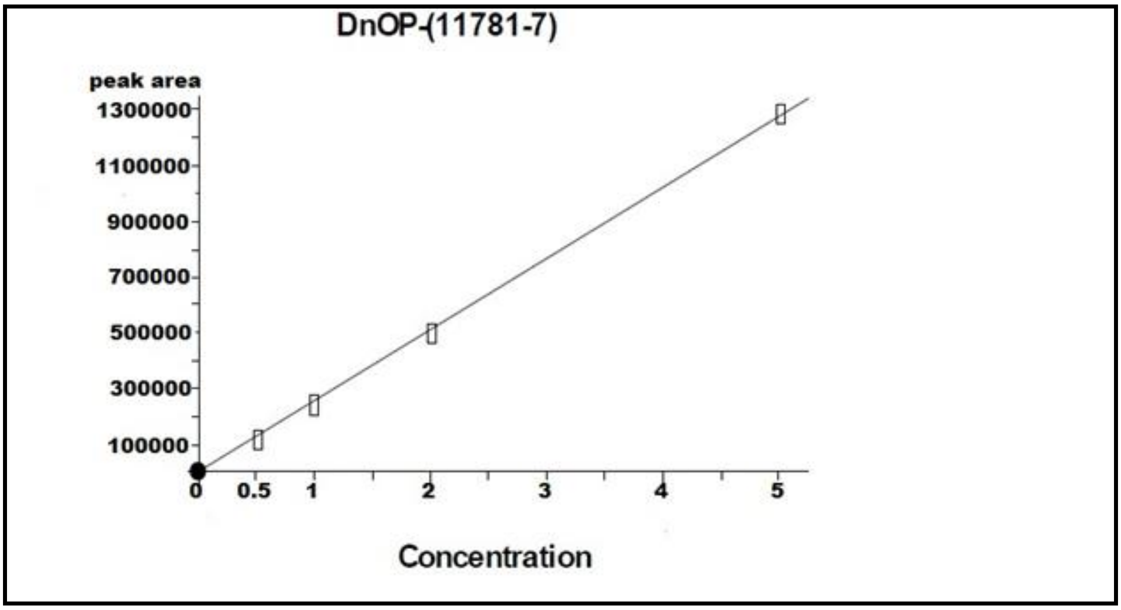


Figure-14 Linearity graph of DnOP

Table -2 Calibration Data of Standard

Standard	Concn 0.5ppm	Peak area	Concn 1ppm	Peak area	Concn 2ppm	Peak area	Concn 5ppm	Peak area
DIBP	0.629	224477	1.0071	359349	2.128	803374	4.878	1741030
DBP	0.607	246838	0.959	390106	2.186	900312	4.908	1996181
BMOEP	0.579	67405	0.962	111991	1.963	229705	5.033	585806
DIPP	0.576	154110	0.942	251987	2.251	591993	4.920	1315644
IPPP	0.560	183404	0.931	304707	2.205	701478	4.948	1619642
DPP	0.507	188633	0.817	304158	2.530	804347	4.917	1850759
DHP	0.495	151585	0.838	256587	2.438	650841	4.972	1523941
BBP	0.514	78668	0.871	133289	2.208	304910	5.037	770583
DEHP	0.506	93061	0.834	153407	2.444	390941	4.978	916132
DNOP	0.629	170243	0.774	209354	2.351	505655	5.110	1282022

LOD and LOQ

The LOD and LOQ that produced the requisite precision and accuracy are reported in table 2. The determination of the limit of detection (LOD) and limit of quantification (LOQ) were based on the characteristic extracted ion mass chromatograms with a peak signal to noise ratio S/N ≥3 for LOD and S/N≥10 for LOQ.

Table -3 Results of Validation

Phthalate	Retention time(min)	Quantitation ion(m/z)	Linearity (mg/L)	Correlation coefficient r2	LOD (mg/L)	LOQ (mg/L)
DIBP	5.656	149	0.1-5	0.9994	1.0	1.0
DBP	6.141	149	0.1-5	0.9991	0.8	1.3
BMOEP	6.358	59	0.1-5	0.9992	0.8	1.0
DIPP	6.753	149	0.1-5	0.9993	1.0	1.0
IPPP	6.969	149	0.1-5	0.9992	1.0	1.0
DPP	7.268	149	0.1-5	0.9994	0.9	1.3
DHP	8.595	149	0.1-5	0.9995	0.9	1.0
BBP	8.702	149	0.1-5	0.9990	1.0	1.0
DEHP	9.670	149	0.1-5	0.9996	1.0	1.0
DnOP	10.664	149	0.1-5	0.9999	1.0	1.0

Accuracy

The accuracy of the proposed method was confirmed by recovery studies. The average recovery was found to be 95-105 % obtained from the results. The results of recovery studies are shown in Table-3.

Precision

The % RSD in precision study of the method was found to be 1.4 to 5.5. The % RSD values were not more than 2%, thus indicating the reproducibility of the method (Table-4).

Table-4 Results of Precision and Recovery

Phthalate	Recovery %	Precision (RSD %)
DIBP	102.0	1.4
DBP	105.0	3.3
BMOEP	95.0	5.4
DIPP	103.0	5.5
IPPP	101.0	2.0
DPP	102.0	1.4
DHP	103.0	5.5
BBP	104.0	4.6
DEHP	101.0	2.0
DnOP	105.0	3.3

Market sample analysis

The proposed method was applied for the detection of phthalates in market samples L1, L2, W1, W2, S1 and S2.

The six commercial liquid samples were prepared by the described sample preparation method for determining possible contamination by phthalate esters. The concentrations of phthalate ester residues present in the commercial liquid samples were analyzed by using above described method and the concentrations of phthalate residues found are shown in Table-4. The proposed method was able to detect and quantify the analyte hence, the method can be considered specific.

The chromatograms of the samples are shown in figures 15 to 20. Mass spectrum was recorded to identify the phthalate residues present in the samples (Figures 22 to 27). The phthalate DEHP was detected in all samples which was confirmed by comparing with the spectrum of standard DEHP (Figure-21).

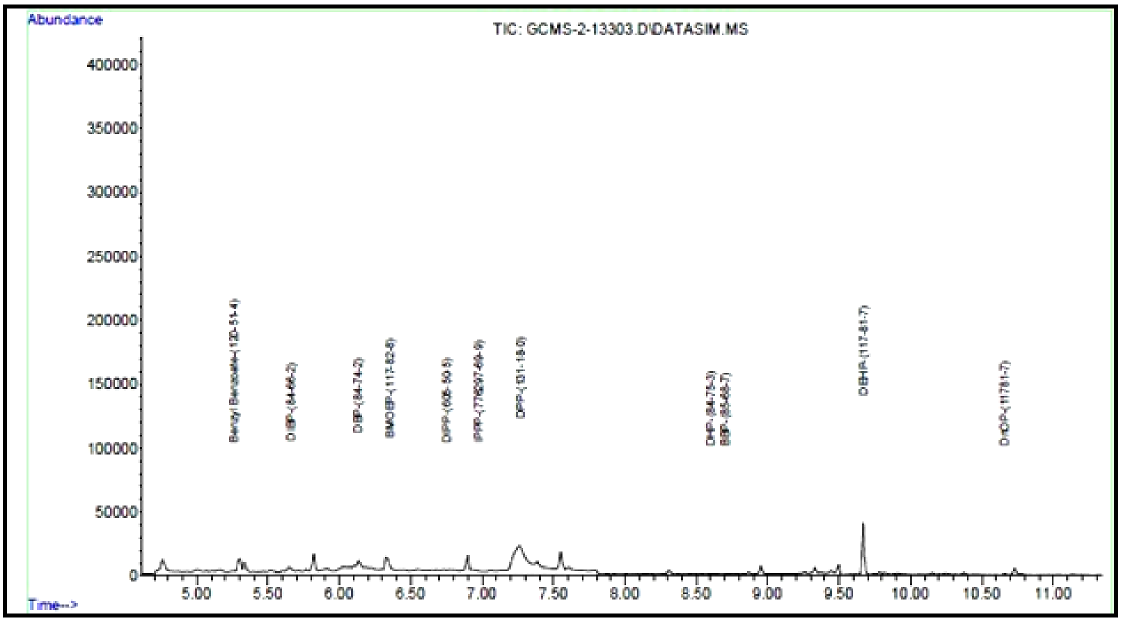


Figure-15 Chromatogram of sample L1

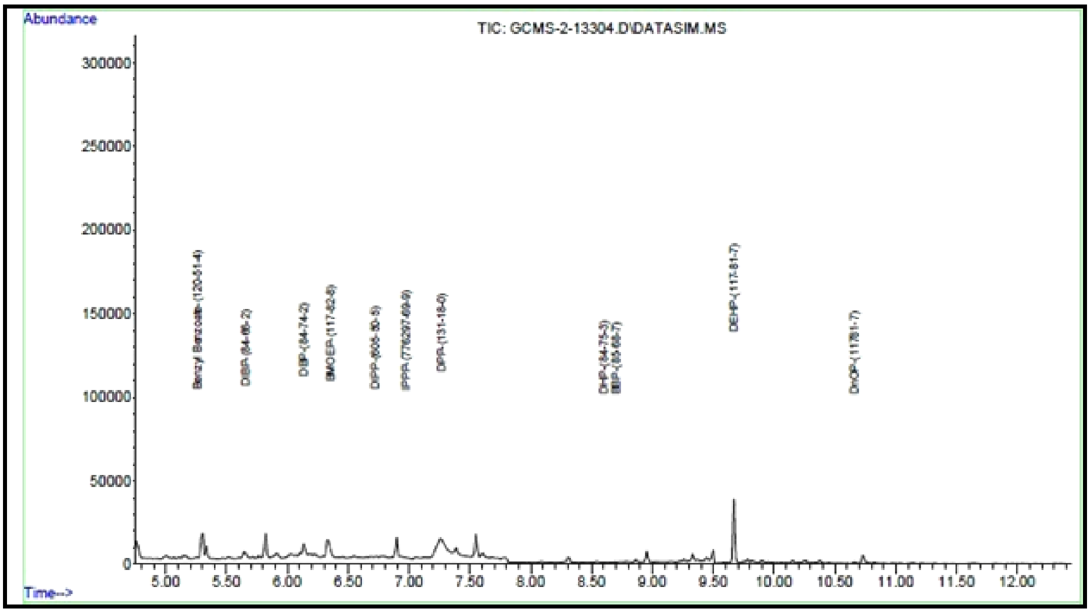


Figure-16 Chromatogram of sample L2

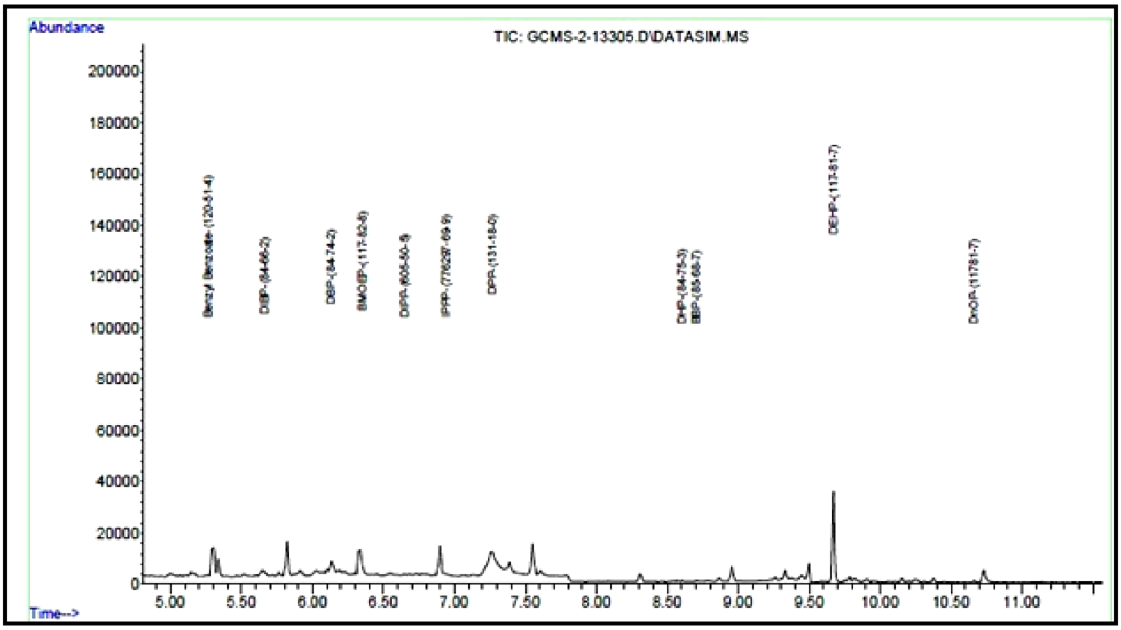


Figure-17 Chromatogram of sample W1

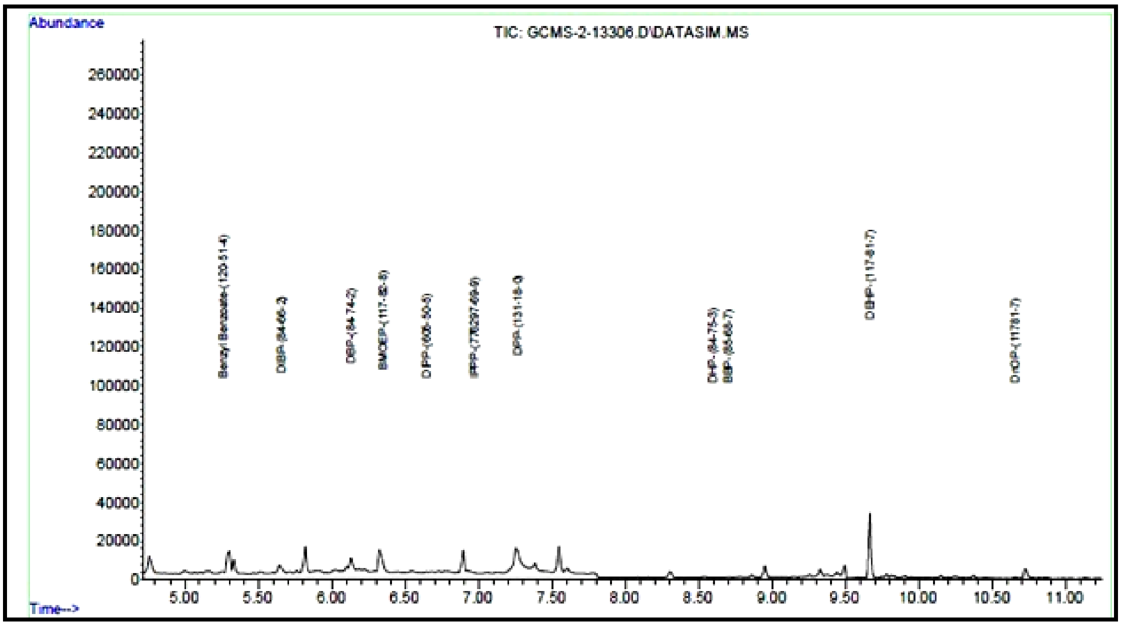


Figure-18 Chromatogram of sample W2

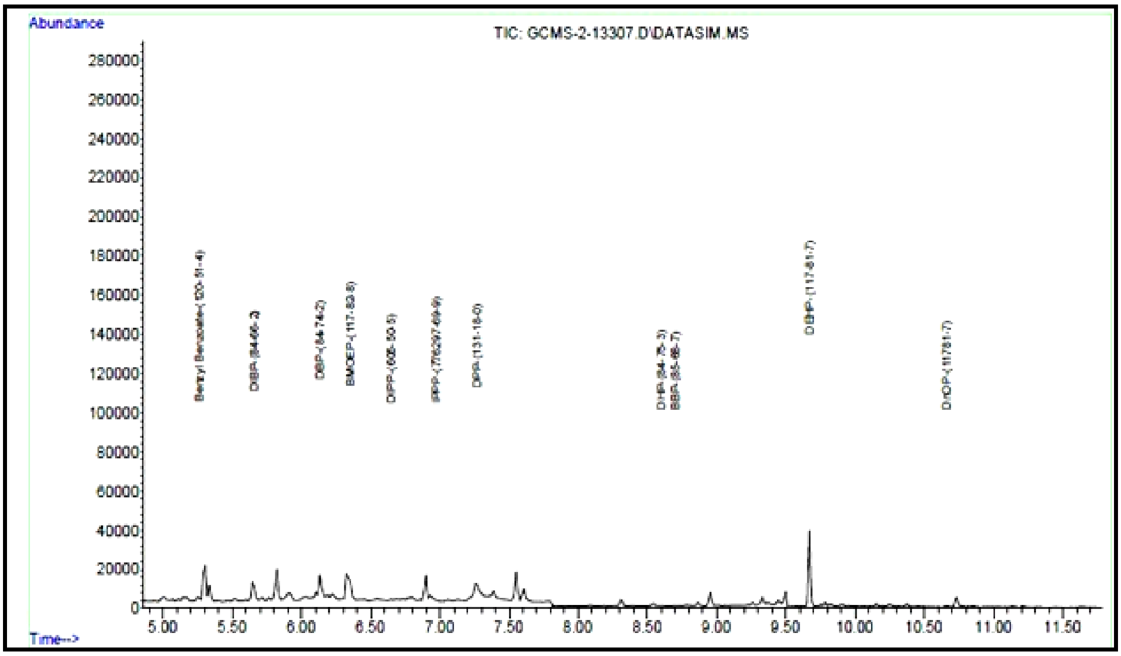


Figure-19 Chromatogram of sample S1

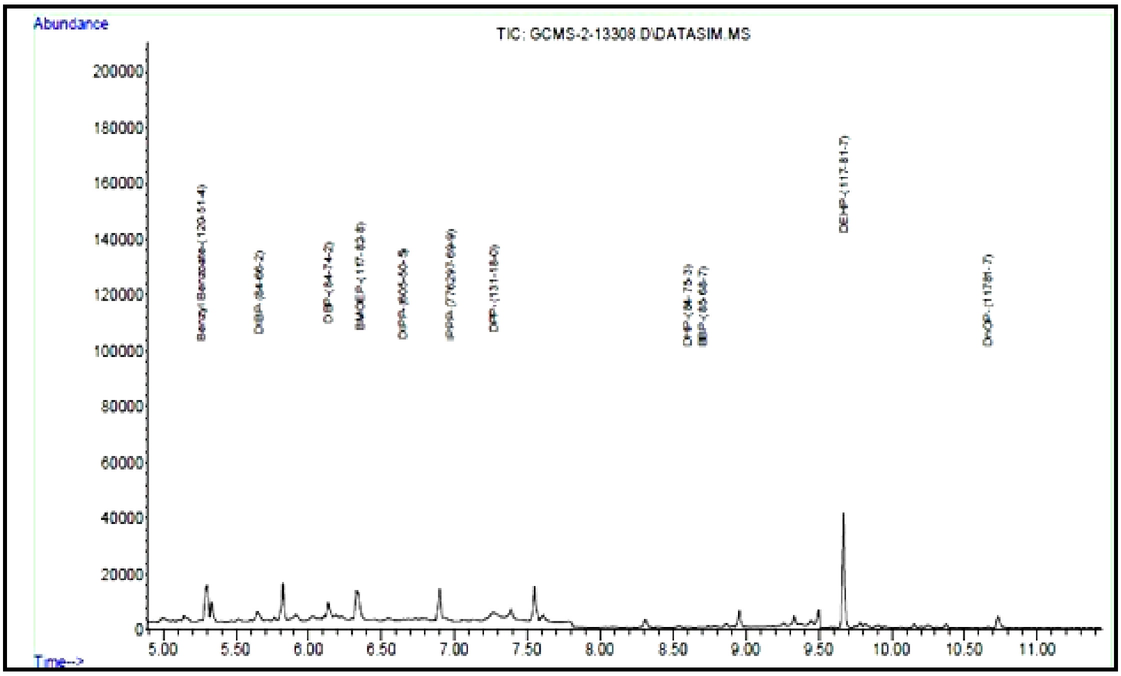


Figure-20 Chromatogram of sample S2

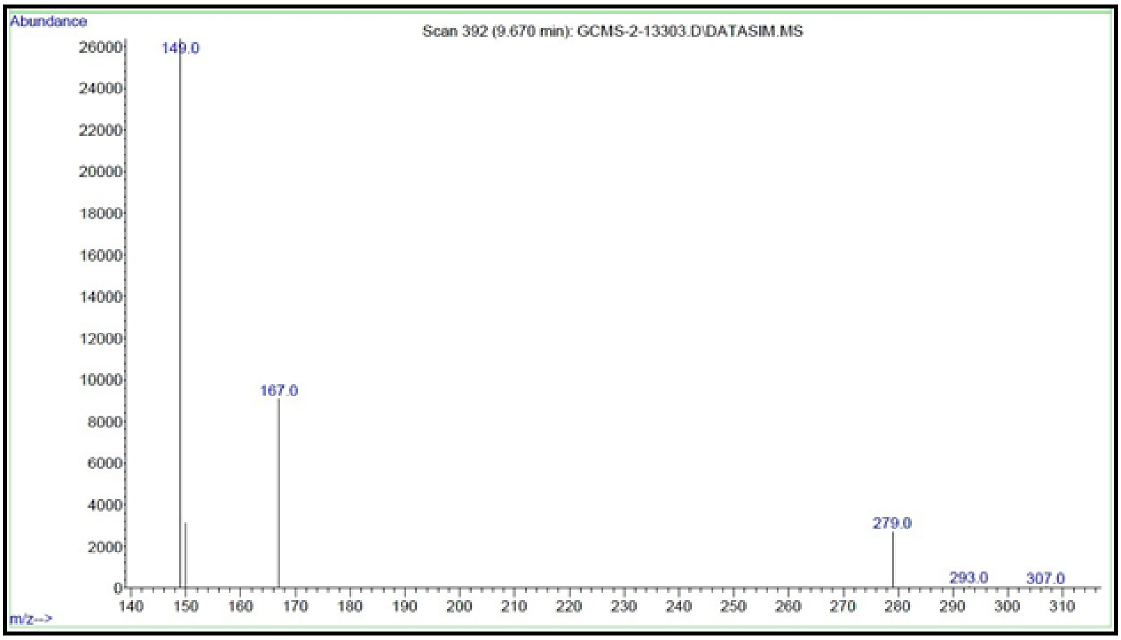


Figure-21 Mass spectrum of DEHP standard

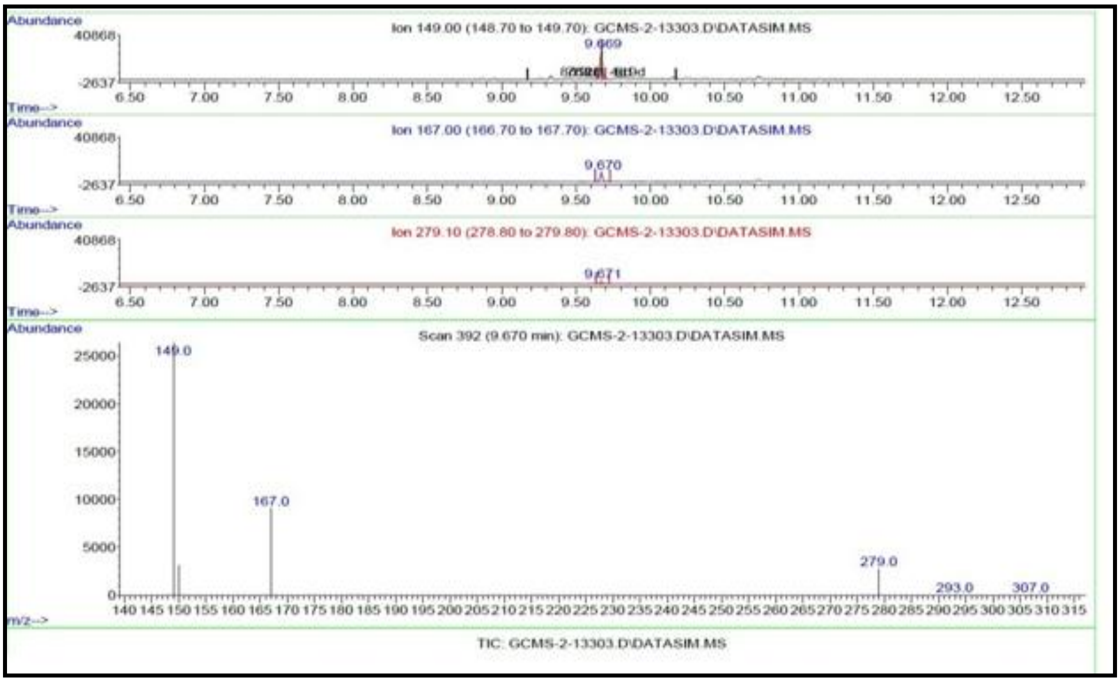


Figure-22 Mass spectrum of sample L1

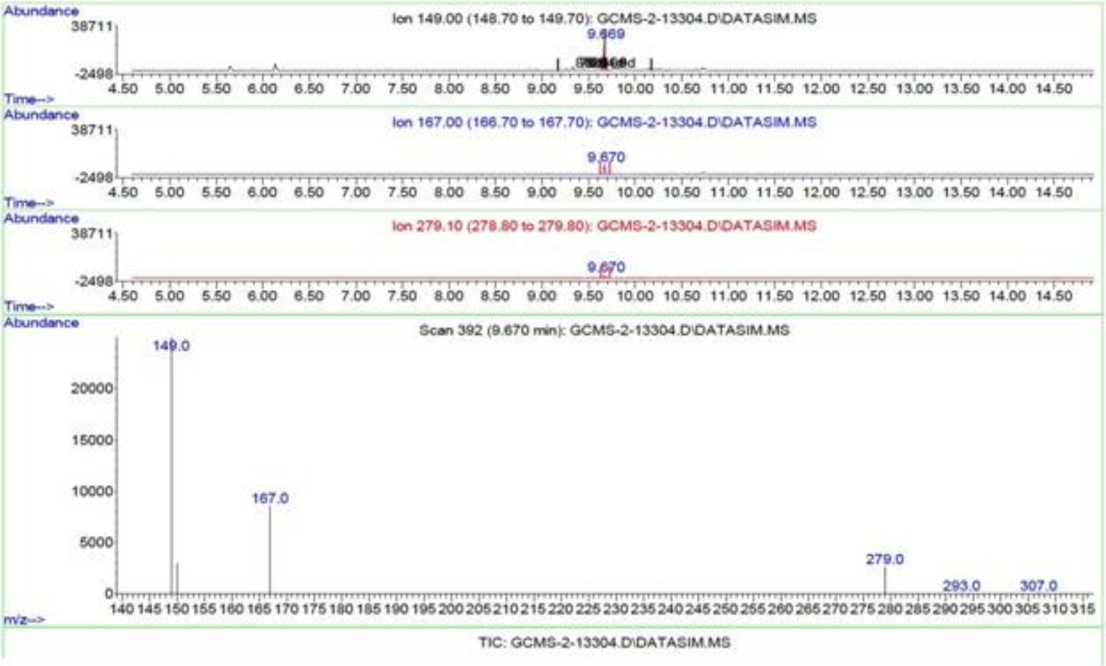


Figure-23 Mass spectrum of sample L2

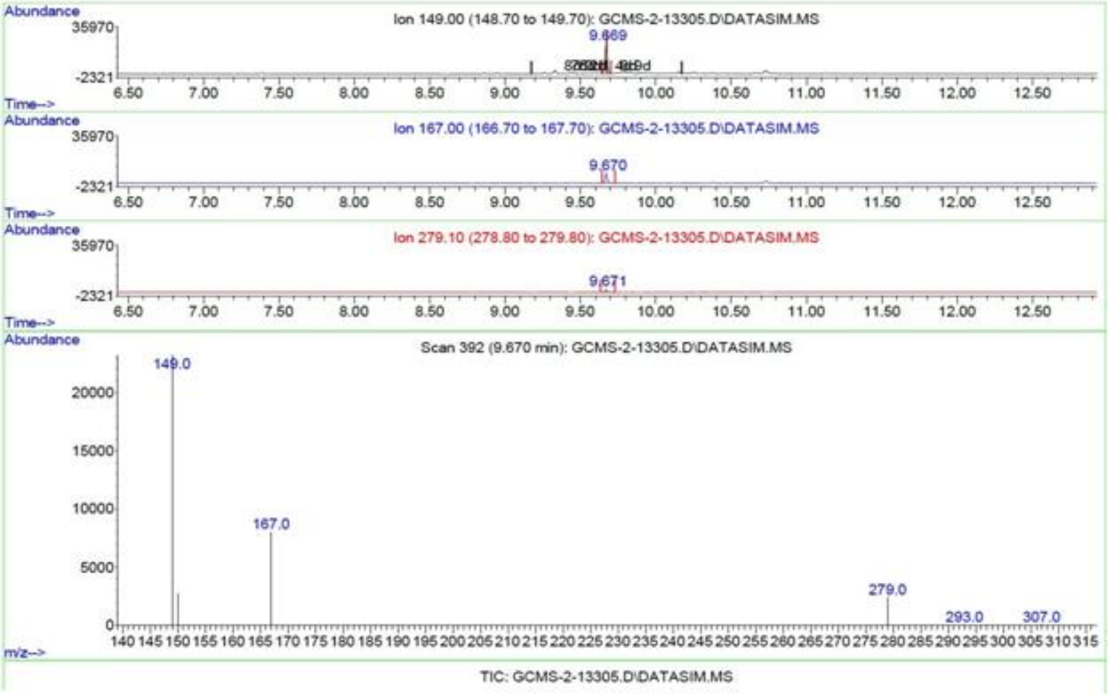


Figure-24 Mass spectrum of sample W1

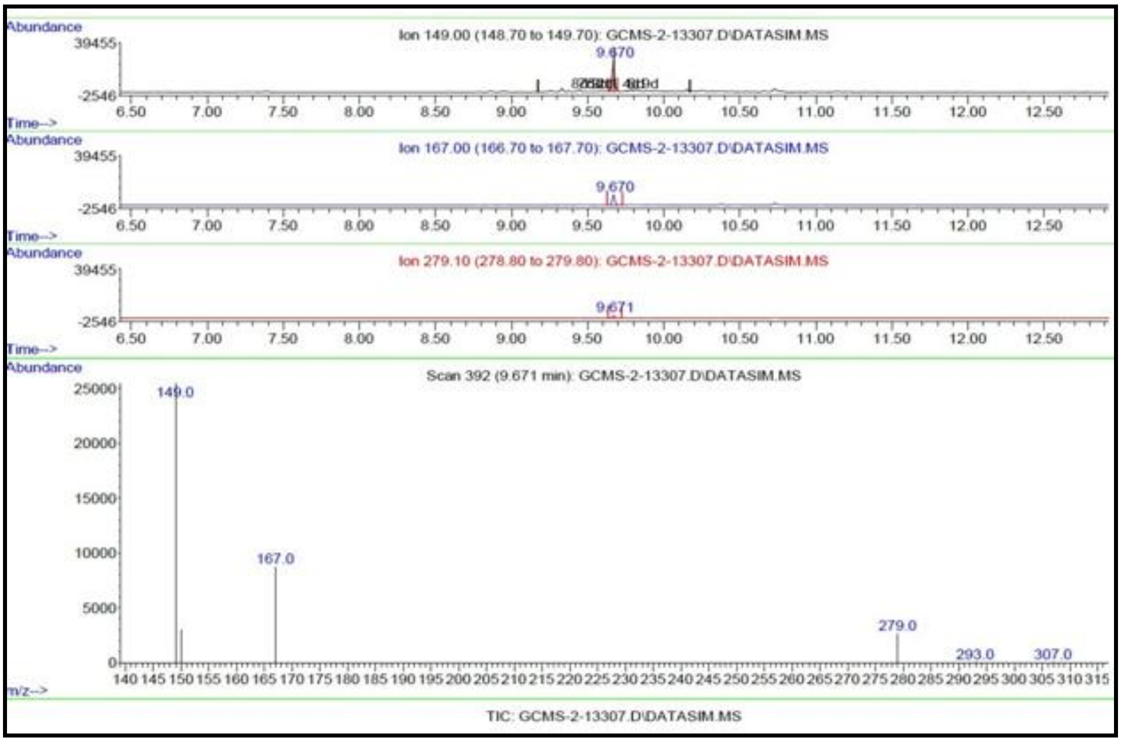


Figure-25 Mass spectrum of sample W2

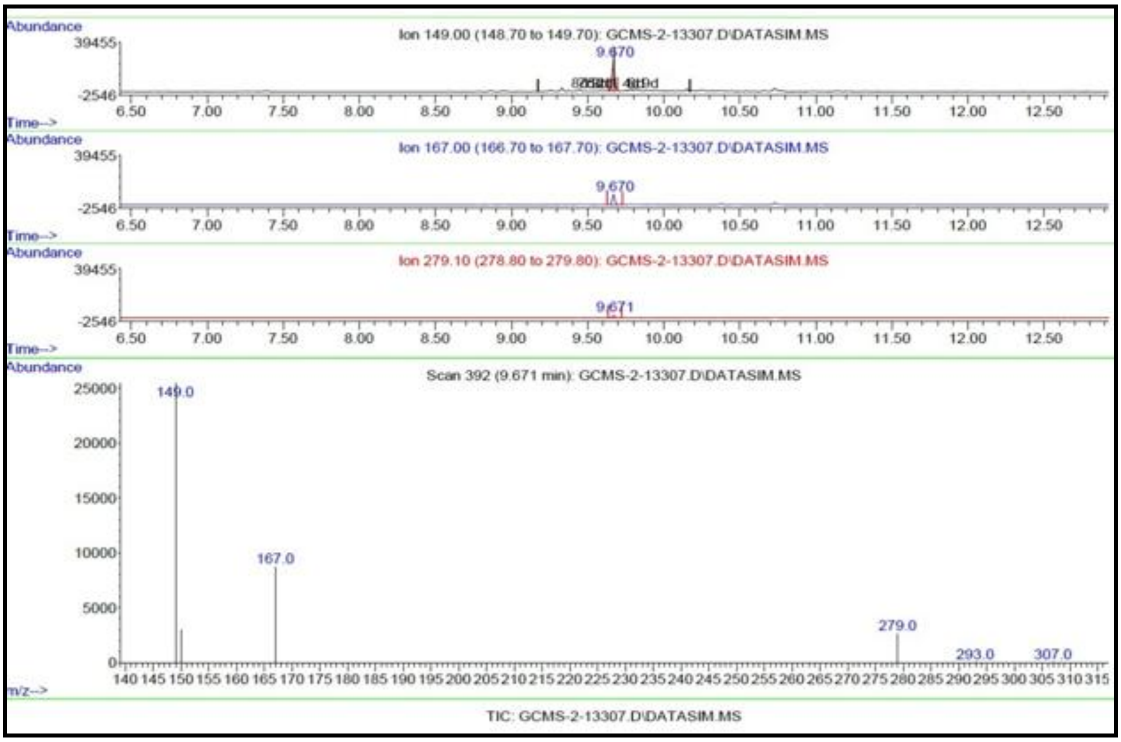


Figure-26 Mass spectrum of sample S1

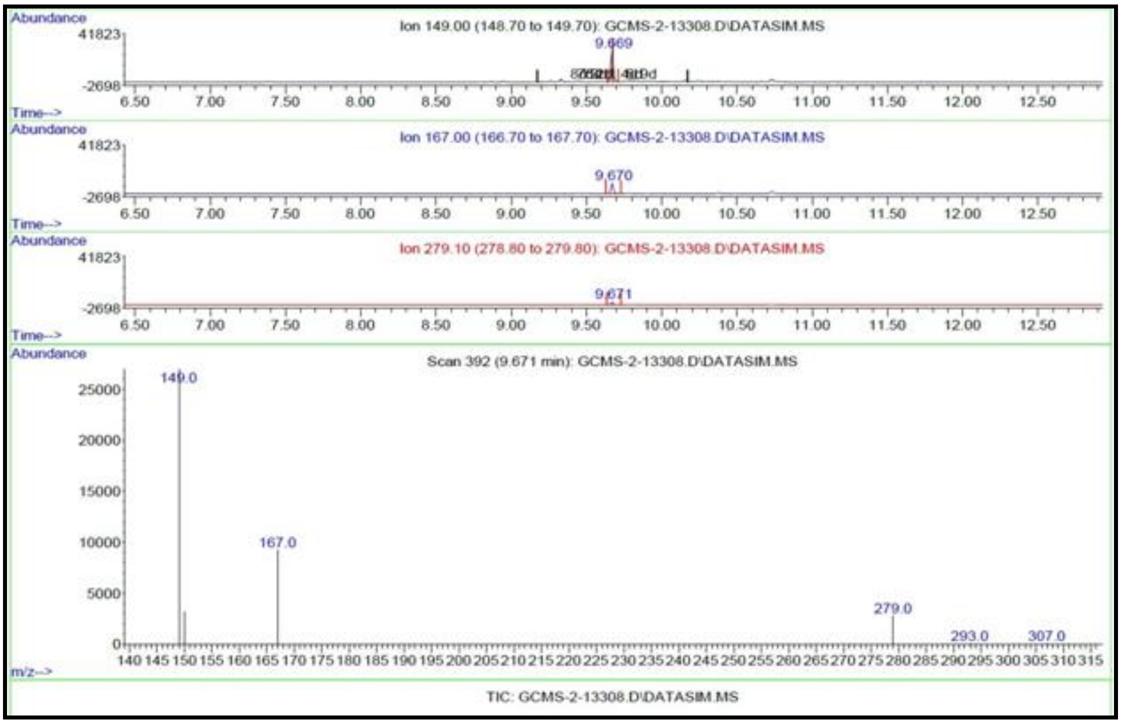


Figure-27 Mass spectrum of sample S2

The developed method was applied for the quantification of phthalates in the selected samples. The results of sample analysis are given in Table-5.

Table-5 Results of Sample Analysis

Phthalate	L 1	L2	W1	W2	S1	S2
DIBP	ND	ND	ND	ND	ND	ND
DBP	ND	ND	ND	ND	ND	ND
BMOEP	ND	ND	ND	ND	ND	ND
DIPP	ND	ND	ND	ND	ND	ND
DEHP	1.52 mg/L	1.50 mg/L	1.32 mg/L	1.31 mg/L	1.47 mg/L	1.65 mg/L
DPP	ND	ND	ND	ND	ND	ND
DHP	ND	ND	ND	ND	ND	ND
BBP	ND	ND	ND	ND	ND	ND
IPPP	ND	ND	ND	ND	ND	ND
DnOP	ND	ND	ND	ND	ND	ND

This work demonstrates the practical utility of GC-MS study in the effective detection and quantification of phthalates. The developed method can be successfully applied for the detection and quantification of phthalates in liquid food products.

The results of market sample analysis suggests the applicability, reproducibility and utility of method for estimation of phthalates in quality control laboratories. The method was found to be precise, accurate, reproducible, specific and robust.

CHAPTER 6

SUMMARY AND CONCLUSION

The present work deals with the development and validation of GC-MS technique for the detection and quantification of phthalates. The validation of the proposed method was carried out as per ICH guidelines. Linearity was obeyed in the concentration range of 0.5 to 5 mg/L. The developed method was successfully applied to the analysis of phthalate esters in liquid food samples.

The sample preparation method for liquid samples was quick and easy to accomplish using n-hexane as extraction solvent which provided constant and high recoveries even at trace level. The average recovery of the sample analysis by this method was 102.1%. Among the phthalates generally present, Di 2-ethyl hexyl phthalate was detected in all the samples in the concentration range of 1.31 to 1.65 mg/L. The GC-MS measurement method is highly accurate as demonstrated with precise calibrations and spiked liquid samples.

The proposed GC-MS method has good usability, provides the necessary sensitivity and delivers the complete spectrum information for identification and confirmation of a wide variety of possible phthalate ester contaminations by comparison with the NIST mass spectral library.

The determination of phthalate plasticizers using the proposed GC-MS method is very sensitive and accurate. It is easy to perform, rapid and covers a wide linear range to meet the need for trace level detections of PAEs in liquid food samples.

The advancement of medical science always has influenced mankind. Science builds on itself over time, each period gives its own contribution. Sometimes the knowledge is not true, but it is in the act of trying that leads to success. This accumulation of medical knowledge over time has allowed society to advance health care to where it is today. In conclusion, the sciences emerged from human curiosity and ingenuity because of the questions and problems the environment has thrust upon us. The information gained is not always equitable but does serve as a medium for correct sciences to appear. Thus, if history repeats itself, the road ahead for science will be intermittently rewritten to serve the future needs of both scientific communities and society at large. This project can be considered as the effect of an activity on the social fabric of the community and well-being of the individuals, thus imparting a social impact in the society.

CHAPTER 7

REFERENCES

1. Richard Coles, Derek Mcdowell and Mark Kirwan J.Food packaging technology. Blackwell publishers; 2003.

2. General principles of food packaging. Packaging materials.2003; 22:1-46.

3. Catala R and Gavara R. Food packaging. Food engineering EOLSS. 2002; 3.

4. David F, Cadogan and Christopher J. Ullmann’s encyclopedia of industrial chemistry. Wiley online library; 2000.

5. Paine FA and Paine HY. A handbook of food packaging, Blackie, Glasgow; 1983.

6. BSI. Sensory evaluation on sensory analysis of food. 1984; 5929:3.

7. Cartwright CD, Thompson P and Burns R. Degradation and impacts of phthalate plasticizers on soil microbial communities. Environ. Toxicol. Chem 2000; 19:1253-1261.

8. Carter SA. The potential health hazard of substances leached from plastic packaging. Journal of Environmental Health. 1977; 40: 73–76.

9. Castle L, Mercer A, Startin JR and Gilbert J. Migration from plasticized films into foods. Food Additives and Contaminants. 1987; 4: 399–406.

10. Nerin C, Cacho J and Gancedo P. Plasticizers from printing inks in a selection of food packagings and their migration to food. Food Additives and Contaminants. 1993; 10: 453–460.

11. Bouma K and Schakel DJ. Migration of phthalates from PVC toys into saliva simulant by dynamic extraction. Food Addit Contam. 2002; 19:602-10.

12. Malveda and Michael P. A handbook of chemical economics report on plasticizers. CBS Publishers and Distributors; 2015.

13. Bornehag CG, Lundgren B, Weschler CJ, Sigsgaard T and Sundell J. Phthalates in indoor dust and their association with building characteristics. Environ Health Perspect. 2005; 113:1399–1404.

14. CPSC. The risk of chronic toxicity associated with exposure to diisononyl phthalate (DINP) in children's products. Bethesda MD. 1998.

15. Etzel RA, Balk SJ, Elk Grove and Village IL. American Academy of Pediatrics, Committee on Environmental Health. Handbook of Pediatric Environmental Health. 1999 expert panel report on the reproductive and developmental toxicity of di-isononyl phthalate.

16. Sapna Johnson, Nirmali Saikia and Ramakant Sahu. Phthalate in toys. Centre for science and environment. 2010:1-40.

17. Sathyanarayana S, Karr CJ, Lozano P, Brown E, Calafat AM, Liu F and Shanna H. Baby Care Products: Possible Sources of Infant Phthalate Exposure. Pediatrics. 2008; 121:e260-e268

18. Jaakkola JJ an d Knight TL. The role of exposure to phthalates from polyvinyl chloride products in the development of asthma and allergies: a systematic review and meta-analysis. Environ Health Perspect. 2008; 116:845-53.

19. Meek ME, Chan PKL. Bis(2-ethylhexyl)phthalate: evaluation of risks to health from environmental exposure in Canada. Environ Carcin Ecotoxicol Rev.1994; C12 :179 –194

20. Lington AW, Bird MG, Plutnick RT, Stubblefield WA and Scala RA. Chronic toxicity and carcinogenic evaluation of diisononyl phthalate in rats. Fund Appl Toxicol.1997; 36:79-89.

21. Voss C, Zerban H, Bannasch P and Berger MR. Lifelong exposure to di-(2-ethylhexyl)-phthalate induces tumors in liver and testes of Sprague–Dawley rats. Toxicology. 2006 ;206: 359–71.

22. Gangolli SD. Testicular effects of phthalate esters. Environ. Health Perspect.1982; 45: 77-84.

23. Duty SM, Silva MJ, Barr DB, Brock JW, Ryan L, Chen Z Herrick RF, Christiani DC and Hauser R. Phthalate Exposure and Human Semen Parameters. Epidemiology. 2003; 14: 269-277

24. Park JD, Habeebu S and Klaassen C. Testicular toxicity of di-(2-ethylhexyl)phthalate in young Sprague–Dawley rats. Toxicology. 2002;171:105–15.

25. Ema M and Miyawaki E. Adverse effects on development of the reproductive system in male offspring of rats given monobutyl phthalate, a metabolite of dibutyl phthtlate, during late pregnanacy. Reproductive Toxicology.2001; 15:189-194.

26. Ema M and Miyawaki E. Effects of monobutyl phthalate on reproductive f unction in pregnant and Pseudopregnant rats. Reprod. Toxicol.2001;15: 261-267.

27. Fickentscher K. Analysis of phthalic acid esters in organic solvents. Praep. Pharm. 1979; 59: 60725.

28. Fishbein L and Albro PW. Chromatographic and biological aspects of the phthalate esters. J. Chromatogr.1972;12: 365.

29. Liang P, Xu J and Li Q. Application of dispersive liquid-liquid microextraction and high performance liquid chromatography for the

determination of three phthalate esters in water samples. Anal. Chim. Acta. 2008;609:53-58.

30. Yasuhara A, Shiraishi H, Nishikawa M, Yamamoto T, Uehiro T andNagasugi O. Determination of organic compounds in leachates from hazardous waste disposal sites in Japan by gas chromatography-mass spectrometry. J. Chromatogr. 1997; 774:321-332.

31. Zitko V. Determination, toxicity and environmental levels of phthalate plasticizers. Fisheries research board of Canada. 1972;344.

32. John Autian. Toxicity and health threats of phthalate esters. Environme1ntal health perspectives. 1973.

33. Charles A, Dennis R, Thomas F and William J. The environmental fate of phthalate esters. Chemosphere. 1997;25(4):667-749.

34. Robert Kavlock et al. NTP center for the evaluation of risks to human reproduction and phthalate expert panel report on the reproductive and developmental toxicity of Di- n- butyl phthalate. Elsevier reproductive toxicology. 2002;489-527.

35. Bart Tienpont. Determination of phthalates in environmental, food and biomatrices. Fculty of sciences. 2004.

36. Kayoko kato et al. Quantitative detection of nine phthalate metabolites in human serum using RPHPLC-EI-MS. Journal of analytical toxicology. 2003;27.

37. Feng Y, Zhu J, Sensenstein R. Development of a headspace solid-phase microextraction method combined with gas chromatography mass spectrometry for the determination of phthalate esters in cow milk. Anal Chim Acta. 2005;538:41-48.

38. Jean C and Donald C. Analysis of consumer cosmetic products for phthalate esters. Journal of cosmetic sciences. 2006;57:127-137.

39. Sorensen LK. Determination of phthalates in milk and milk products by liquid chromatography/tandem mass spectrometry. Rapid Commun Mass Spectrom.2006; 20:1135–1143.

40. Koch HM, Wittassek M, Anjerer J, Drexler H, Kafferlein HU and Bruning T. Fast HPLC-MS/MS quantification of 22 phthalate metabolites in urine. Biological monitoring in occupational and environmental health. 2007.

41. Carrillo JD, Salazar C, Moreta C, Tena MT. Determination of phthalates in wine by headspace solid-phase microextraction followed by gas-chromatography mass spectrometry. Journal of chromatogr. 2007;1164:248-261.

42. Del Carlo M, Pepe A, Sacchetti G, Compagnone D, Mastrocola D and Cichelli A. Determination of phthalate esters in wine using solid –phase extraction and GC-MS. Food chem. 2008;111:771-777.

43. Debajit Sircar, Sargon Albazi J, Yousef Atallah and William Pizzi. Validation and application of an HPLC method for determination of DEHP and MEHP in liver samples. Journal of chromatographic science. 200;46.

44. Cao. Determination of phthalate and adipate in bottled water by using headspace solid –phase micro extraction and gas chromatography with mass spectrometry. Journal of chromatogr. 2008;1178:231-238.

45. Thomas Wenzl. Methods for the determination of phthalates in food. JRC scientific and technical reports. 2009:978-992.

46. Kim M, Yun SJ, Chung GS. Determination of phthalates in raw bovine milk by gas chromatography/time-of-flight mass spectrometry (GC/TOF-MS) and dietary intakes. Food Addit Contam. 2009; 26:134–138.

47. He J, Lv R, Zhu J, Lu K. Selective solid-phase extraction of dibutyl phthalate from soybean milk using molecular imprinted polymers . Anal Chim Acta. 2010; 661:215–221.

48. Guo Z et al. Development and application of a method for analysis of phthalates in ham sausages by solid-phase extraction and gas chromatography– mass spectrometry. Meat Sci. 2010; 84:484–490.

49. Michael A. Overview of phthalates toxicity. The United States consumer product safety commission. 2010.

50. Chong Kian W, Loke Chui F and Michelle P. Determination of six phthalates in polypropylene consumer products. The Malaysian journal of analytical sciences. 2011;15(2):167-174.

51. Melissa Dunkle, Gerd V, Frank D and Pat S. Determination of phthalate migration from toys. Agilent technologies. 2012;9597.

52. Li Z et al. Simultaneous determination of nine types of phthalate residues in commercial milk products using HPLC-ESI-MS-MS. Journal of chromatogr. 2011;49:338-343.

53. Kanchanamayoon W, Prapatpong P, Chumwangwapee S and Chaithongrat S. Analysis of phthalate esters determination in drinking water samples. African journal of biotechnology. 2012;11(96):16263-69.

54. Jimmy Chan. Rapid, sensitive and robust detection of phthalates in food using GC-MS or LC-MS. Agilent technologies. 2012.

55. Ying Guo and Kurunthachalam Kannan. Challenges encountered in the analysis of phthalate esters in food stuffs and other biological matrices. Analytical and bioanalytical chemistry. 2012;404(9):2539-2554.

56. Russo MV, Notardonato I, Cinelli G and Avino P. Evaluation of an analytical method for determining phthalate esters in wine samples by solid-phase extraction and gas chromatography coupled with ion-trap

mass spectrometer detector. Analytical and bioanalytical chemistry. 2012;402:1373-1381.

57. Jianxia L, Lina L and Hans J. Determination of phthalates in liquor beverages by single quadrupole GC-MS. Thermo scientific. 2012:07.

58. Fierens T, Van Holderbeke M, Willems H, De Henauw S and Sioen I. Transfer of eight phthalates through milk chain. Elsevier environmental international. 2013:1-7.

59. Chen jing, Xu Qun and Jeffrey Rohrer. Determination of phthalates in drinking water by UHPLC with UV detection. Thermo fisher scientific. 2013.

60. Anila I Khan. Determination of phthalate esters in soft drinks by GC-MS. Thermo scientific. 2014: 1114-16.

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Human Welfare

Introduction

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References