ANALYSIS OF PHTHALATES IN FOODS BY USING GAS CHROMATOGRAPHY MASS SPETROMETRY (GC-MS)

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DEPARTMENT OF CHEMISTRY

FACULTY SCIENCE

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RESEARCH REPORT SUBMITTED IN FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE (ANALYTICAL CHEMISTRY & INSTRUMENTAL ANALYSIS)

DEPARTMENT OF CHEMISTRY
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ABSTRACT

Migration of phthalates from food packaging into food has caused exposure of human on these compounds. Also, these compounds may cause adverse effect on human and thus, identification and quantification of these compounds in food become more particular. Simple and rapid methods have been developed for the simultaneous determination of six phthalate compounds in the food samples including drinking water, milk, cereal and instant noodles by using gas chromatography mass spectrometry (GC-MS). The six phthalate compounds been examined were dimethylphthlate (DMP), diethylphthalate (DEP), dibutylphthalate (DBP), benzyl butyl phthalate (BBP), diethylhexylphthalate (DEHP) and dioctylphthlate (DNOP). For both liquid and solid samples, extractions were done by using solvent n-hexane. For liquid samples, the limit of detection (LOD) and limit of quantification (LOQ) were 3.3 μg/L and 10 μg/L respectively. While for solid samples, the LOD and LOQ were 0.33 mg/kg and 1 mg/kg respectively. The sample spiked recoveries were in the range of 72.9-124.9 % with relatively low relative standard deviation (RSD) that was below 3.30 %.

ABSTRAK

Migrasi phthalate dari bungkusan makanan ke dalam makanan telah menyebabkan manusia terdedah kepada analit tersebut. Phthalate akan memberi kesan buruk kepada manusia. Oleh itu, pengenalan dan kuantifikasi phthalate dalam makanan adalah sangat penting. Kaedah yang mudah and cepat telah dibangunkan untuk menkaji enam jenis phthalate dalam sampel makanan termasuk air minuman, susu, bijirin and mi segera dengan menggunakan kromatografi gas spectrometri jisim (GC-MS). Phthalate yang dikaji adalah dimethylphthlate (DMP), diethylphthalate (DEP), dibutylphthalate (DBP), benzyl butyl phthalate (BBP), diethylhexylphthalate (DEHP) dan dioctylphthlate (DNOP). Pengekstrakan phthalate dari sampel cecair dan pepejal telah dilakukan dengan menggunakan n-hexane. Had pengesanan dan had kuantifikasi untul sampel cecair adalah 3.3 μg/L dan 10 μg/L masing-masing. Had pengesanan dan had kuantifikasi untuk sampel pepejal adalah 0.33 mg/kg dan 1 mg/kg masing-masing. Perolehan kembali analit untuk sampel adalah dalam linkungan 72.9-124.9% dengan relatif sisihan piawai rendah daripada 3.3 %.

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CHAPTER 1

INTRODUCTION

1.1 Background of study

Plasticizers have become important in the industry due to its effectiveness to producing pliable plastics for application ranging from consumer and medical products to automotive industries (Dunming et al., 2014). The earlier usage of plasticizer was to plasticize celluloid by using camphor. Camphor was then substituted by tricresyl phosphate, and this compound is still in use for the production of polyvinyl chloride (PVC) until now. In the year 1920, phthalic acid esters were introduced into the market and since then has become the most important class of plasticizers (Fink, 2010) .

Since the year of 1980s, phthalate compounds have raised public concern due to the migration issue of phthalic acid esters and its effect on human health. Phthalate compounds are one of the most abundantly used chemicals in the global. According to Toxics Use Reduction Institude (TURI), the most ubiquitous phthalates was di(2-ethylhexyl) phthalate (DEHP) with over one billion pounds produced at Western Europe in year 2006. According to European Chemicals Bureau (ECB), 2008 reports and summary, estimated annual global production of DEHP was around 2.2 to 8.8 billion pounds in year 1994.

As the report stated on the European Chemicals Agency (ECHA) DEHP, 2009, the production of DEHP in Europe (EU) for year 1997 and 2007 were 1.2 billion pounds and 750 million pounds respectively. There is a drastic fall in the production size of DEHP in EU. This is due to the concerns of the community on the potential health risks. The market was still dominated by high molecular weight phthalates in the year of 2010. However, the demand on these plasticizers has decrease due to the growing environmental perception and awareness and legal provisions. This has force the producers to use non-phthalates plasticizers.

Phthalate compounds are widely used in the production of medical device, building materials, children's toy, food packaging, personal care products and clothing (Kavlock et al., 2002). Migration of phthalate compounds from food packaging material into foodstuffs has raise public concerns as phthalates are hazardous to human being.

1.2 General

1.2.1 Phthalates

Phthalates are also known as ester of phthalic acids or phthalate esters or diesters of benzenedicarboxylic acid with the general chemical structure shown in Figure 1.

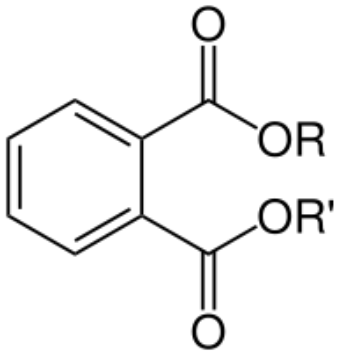

Figure 1: General chemical structure of phthalate (R and R' both are alkyl groups)

Physical and chemical properties of phthalate compounds are based on the ester side chains (represented by R and R'). The list of common phthalate compounds in used is shown in Table 1.

Table 1: List of phthalate compounds

Phthalate		CAS Registry number	Molecular weight
Dimethylphthalate	DMP	131-11-3	194
Diethylphthalate	DEP	84-66-2	222
Di-n-butyl phthalate	DBP	84-74-2	278
Butyl benzyl phthalate	BBP	85-68-7	312
Di(2-ethylhexyl) phthalate	DEHP	117-81-7	390
Di-n-octyl phthalate	DNOP	117-84-0	390

Basically, phthalates are divided into two distinct groups that are high and low molecular weight phthalate esters due to the difference in their application, properties, toxicology and classification which based on the R and R' group ("Phthalate,"). For low molecular weight phthalates, it is usually has three to six carbons backbone on the side chain (both R and R' group). Low molecular weight phthalates are primarily use as plasticizers in some non-vinyl resins which including cellulosics, acrylics and urethanes. The wide usage of low molecular weight phthalates can be ranging from pharmaceutical to consumer products. However, due to the concerns of the compounds to health, it has been slowly replaced by high molecular weight phthalate (Fink, 2010).

Phthalate compounds which have more than six carbons on the side chain (both R and R' group) are categorized as high molecular weight phthalates. This type of phthalates are used in high volume if compare to low molecular weight phthalates. It is used as additive to enhance the processability, softness and pliability of vinyl compounds. High molecular weight phthalates are better as it will increased the durability and permanency on vinyl compounds, and its broad usage in production of vinyl goods ranging from consumer to commercial products

DMP is dimethyl ester of phthalic acid with the simplest chemical structure amongst all the phthalates and it appear to be in clear oily liquid in room temperature. It is used as ectoparasiticide and the other usage of this plasticizer is in production of plastics, solid rocket propellants and insect repellents ("Dimethyl phthalate,"). The chemical structure of DMP is shown in Figure 2.

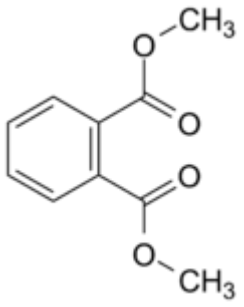

Figure 2: Chemical structure of DMP

DEP is diethyl ester of phthalic acid with the chemical structure as shown in Figure 3. It appears to be in clear liquid form at room temperature, slightly denser than water and has low volatility. It has faint unpleasant smell and the smell can be released from the plastics contained it. When it is exposed to flame, it will produce irritating and toxic fumes. There is a broad usage of DEP worldwide such as in cosmetic and fragrance formulations ("Diethyl phthalate,"), plastic packaging films, toiletries, aerosol sprays, and medical treatment tubing (Jie Guo, 2013). Thus, human are expected to be exposed more on this compound.

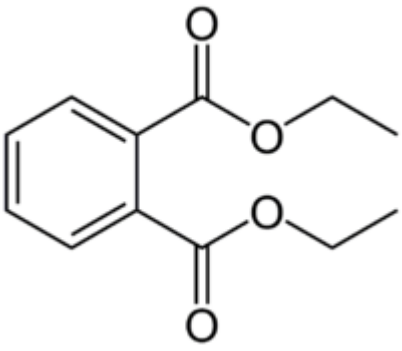

Figure 3: Chemical structure of DEP

DBP is a clear and odourless oily liquid at room temperature and soluble in various organic solvents. The chemical structure is shown in Figure 4. It is generally used as plasticizer to soften hard plastics. DBP can be used in manufacturing of products such as personal care products, adhesives, cosmetics, lacquers and dyes (ATSDR, 2001). This compound has been banned in the production of children's toy in United States (U.S.) as the present in the toy must be less than 0.1% of the mass of toy according to Section 8 of the Consumer Product Safety Improvement Act of 2008 (CPSIA).

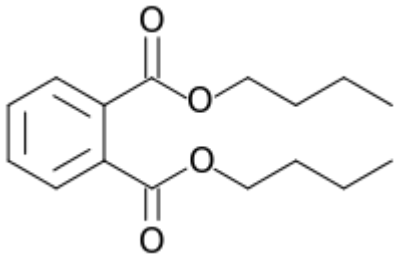

Figure 4: Chemical structure of DBP

BBP is a synthetic phthalate ester which appears to be in clear and slightly viscous liquid. The chemical structure of the compound is shown in Figure 5. It comes under several trade names such as Santicizer 160, Unimoll BB, Sicol 160 and Palatinol BB. It is mostly used in as plasticizer in vinyl tiles (NTP BBP, 2003). Other common uses are in products such as traffic cones, conveyor belts, carpet, weather stripping and automotive trims. BBP is also banned in US especially in children's toy as the present in the toy must be less than 0.1% of the mass of toy (CPSIA, 2008)

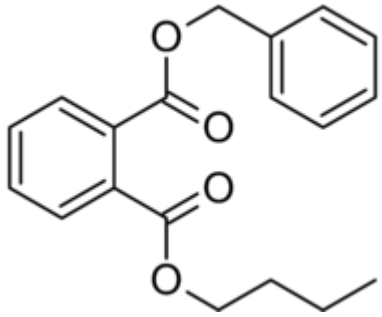

Figure 5: Chemical structure of BBP

DEHP also known as bis(2-ethylhexyl) phthalate, and diethylhexyl phthalate is compound with molecular formula of $C_6H_4(C_8H_{17}COO)_2$. The general chemical structure is shown in Figure 6. It is insoluble in water, but soluble in oil. This is the most heavily used phthalate as plasticizers for PVC in products such as medical products, building materials, car products, clothing and food packaging. In vinyl products, it can contain up to 40% of DEHP (ATSDR, 2002). The high usage of DEHP causes human and environmental are heavily expose to this compounds. Same to DBP and BBP, DEHP is also banned in children's toy as the present in the toy must be less than 0.1% of the mass of toy (CPSIA, 2008).

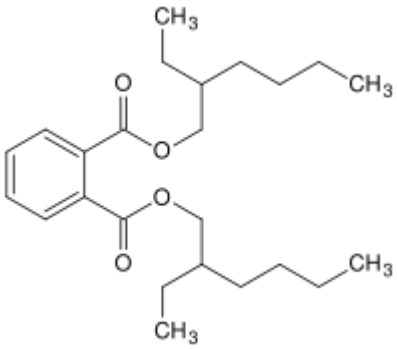

Figure 6: Chemical structure of DEHP

DNOP is an oily substance and the chemical structure is shown in Figure 7. It is also act as plasticizers and used in the production of PVC plastics. However, this compound is slightly different from those which have been discussed previously as it has been approved by U.S. Food and Drug Administration (FDA) on its usage on indirect food additive. DNOP also used in conveyor belts, seam cements, bottle cap liners, garden hoses, carpet tiles and tarps (NTP-CERHR DNOP, 2003). Also, it has been banned in U.S. in children's toy with the scenario as discussed earlier (CPSIA, 2008).

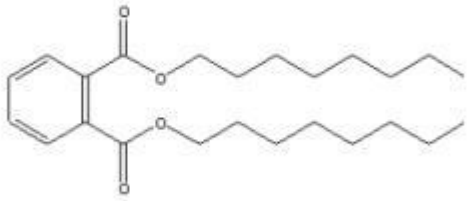

Figure 7: Chemical structure of DNOP

1.2.2 Analytical Method for accessing phthalate compounds

In the older days which are around end of 1980s, the analytical techniques used to access phthalates were thin later chromatography, packed-column gas chromatography, fluorescence measurement and ultraviolet (UV) spectrometry. However, the most commonly methods used to analyze phthalate compounds these days are by using gas chromatography (GC) and liquid chromatography (LC) coupled with mass spectrometry detector (MS) detector. This is because chromatography system coupled with MS can provide sufficient detection resolution for mostly all important phthalate compounds (Staples, 2003). While for LC, it is usually used for the analysis of isomeric mixtures.

The major problem encountered by the phthalate analysis is not the analysis itself, but is the possibility of contamination in almost every stage of the analysis procedure which is from sampling till stage of analysis (Wenzl, 2009). Thus, in order to avoid bias in the analysis, the original food packaging is used in sample storage stage. This is because others storage materials such as aluminium foil and glass container might contaminated with trace amount of phthalates which will give bias in the analysis.

The samples need to go through extraction process prior to the identification and quantification by using GC-MS or LC-MS. For extraction stage, both liquid-liquid extraction (LLE) and solid phase extraction (SPE) have been used ((Dunming Xu, 2014). Isolation of phthalate compounds from the matrix can be done with the used of solvents such as *n*-hexane, chloroform, isooctane or *n*-heptane. Then, extraction can be accomplished by shaking the extractant mixture, vortex or even using ultrasonic water bath. The extract of n-hexane can be directly sent for the qualification and quantification of

phthalates by using GC with detector such as MS, flame ionization detection (FID) or electron capture detection (ECD).

1.2.3 Hazards of phthalates

Phthalates can be found anywhere in the environment, and thus, human exposures on these compounds are high. Exposure of human on phthalates can be occurred when they consumed food that has been in contact with products and containers that contained phthalates. In addition, minor exposure can be occurred when human breathing in air that contains phthalates vapours and dust contaminated particles. After all, children exposure on phthalates is more critical. This is due to their hand-to-mouth behaviors especially when they are in contact with plastic toys. The effects of human exposure to low levels of phthalates are unknown. However, some phthalates cause adverse effect on human health especially DEHP and diisononyl phthalate (DINP).

Since year of 2000, there are plenty of data have shown that 3-10 carbon chain phthalates will cause development of toxicity on living beings (Benson, 2014). In rats and humans, diester phthalates that entered the organisms will be converted into monoester phthalates by de-esterification of one alkyl linkage. Monoester metabolites are believed to give toxic developmental effects. Presence of monobutyl phthalates (MBP) in urine was related with lower sperm motility and sperm concentration (Mariko Matsumoto, 2008). Diester phthalates and the corresponding monoester phthalates are shown in Table 2.

Table 2: Diester phthalates and the corresponding monoester phthalates (Benson, 2014)

Diester phthalates	Monoester metabolites
Dibutyl phthalate	Monobutyl phthalate (MBP)
Diisobutyl phthalate	Monoisobutyl phthalate
Butylbenzyl phthalate	MBP and Monobenzyl phthalate
Diethylhexyl phthalate	Monoethylhexyl phthalate
Dipentyl phthalate	Monopentyl phthalate
Diisononyl phthalate	Monoisononyl phthalate

According to the National Toxicology Program (NTP), diisononyl phthalate (DINP) may cause adverse effect on human development and reproduction (NTP-CERHR DINP, 2003). Also, phthalates in related products and in dust will cause respiratory with bronchial obstruction (such as asthma) and allergic to the people who inhale or expose to the chemical for certain limits (Yu Aint Bamai, 2014).

1.3 Objectives of study

1. To extract phthalates from its matrix by using LLE and determine its content by using GC-MS.
2. To validate the developed method.

CHAPTER 2

LITERATURE REVIEW

2.1 Phthalates in food

Tsumura et al., (2000) investigated the phthalates content in packed lunches from the market and set lunches from the restaurant. All the samples were detected for DEHP with the found out showed that packed lunches contain higher concentration of DEHP than set lunches. The concentrations of DEHP in packed lunched and set lunches were in the range of 0.80-11.8 mg/kg and 0.012-0.30 mg/kg respectively. Uses of PVC gloves in food preparation were the major cause of phthalates contamination.

Cirillo et al., (2013) study the exposure of DEHP and DBP to hospital packed meals provided to the patient. DEHP and DBP were migrated to the food through its food packaging which was consisted of polyethylene terephthlate (PET) and polypropylene (PP). The result found to be in the range of 0.012-0.420 ug/g and 0.008-0.273 ug/g for DEHP and DBP respectively. Thus, the daily total intakes estimated were 11% and 24% for DEHP and DBP respectively. This estimation is based on the TDI established by European Food Safety Authority (EFSA).

Tsumura et al., (2001) identified di(2-ehtylhexyl) adipate and eleven phthalate compounds in the diet samples from the hospital. Analyses of phthalates from the samples were done by using GC-MS with selective ion monitoring mode (SIM). The highest concentration of phthalates detected were DEHP which in the range of 10-4400 ng/g of all sample. The ratio of the mean DEHP to the daily total intakes (TDI) was found to be 28% which means it has exceeded the TDI established by European Commission. DEHP contamination in food was suspected from the PVC gloves during meals preparation.

Sui et al., (2014) determined the amount of DEHP in food samples collected from the markets and supermarkets in 16 regions in China. Food samples collected were segregated into 12 categories which were jelly, jam, instant noodles, vegetables oil, drinking water, beverages, aquatic products, leaf vegetables, root vegetables, milk, meat and cereals. From the result obtained the main sources of DEHP were from cereals, drinking water and meat.

Wu et al., (2012) reported the phthalates tainted food happened in Taiwan in year of 2011 and the effect on people's health. Phthalates were added into the production of food as a replacement of emulsifier. The foods were mostly contaminated with DEHP and diisononyl phthalate (DINP). This issue was taken seriously by Taiwan government by created and enforcing several policies.

2.2 Analytical method for phthalates

Ostrovsky et al., (2011) studied a method for determination of total phthalates by using gas chromatography flame ionization detector (GC-FID). This was done by alkaline hydrolysis to phthalic acid at optimum temperature of 80°C for 20 hours, and then followed by selective removing of lipophilic interferences by n-hexane at pH 1.

Li et al., (2011) presented a method for determination of multi-residue phthalates in milk simultaneously by using high performance liquid chromatography electrospray ionization tandem mass spectrometry (HPLC-ESI-MS-MS). Phthalates were isolated from the samples by using acetonitrile. Qualifications of the compounds were done by comparing the mass spectrum and retention time.

Guo et al., (2013) studied the determination of dibutylphthalate (DBP) at pictogram level in wine by using flow injection chemiluminescence (FL-CL). This analysis was based on the theory of quenching effect of DBP in the luminal-myoglobin chemiluminescense (CL) system. Quantification can be done because the decrement of CL intensity was directly proportional to the logarithm of DBP concentration. The quantification range was from 0.1 to 100pg/mL.

Hao et al., (2004) performed sonication assisted extraction followed by analysis using GC-MS for simultaneous determination and screening of eight phthalic acid esters in plastic products used for food packaging. Ionization mode used was electron ionization (EI) with selective ion monitoring (SIM) data acquisition method. Detection limit of this method was 10 μg/kg. Also, it has shown relatively good recoveries that were in the range of 82 to 106 % with relative standard deviation (RSD) of 3.8 to 10.2 %.

Xu et al., (2014) has used liquid chromatography mass spectrometry coupled with mass spectrometry (LC-MS-MS) with electrospray ionization (ESI) ion source for the determination of 23 types of phthalates in food samples. Liquid samples were extracted by using acetonitrile and solid samples were extracted by using QuEChERS (Quick Easy Cheap Effective Rugged and Safe) or glass based solid phase extraction (SPE).

2.3 Hazards of phthalates

Benson., (2009) reviewed the effect of phthalates on male reproductive system based on the reference dose (RfD) for each phthalate compounds. Each of the phthalate compounds caused similar adverse effect on male reproductive system that is cause decrease in testosterone synthesis in fetal Leydig cells.

Colborn et al., (1993) reported that phthalates have endocrine disrupting and reproductive effects. The compounds will develop irreversible and permanent effect on humans and wildlife especially indirectly exposure during prenatal and postnatal life.

Heudorf et al., (2007) presented current risk and exposure evaluation data were done by expert based on the current and ambient human biomonitoring results. Few phthalates are developmental and reproductive toxicants in animal and cause endocrine disrupting in humans. Exposure evaluation was done based on the ambient data and it has shown that phthalates exposure of children is higher than adult.

Ian et al., (2010) evaluated the influence of phthalates on human immune system and allergic development. An increase of the rate of allergic development and asthma on humans were caused by the exposure to phthalates. For the investigation of effect of immune systems, it was done according to the measurement of antibody response. Exposure of mice to DEHP and DINP has triggered an increase in the production of Th2 type cytokines.

CHAPTER 3

METHODOLOGY

3.1 Reagents and materials

The entire chemicals used were analytical grade or HPLC grade unless otherwise specified. Solvent used for the extraction is *n*-hexane (Merck). Deionized water used was produced from Milli-Q Ultrapure Water System with resistivity > 18 M ohm.cm and TOC ≤ 10 ppb.

Magnesium sulphate, $MgSO_4$ (Merck) and sodium sulphate, $NaSO_4$ (Merck) were purified by heating in the oven at 400°C for 4 hours. Mixture of $MgSO_4$ and $NaSO_4$ with the ratio of 5 to 1 respectively was prepared.

3.2 Standard solution

Semi volatile internal standard with concentration 4000 ppm in methylene chloride was used. The mixture of internal standard consist of naphthalene [$^2H_8$] (naphthalene-d_8), acenaphthene [$^2H_{10}$] (acenaphthene-d_{10}), phenanthrene [$^2H_{10}$] (phenanthrene-d_{10}), and chrysene [$^2H_{12}$] (chrysene-d_{12}) (SUPELCO).

Standard solution containing phthalates mixture of dimethylphthlate (DMP), diethylphthalate (DEP), dibutylphthalate (DBP), benzyl butyl phthalate (BBP), diethylhexylphthalate (DEHP) and dioctylphthlate (DNOP) (SUPELCO) with the concentration of 1000 ppm in methylene chloride was used.

3.2.1 Preparation of internal standard

200 ppm of internal standard was prepared by diluting 500 uL of 4000 ppm semivolatile internal standard solution in 10 mL volumetric flask. The solvent used for dilution is methylene chloride (Merck).

3.2.2 Preparation of calibration standard

100 ppm of stock solution containing 6 six phthalate compounds as stated previously was prepared. The stock solution was kept in the fridge at 4±2°C for not longer than three months.

Two sets of calibration standards were prepared for full scan and selective ion monitoring (SIM) mode respectively. For SIM mode, series of calibration standard prepared with concentration of 0.05, 0.1, 0.2, 0.5, 1 and 2 ppm. While for the full scan mode, series of calibration standard prepared with concentration of 0.5, 1, 2, 5, 10 and 20 ppm. The calibration standards were prepared by dilution from 1000 ppm standards phthalates mixture. All the standards were spiked with 5 ppm of internal standard.

3.3 Instrumentation

3.3.1 Gas Chromatography Mass Spectrometry (GC-MS)

An Agilent Tehcnologies 7890A GC system used for separation of compounds coupled with 5975C inert MSD with triple Axis Detector which used for qualitative and quantification purposes. The system was operated with electron impact ionization mode (EI, 70 eV). The system is automated with Agilent Technologies 7693 automatic liquid sampler (ALS) and it was programmed for three washes in each of two solvents to minimize carryover. The GC and MS operating parameters were shown in Table 2 and Table 3 respectively.

Table 3: Gas Chromatography operating parameters

GC Injection Parameters	
Inlet type	Multimode inlet He
Injection mode	Pulsed splitless
Injection volume	1uL
Injection port temperature	280°C
Pulse pressure and time	50.0 psi, 1.00 min
Purge flow and time	54.4 mL/min, 1.00 min
Gas saver flow and time	20.0 mL/min, 5.00 min
Gas saver flow and time	20.0 mL/min, 5.00 min
Carrier gas	Helium
DB-5.625 Column, and Oven Parameters	
GC column	J &W Scientific DB-5.625 (Dimensions: 20 m × 0.18 mm i.d. x 0.36 μm film thickness)
Flow and mode	1.5 mL/min, Ramp flow
Detector and outlet pressure	MSD, Vacuum
Oven temperature program	From 50 °C (holding time 2.00 min) to 330 °C at 25.00 °C/min, keeping the final temperature for 1.80 min.
Oven equilibrium time	0.10 min
Total program time	15.0 min
MSD transfer line temperature	280 °C

Table 4: Mass Spectrometry Operating Parameters

Mass Spectrometer Parameters	
Tune parameters	Autotune
Solvent delay	3.50 min
Quadrupole temperature	200°C
Source temperature	250°C

3.4 Analytical method

3.4.1 Determination of limit of detection (LOD) and limit of quantification (LOQ)

The limit of of quantitation (LOQ) was established by the lowest calibration point and limit of detection (LOD) was 3 times lower than LOQ.

3.4.2 Extraction of phthalates from liquid samples

25 mL of liquid sample was measured in a 40 mL vial by using measuring cylinder. Then, followed by the addition of 5 mL hexane and 2 grams of $MgSO_4$:Na_2SO_4 (5:1). The vial containing the sample was then vortex for 10 minutes and followed by centrifugation at 2500 rpm for 10 minutes. Lastly, 1 mL of solvent layer was pipette into 2 mL vial and then followed by addition of 25 uL of 200 ppm internal standard. Figure 8 shown the liquid sample and *n*-hexane layer after extraction process.

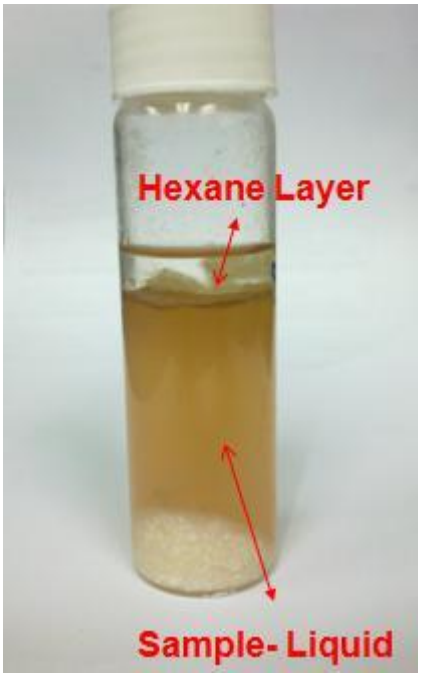

Figure 8: Liquid sample and n-hexane layer after extraction process

3.4.3 Extraction of phthalates from solid samples

1 gram of solid sample was weighed in a 40 mL glass vial using analytical balance. Then, followed by addition of 20 mL hexane and 5 grams of $MgSO_4$:Na_2SO_4 (5:1). The 40 mL vial was then capped and sonicated in water bath at ambient temperature for 1 hour to ensure all the phthalates were extracted to the solvent layer. To separate the components in the mixture, the vial was sent for centrifuged at 2500 round per minute (rpm) for 10 minutes. After centrifudge, two layers were obtained, and both layers were represented by *n*-hexane and solid sample as shown in Figure 9. Finally, 1 mL of solvent layer was pipette into 2 mL vial and followed by addition of 25 uL of 200 ppm internal standards.

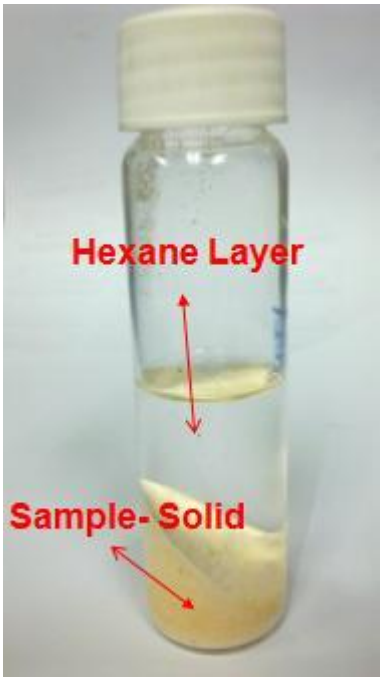

Figure 9: Solid sample and n-hexane layer after extraction process

3.5 Qualification and Quantification

Compounds qualification and quantification were carried out using ChemStation software. Quantification of compounds was performed in full scan and selected ion monitoring (SIM) modes. Internal standard quantification was performed using the deuterated compounds with the number of the deuterium atoms in range of 8 to 12 which present in each elution window.

The data for full scan mode were collected over a wide target range of mass fragment that was from m/z of 45 to 450. The identification of phthalate compounds were done according to the retention time and m/z ratios. Retention time of the interest compounds must fall in the predetermined windows that were set to be ± 0.1 minutes. Selection of fragment ions was based on those fragment ions with higher abundance. This can eliminate interferences on interest compound's peak. In this experiment, three to four ions (one target ion and two or three qualifier ions) were chosen for qualification purposes. Both retention time and ion ratios of samples were compared with those of the phthalate standards in the calibration.

SIM mode is more selective than full scan mode. In SIM mode, target ion fragments were entered into the instrument method. Thus, only those fragment ions will be selected and detected in mass spectrometer. Target ions and qualifier ions of phthalate compounds are shown in Table 5.

Table 5: Target and qualifiers ions for Phthalate analytes and internal standards

Time window (min)	T_R (min)	Compounds	Target ions (m/z)	Qualifying ions (m/z)		
				Q1	Q2	Q3
4.00	6.16	Napthalene-d8	136	108	134	137
	7.36	Dimethylphthalate	163	50	77	
7.65	7.91	Acenaphthene-d10	164	80	160	162
	8.34	Diethylphthalate	149	65	76	150
9.15	9.39	Phenantherene-d10	188	184	189	
	9.98	Di-n-butylphthalate	149	150	205	223
	11.49	Benzyl butyl phthalate	149	91	206	238
11.75	12.04	Diethylhexylphthalate	240	150	167	279
	12.05	Crysene-d12	149	118	120	236
	12.66	Di-n-octylphthalate	149	150	261	279

3.6 Determination of phthalates in foods

Four types of sample were selected to be analyzed by this method. The samples were drinking water, milk, cereal and instant noodle. The final concentration of phthalates in the sample will be calculated according to the formula shown below:

For solid samples,

$$Concentration\ (mg/kg) = \frac{A\ \times B\ \times DF}{C}$$

Where,

A = Reading from instrument (mg/L)

B = Final extract volume (mL)

C = Mass extracted (gram)

DF = dilution factor

For liquid samples,

$$Concentration\ (ug/L) = \frac{A \times B\ \times DF}{D}$$

Where,

A = Reading from instrument (mg/L)

B = Final extract volume (mL)

D = Volume extracted (L)

DF = dilution factor

3.7 Recovery of phthalates in Foods

Recoveries of phthalates from solid and liquid samples were performed by spiking the samples with appropriate amount of standards prior to extraction. Then, the percentage (%) recoveries of samples were calculated by using the formula shown below:

$$\%\ Recovery = \frac{X - Y}{Z} \times 100$$

Where;

X = Concentration of spiked samples (mg/kg or ug/L)

Y = Concentration of samples (mg/kg or ug/L)

Z = Spike concentration (mg/kg or ug/L)

CHAPTER 4

RESULT AND DISCUSSION

4.1 Analytical method

4.1.1 Limit of Detection (LOD) and Limit of Quantification (LOQ)

LOQ was the lowest concentration of analytes that can be detected reliably with acceptable precision and accuracy by the method. In this analysis, LOQ was based on the lowest concentration in the calibration curve that is 0.05 mg/L for SIM mode. For solid sample, the calculated LOQ was 1 mg/kg according to 1 gram of sample and 20 mL solvent used. While for liquid sample, calculated LOQ was 10 μg/L according to 25 mL of sample and 5 mL solvent used. LOD is three times lower than LOQ and thus, LOD for solid and water samples were 0.33 mg/kg and 3.3 μg/L respectively.

While for full scan mode, the LOQ is significantly higher than SIM mode as it has lower sensitivity. The lowest concentration in the full scan mode calibration curve was 0.5 mg/L. The calculated LOQ for solid and liquid samples were 10 mg/kg and 0.1 mg/L respectively. The LOD was calculated same as SIM mode, that is three times lower than LOQ. Thus, the LOD for solid and liquid samples were 3.3 mg/kg and 0.033 mg/L respectively.

4.1.2 Linearity of Calibration Curve and Working Range

Linearity was the capability of a method to obtain the concentration of the analyte which was proportional to the measured signal within the working range. Linearity of the method was determined by the measurement of the instrument signal versus concentration data. The acquired results were reported as the variance of the regression line slope. A simple linear regression equation applied to the results should have an intercept and slope that will be used to calculate correlation coefficient. The linear regression equation was as follow:

$$y = mx + c$$

Where,

y = dependent variable (instrument response)

x = explanatory variable (concentration, mg/L)

m = slope of the line

c = *y*- intercept (the value of *y* when *x* is zero)

The correlation coefficient, R^2 was calculated by using the formula shown in the equation below and the correlation coefficient must be greater than 0.990 to provide confident reading.

$$R^2 = \left[\frac{\sum(x - \bar{x})(y - \bar{y})}{\sqrt{\sum(x - \bar{x})^2 \sum(y - \bar{y})^2}}\right]^2$$

Six points of calibration standard were performed and analyzed by the instrument for the selected phthalate compounds prior to the sample analysis. The SIM mode calibration curve of the six phthalate compounds were range from concentration of 0.05 to 2mg/L. The obtained signals represent the respective concentrations were plotted and a linear curve is obtained for each of the phthalate compounds. The R^2 obtained for each of the linear curves are shown in the Table 5. All the R^2 values are more than 0.990 and thus, the calibration curves show good linearity over the calibration range which was from 0.05 to 2 mg/L. The calibration curve for each of compounds can be found in appendix 1 to 6.

Table 6: Correlation coefficient, R^2 of the calibration curve (SIM mode)

Analytes	Correlation coefficient, R^2
Dimethylphthalate	0.9989
Diethylphthalate	0.9989
Di-n-butylphthalate	0.9996
Butyl benzyl phthalate	0.9993
Di(2-ethylhexyl) phthalate	0.9998
Di-n-octyl phthalate	0.9993

For full scan mode, linear calibration curve with relatively excellent R^2 values were obtained for all the six phthalate compounds with the concentration range from 0.5 to 20.0 mg/L. The calibration curve for each of compounds can be found in appendix 7 to 12.The R^2 values were shown in Table 6.

Table 7: Correlation coefficient, R^2 of the calibration curve (full scan mode)

Analytes	Correlation coefficient, R^2
Dimethylphthalate	0.9994
Diethylphthalate	0.9997
Di-n-butylphthalate	0.9997
Butyl benzyl phthalate	0.9999
Di(2-ethylhexyl) phthalate	0.9993
Di-n-octyl phthalate	0.9992

Working range was the range where the concentration of analytes can be determined confidently as it was proportional to the measured signals from instrument. The limiting factor at the lower end of the working range is the value of LOD. While for the upper end of working range, the limitation was usually depends on the response provide by the instrument.

Basically, working range was limited by the lowest and highest calibration concentration which was from 0.05 to 0.2 mg/L for SIM mode. In this study, lowest point was determined from the LOQ which was 1mg/kg and 10 μg/L for solid and liquid samples respectively. While for the highest point was determined from the highest point of the calibration standard which was 40 mg/kg and 400 μg/L for solid and liquid samples respectively.

For full scan mode, the working range was higher than SIM mode and the range obtained was from 0.5 to 20.0 mg/L in the calibration curve. After calculation with the

respective amount of sample and solvent used, the working range for solid and liquid samples were 10 to 400 mg/kg and 0.1 to 4.0 mg/L respectively.

4.1.3 Extraction of phthalates from liquid and solid samples

The extraction method used was adopted from JRC European Commission EUR 23682 EN – 2009. It was a method for the determination of phthalates in food. The most critical stage in phthalates analysis was the sample handling and storage. The sample must be handled carefully prior to extraction steps. Residue of phthalates can be found anywhere especially trace level of it may found in the glassware and aluminium foil. In order to avoid any contaminations, all the glassware were washed thoroughly and rinsed with acetone and *n*-hexane, then heated at 250°C prior to storage.

Extraction of phthalates were tried on others solvents such as trichloromethane, 2-propanol, 1-propanol, acetonitrile, methanol and *n*-hexane, and among all the solvent used, n-hexane provides the better extraction efficiency (Guo et al., 2010). The solvent used in the extraction is n-hexane due to its ability to dissolve phthalates. For liquid samples, samples were homogenized by shaking prior to extraction. While for solid samples, samples were pulpified by using a mixer. The usage of $MgSO_4$ and Na_2SO_4 were to remove excess water in the sample as it will prevent interference on the result. Actually, $MgSO_4$ was more effective and can remove water faster, but it was slightly acidic. Thus, it was mix with Na_2SO_4 which was more neutral. These compounds will bind with any excess water in the organic solvents to form cluster when they react.

In order to ensure the sample were mix thoroughly with solvents, sample with solid matrices were sent for sonication. Liquid samples were vortex in order to ensure phthalates were extracted out from the sample matrices. Centrifugation of mixture was to ensure that the solvent layer was fully separated from the samples. This step is very important as the solvent layer will be sent for analysis by GC-MS. Phthalates which were extracted from non-fatty samples with n-hexane can be directly measure by the instrument without additional cleanup steps (EUR 23682 EN – 2009).

4.2 Comparative study on MS data acquiring modes (Full scan and SIM mode)

There was two ways for the MS to acquire data which was known as full scan and SIM mode. Full scan mode will monitor a range of masses which in this study was from m/z of 45 to 450 and gives total ion current (TIC). It will scan stated range of masses four times per second and detect compound's fragments within that range over a set time period. Mass scanning and data will be recorded continually even as the impurity peaks are eluted out from the GC. From the scanning and data accumulation, TIC chromatogram can be plotted as the summed ion intensity as a function of time. In full scan mode, it will detect all the compounds delivered by GC in which it must fall in the m/z range. Thus, full scan mode can be used to identify unknown compounds. In method development, it was very common to analyze the sample in full scan mode first. This was to determine the retention time and the target and fragment ions of the compounds before quantification was made using SIM mode analysis.

In SIM mode, scanning and data acquisition were done on selected ion masses. Thus, only selected ion masses will be monitored and the scanning of MS can be programmed to "hop" rapidly from mass to mass all the way through the GC run. The main advantage of SIM mode over full scan mode was the improvement of the signal to noise ratio. This was because only selected ion masses are monitored and thus, only signal from the selected masses were accumulated and use to plot the chromatogram. SIM mode was relatively more sensitive than full scan and it was very useful in trace level analysis and eliminates difficult matrix interferences.

In comparison with the LOQ of the SIM and full scan mode, SIM mode gives relatively better sensitivity in the analysis. SIM mode can detect concentration which was 10 times lower than full scan mode. This was because only selected masses are monitored and more scans can be done at each second.

4.3 Quantification

Several quantification methods such as external standard approach, standard addition method approach and internal standard approach have been deduced. Among all of them, internal standard approach was the most suitable in this analysis as more selective detection was needed. Basically, internal standardization was based on the comparison between response factors of the analytes of interest with the internal standard's compound.

The aim of using internal standards was to improve the overall precision of the data. All the samples and standards in this analysis were spiked with 5 ppm of internal standard mixture. It is basically used in the calibration for plotting the ratio of the analyte signal to the internal standard signal as a function of the analyte concentration of the standards. An

advantage of using this is to compensate instrument instabilities and drift. Response factors (RF) for each analyte relative to one of the internal standards were calculated according to the formula shown below:

$$\text{Response Factor} = (A_S \times C_{IS}) / (A_{IS} \times C_S)$$

Where,

A_S = Peak area (or height) of the analyte

A_{IS} = Peak area (or height) of the internal standard

C_S = Concentration of analyte, in µg/L

C_{IS} = Concentration of internal standard, in µg/L

4.4 Determination of phthalates in foods

Foods that were selected for the study were drinking water, milk, cereal and instant noodles. Phthalates have become one of the most common interferences in food due to its function to make the polyvinyl chloride (PVC) more flexible especially in food packaging. In addition, many food packaging were made from PVC, and thus this has caused the contamination of phthalates in food become more severe. Among all the samples analyzed, there was no sample detected for the presence of phthalates. All the phthalates were found to be less than limit of reporting (LOR) or less than LOQ. The chromatograms can be found in Appendix 13 to 16.

4.5 Recoveries of Phthalates in foods

Foods from different type of samples were selected to be analyzed by the method. This was to determine suitably of the method to isolate phthalates from the food matrices. Four types of food products that were drinking water, milk, cereal and instant noodle were selected for the analysis. These food samples were fortified with 0.5 mg/L phthalates for recoveries test and triplicate (n=3) of analysis were done. The recoveries results for drinking water and milk were shown in Table 7.

The percentage of recoveries obtained for drinking water was near to 100 %. This was because the matrix was clean in nature and thus, no interferences observed. While for the milk sample, the low recoveries for DMP, DEP, DBP, BBP and DEHP which might due to the interferences from sample matrix as it contain lipophilic components inside. Overall, the repeatability was less than 2 % which means this method gives high repeatability of

data. While for DNOP, higher recovery of DNOP obtained which possibly might due to the low recovery of crysene-d12.

Table 8: Recoveries of phthalates in liquid samples (n=3)

Phthalates	Drinking water % mean ± RSD	Milk % mean ± RSD
Dimethylphthalate	93.27 ± 0.97	75.00 ± 1.93
Diethylphthalate	92.53 ± 1.61	72.93 ± 0.61
Di-n-butylphthalate	92.33 ± 0.14	73.67 ± 0.84
Butyl benzyl phthalate	95.40 ± 0.62	76.67 ± 1.27
Di(2-ethylhexyl) phthalate	96.00 ± 1.41	86.47 ± 1.54
Di-n-octyl phthalate	106.67 ± 1.36	124.93 ± 0.60

As for solid samples, the recoveries result for cereal and instant noodle are shown in Table 8. The recoveries obtained were range from around 72 to 119 %. The repeatability of the analytical method was good as the relative standard deviation values obtained were less than 3.30 %. The recoveries of DNOP obtained was over 100 % for both samples (cereal and instant noodle), this might also due to the low recovery of crysene-d12.

Table 9: Recoveries of phthalates in solid samples (n=3)

Phthalates	Cereal % mean ± RSD	Instant noodle % mean ± RSD
Dimethylphthalate	77.11 ± 2.79	79.33 ± 1.78
Diethylphthalate	75.59 ± 1.22	78.27 ± 1.73
Di-n-butylphthalate	79.09 ± 1.97	74.38 ± 1.23
Butyl benzyl phthalate	74.30 ± 2.10	72.98 ± 1.41
Di(2-ethylhexyl) phthalate	83.21 ± 1.57	86.69 ± 0.45
Di-n-octyl phthalate	116.4 ± 3.30	118.87 ± 0.09

CHAPTER 6

CONCLUSION

Simple and rapid extraction methods for phthalates have been developed. This method can simultaneously determine 6 phthalate compounds that were dimethylphthlate (DMP), diethylphthalate (DEP), dibutylphthalate (DBP), benzyl butyl phthalate (BBP), diethylhexylphthalate (DEHP) and dioctylphthlate (DNOP) by using liquid liquid extraction followed by analysis using GC-MS.

Both of the extraction methods were successfully used to extract phthalates from the different types of food matrices. *n*-hexane was the suitable solvent for the extraction of phthalates mainly for the low fat content food. The food matrices investigated were drinking water, milk, cereals and instant noodles. Phthalates in all the samples were detected to be less than limit of reporting or LOQ. Spike samples have proof the method shows good repeatability of data with the RSD less than 3.30 %.

Although this method produces good recoveries and repeatability of data, however study can be made further to increase the sensitivity as trace level analysis is very particular in food analysis.

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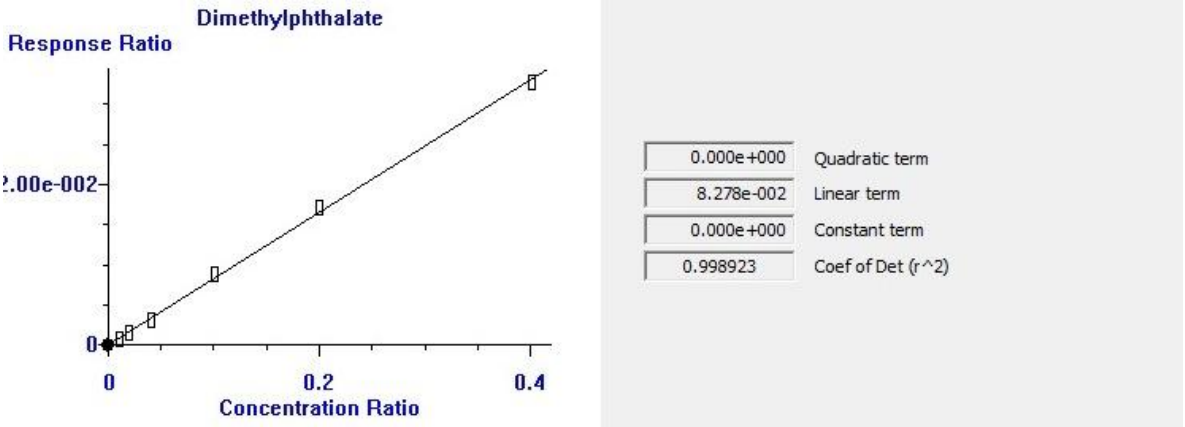
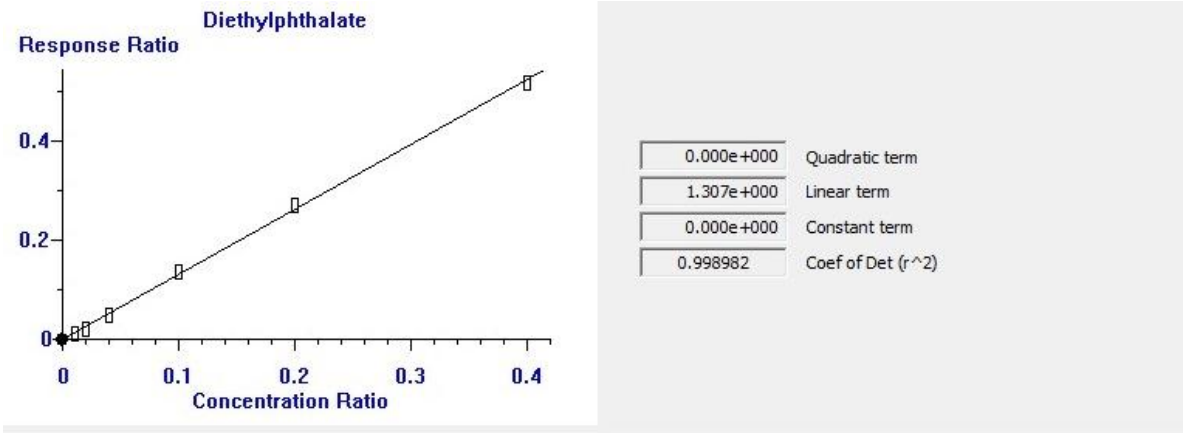
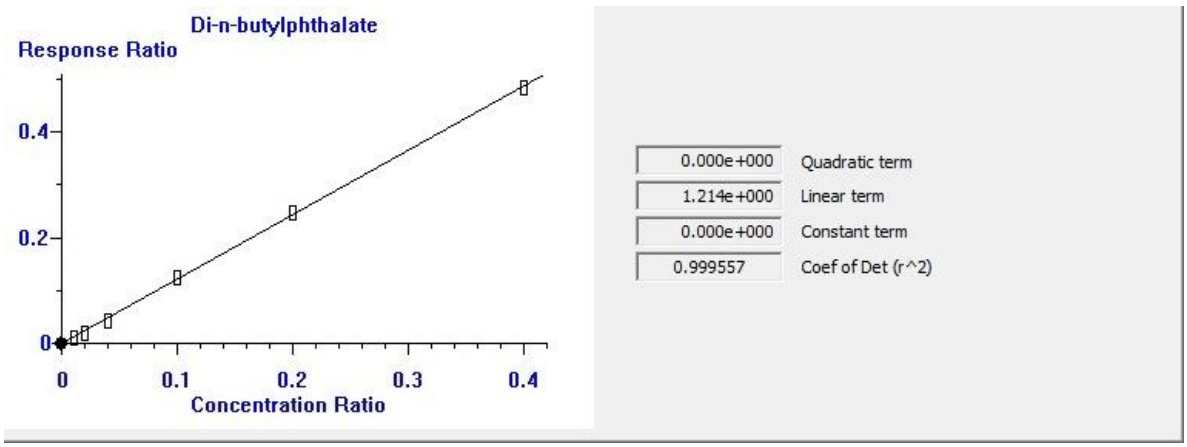
APPENDICES



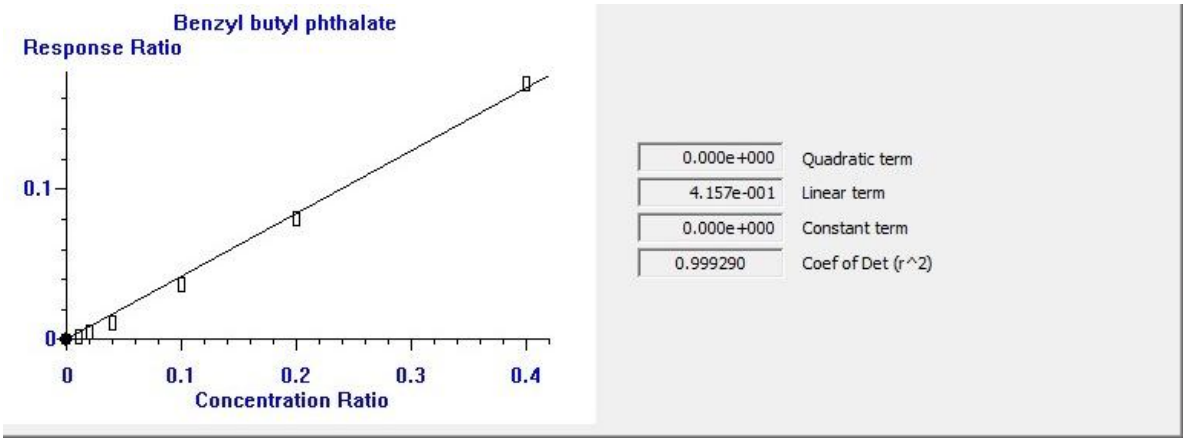
Appendix 1: DMP calibration curve (SIM mode)



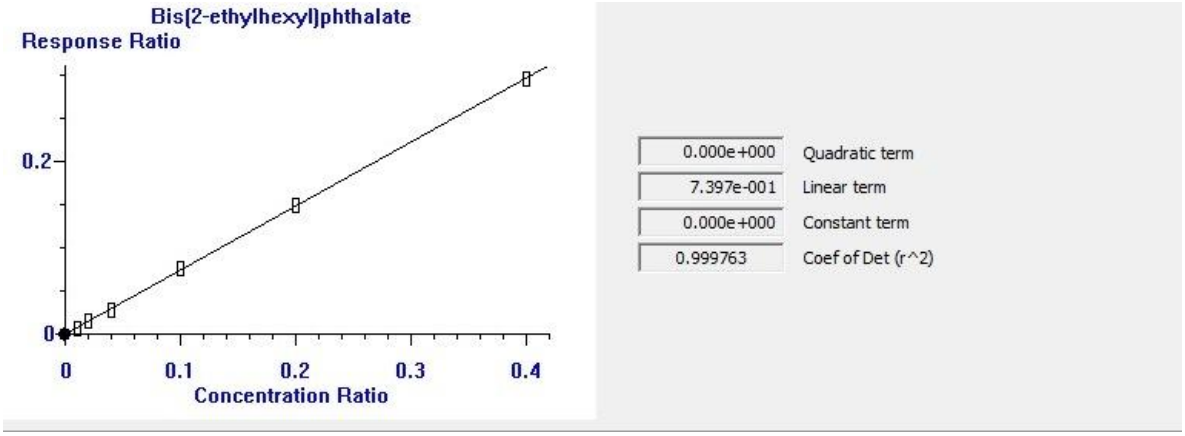
Appendix 2: DEP calibration curve (SIM mode)



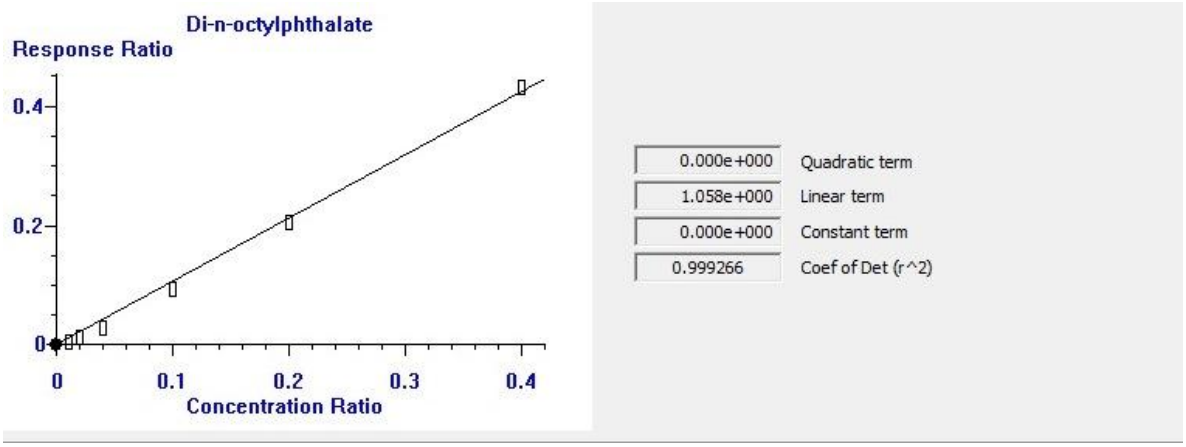
Appendix 3: DBP calibration curve (SIM mode)



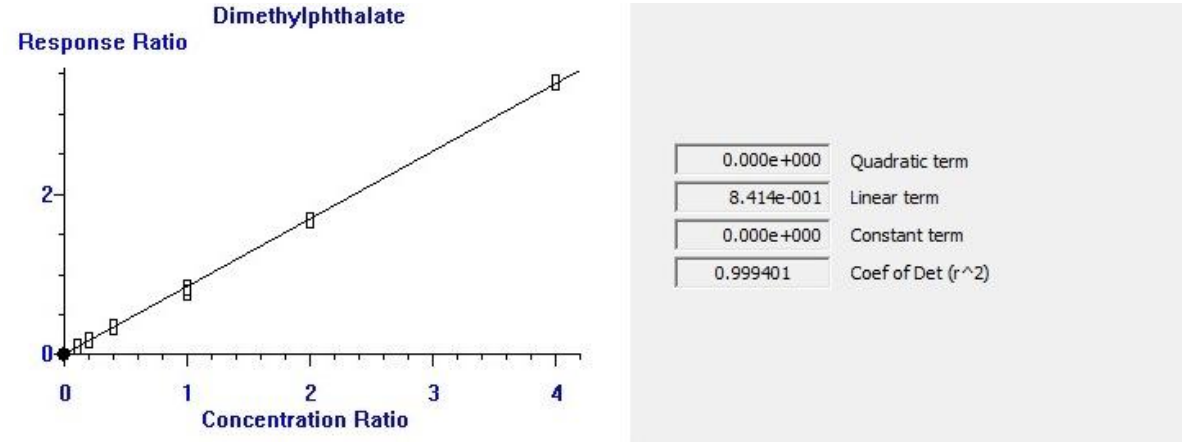
Appendix 4: BBP calibration curve (SIM mode)



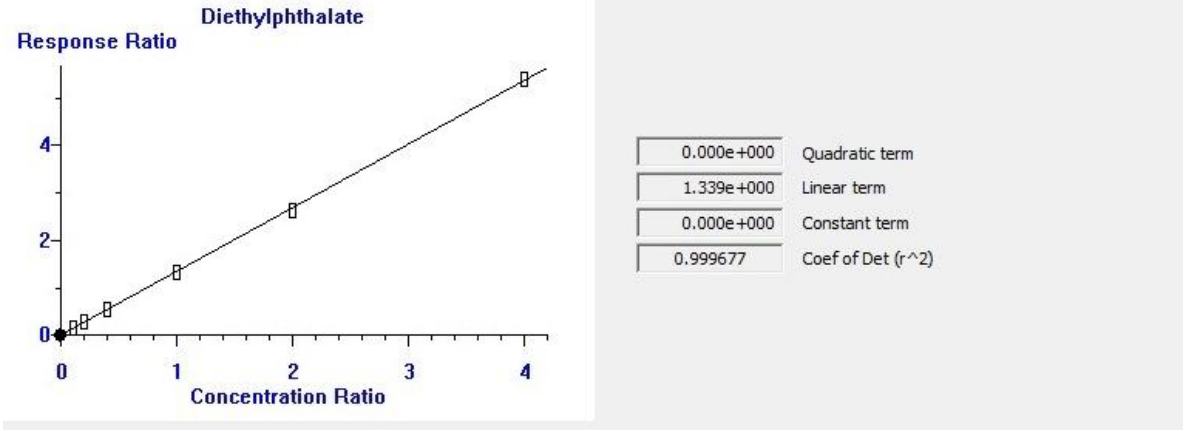
Appendix 5: DEHP calibration curve (SIM mode)



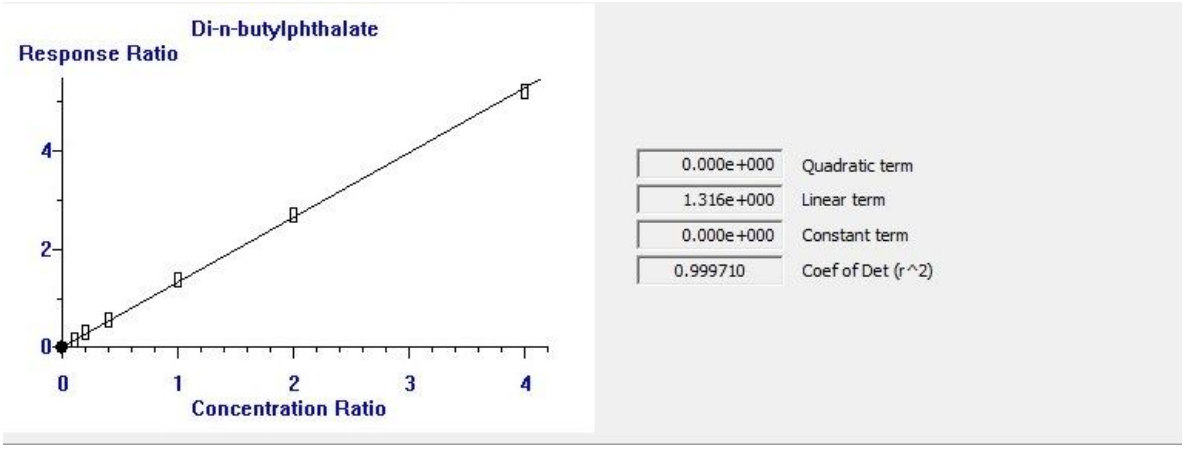
Appendix 6: DEHP calibration curve (SIM mode)



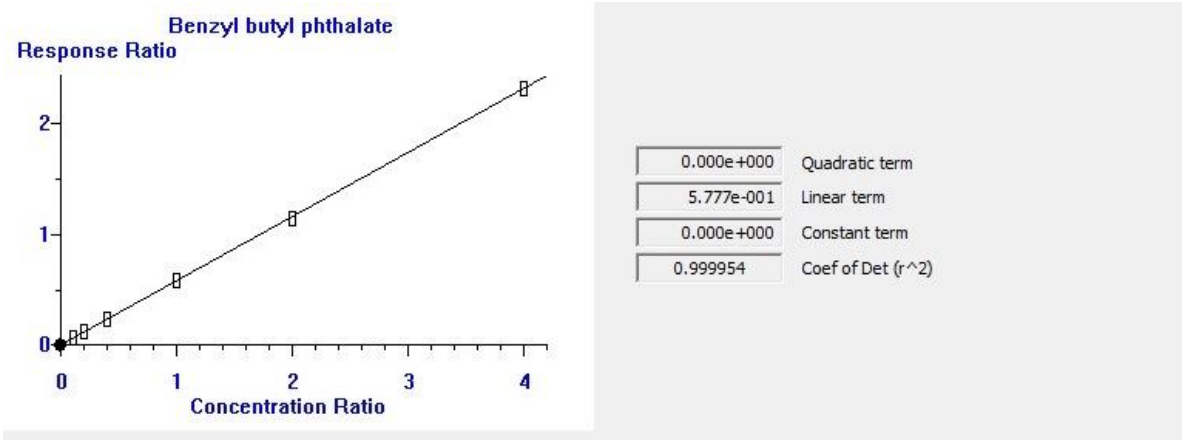
Appendix 7: DMP calibration curve (full scan mode)



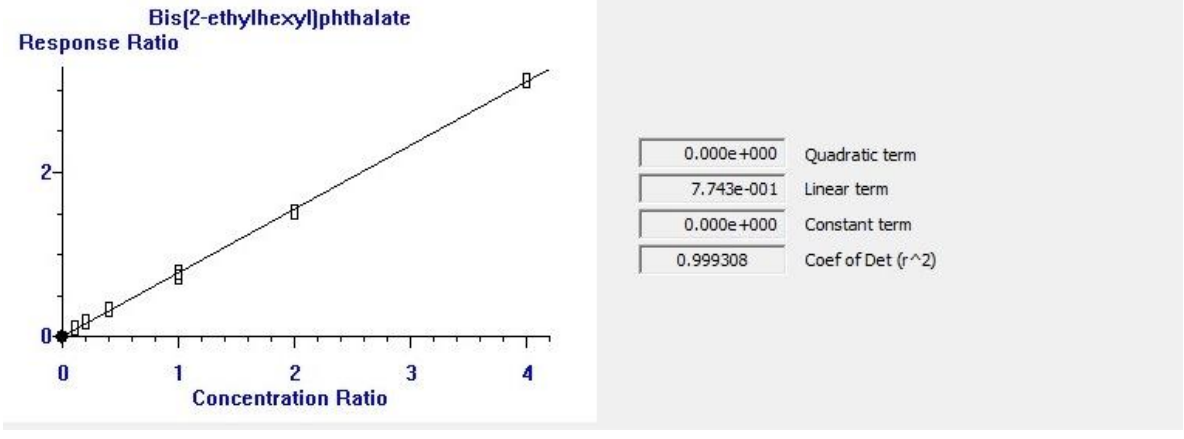
Appendix 8: DEP calibration curve (full scan mode)



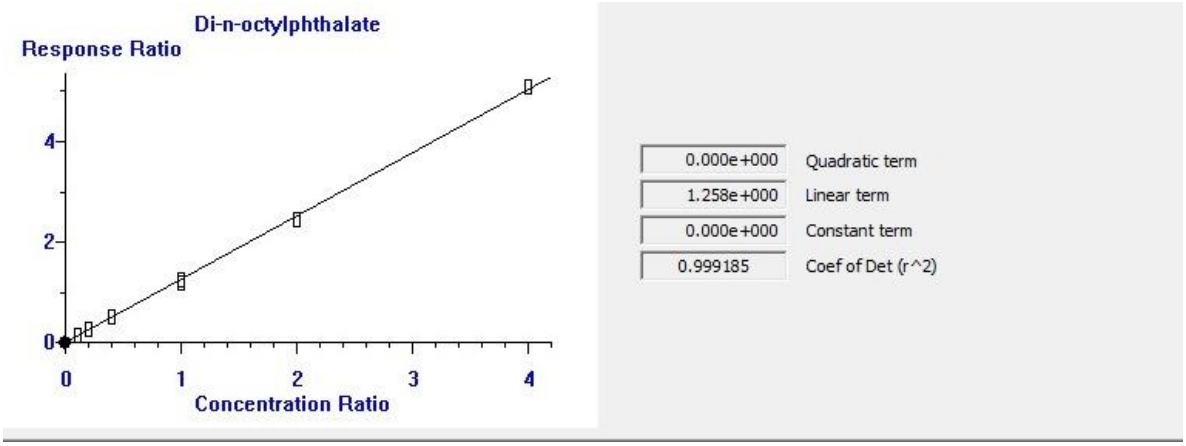
Appendix 9: DBP calibration curve (full scan mode)



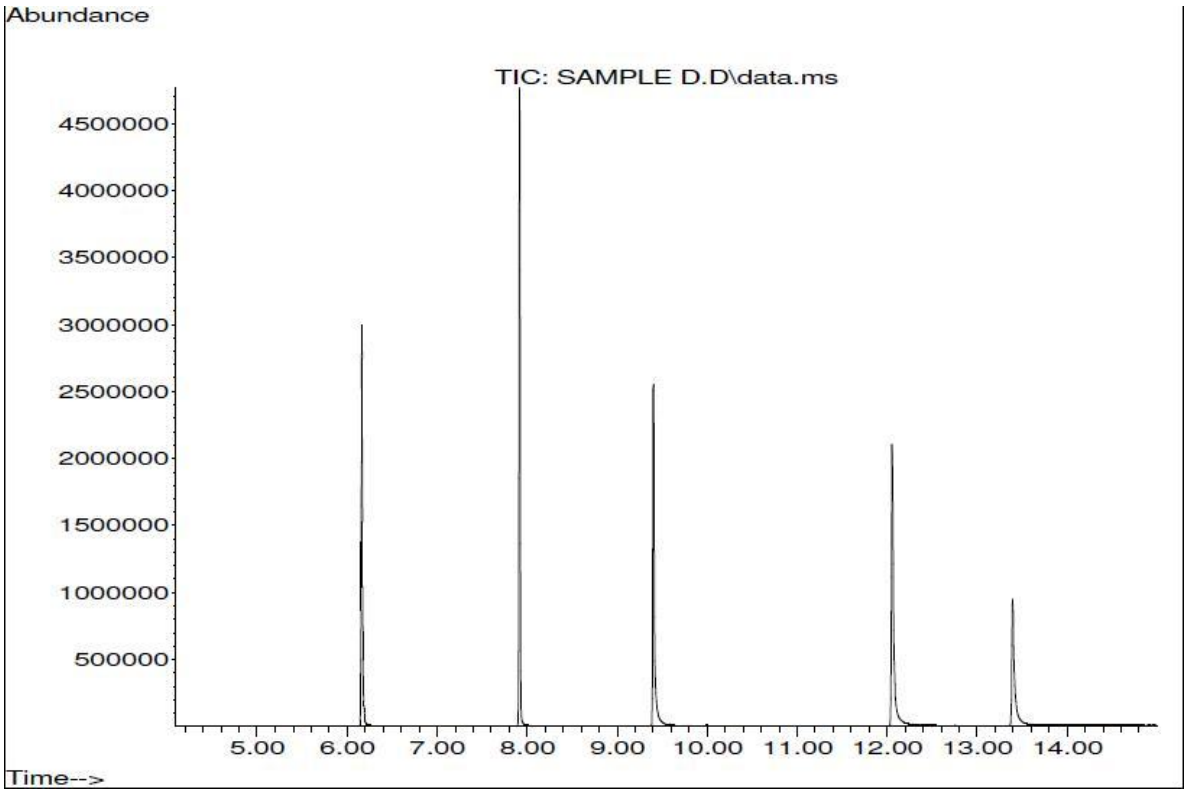
Appendix 10: BBP calibration curve (full scan mode)



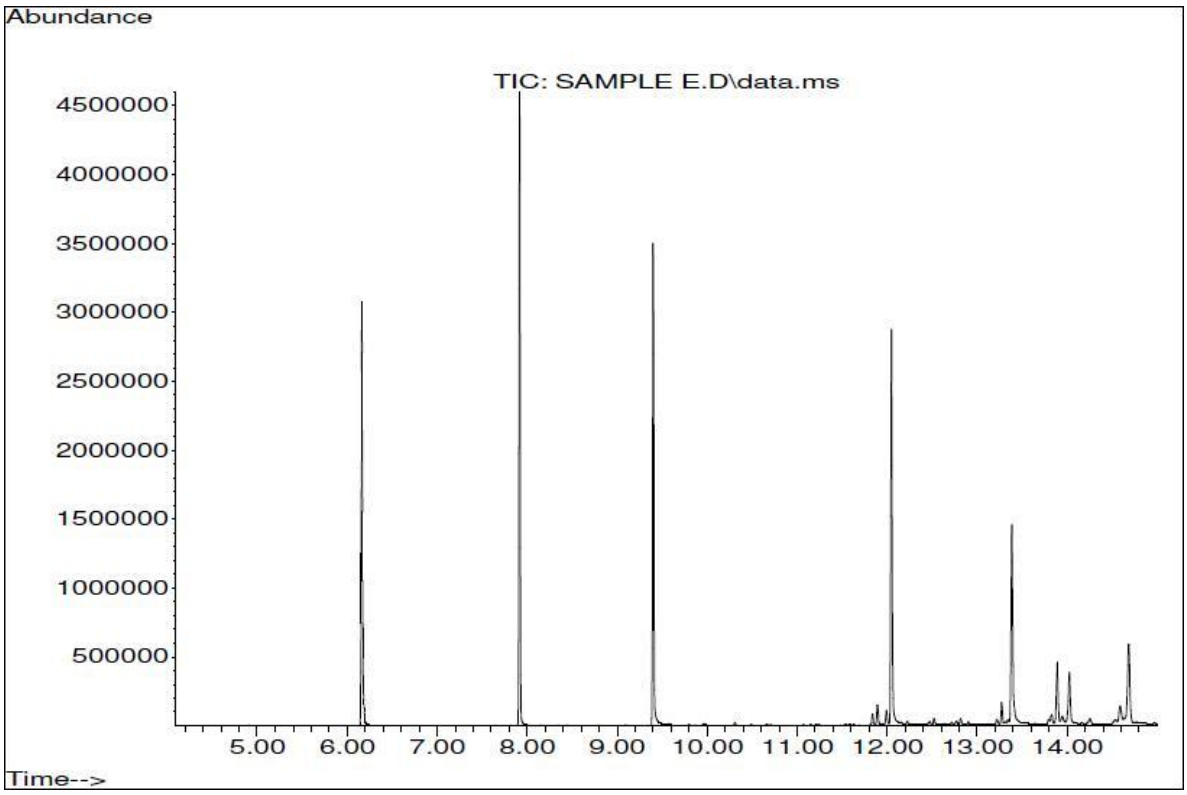
Appendix 11: DEHP calibration curve (full scan mode)



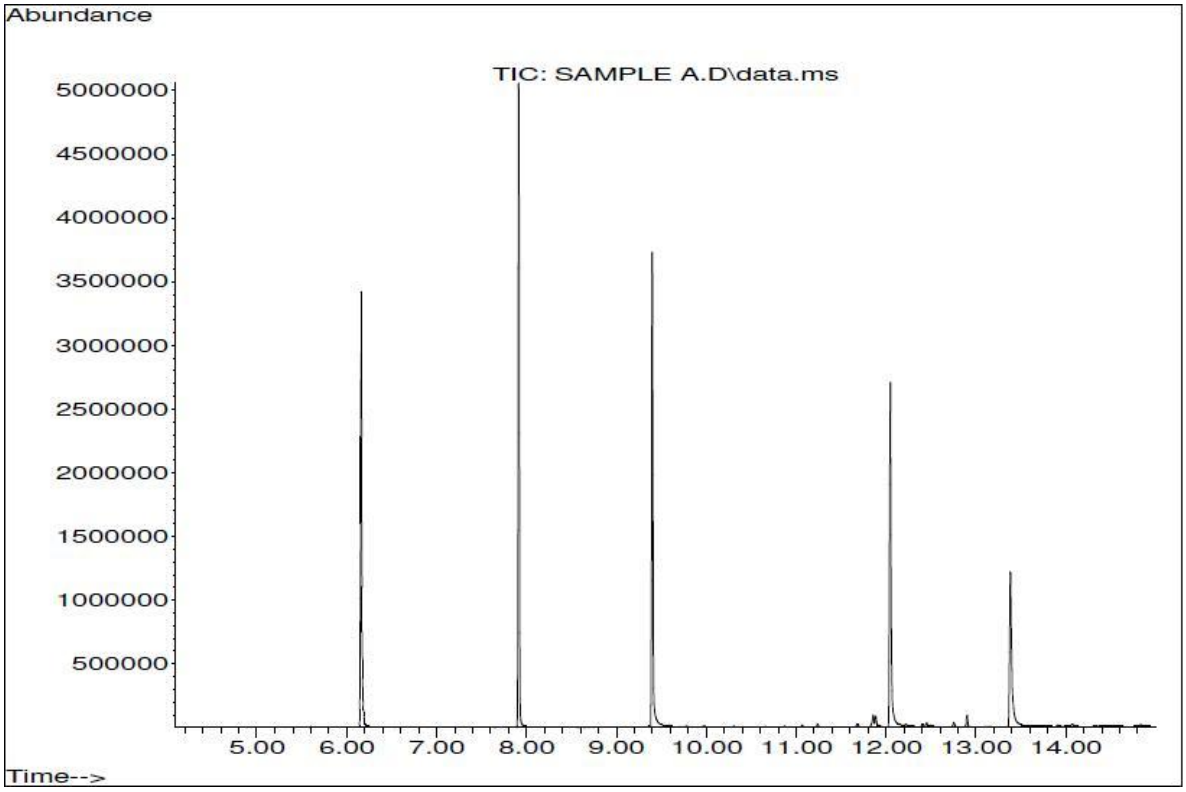
Appendix 12: DNOP calibration curve (full scan mode)



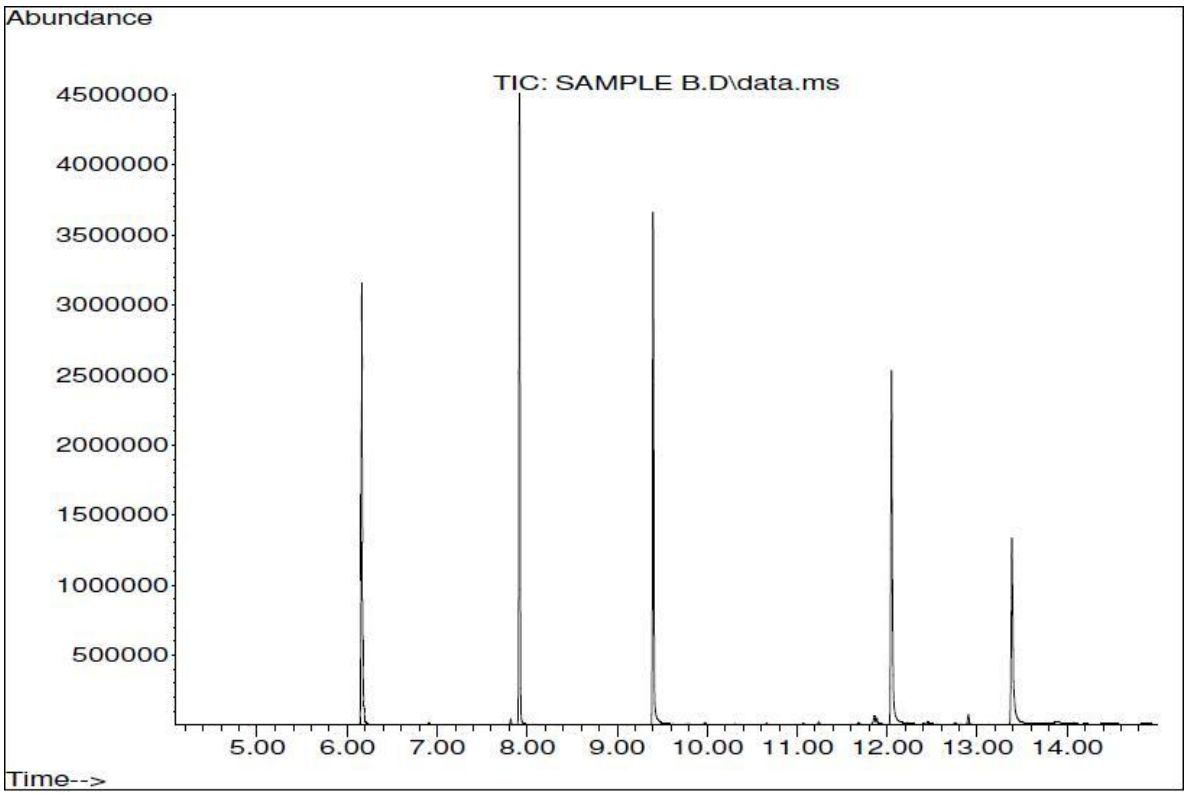
Appendix 13: Chromatogram for drinking water sample



Appendix 14: Chromatogram for milk sample



Appendix 15: Chromatogram for cereal sample



Appendix 16: Chromatogram for instant noodles sample