EVALUATION OF PHTHALATE ESTERS IN PASTEURIZED MILK SAMPLES AND THEIR PACKAGES BY GAS CHROMATOGRAPHY-MASS SPECTROSCOPY (GC-MS)

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I hereby declare that all information in this document has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all material and results that are not original to this work.

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ABSTRACT

EVALUATION OF PHTHALATE ESTERS IN PASTEURIZED MILK SAMPLES AND THEIR PACKAGES BY GAS CHROMATOGRAPHY AND MASS SPECTROSCOPY (GC-MS)

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In this study, the Phthalate Esters, which are specifically Dimethyl Phthalate (DMP), Diethyl Phthalate (DEP), Dibutyl Phthalate (DBP), Butylbenzyl Phthalate (BBP), Diethlyhexzyl Phthalate (DEHP), and Dioctylphthalate (DOP), were evaluated in the 5 different pasteurized milk samples and their packages that were chosen from the Turkish market. As validated extraction methodology Ultrasound Assisted Dispersive Liquid Liquid Micro Extraction (UA-DLLME) and Ultrasonic Bath Extraction (UBE) were used for the milk samples and packages, respectively. Gas Chromatography-Mass Spectroscopy (GC-MS) was used as the analytical instrumentation. Extraction efficiencies of the UA-DLLME were in between 66-100% while the ones that belong to UBE were in between 115-127%. For both the pasteurized milk samples and their packages, DBP and DEHP were found as the most common phthalate esters in each of five milk and package samples. DBP values that are obtained after the analysis of milk samples were between 3.08-5.03 ng/g while DEHP values were in between 0.41-4.00 ng/g. Concentration of DBP in milk packages were in between 1.05-2.03 ng/g while concentration of DEHP values were in between 30.0-62.6 ng/cm^2. Concentration of DOP was found as the lowest concentrated phthalate ester in milk packages. It is only

found in Milk Package Sample E as 2.42 ng/g while no DOP was found in milk samples. In addition, the results of this study and the found values of the phthalate levels were compared with the other studies performed in other countries.

Keywords. Phthalate Esters, Pasteurized Milk Samples and Their Packages, UA-DLLME, GC-MS

ÖZ

PASTÖRİZE SÜT ÖRNEKLERİ VE PAKETLERİNDEKİ FTALAT ESTERLERİN GAZ KROMATOGRAFİ- KÜTLE SPEKTROSKOPİSİ İLE DEĞERLENDİRİLMESİ

Şenlik, Damla
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Ocak 2014, 92 sayfa

Bu çalışmada altı farklı ftalat ester, Dimetil Ftalat (DMP), Dietil Ftalat (DEP), Dibütil Ftalat (DBP), Bütil Benzil Ftalat(BBP), Dietil Hekzil Ftalat (DEHP), Dioktil Ftalat (DOP) beş farklı pastörize süt örneğinde ve paketlerinde incelenmiştir. Ultrason destekli-dağıtıcı sıvı sıvı mikroektraksiyonu (UA-DLLME) ve ultrasonik banyo ekstraksiyonu (UBE) sırasıyla süt örnekleri ve paketlerindeki ftalatların eldesi için kullanılmıştır. Ayrıca, Gaz Kromatografi Kütle Spekroskopi (GC-MS) ise analitik cihaz olarak kullanılmıştır. Süt örneklerindeki ftalatların ekstraksiyon verimi %66-100 arasında bulunurken süt paketlerindeki ftalatların ektraksiyon verimi %115-127 olarak sonuçlandırılmıştır. Hem pastörize süt örnekleri hem de paketleri için DBP ve DEHP en çok rastlanan ftalatlar olarak gözlemlenmişlerdir. Süt örneklerinin analizlerinden elde edilen DBP değerleri 3.08-5.03 ng/g olarak değişirken DEHP değerleri 0.41-4.00 ng/g aralığında bulunmuştur. Ek olarak, süt paketlerinin analizleri sonucu DBP miktarı 1.05-2.03 ng/g olarak tayin edilirken DEHP aralığı 3.00-6.26 ng/g olarak belirlenmiştir. DOP hem süt ürünlerinde hem de paketlerinde en az rastlanan ftalat olarak kaydedilmiştir. Süt örneklerinde ölçülebilir miktarda bulunamazken, süt paketlerinin sadece bir tanesinde (örnek E) 2.42 ng/g olarak bulunmuştur. Ayrıca bu

çalışmanın sonucunda bulunan ftalat konsantrasyon seviyeleri başka ülkelerdeki başka çalışmalarla kıyaslanmıştır.

Anahtar Kelimer: Ftalat Ester, Pastörize Süt Örnekleri ve Paketleri, UA-DLLME, GC-MS

To My Grandmother

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LIST OF ABBREVIATIONS

UA-DLLME. Ultrasound Assisted Dispersive Liquid Liquid Microextraction

BBP. Butyl Benzyl Phthalate

BLOQ. Below Limit of Quantification

BW. Body Weight

DBP. Dibutyl Phthale

DEHP. Diethyl Hexyl Phthalete

DEP. Diethyl Phthalate

DMP. Dimethyl Phthalate

DOP. Dioctyl Phthalate

ECD. Electron Capture Detector

GC. Gas Chromatography

HS-SPME. Head Space-Solid Phase Micro Extraction

K_{ow}. Octanol-water Coefficient

LD_{50}. Lethal Dose 50%

LC_{50}. Inhalation Toxicity

LOD. Limit of Detection

LOQ. Limit of Quantification

MS. Mass Spectrometry

NIST. National Institute of Standards and Technology

QA. Quality Assurance

QC. Quality Control

SIM. Selected Ion Monitoring

S/N. Signal to Noise Ratio

SRM. Standard Reference Material

UBE. Ultrasonic Bath Extraction

% RSD. Percent Relative Standard Deviation

CHAPTER 1

INTRODUCTION

1.1. Phthalates

1.1.1. Structural Properties of Phthalates

Many industrial processes and products contain phthalates that are a kind of chemicals.

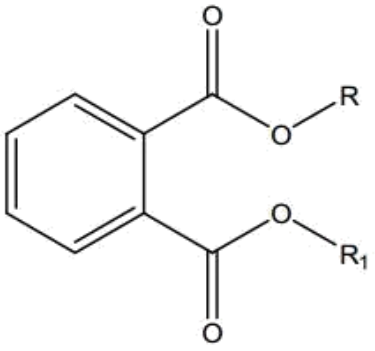


Figure 1. General Structure of Phthalate Esters

As can be seen in Figure 1, phthalates can be categorized as a di-ester with the existence of a benzenedicarboxylic acid head group which is bound to two ester side chains. Phthalates have three isomeric forms. ortho-phthalates (phthalates), meta-phthalates (isophthalates) and para-phthalates (terephthalates). Mostly, ortho-structural configuration of these chemicals are called as phthalate or phthalate esters. Meta-phthalates have one carbon atom, para-phthalates have two carbon atoms between two carboxylic acid functional groups and ester side chains (NINCAS, 2008a).

Mostly, phthalate esters have ester side chains differing between one carbon to thirteen carbons (C1-C13). Side chains may be linear, branched or a combination of linear branched and ringed structures. Commonly, both sides of the side chains are identical, but for some phthalates might be different. In other words, the level of existence of branched or linear side chains make a distinction for the structural property of the phthalate esters.

Phthalates are the most widespread set of chemicals used as universal plasticisers (plastic softeners) in some polymers (e.g. PVC). Moreover, they are often used as solvents. It is possible to say, as a chemical group, they are everywhere (NINCAS, 2008a).

Although the low molecular weight phthalate esters are used for their extensive emulsifying properties; DEHP, DiNP and DiDP are mainly integrated in polymers since they can be used as plasticizers. In Table 1, the most commonly used phthalates are listed with their molecular formulas and structures (Tienpont, 2004).

Table 1. List of Phthalates (NINCAS, 2008a)

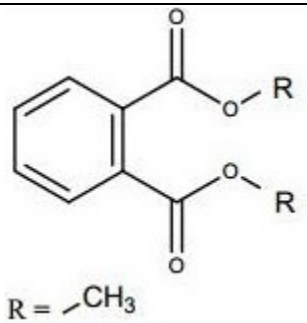
Backbone Carbon	Chemical Name	Common Name	CAS no.	Molecular Formula	MW	Structure
C1	1,2-Benzenedicarboxylic acid, dimethyl ester	Dimethyl phthalate (DMP)	131-11-3	$C_{10}H_{10}O_4$	194.19	
C2	1,2-Benzenedicarboxylic acid, diethyl ester	Diethyl phthalate (DEP)	84-66-2	$C_{12}H_{14}O_4$	222.30	
C3 (double bond)	1,2-Benzenedicarboxylic acid, di-2-propenyl ester	Diallyl phthalate (DAP)	Diallyl phthalate (DAP)	$C_{14}H_{14}O_4$	246.27	

Table 1. List of Phthalates (NINCAS, 2008a) (Cont'd)

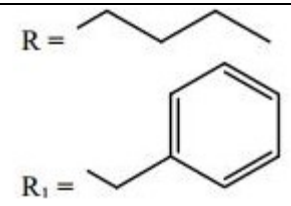
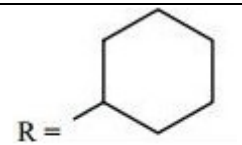
C3	1,2-Benzenedicarboxylic acid, bis(2-methoxyethyl) ester	Bis(2-methoxyethyl) phthalate (DMEP)	117-82-8	$C_{14}H_{18}O_6$	282.30	
C3	1,2-Benzenedicarboxylic acid, bis(2-methylpropyl) Ester	Diisobutyl phthalate (DIBP)	84-69-5	$C_{16}H_{22}O_4$	278.35	
C4	1,2-Benzenedicarboxylic acid, dibutyl ester	Dibutyl phthalate (DBP)	84-74-2	$C_{16}H_{22}O_4$	278.35	
C4, C5	1,2-Benzenedicarboxylic acid, butyl phenylmethyl Ester	Butylbenzyl phthalate (BBP)	85-68-7	$C_{19}H_{20}O_4$	312.35	
C4 (ring)	1,2-Benzenedicarboxylic acid, dicyclohexyl ester	Dicyclohexyl phthalate (DCHP)	84-61-7	$C_{20}H_{26}O_4$	330.46	

Table 1. List of Phthalates (NINCAS, 2008a) (Cont'd)

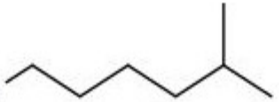
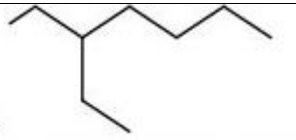
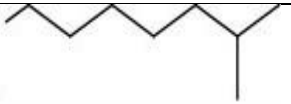
C5	1,2-Benzenedicarboxylic acid, dihexyl ester, branched and linear	Diisohexyl phthalate (DIHP)	68515-50-4	$C_{20}H_{30}O_4$	334.00	R = 
C6	1,2-Benzenedicarboxylic acid, dihexyl ester	Di-n-hexyl phthalate (DnHP)	84-75-3	$C_{20}H_{30}O_4$	334.40	R = 
C6-rich	1,2-Benzenedicarboxylic acid, di-C6-8-branched alkyl esters, C7-rich	Diisoheptyl phthalate (DiHepP)	71888-89-6	$C_{22}H_{34}O_4$	363.00	R = 
C6	1,2-Benzenedicarboxylicacid, bis(2-ethylhexyl) Ester	Diethylhexyl phthalate (DEHP)	117-81-7	$C_{24}H_{38}O_4$	390.56	R = 
C7	1,2-Benzenedicarboxylic acid, diisooctyl ester	Diisooctyl phthalate (DIOP)	27554-26-3	$C_{24}H_{38}O_4$	390.62	R = 

Table 1. List of Phthalates (NINCAS, 2008a) (Cont'd)

C8	1,2-Benzenedicarboxylic acid, dioctyl ester	Di-n-octyl phthalate (DnOP)	117-84-0	$C_{24}H_{38}O_4$	390.60	R= 

1.1.2. Physicochemical Properties of Phthalates

Although the structural properties of the phthalate esters mainly affect the physicochemical properties of phthalates, they are all in the liquid state at ambient temperatures and they have melting points below 0 °C.

Pure phthalates boil between 230 °C(boiling point of DMP) and 486 °C (boiling point of DiDP), but high molecular weight phthalates have a tendency to be decomposed when they are subjected to high temperature processes. When the boiling points of phthalate mono-esters are compared to boiling points of phthalates, due to the hydrogen bridging of the polar groups, phthalate mono-esters have higher boiling points (Tienpont, 2004).

In addition, although phthalates have comparatively low vapor pressures, phthalates might be classified as semi-volatile compounds and they exist in the vapor phase. Moreover, the densities of phthalates at 20 °C are all almost equal to unity within the complete range of compounds. Another point that must be explained in physicochemical properties is octanol-water partition coefficient. It can be showed as $K_{o/w}$. Fundamentally, it shows the equilibrium distribution of a solute between water and octanol. That is why it can be classified as a physical constant which is related to the hydrophobicity. Formerly, it is required to measure the equilibrium concentrations of the both octanol and water phase to be able to obtain the ratio of $K_{o/w}$.

Commonly, the $K_{o/w}$ is enhanced proportionately to the molecular weight of alkyl phthalates or the side chain length. That means high molecular-weight phthalates that are used as plasticizers (DEHP, DiNP, DiDP) exhibit high affinity for non-polar matrix substances that are fat, bio-matrices or (suspended) solids in surface or waste water. Physicochemical data are summarised in Table 2. Unless stated, values are for standard pressure and temperature conditions of 101.325 kPa and 25 °C. All these physicochemical properties of phthalates allow migration and leaching of phthalates from polymer substrates when they are used as plasticizers. This possibility of leaching of phthalate esters from ready to use products that are made up of plastics has led to health effects (NINCAS, 2008a).

Table 2. Physicochemical Properties of Phthalate Esters (NINCAS, 2008a)

Backbone C Length	Phthalate	Melting Point (ºC)	Boiling Point (ºC)	Density (kg/m^3)	Vapour Pressure (kPa)	Water Solubility (g/L)
C1	DMP	5.5	284	1190	8.0×10^{-4} (20ºC)	4.3 (20ºC)
C2	DEP	No data	298	1120	2.19×10^{-4}	1
C3 (double bond)	DAP	-70	157 (0.67 kPa)	1120	2.13×10^{-5}	1.48×10^{-1} (20ºC)
C3	DMEP	-40	340	1170 (15 ºC)	$<1.30 \times 10^{-2}$ (20º C)	9×10^{-1} (20°C)

Table 2. Physicochemical Properties of Phthalate Esters (NINCAS, 2008a) (Cont'd)

C3	DIBP	-37	320	1038	1×10^{-5} (20°C)	1×10^{-3}
C4	DBP	-69	340	1045 (20 °C)	9.7×10^{-6}	1×10^{-2} (20˚C)
C4, C5	BBP	<-3.5	370 (1.01 kPa)	1114-1122	8.0×10^{-8}	2.8×10^{-3}
C4 (ring)	DCHP	66	222-228 (0.5 kPa)	1383 (20ºC)	13.3×10^{-3} (150ºC)	4×10^{-3} (24ºC)
C5	DIHP	No data	No data	No data	No data	No data

Table 2. Physicochemical Properties of Phthalate Esters (NINCAS, 2008a) (Cont'd)

C6	DnHP	-27.4	350	1011	6.67×10^{-7}	5×10^{-5}
C6-rich	DiHepP	-45	398	994 (20°C)	9.33×10^{-8}	1.7×10^{-5} (22°C)
C6	DEHP	-47	384	984 (20°C)	1.33×10^{-8}	4.1×10^{-5}
C7	DIOP	-45	230 (0.53 kPa)	986 (20°C)	1.33 (200°C)	<0.1 (20°C)
C8	DnOP	-25	390	978	1.92×10^{-5}	3×10^{-3}

As a summary (NINCAS, 2008a):

- Phthalates are generally clear to yellow colored chemical compounds,
- At ambient temperatures they have high boiling points,
- Almost all of the phthalates have melting points below -25ºC,
- While octanol-water partition coefficient increases with increasing carbon, there occurs a decrease in vapor pressure,
- Water solubility is also inversely related to molecular weight and backbone length of the phthalate ester.

1.1.3. Legislation and Regulation of Phthalates

Regulations in the United States have been taking place since 1976 about phthalate usage. Although phthalates are categorized as hazardous waste and pollutant for the environment, there is no enough regulation for the usage of these chemicals in consumer products. Even though many states in the US tried to ban the phthalates, except for California, all of them failed. In California, use of the 6 phthalates (DEHP, DBP, BBP, DINP, DIDP and DNOP) only in children's toys was banned in 2007. Moreover, they were also banned in the European Union in 2005 and by following it fourteen other countries either restricted or did not allow the use of it in children's toys (ACAT).

In the case of Turkey, Ministry of Health started to make some regulations for the usage of phthalates in children's toys and childcare products in 2005. However, application of the law to decrease the limit of phthalates to 0.1% by mass in the mentioned products started in 2008. Before this law, its mass fraction could be even 30% by weight (İTKİB).

1.2. Usage and Toxicological Effects of Phthalates

The yearly production of phthalates are approximately 5 million tons and all these are used for medical devices, food wrap, building materials, packaging, automotive parts, children's toys, and childcare products. Roughly, phthalates are classified as reproductive and developmental toxicants. In the below part, more detailed information is given for only the phthalates that are going to be investigated during this study (Lowell Center for Sustainable Production, 2011).

1.2.1. Diethyl Phthalate (DEP)

DEP can be classified as a plasticizer generally applied to use in tools, automotive parts, tooth brushes, food packaging, cosmetics and insecticide, personal care and pharmaceutical products, children's toys, and perfumes. Moreover, DEP is also used in fragrance of household cleaning and personal care products. In addition, it is also used in biotechnological research laboratories (NINCAS, 2008b).

Since there are no studies that the people are exposed to DEP, it can be said that there is no information about the possible effects of DEP on human health. However, there are studies on laboratory animals.

When DEP was given to animals with high doses by mouth, it was a reason of death of laboratory animals. When they digest high dose of DEP for long time periods, a decrease in animal weight was observed. There are two possible reasons of weight loss of the animals. Either they ate less food, or because they discarded more of the food they ate. Furthermore, some other studies indicated that when the mother animal was exposed to high level of DEP, the presence of an extra rib in the fetus was distinguished. In addition to all those, when the female animals were subjected to the injection of DEP (approximately 3 g/kg) both a decrease in the number of the live-born babies and increase in birth defects were detected. Moreover, DEP is irritating when the animal skin and eyes are subjected to it (U.S. Department of Health and Human Services, 1995).

Lethal Dose 50% can be shortened as LD_{50}. LD_{50} value is the statistical of the evaluation of a phthalate that can kill 50% of the test animals (e.g. rat, guinea pig, mouse, rabbits…) in a time period. Because phthalates can go into the body by oral, dermal, respiratory ways, for each option lethal dose must be measured. Phthalates that have low LD_{50} values are tremendously toxic. That means, even only a very small amount of these low LD_{50} phthalates are adequate to harm the living organisms.

Inhalation Toxicity LC_{50} can be expressed as the phthalate concentration in the air that will kill 50% of the test animals by breathing in period of time. Both LD_{50} and LC_{50} are used to obtain information about acute toxicity.

Chronic toxicity which illustrates the unpleasant health effects of phthalates over a longer time period should be completed with acute toxicity. LD_{50}-LC_{50} values of DEP are given in Table 3 (Connell, 2005).

Table 3. Accute Toxicity of DEP in animals (NINCAS, 2008b)

Route of administration	Species	Results (LD_{50}/LC_{50})
Oral (LD_{50})	Mouse Rat Guinea Pig Rabbit Dog	$6.2x10^2$ mg/kg bw $>5.6x10^2$ to $3.1x10^3$ mg/kg bw $>4.0x10^2$ to $8.6x10^2$ mg/kg bw $1.0x10^2$ mg/kg bw $5.0x10^2$ mg/kg bw
Dermal (LD_{50})	Rat Guinea Pig	$>1.1x10^3$ mg/kg bw $3.0x10^2$ mg/kg bw
Inhalation (4h) (LC_{50})	Mouse Rat	4.9 mg/L 7.5 mg/L

1.2.2. Di-2-ethylhexyl Phthalate (DEHP)

DEHP is the most extensively used phthalates worldwide. In many parts of the world, it is mainly used as a plasticizer in PVC. DEHP is used as a plasticizer in many products like toys, automotive components, furniture, shoes and boots, outdoor and rainwear, building material such as flooring, cables, profiles and roofs. As well as, DEHP is used as an ingredient in cosmetics, it is also used in health area in medical products like blood bags, dialysis equipment (NINCAS, 2008c).
DEHP level in the normal environment, is not expected to lead to unpleasant health effects in humans. Nonetheless, a man who willingly consumed 10 g of DEHP at once had stomach irritation and diarrhea.

There are not so many studies on humans about DEHP. However, most of what is known about the health effects of DEHP on humans is from the experiments that were performed on rats and mice. They were exposed to DEHP either by their food or by a tube that was placed in their stomach through their mouth. Receiving high doses of DEHP for a long time was concluded with liver cancer in rats and mice.

Moreover, it is also known that since kidney is subjected to DEHP during its dialysis, structural and functional changes were observed in rats that were exposed. However, since the kidney functions of rats and humans are not the same, in other words, humans absorb and breakdown DEHP in the body differently than rats and mice, it cannot be stated with certainty that DEHP is damaging the human kidney functionality.

By the route of breathing, DEHP is unlikely to have dangerous effects. Experiments by rats have indicated that DEHP in the air has no effect on reproduction. However, oral exposures of DEHP in short time periods decreased the sperm formation in male rats. Moreover, exposure to DEHP postponed the sexual maturity in young rats. In addition to all those, since the skin does not let the DEHP go into the body easily, by skin contact there is no health effect of DEHP (U.S. Department of Health and Human Services, 2002).
The following table, Table 4, gives information about the acute animal toxicity studies of DEHP.

Table 4. Acute Animal Toxicity Studies of DEHP (NINCAS, 2008c)

Study Type	Species	Results (LD_{50}/LC_{50})
Oral (LD_{50})	Rat	>$4.0x10^4$ mg/kg bw
	Mouse	>$2.0x10^4$ mg/kg bw
	Guinea pig	$2.6x10^4$ mg/kg bw
	Rabbit	$3.4x10^4$ mg/kg bw
Dermal (LD_{50})	Rabbit	$2.5x10^4$ mg/kg bw
Inhalation (4h) (LC_{50})	Rat	>10.6 mg/L

1.2.3. Dibutyl Phthalate (DBP)

DBP is another kind of plasticiser that is used in resins and polymers. Moreover, it is used as a softener in adhesives, lacquers, varnishes and printing inks. It also has wide range of usage in cosmetics as perfume solvent and fixative. In addition, it has various usage purposes that can be counted as; a suspension agent for solids in aerosols, a lubricant for aerosol valves, an anti-foamer, and a plasticiser in nail polish and fingernail elongators. Besides of all those, DBP is also used for personal care and cosmetic products, children's toys, exercise balls, hoses and rubber sheets (NINCAS, 2008d).

During the studies with DBP on laboratory animals, it is exhibited that dietary exposure of it causes a decrease in sperm production. However, it is observed that when the exposure of DBP on animals is stopped sperm production comes to normal levels. But this kind of adverse effect is seem when the animal is subjected to 10,000 times higher than the levels of DBP that is in the air. Although there is no available data on whether DBP causes any kind of cancer or not, it can also be said that when large amounts of DBP is applied to the skin, it may cause some skin irritation (U.S. Department of Health and Human Services, 2001). Table 5 gives information about the acute animal toxicity studies of DBP.

Table 5. Acute Animal Toxicity Studies of DBP (NINCAS, 2008d)

Study Type	Species	Results (LD_{50}/LC_{50})
Oral (LD_{50})	Rat Mouse	$6.3x10^3$ mg/kg bw $4.8x10^3$ mg/kg bw
Dermal (LD_{50})	Rabbit	$>2.0x10^4$ mg/kg bw
Inhalation (4h) (LC_{50})	Rat	>15.7 mg/L

1.2.4. Dioctyl Phthalate (DOP)

DOP is usually used in PVC for the production of a wide range of products like flooring and carpet tiles, swimming pool liners, notebook covers, traffic cones, toys and dolls, vinyl furniture, shower curtains and gloves, garden hoses, weather stripping, and shoes.

In many food purposes like production of bottle cap liners, and conveyor belts, PVC which contains DOP is used. DOP is also used as a dye carrier in plastics production, an active pesticide ingredient, as a colorant in cosmetics (NINCAS, 2008e).

There are not sufficient number of experiments that are performed on people or animals with DOP. However, some basic knowledge exists about the effects of DOP on the liver. It is known that short oral exposures of DOP commonly lead to no harmful effects. Additionally, DOP can also be classified as slightly mildly skin and eye irritating. However, there is no enough information about the DOP when the skin is exposed to it for long time periods.

On the other hand, it is reported that DOP caused some birth defects on new-born rats since their mothers got high doses of DOP –around 5 g/kg bw during their pregnancy. However, it is not known whether DOP has an unpleasant effect on developing fetus. It is not exactly known that DOP leads to cancer in humans or animals. That is why Department of Health and Human Services, the International Agency for Research on

Cancer and the EPA have not classified DOP as carcinogenic. In addition, it does not affect the reproducibility of male animals unlike DEHP (U.S. Department of Health and Human Services, 1997). Table 6 gives information about acute animal toxicity studies of DOP.

Table 6. Acute Animal Toxicity Studies for DOP (NINCAS, 2008e)

Study Type	Species	Results
Oral (LD_{50})	Rat Mouse	$5.4x10^4$ mg/kg bw $1.3x10^4$ mg/kg bw
Dermal (LD_{50})	Guinea Pig	75.0 mL/kg bw

1.2.5. Butylbenzyl Phthalate (BBP)

According to American Chemistry Council, BBP is most widely used in vinyl floor covering. In addition, food conveyor belts, carpets, non-natural leather, and vinyl gloves include BBP as a kind of plasticizer. Moreover, BBP is used in the production of food wrap. It is also reported that in baby equipment and children toys BBP is detected at low concentrations as impurity. Due to the current EU legislation rules, BBP cannot be found in cosmetics (NINCAS, 2008f).

A Swedish study reported that increased concentration of BBP in house dust caused increase in eczema in children.

BBP can also be classified as endocrine disruptor since it affects the sexual development of males. It is also seen that when a pregnant laboratory animal is exposed to ingestion of BBP, her male offspring has decreased testicular and plasma

testosterone levels, and female like nipples. Additionally, prenatal exposure of rats to BBP also caused skeletal malformations and decreased live births.

When female rats are exposed to BBP, this exposure leads to cell leukemia and increase in liver size. That is why United States EPA classified BBP as a possible carcinogen.

BBP caused low skin and eye irritation in animals with little acute oral, dermal toxicity on animals. Table 7 gives information about acute toxicity studies for BBP.

Table 7. Acute Toxicity Studies for BBP (NINCAS, 2008f)

	Species	Results (LD_{50}/LC_{50})
Oral (LD_{50})	Rat	$2.0x10^4$ mg/kg bw
	Guinea pig	$>1.4x10^4$ mg/kg bw
	Mouse	$6.2x10^3$ mg/kg bw
Dermal (LD_{50})	Mouse	$3.2x10^3$ mg/kg bw
	Rat	$6.7x10^3$ mg/kg bw
	Rabbit	$>1.0x10^4$ mg/kg bw

1.2.6. Dimethyl Phthalate (DMP)

DMP has been used in automotive parts, encapsulation of electrical wiring, mining and construction, fabrication of fibreglass, paints, nitrocellulose, cellulose acetates, plasticizer in children's toys, and rubber (NICNAS, 2008). Other uses include solvent for cosmetics, plasticizer, creams, perfumes, candles, hair sprays, and shampoos, and formerly as an insect repellent. Lower molecular weight phthalates are also reported to be used as solvents in fragrance bases for household cleaning products (NICNAS,

2008). According to the Cosmetic Ingredient Review (CIR) panel, the highest reported concentration of DMP in cosmetics was 2%.

Oral exposure to DMP resulted in LD_{50}values of 8,200, 5,200, 2,900, 10,100, and 8,600 mg/kg for rats, rabbits, guinea pigs, chicks, and mice, respectively. Evidence supported the conclusion that DMP was a subchronic toxicant. Short- to intermediate-term exposure to DMP induced body weight gain, changes in hemoglobin, and increases in absolute and relative liver weight (Carlson, 2010).

1.3. Importance of Food Safety and Analytical Chemistry

Food safety is one of the most important issue of the developing world in recent years. Food safety begins when seed goes into the ground and continues through all phases of food production and preparation. Many different parts of the food system share the responsibility for food safety; farmers, processors, wholesalers, retailers, and consumers. It is also important to express the role of regulatory agencies like United States Department of Agriculture (USDA), the Food and Drug Administration (FDA), the Environmental Protection Agency (EPA), and other state agencies whose duties are generally authorization, licensing, inspection, sampling, testing, and enforcement.

Inside these agencies analytical chemists also take place and they have important responsibilities since food safety is not only interested in foodborne pathogens. Food safety is also related to heavy metals, pesticides, food additives, persistent organic pollutants, and food contaminants. All these chemicals are mostly in trace amounts inside the food samples and in case of their accumulation inside the body, they have harmful effects.

For the purpose of quantitation and qualification of these chemicals, analytical chemistry becomes important. Not only detecting the harmful chemical is essential but also finding the suitable method for qualification/quantitation and validation of the found method is also crucial. So the criteria for a reliable analytical method can be ordered as following and an analytical chemist is responsible for finding the method that has:

- Good selectivity to the harmful chemical,
- High accuracy,
- High precision-Repeatability intra laboratory (within a lab) and reproducibility inter laboratory (within a lab and between labs)
- Low limit of detection,
- High sensitivity,
- Practicability and applicability under normal laboratory conditions.

1.4. Phthalates and Food Industry

Phthalates have widespread application. People are subjected to phthalates via air, water, pharmaceutical products, cosmetics, and food. Food can be contaminated by phthalates by means of processing equipment, environmental sources, and packaging materials. Since foods have lipophilic characteristics due to their fatty and oily content, they tend to be contaminated by phthalates that are lipophilic chemicals (Wenzl, 2009).

1.4.1. Phthalates and Milk Production

There are two main reasons to study the contamination of milk with phthalates.

- Firstly, especially for children, milk is a significant consumer product. In order to quantify the phthalate amount that humans are exposed to by means of their dietary, it is important to know the phthalate content of such food products.
- Secondly, phthalates are likely to be concentrated in the lipid phase of the foods due to their lipophilic characters. Since dairy products like milk can be classified as high-fat foods, they have higher tendency to be contaminated by phthalates than low-fat content foods (Fierens, 2013).

In order to comprehend the contamination of milk with phthalates, milk processing materials and steps must be investigated closely. In milking process or in the bulk transfer of milk between storage tanks, PVC tubing is used. In order to increase the flexibility of PVC products, plasticizers like phthalates are mainly used. Principally at

high temperatures of pasteurization step, plasticizers tend to migrate from the PVC materials to the milk since they are not chemically bonded to the polymer (Cao, 2010)

Possible phthalate contamination in an entire milk chain can be discussed as following. During the production of pasteurized milk chain, there are some points that are possible to cause the contamination of milk by phthalates. These can be listed as.

- The feed of the cattle might have already been contaminated and as a result of ingestion of phthalate containing feed, there might take place an increase in the phthalate level of the milk.
- Contact materials (e.g. PVC tubings) that are used during the mechanical milking process can be responsible for the occurrence of phthalates in milk,
- Separation, pasteurisation, standardisation and cooling can be other steps that might increase the phthalate level in milk due to the phthalate containing food contact materials (e.g. tubings and sealants) and due to the acceleration of phthalate migration by the applied heat during pasteurization.
- After the packaging step phthalate levels may raise considerably according to the kind of food packaging material (Fierens, 2013).

Because of the health effects of phthalates, many countries have already started to obey some regulation rules. These rules are especially about the usage of DEHP in PVC tubing for milking purpose.

On the other hand, PVC tubing plasticized with DEHP may still have been used for milking purpose in some other countries' pasteurized milk production plants. For example, according to a Canadian study, DEHP levels in cow milk which was collected with a machine using 28% DEHP containing PVC tubing are firstly measured. Then, DEHP level in cow milk that was obtained manually instead of mechanical milking was quantified. Finally, when the results of these two measurements were compared to each other, it is indicated that milk that obtained by usage of milking tubes have phthalate level that is 15 times higher than the average phthalate amount of cow milk manually collected without using PVC tubing. This

study shows that PVC tubing in milk processing can be the main source of DEHP in Canada (Cao, 2010).

1.4.2. Phthalates and Milk Packages

In order to keep the food fresh and protect it from various environmental effects, it is required to wrap it in the packaging films. PVC (polyvinyl chloride) film, polyethylene (PE) film, regenerated cellulose film (RCF), cellulose acetate film are just a couple examples of the food packaging materials.

During the production of some packaging materials such as PVC, in order to increase the flexibility, phthalates are used as plasticizers that are not chemically bonded to the polymer and can leach inside the food from the packaging material when they are in contact. For example, although DEHP is used as a plasticizer to increase the flexibility of PVC film, in some cases plasticizers are not required to be used during the production of food packaging materials. For instance, PE film is naturally flexible and thus does not contain plasticizers (Cao, 2010).

In the case of milk cartons, they are made up of a packaging material that is called as liquid paperboard (LPB). It contains layers of plastic and cardboard. Moreover, for longer life products like Ultra High Temperature (UHT) milk, a thin aluminum layer is also added. For example, 1 L fresh carton milk container has 88% of its mass as cardboard.

There are two different kinds of containers to keep the milk. Figure 2 shows these two kinds of food packages (Planet Ark, 2012).

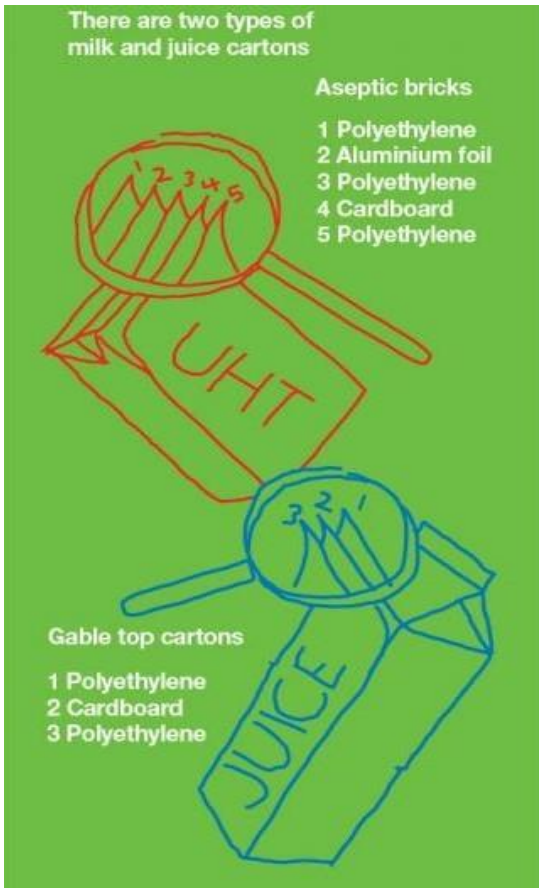


Figure 2: Most Common Two Kinds of Milk Packages

First one is aseptic packages that can also be called as UHT packs.
These are a newer type of carton. They have five layers: three of plastic, one of Al foil and one of cardboard. The product that is served in these food packaging materials is firstly heat treated and there is no microorganisms left after that kind of heat treatment. That is why, it can be said that UHT packs seal the product fully and then the milk in UHT pack can be kept at room temperature. Second one is gable top cartons. Gable top cartons are made from a layer of cardboard sandwiched between two layers of very thin plastic (Planet Ark, 2012).

Although these milk packs look like safer than PVC or other food materials since they contain PE without any plasticizer, the cardboard layers of these Tetra Pak containers

are source of phthalate contamination. Because they have printing inks (sources of DEHP and DBP) on these materials and they are re-used after their recycling procedure, the cardboard inside the Tetra Pak is possible phthalate contamination source for milk (Cao, 2010).

1.5. Analytical Methods For Determination of Phthalates in Food and Food Packages

1.5.1. Extraction and Separation Methods

For the extraction of phthalates from food and food packages, Solid Phase Extraction (SPE), Direct Immerge Solid Phase Micro Extraction (DI-SPME), Head Space Solid Phase Micro Extraction (HS-SPME), Ultra Sound Assisted Liquid Liquid Micro Extraction (USA-LLME), Gel Permeation Chromatography (GPC), Ultrasonic Bath Extraction are the most common extraction techniques.

1.5.1.1. Solid Phase Micro Extraction (SPME)

In the beginning of 1990's, Solid-phase micro extraction (SPME) was found (Feng, 2005).

Solid-phase micro extraction (SPME) is mostly used in trace analysis. It can be classified as a modern, solvent-free sample preparation technique. In SPME, sampling and sample preparation are combined in one step (Wardencki, 2004).

It can be used in the sample preparation of volatile and semi-volatile compounds like aromas, flavors, fragrances, trace levels of analytes in air, and water screening for pollutants.

In many sectors, SPME is used because it is solventless, and sample preparation and injection can be performed in only one step. That is why it can be called as a fast extraction method. Moreover, since SPME fibre is reusable (100+ times), it is

inexpensive. In addition, it is a quite suitable method for GC instrument (Sigma Aldrich Co., 2009)

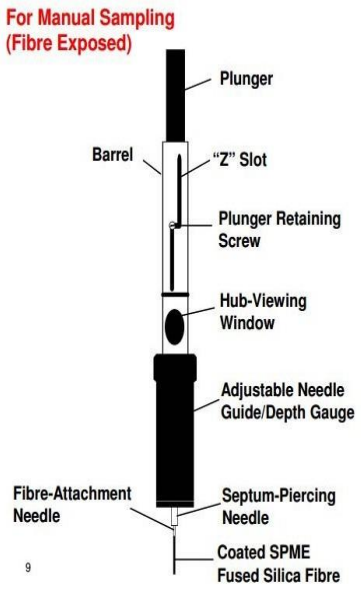


Figure 3. Parts of a SPME Apparatus and Fibre (Sigma Aldrich Co., 2009)

SPME fibres can be classified as absorbent-type and adsorbent-type. In absorbent type SPME fibre, coating is a liquid film, which cross-linked to the silica rod. In this kind of fibres, extraction is based on partition of analytes into a 'liquid-like' phase. Absorbent type fibres can have high capacity.

Although coating is a liquid film in absorbent type SPME fibres, adsorbent type fibres are solid particles with pores on the surface. In the case of adsorbent type fibres the particle surface interacts physically with the analytes. Adsorbent type fibres have limited capacity (Sigma Aldrich Co, 2009).

In SPME method, the extraction from sample to the fibre can be performed by two ways. First one is direct SPME. In this kind of SPME, the coated fibre is directly

immersed in the liquid sample. However, in second kind of SPME, which is called as Head Space Solid Phase Microextraction (HS-SPME), extracting fibre is suspended above the sample. The main difference between both of these SPME methods is that direct SPME cannot be used for complex matrices. That is why HS-SPME is essential to extract the desired analytes from the complex sample matrix (Feng, 2005).

There are many parameters that are used to the effectiveness of analyte extraction by SPME method. These parameters are type of fiber, sample volume, temperature and extraction time, salting, mode of extraction, desorption of analytes from the fiber (Wardencki, 2004).

Type of fibre

The effectiveness of the SPME depends on a distribution constant that can be describes as the partition value between the sample and the stationary phase of the fibre. This constant can be showed as K_{fs}. Since K_{fs} changes with the type of the fibre, extraction efficiency also depends on the type of the fibre. In addition to K_{fs}, preconcentration efficiency depends on the thickness of the fibre. When the thick films are compared to the thin ones, thick fibres allow the extraction of higher amounts. They are also more useful for the volatile compounds since they enable better transport to the chromatic injector without any loss. However, thin film fibres are used for high boiling point substances. Because with these thin layer fibres, extraction and desorption steps can be performed in a shorter time period.
The following table shows the effect of SPME fibre coating thickness on analyte recovery. The used material and the conditions are PDMS fibres, immersion sampling, in 15 minutes time interval (Wardencki, 2004).

Table 8. Fibre coating thickness on relative recovery (%)

Analyte/Fiber Thickness	**100 µm**	**30 µm**	**7 µm**
Benzene	2	1	1
Toluene	5	1	1
Ethylobenzene	6	4	1
Naphthalene	13	4	1
Fluorene	29	18	6
Phenanthrene	37	27	16
Anthracene	49	38	32
Pyrene	69	54	47
Chrysene	100	100	100
Benzo(a)anthracene	105	91	96
Benzo(a)pyrene	119	127	131

Sample volume

Sample volume is another parameter that affects the extraction efficiency. If the volume of the sample in the vial is minimized, the extraction efficiency increases. For example, it was reported that in the case of 1 cm^3 of liquid is placed into the 5 cm^3 vial, the system got the equilibrium three times quicker than the case that 10 cm^3 liquid is placed in a 50 cm^3 vial (Wardencki, 2004).

Temperature and extraction time

The distribution constant (Kfs) does not only change with the type of the fibre like mentioned in the above part, it also changes with the extraction temperature. Raise in the temperature can increase the transfer of analyte from the sample to the fibre. However, an excess increase in temperature can also cause the early desorption of the

analytes. Because of that reason, it is an essential task to find the optimized extraction temperature with the required exposure time that is another significant extraction efficiency parameter. When the time is longer, more sites by the analyte on the fibre can be occupied. Nevertheless, if more time than required is spent on the extraction, desorption can be caused.

As a result of the evaluation of these two parameters, it can be said that time and temperature parameters are quite related to each other since an increase in temperature leads to shorter exposure time (Wardencki, 2004).

Salting-out agents

When salting-out agents are used in SPME, usage of them increases the extraction efficiency since addition of them decreases the solubility of analytes in the solution. That leads to increase in sorbed analytes on the fibre (Wardencki, 2004).

Mode of extraction

There are two different modes that the fibre is exposed to sample. They are direct dipping of SPME fibre into the sample and head space SPME that is generally used for the samples with the complex matrix. In the case of HS-SPME method fibre is inserted in a vial above a liquid or a solid sample. The HS-SPME technique increases the life of the fibre used since it is not subjected to sample directly. Therefore, it is better to use HS-SPME as much as possible since usage of this method decreases the cost (Wardencki, 2004).

Desorption of analytes from a fibre

Determination of desorption parameters are also important as much as determination of extraction conditions. Usually, since SPME is a method combined with GC-MS, thermal desorption in a gas chromatograph is used as the desorption technique. It is important to care about choosing a desorption temperature that is higher than the

boiling point of the analyte. However, this can be restricted by the thermal resistance of the fibre that is used (Wardencki, 2004).

1.5.1.2. Ultrasound Assisted Dispersive Liquid Liquid Micro Extraction (UA-DLLME)

Recently, a novel microextraction technique, termed dispersive liquid–liquid microextraction (DLLME) is developed, which is based on a ternary solvent system like homogeneous liquid–liquid extraction and cloud point extraction. In this method, the appropriate mixture of extractant and dispersant is injected rapidly into an aqueous sample by syringe, and then a cloudy solution is formed, which markedly increase the contact surface between phases and reduce the extraction times with increasing enrichment factors. After extraction, the phase separation is performed by centrifugation, and the analytes in the sediment phase are determined by chromatography or spectrometry methods. As the advantages of simplicity, rapidity, low cost, and enrichment factors, the DLLME method had been widely applied for the determination of polycyclic aromatic hydrocarbons, organophosphorus pesticides, chlorobenzenes, tri-halomethanes, chlorophenols, and metals ions in aqueous samples. However, it still suffered from low repeatability and lack of special selectivity, so its application for complex samples than water was rare (Yan, 2011).

1.5.1.3. Ultrasonic Bath Extraction (UBE)

In order to separate the non-volatile and semi-volatile organic compounds from soil, sludge, and sediment samples, ultrasonic bath extraction method is widely used since this method increases the contact of sample matrix with the extraction solvent.

In ultrasonic bath extraction method, expansion and compression cycles in the samples are created by the movement of sound waves whose frequency is higher than the waves that are audible to the humans. The lowest ultrasonic frequency is accepted as 20 kHz. In the expansion cycles, molecules move apart from each other while in the compression cycles they become closer to each other. During the expansion cycle, there might take place a negative pressure which leads to bubbles and cavities in the liquid. Cavitation means growing and undergoing implosive collapse of bubbles

created by negative pressure during expansion of sound waves. During the cavitation, it is estimated that some spots can reach 5000 ^{o}C and the pressure may reach 1000 atm. However, these changes do not affect the bulk conditions, since the bubbles are very tiny and it is estimated that the rate of cooling of the bubbles are 10 billion °C per seconds. Due to the creation of very high effective temperatures that increase the solubility of analytes, and due to the high pressures that lead to the transfer of analytes to the desired phase, ultrasonic bath extraction is a useful technique (Özcan, 2006).

However, there are important steps that must be cared while using this extraction technique. First of all, it is essential to get rid of all the moisture from the sample, like in the case of other extraction methods that are performed with organic solvents. In order to dry the samples, Na_2SO_4 can be used. The samples are put into the bath. Then the sound waves disrupt the analyte particles by increasing their solubility in a few minutes. Lastly, the sample is centrifuged or filtered to complete the extraction of analyte (United States Environmental Protection Agency, 1996).

1.5.2. Analysis Techniques

1.5.2.1. Gas Chromatography

The basic aim of chromatography is to separate an analyte from a complex mixture of compounds.

It is used with a variety of detectors to scan compounds sensitively and selectively. In the working principle of chromatography, the sample analyte is obtained due to its volatility difference and also the difference between its interaction with the stationary phase.

A carrier gas, an injection system, a temperature controlled column, and a detector are the four essential elements of the chromatographic system. Figure 4 shows a scheme of a typical gas chromatograph (Kebbekus, 1998).

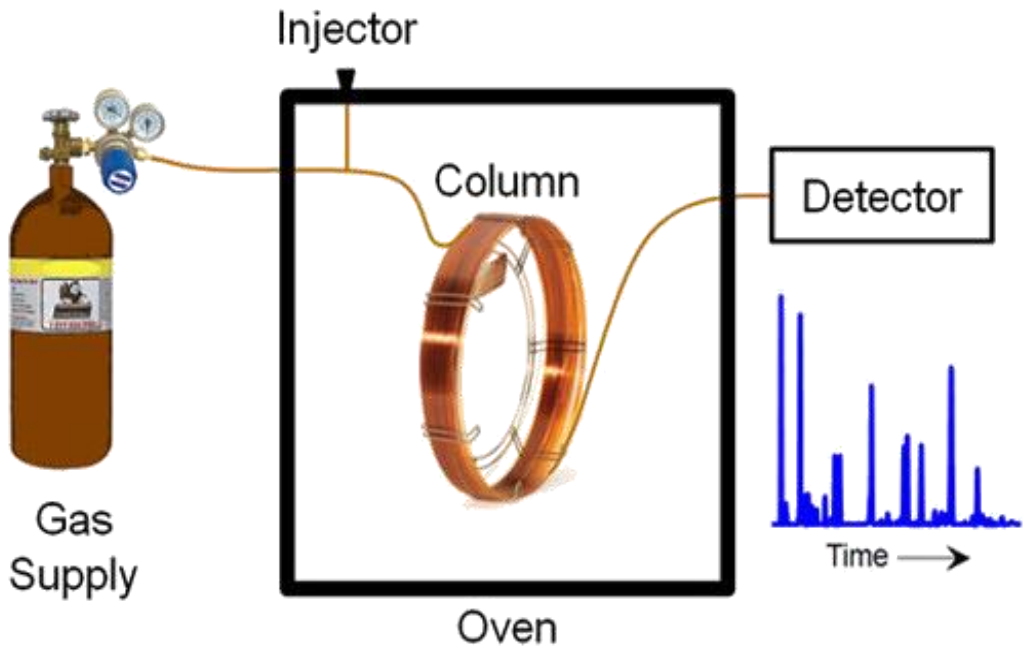


Figure 4. A scheme of a typical gas chromatography

Extremely pure helium due to its inertness is used as the carrier gas or mobile phase in a Gas Chromatography (GC) System. Moreover, the shape of its van Deemter curve, which shows per unit length of a separation column to the linear mobile phase velocity, lets wider range of optimum mobile phase linear velocities compared to Nitrogen gas as seen on figure. Hydrogen is less frequently encountered due to its explosive nature.

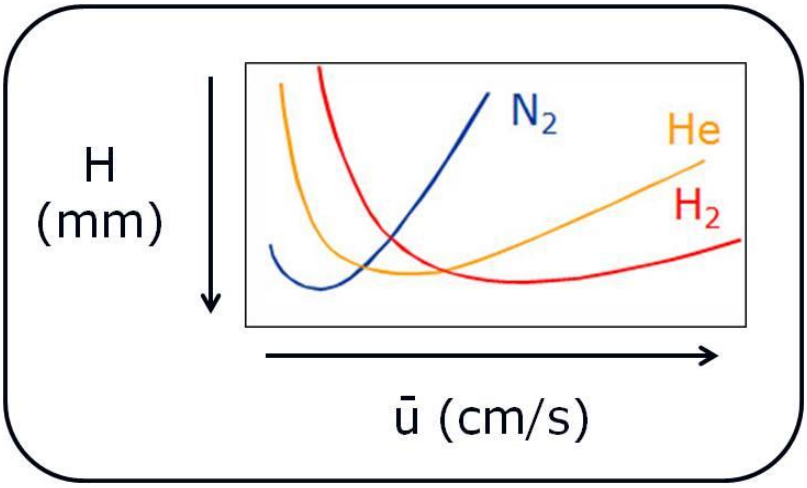


Figure 5. Van Deemter Plot

Hence through the separation column, helium gas is chosen as the mobile phase to set the analytes in the motion. For the injection purpose, the most common injector is a split-splitless injector. For the solutions that are highly concentrated, the injector is performed under the split mode. In this case, the injected sample is divided and the majority of the sample is sent to the purge flow. The rest is sent to the column. However, for the solutions with low levels of analytes (ppm,ppb), the injector gets in action in splitless mode. By means of this method, all injected sample runs through the column.

Separation columns are the most important part of the GC. The temperature of the columns is controlled by an adjustable oven. It can control the temperatures with 0.5 ^{o} C temperature fragments. Today, the most commonly used columns are fused silica capillary columns. While their inner diameters range between 0.25 to 0.53 mm, their length ranges between 5 to 100 meters.

When the column gets longer, it holds more theoretical plates. Therefore, more theoretical plates mean better separation of analyte samples. Film thickness of the columns also may change between 0.25 to 3.00 µm. Thicker films mostly lead to better

resolution although they require longer analysis time. In addition, when the cross-linking of the column films increases, less "column bleed" occurs.

Next, according to the volatility and the interaction with the stationary phase difference, the analytes reach to the detectors. In the following Table 9, the most common detectors that are used in GC are listed with their some specific properties (Dunnivant, 2010).

Table 9. The most common detectors that are used in GC

Detector	**General Type**	**Analytes Used to Measure**	**Typical Detection Limits**
Flame Ionization Detector	Selective	Any chemical that will burn in a H_2/O_2 flame	parts per million
Thermalconductivity Detector	Universal	Any chemical with a thermal conductivity (~specific heat) different from He	Parts per thousand or hundred
Electron Capture Detector	Selective	Electrophores such as halogenated hydrocarbons	parts per billion or less
Flame Photometric	Specific	P and S containing compounds	parts per million or less

Table 9. The most common detectors that are used in GC (Cont'd)

Fourier Transform Infra-Red	Specific	Chemicals with specific molecular vibrations	parts per thousand or hundred
Mass Spectrometry	Universal	Any chemical Species	parts per million or less

1.5.2.2. Mass Spectroscopy

In gas chromatography, the identification of the compounds is done by the help of their retention times. Nevertheless, in some cases, two or more analytes might have retention time that are very close to each other. Then, identification becomes more difficult and it is required to use mass spectrometer for further structural information of the analytes (Kebbekus, 1998).

Mass Spectrometry is an analytical technique that produces a magnetic field in order to identify the charged particles according to their mass to charge ratios. Mass spectrometry is used for many purposes like determination of the molecular mass, shaping the structure of an unknown, getting data on isotopic abundance.
A representative mass spectrometer system is shown in Figure 6.

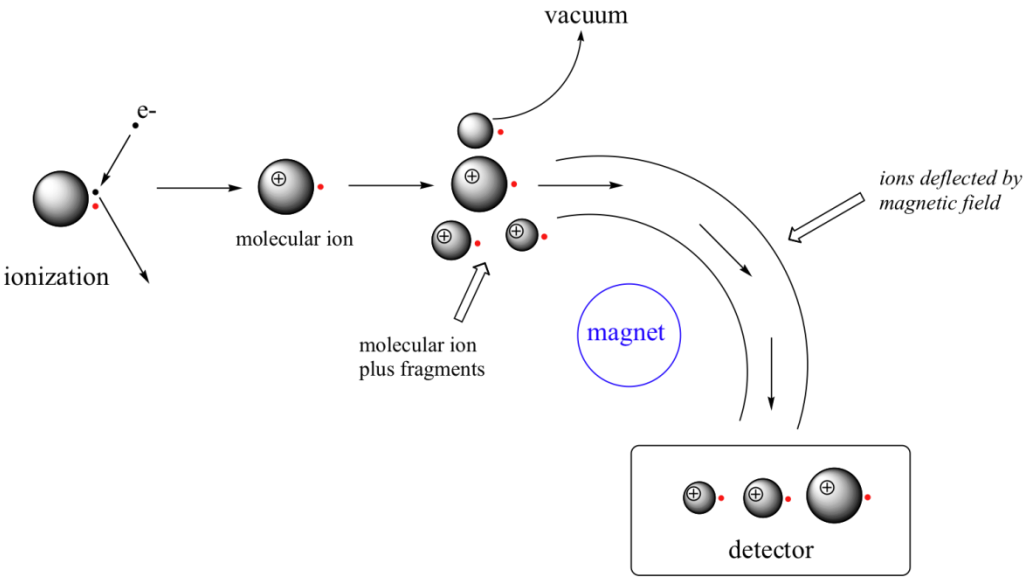


Figure 6. A Representative Mass Spectrometer System

The working principle of a mass spectrometer can be summarized as following:

Step 1. In order to produce ion fragments, the sample is vaporized and bombarded with electrons in ion source.

Step 2. In the ionization chamber, there exist one positively and one negatively charged particles. The positive one causes the cation's movement to the analyzer tube.

Step 3. Since the analyzer tube is covered by a magnetic field, fragments are separated according to their m/z ratio. Only the specific mass can hit the detector and be recorded.

Step 4. Lastly, MS gives the mass spectrum of the analytes that could reach to the detector (UCLA Chemistry and Biochemistry)

1.6. Literature Research

A variety of analysis methods for phthalate esters are available in literature. The followings are some of them.

Fierens (2012c) studied on the contamination of dimethyl phthalate (DMP), diethyl phthalate (DEP), diisobutyl phthalate (DiBP), di-n-butyl phthalate (DnBP), benzylbutyl phthalate (BBP), di(2-ethylhexyl) phthalate (DEHP), dicyclohexyl phthalate (DCHP) and di-n-octyl phthalate (DnOP) in raw cow's milk. In this study, the most possible contamination pathway of the milk and dairy products was investigated. The contamination chain started with the feed of the castle and ended with packaging of the food product. Although DMP, DEP, DnBP, DEHP and DnOP were measured in various feed samples, they were not found in raw cow's milk. The reason of non existence of these phthalate might be due to the rapid metabolism of them in cows. It is also observed that the amount of DiBP and BBP in the milk samples also changes with seasons. This change might be because of different feed compositions during summer and winter. Moreover, it is revealed that contact materials like PVC tubings used during the mechanical milking process are additional important contamination points. As a result of this study, decrease of DEHP level in European cows' milk is also observed because of the substitution of other kind of plasticizers instead of DEHP into the polymers (Fierens, 2012a).

In another study, it is investigated that the phthalates in 400 Belgian food products and their packages under the root of PHTAL Project, which is the first project that gives information about the phthalate content of the Belgian food products. He divided the samples in eleven groups. He aimed to find the target phthalates that were dimethyl phthalate (DMP), diethyl phthalate (DEP), diisobutyl phthalate (DiBP), di-n-butyl phthalate (DnBP), benzylbutyl phthalate (BBP), di (2-ethyl-hexyl) phthalate (DEHP), dicyclohexyl phthalate (DCHP) and di-n-octyl phthalate (DnOP). For this reason, he classified the samples as high-fat foods, low-fat food products, aqueous-based beverages and packaging materials. Then it was applied some different extraction techniques for each different class. Ultrasonic bath extraction was used to extract the phthalates from food packages and Gel Permeation Chromatography was used to

purify the food samples. The quantification of the target phthalates in the samples were done by gas chromatography–low resolution-mass spectrometry with electron impact ionisation (GC–EI–MS). DEHP was the most found phthalates. DiBP, DnBP and BBP were the following phthalates of the DEHP (Fierens, 2012b).

Betlej (2001) worked on Solid-phase microextraction (SPME) and drinking water samples. During the experiment, six different non-polar and polar fibres were tried to extract seven phthalate esters then the quantification was performed by gas chromatography–mass spectrometry. In terms of extraction efficiency and repeatability of the extractions, the 70-mm Carbowax–divinylbenzene fibre was quite convenient to use for the determination of the amount of the desired phthalates. After the analysis of the drinking water samples from Leipzig (Germany) and Katowice (Poland) diethyl phthalate (DEP), di-n-butyl phthalate (DnBP), butylbenzyl phthalate (BBzP) and di (2-ethylhexyl) phthalate (DEHP) were detected to be between 0.02 and 0.6 mg/L. During the study, it is concluded that all fibres except 7 μm PDMS were suitable for the determination of the amount of BBP in the drinking water samples. In addition, DMP, DEP and DBP that are the phthalates that can be classified as low molecular weight ones were extracted by DVB–Carboxen–PDMS in a better manner. By the help of this study, it was able to be detected the phthalates by means of optimized SPME-GC-MS and the usage of CW-DWB fibres at very low concentrations (Luks-Betlej, 2001).

Fierens (2012c) obtained the Belgian milk and dairy product samples from various farms, a dairy factory and from different shops to be able to determine the phthalate contamination “from farm to fork”. In this study, it is investigated the levels of eight phthalates–i.e. dimethyl phthalate (DMP), diethyl phthalate (DEP), diisobutyl phthalate (DiBP), di-n-butyl phthalate (DnBP), benzylbutyl phthalate (BzBP), di(2-ethylhexyl) phthalate (DEHP), dicyclohexylphthalate (DCHP) and di-n-octyl phthalate (DnOP)–in several Belgian milk and dairy products. Contamination of product with phthalates, especially DiBP, DnBP, BzBP and DEHP, at some stages of the milk chain was observed. The possible sources of the contamination were labeled as mechanical milking process and intake of the feed by the cattle. However, it is stated that almost no extra phthalate contamination took place during the transportation of

milk from the farm cooling tank to the dairy plant cooling tank. During pasteurisation, standardisation and cooling, the DEHP content in milk increased from 364 to 426μg/kg fat (median level) and the reason of this increase was most likely to be the DEHP containing food contact materials. Tubings and sealants used during processing can be counted as the examples of these materials. In addition to those, packaging materials were also identified as another source of contamination. The used packaging materials increased the detected amount of DiBP, DnBP, BzBP and DEHP in milk (powder) (Fierens, 2013).

Tena and others (2008) developed a SPME method in order to determine the phthalate esters in wine and they combined it with GC-MS. To be able to screen the extraction efficiency of the best method, six different SPME fibres were used under different temperatures and sample volume conditions. As a result, carbowax-divinylbenzene (CW-DVB), and polydimethylsiloxane-divinylbenzene (PDMS-DVB) were selected as the best fibres. For these fibres, temperature, sample volume and sodium chloride concentration were optimized. The optimal values were found as 70 ^{o}C, a NaCl concentration of 3.6 and 5.5 M for CW-DVB and PDMS-DVB fibres, respectively, and sample volumes of 3.5 and 3.0 mL. Next, for the mentioned SPME fibres and different wine samples it is concluded that:

- high temperatures helped increase in extraction efficiency,
- when the polarity of the fibre increases, optimal sample volume decreases,
- when the fibre polarity increases, optimal value for salt concentration increases.

As a result, total phthalate concentrations in the wine samples were found as ranging between 7 to 12 ng mL^{-1} (Tena, 2008).

Hongyuan and his colleagues (2011) from Hebei University, China developed a new method for the determination of six phthalate esters in bottled milks simultaneously by the help of ultrasound assisted dispersive liquid-liquid micro extraction (UA-DLLME). It is followed by Gas Chromatography-Flame Ionization Detection (GC-FID). During the sample preparation for GC-FID, 0.8 ml of methanol as dispersant and 40 μL of CCl_4 were added into the 8.0 ml of milk sample. The mixture was exposed

to ultrasound for 2.0 min. After all the factors were optimized, the recovery of the method ranged from 93.2% to 105.7%. The limits of detection (LODs) when the signal to noise ratio is equal to 3 were found as 0.64–0.79 ng g^{-1}. In conclusion, in this study a simple UA-DLLME–GC method to determine the level of six phthalates, Dimethyl phthalate (DMP), diethyl phthalate (DEP), dibutylphthalate (DBP), butyl benzyl ester (BBP), diisooctyl phthalate (DIOP), dioctyl phthalate (DNOP) in plastic bottled milk products has been developed. At the end of the experiment, adequate repeatability, high recoveries and high enrichment factors proved that the method is suitable for quantitative analysis of phthalate esters in real milk samples (Yan, 2011).

Fierens and others (2012c) stated that food products might be contaminated by different ways. A possible way of this kind of contamination can be from food contact materials into foods during their processing. Then, in their study, it was tried to be found how cooking at home affects the levels of the phthalates in many food types that were classified as starchy products, vegetables, meat, and fish. During the study, eight phthalates, namely dimethyl phthalate (DMP), diethyl phthalate (DEP), diisobutyl phthalate (DiBP), di-n-butyl phthalate (DnBP), benzylbutyl phthalate (BBP), di(2-ethylhexyl) phthalate (DEHP), dicyclohexyl phthalate (DCHP) and di-n-octyl phthalate (DnOP) were investigated by the help of Gel Permeation Chromatography (GPC) and Gas Chromatography Electron Impact Mass Spectroscopy (GC-EI-MS). Food products were analysed before as well as after cooking. According to the kind of the food product, they were boiled, steamed, deep-fried or grilled. Generally, phthalate concentrations in foods decreased after cooking process. However, DEHP, DiBP, and BBP were the most affected ones. Throughout this study not only how cooking affects the phthalate levels but also how the kind of packaging affects the phthalate content was investigated. For example, food which was packed in cardboard resulted in more DiBP, DnBP, BBP and DEHP than the food that was packed in plastic (Fierens, 2012c).

1.7. Purpose of the Study

In order to determine the phthalates in different matrices, obviously there are various methods. However, each method has different method performance. This research has the following aims:

- Application of Head Space Solid Phase Micro Extraction (HS-SPME) and Ultrasound Assisted Dispersive Liquid-Liquid Microextraction (UA-DLLME) methods for the determination of six phthalates (DMP, DEP, DBP, BBP, DEHP, DOP) in milk samples that are selected from the market,
- Application of ultrasonic bath extraction method for the determination of phthalate levels in milk packages,
- Analysis of the both milk and milk package samples in terms of phthalate concentrations using GC-MS system,
- Validation of used extraction and analysis methods,
- Investigation of the contamination levels of the milk samples in the market by phthalates and comparison of the recorded levels in the samples with the health limits and rest of the world.
- A contribution to food safety problem in our country.

CHAPTER 2

EXPERIMENTAL

2.1. Sample Collection and Sample Storage

For this study, samples of pasteurized milk were bought from several stores in Ankara. These samples (n=5) were selected among the brands which are with the same fat content (3%).

The milk samples were either in milk carton that is made up of printed polyethylene (PPE) or high density polyethylene (HDPE). Until the analysis day, all milk samples were kept in glass bottles in order to protect additional phthalate ester transfer from the milk package to product if there is any. Then, they were stored at -18 ^{o}C prior to analysis (Fierens, 2012c).

2.2. Reagents and Materials

The phthalate ester standards (EPA Method 606-Phthalate Esters Mix 1, 200 ng/µl each component in methanol, 1 mL), and Surrogate Standards that are in some cases used as Internal Standards (Dibenzyl phthalate, Diphenyl phthalate, Diphenyl isophthalate, 500 ng/µL each component in acetone 1 mL) were purchased from Dr. Ehrenstorfer (Ausburg, Germany).

The intermediate standard solutions were prepared from the stock solutions with Dichloromethane to be able to draw the calibration curve of UA-LLME milk samples, and carbon tetrachloride to draw the calibration curves of the UBE milk package samples since DCM and CCl_4 exhibit high performance with GC-MS instrument.

To perform the Head Space Solid Phase Microextraction trials, a Supelco brand 100 μm Polydimethylsiloxane (PDMS) fibre was purchased from USA.
All the solvents that were used during UA-DLLME and UBE were chromatographic grade and purchased from Merck Company (Germany).

All the other reagents (Trichloroacetic acid, Lead acetate, Sodium Chloride) used in the experiment were of the highest grade available.

After preparation of all the stock, intermediate and standard solutions, they were kept in refrigerator at +4 ^{o}C. 500 μL, 100 μL, 10 μL Hamilton brand micro syringes were used in order to prepare the standards in 1.5 mL glass vials (Supelco).

All the ultrasonic bath extractions were performed in Branson brand ultrasonic bath. Moreover, in order to clean the fat and protein content of the milk samples, LF 200 brand centrifuge is used.

Heidolph rotary evaporator (Laborota 4000) was used to evaporate the solvents of both standards and samples. A Supelco minivap evaporator was used to reduce the volumes of extracts. The extracted samples were transferred to 1.5 mL glass vials for further reduction of the volume. As the carrier gas for GC-MS instrument, ultra pure He gas was used.

2.3. Cleaning of Glassware

Throughout the study, cross contamination from the chemicals, materials and laboratory equipment was one of the most important problem since trace analysis of phthalates in milk samples would be performed.

Due to that reason, all the solvents that were used during UA-DLLME and UBE were chromatographic grade and all the other reagents (Trichloroacetic acid, Lead acetate, Sodium Chloride) used in the experiment were of the highest grade available.

Moreover, in order to avoid further phthalate contamination, laboratory equipment was cleaned carefully. All glassware used in the study were soaked and washed in acetone. Next, they were dried at 140 ^{o}C for at least 4 hours.

Then, to analyze whether there is any contamination from the reagents and glassware, all them were checked by GC-MS system by washing the equipment by DCM (Russo, 2013).

2.4. Instrument and Apparatus

An HP (Hewlett Packard) 6890 series gas chromatograph coupled with HP 5973 mass spectrometer was used for the phthalate analysis. A 30.0 m*0.250 mm*0.25 μm film thickness, crosslinked 5% Phenyl-methylpolysiloxane, HP-5, capillary column (Agilent Tech.) was used for the analysis of phthalates throughout the study.

2.5.Optimization of GC-MS System

The parameters of GC-MS for phthalates were formerly optimized by Y. Feng (2005) and K. Luks-Betlej (2001) for the phthalates that are extracted by HS-SPME, UA-DLLME, and UBE respectively. GC-MS operating parameters for analysis of the phthalates from the samples that are extracted by different extraction methods are indicated in Table 10 and 11, respectively..

Table 10. Operating parameters of GC-MS system for phthalate determination by HS-SPME

Injector.	Splitless
Inlet temperature.	280 ^{o}C
Column.	HP-5 (5% Phenyl)-methylpolysiloxane 30.0 m*0.250 mm*0.25 µm)
Oven temperature.	Initial oven temperature is 55 ^{o}C (1 min), increased at 15 ^{o}C/min to 280 ^{o}C (15 min).
Desorption temperature.	280 ^{o}C (10 min) Purge gas is off.
MS source temperature.	290 ^{o}C
MS quadrupole temperature.	150 ^{o}C
Injection volume.	1 µL
Carrier gas flow rate (He).	1.2 mL/min

Table 11. Operating parameters of GC-MS system for phthalate determination by UA-DLLME and UBE

Injector.	Splitless
Inlet temperature.	280 ^{o}C
Column.	HP-5 (5% Phenyl)-methylpolysiloxane 30.0 m*0.250 mm*0.25 µm)
Oven temperature.	Initial oven temperature is 60 ^{o}C (5 min), increased at 15 ^{o}C/min to 280 ^{o}C (10 min).

Table 11. Operating parameters of GC-MS system for phthalate determination by UA-DLLME and UBE (Cont'd)

MS source temperature.	290 ^{o} C
MS quadrupole temperature.	150 ^{o} C
Injection volume.	1 μL
Carrier gas flow rate (He).	1.2 mL/min

Throughout the study, gas chromatography combined with mass spectrometer with Selected Ion Monitoring (SIM) mode allowed detection of very small quantities of phthalates. SIM mode increases the sensitivity of the measurements by detecting only known ions. Therefore, SIM mode eliminates the noise and irrelevant peaks of scan mode. The SIM Windows for phthalate determination is given in Table 12. Moreover, sample chromatogram for the standards used for phthalates are given in Figure 7.

Table 12. Adjustment of SIM parameters for phthalate determination

Windows	Time Period	Ions Monitored
1	3 - 14.50	77, 105, 149, 163, 177, 194
2	14.50 – 17.50	104, 149, 223
3	17.50 – 20.10	77, 91, 104, 149, 167, 206, 225, 279
4	20.10 – 30.00	76, 91, 104, 107, 149, 225, 279

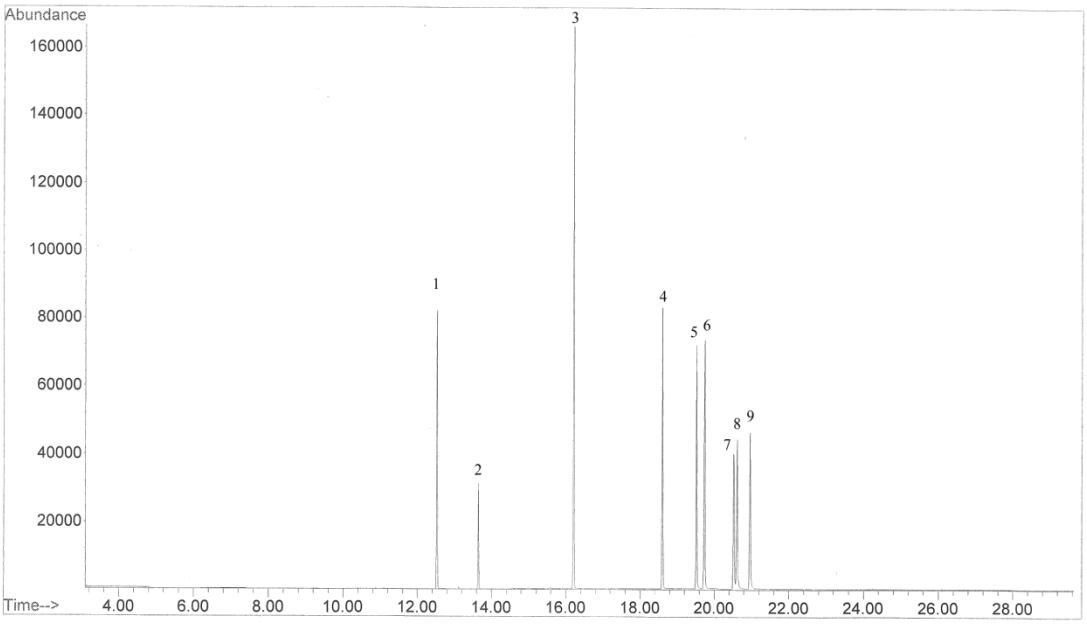


Figure 7. Certified Phthalate Standard Mix Chromatogram (1 ppm in dichloromethane)

(1. DMP, **2.** DEP, **3.** DBP, **4.** BBP, **5.** DEHP, **6.** Bis-Phenyl Ester, **7.** DOP, **8.** Bis-Benzylester, **9.** Bis-Phenyl Ester**)**

2.6.Calibration of the Analysis Systems

Before starting the analysis of the unknown concentration analytes, calibration curves are prepared by external standards with known concentration. However, since the quantitation and qualification are done with very small quantities of extracts like 1 µL, there might happen some uncertainty. Due to that reason, to eliminate that kind of uncertainty, use of internal standard was a must since the peak area ratio between internal standard and analytes are used as an analytical parameter. In this study, internal standard calibration method was used for quantification of phthalates. Table 13 indicates the calibration parameters for the determination. Moreover, calibration curves for phthalates that are extracted by UA-DLLME and UBE are given in Figure 8 and Figure 9, respectively. However, no calibration curve was drawn for HS-SPME part of the experiment. Only peak areas of the same milk sample measurements are compared after seven replicates of the method to be able to get an idea about the precision of the method.

Table 13. Calibration Parameters

Extraction Method of the Phthalates	Internal Standard (Concentration, µg/mL)	Surrogate Standard (Concentration,µg/mL)	Standard Concentrations, (µg/mL)
HS-SPME	Bisphenyl Ester 0,50	-	1.0
UA-DLLME	-	-	0.025, 0.050, 0.100, 0.250, 0.500, 1.000
UBE	-	1.0	0.050, 0.100, 0.250, 0.500, 1.000, 2.500, 5.000

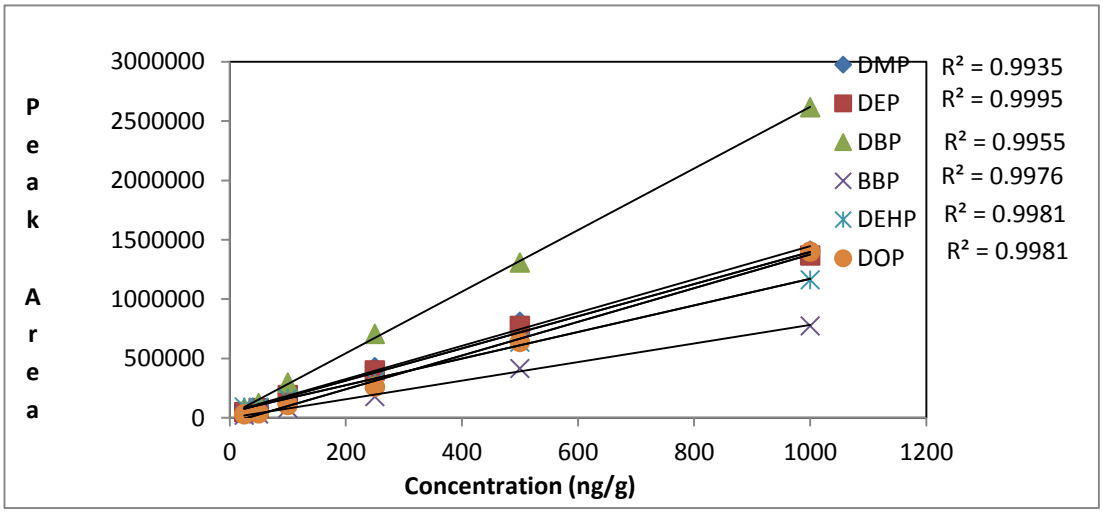


Figure 8: Calibration curves for phthalate standards that are in CCl_4 for UA-DLLME

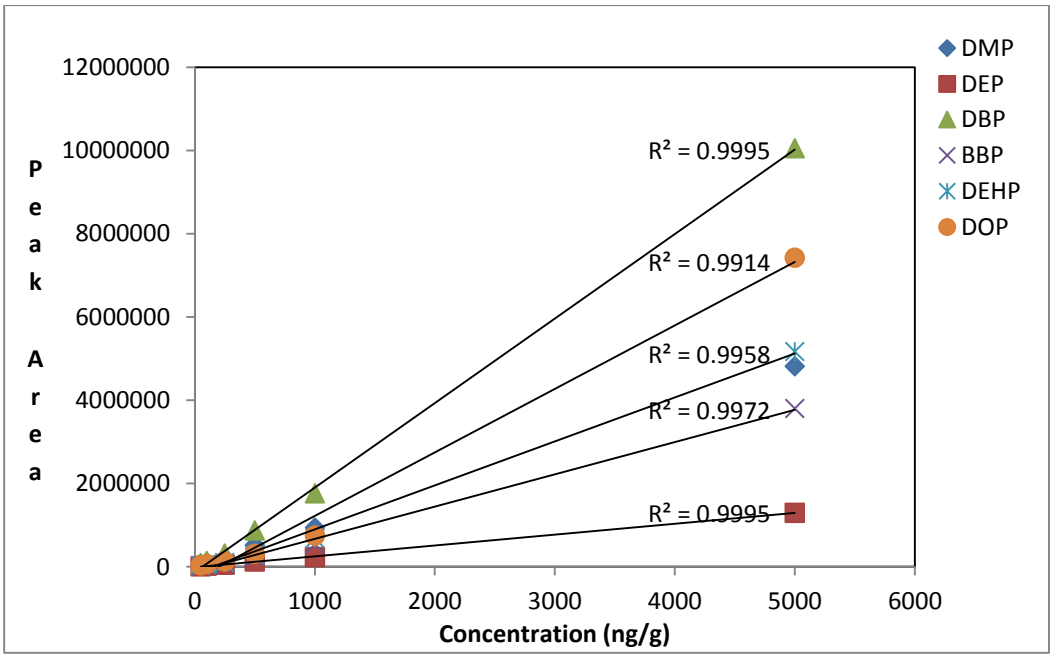


Figure 9: Calibration curves for phthalate standards that are in DCM for UBE

As seen from the Figure 8 and Figure 9, correlation coefficient, R^2, values obtained were greater than 0.99 for phthalates.

2.7.Extraction Procedures.

Throughout the study, several extraction methods were tried in order to analyze the milk and milk package samples. For milk samples, firstly HS-SPME was performed. Next, due to unsuitability of HS-SPME, UA-DLLME was decided as a better method for the detection of phthalates in milk samples. In addition, to be able to detect the phthalates in milk cartons UBE method was performed.

2.7.1. HS-SPME

- Five grams of cow's milk that is spiked with 0.5 µg/g internal Standard was weighed into a 20 mL SPME vial.
- 2.5 g of sodium chloride were added into the vial with a magnetic stirring bar. In order to avoid possible leakage of gas, the vial was tightly closed.

- Next, the vial was put into 90 ^{o}C oil bath on a hot plate. The stirring speed was adjusted to make the solution well stirred.
- After 2 min, the SPME needle was put inside the vial for 60 min. The SPME holder should be placed at a height that it is suspended about 1.5 cm above the milk sample.
- After the sampling was finished, the fibre was retracted into the protection needle. Then, put inside an empty vial to protect its contamination from laboratory air.
- The needle was then immediately inserted into the GC injection port for GC/-MS analysis (Feng, 2005).

2.7.2. UA-LLME

- 6.7 mL of 16% (w/v) trichloroacetic acid solution was added on 40 g of the bottled milk sample. Next, the mixture was centrifuged at 4000 rpm for 10 min.
- The supernatant part of the mixture was removed and mixed with 4.0 mL of 4% (w/v) lead acetate solution. Moreover, the mixture was centrifugated one more time at 4000 rpm for 10 more min.
- After centrifuging, 8 mL supernatant was put into a 10.0 mL conical centrifuge tube. Then, 1.1 mL, 6 % NaCl (w/v) was added on the mixture. Next, 0.8 mL isopropanol and 100 µL CCl_4 were added respectively.
- Furthermore, the mixture was gently shaken for several seconds and further emulsified by ultrasound for 2.0 min to get the cloudy solution.
- Finally, the phase separation was performed by a rapid centrifugation at 4000 rpm for 5.0 min and 1.0 µL of sediment phase was injected into GC for analysis.

2.7.3. UBE

- Packaging materials were cut into pieces of about 1 cm^2.
- Next, 5 cm^2 of the packaging materials were extracted for 60 min with 40 mL of n-hexane in an ultrasonic bath. However, before the extraction process, 100 µg/ mL surrogate standard mix was added to 40 mL hexane from 10 ppm surrogate standard mix stock solution.
- Then, a solvent exchange to 20 mL of dichloromethane took place and the extract was evaporated under nitrogen atmosphere to a volume of 1.0 mL.
- No purification step was required (Fierens, 2012a).

2.8. Analysis of the Samples.

For HS-SPME.

Another important step for HS-SPME was desorption of the phthalates. The desorption of SPME fibre in the GC injection port was 10 min at 280 ^oC. Purge gas was off during the desorption process of the experiment.

After sample desorption, the fibre was heated 30 minutes more at 280 ^oC in the injection port in order to remove the trace residues in the fibre. This step can be called as cleaning procedure of the fibre for the next measurement with the SPME fibre. However, during this 30 min period purge gas is required to be on.

The fibre was then put inside the protection needle back and the needle was inserted into a clean vial, and the fibre is ready for the next sample extraction.

For UA-DLLME of Milk Samples and UBE of Milk Packages.

The 1.0 µL of the extracted sample was taken with the Agilent gas tight glass syringes. Three replicates of the measurements were performed and the average value of these three measurements was used as the result. For the corrections of

the measurements of the phthalate concentrations, blank samples were also analyzed. Another correction was done by measuring the percent extraction recoveries of each analyte. The final concentrations were calculated after all these corrections.

Table 14 shows the retention times, target and confirmation ions of the target phthalates. The sample chromatograms for phthalates are given in Figure 10 and Figure 11.

Table 14. Retention times and ions of the analytes

	Retention Time for SPME method (min)	Retention Time for UA-LLME and UBE	Target and Confirmation Ions
Dimethyl Phthalate (DMP)	8.80	12.53	163, 77, 194
Diethyl Phthalate (DEP)	9.95	13.64	149, 177, 105
Dibutyl Phthalate (DBP)	12.54	16.22	149, 223, 104
Butylbenzyl Phthalate (BBP)	14.93	18.61	149, 91, 206
Di(2-ethyl-hexzyl) Phthalate (DEHP)	15.85	19.53	149, 167, 279
Di-isophenyl Phthalate (SS1 or IS)	16.06	19.74	225, 77, 104
Dioctyl Phthalate (DOP)	16.84	20.52	149, 279, 104

Table 14. Retention times and ions of the analytes (Cont'd)

Dibenzyl Phthalate (SS2)	16.93	20.61	225,76, 104
Diphenyl Phthalate (SS3)	17.29	20.97	149, 107, 91

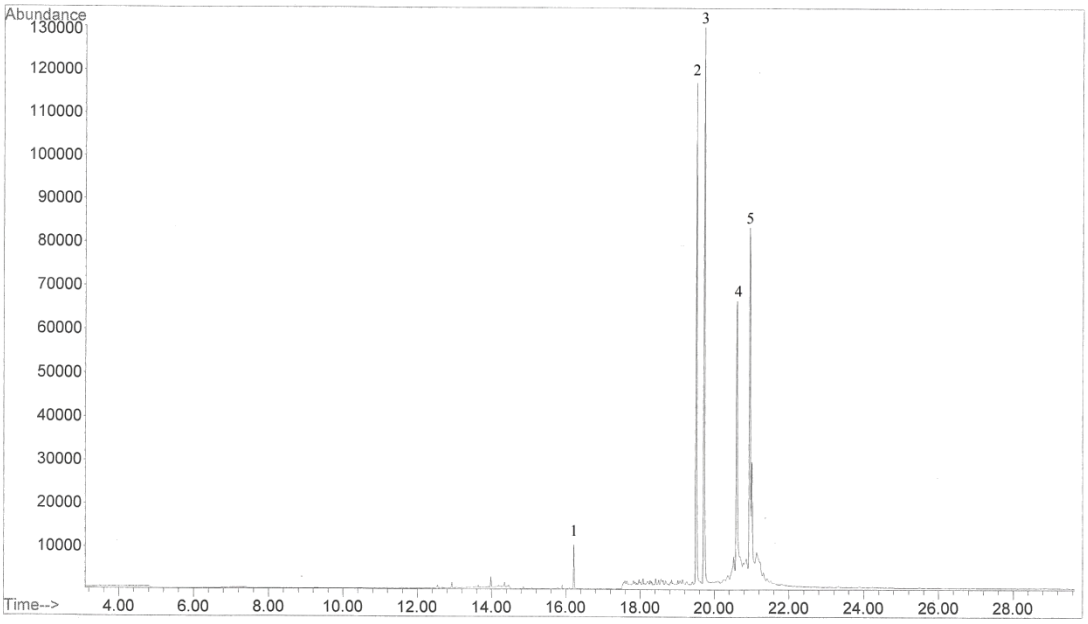


Figure 10. Sample Chromatogram for Milk Sample C

(**1**.DBP, **2.** DEHP, **3.** Bis-Phenyl Ester, **4.** Bis-Benzylester, **5.** Bis-Phenyl Ester)

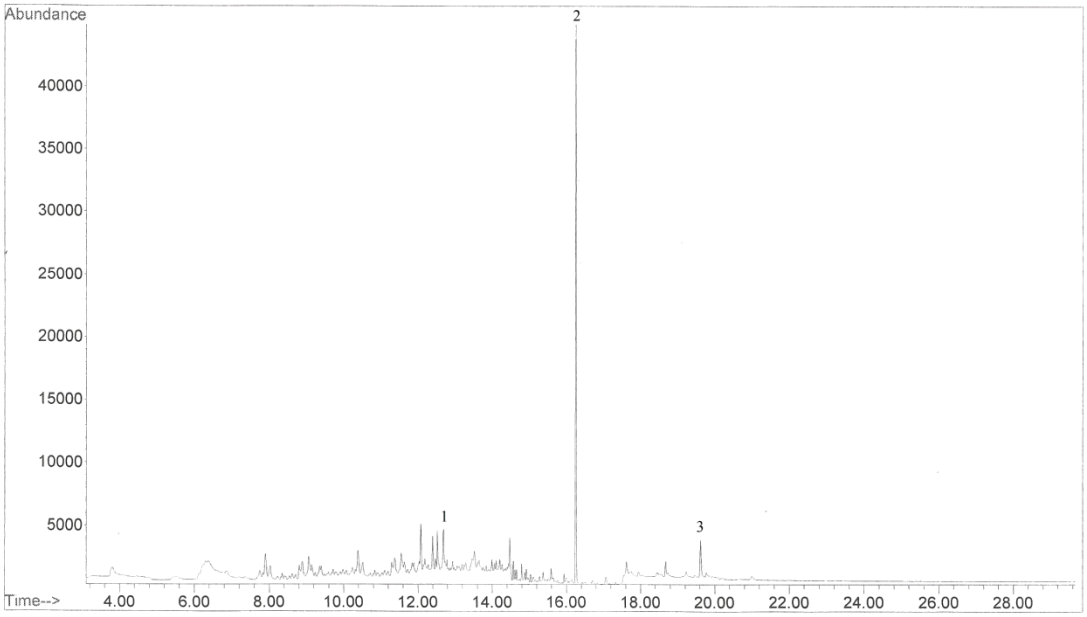


Figure 11. Sample Chromatogram for Milk Package C

(1. DMP**, 2.** DBP**, 3.** DEHP**)**

CHAPTER 3

RESULTS AND DISCUSSION

3.1. Method Validation

In this chapter the Quality Assurance (QA) and Quality Control (QC) tests during extraction process, during the analysis and evaluation of the data set of Phthalates are discussed.

3.1.1. Head Space-Solid Phase Microextraction

As the first trial, HS-SPME was decided to be applied for the analysis of phthalates that the pasteurized milk samples contain. The experimental conditions that the Fierens mentioned in his paper which are about the HS-SPME of the phthalates from the milk samples were applied for the extraction (Fierens, 2013). As the first step, it was required to measure the precision of method to be able to check the validation of it. For that reason 500 µg/L spiked milk sample was extracted by HS-SPME and injected into the GC-MS system for seven times. Although the peak areas were supposed to be same for each phthalate, they were very different from each other. As can be seen on the Figure 12, a low precision was obtained. In addition, the SPME fibres were quite fragile. Because of that reason, it was needed to change the fibre after each 15-20 measurements. Then, the method turned out to be very expensive one. Due to its low precision, the extraction method was changed into the UA-DLLME.

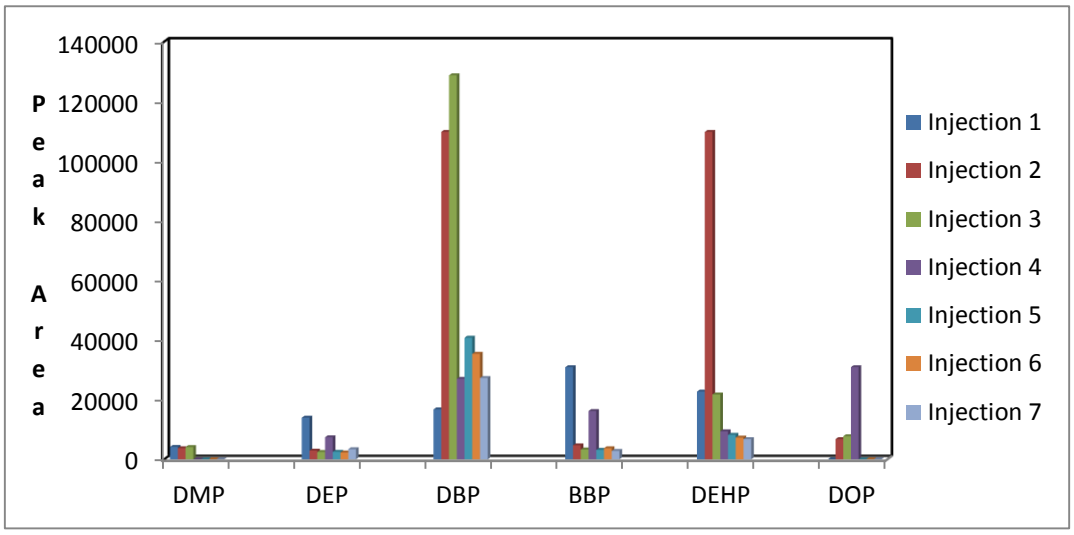


Figure 12: Precision of HS-SPME Procedure

3.1.2. Optimization of the Ultrasound Assisted Dispersive Liquid-Liquid Microextraction (UA-DLLME)

UA-DLLME requires many steps unlike HS-SPME. That is why it is needed to apply many different experimental steps.

As the first step of the UA-DLLME method optimization, it was required to choose the dispersant solvent which increases the surface area between extraction solvent and milk. Due to that reason acetone, acetonitrile, isopropanol, and methanol were tried. When the extraction recovery results of the measurements are calculated, as can be seen on the Figure 13, isopropanol was picked up as the dispersive solvent of the UA-DLLME due to its higher dispersing capability of the extractant and relatively less loss of analytes.

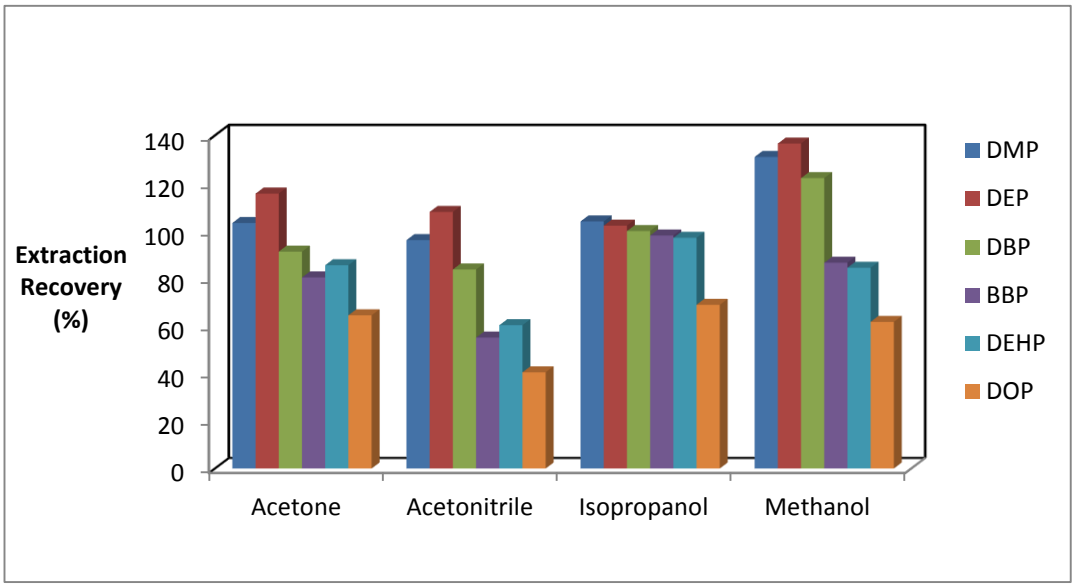


Figure 13: Effect of Dispersant Solvent on UA-DLLME

Next, 50, 100, 150, 200 µL of CCl_4 were tried, respectively. Then the amount of extraction solvent, which is CCl_4, was chosen as 100 µL. It was too difficult to separate the organic phase when 50 µL of extraction solvent is used. Extraction recovery increased with the increasing volume of extraction solvent 50 to 100 µL. Then, it kept constant even further increase of the volume of CCl_4 to 200 µL due to the completed extraction recovery as can be seen on the Figure 14.

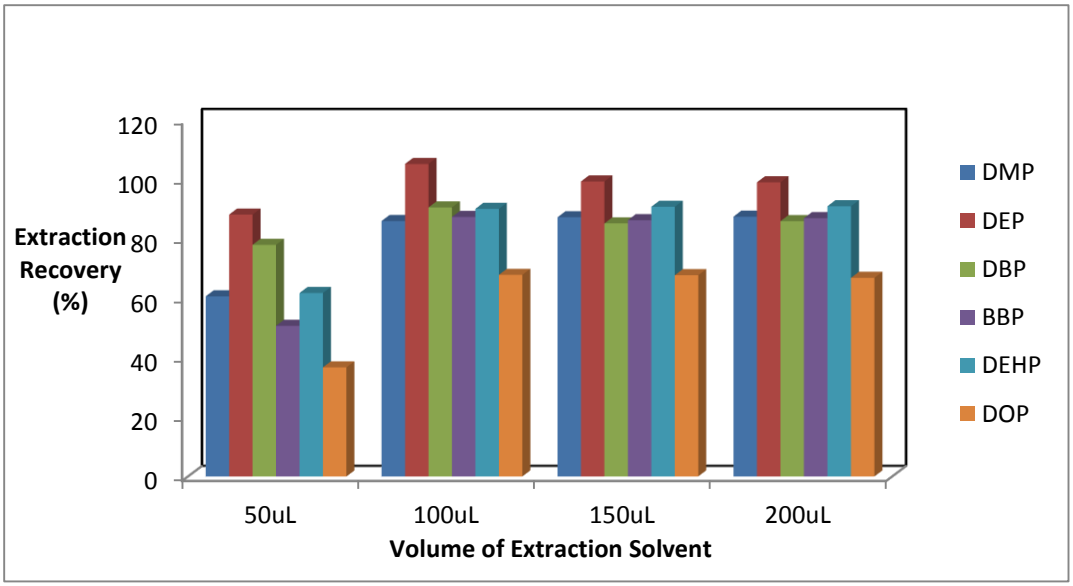


Figure 14: Effect of Extraction Solvent on UA-DLLME

As the next step, the volume of the dispersant solvent, which is decided as the isopropanol in the previous steps, was decided as the 0.8 mL. For the determination of this amount 0, 0.4, 0.8, 1.2, 1.6 mL were tried for the optimization of the dispersant solvent. As seen on the Figure 15, 0.8 mL is the optimized value. Then the extraction efficiency becomes constant when the amount of isopropanol is increased until 1.2 mL.

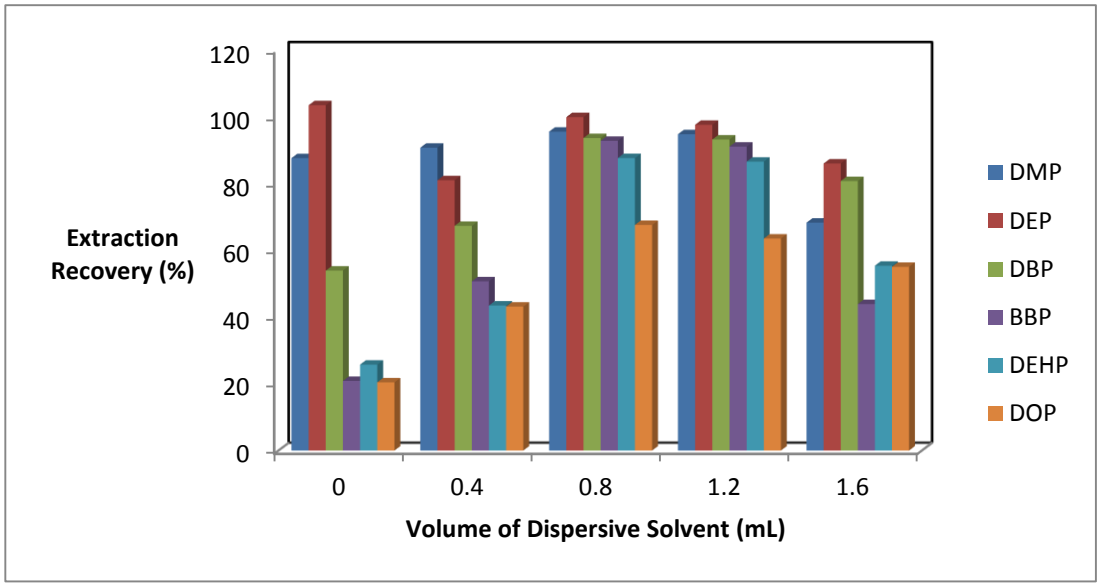


Figure 15: Effect of Dispersive Solvent Volume on UA-DLLME

Lastly the concentration of the NaCl solution, which is responsible for the increase of salting out effect, were supposed to be decided. Due to this purpose, 0%, 6%, and 12% of NaCl solutions were used for the optimization. According to the Figure 16, 6% (w/v) NaCl solution is decided as the suitable one for the experimental system.

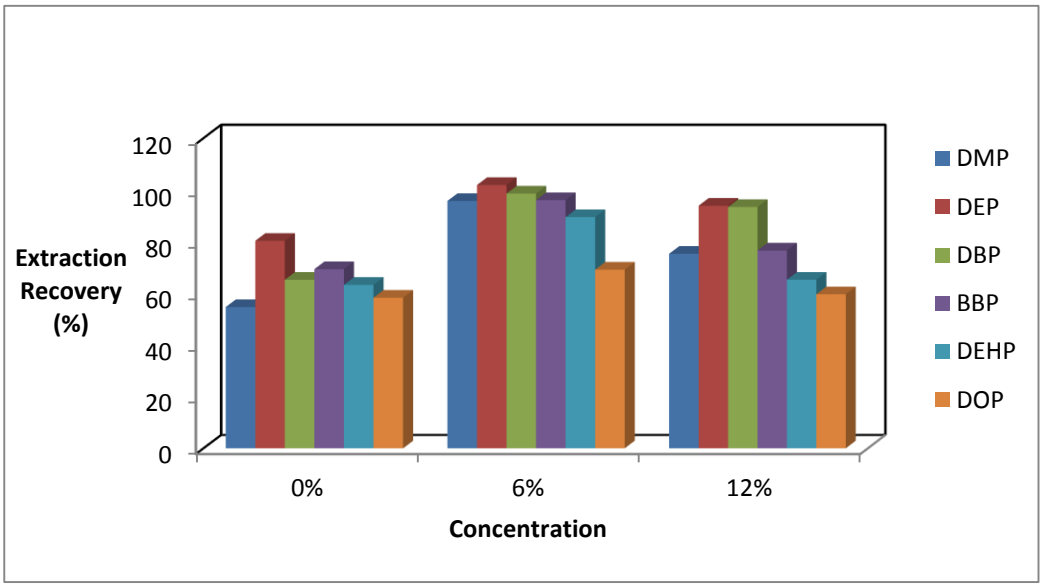


Figure 16: Effect of NaCl Concentration on UA-DLLME

3.1.3. Quality Assurance (QA) and Quality Control (QC) Tests During Extraction of the Samples

One of the purpose of the study is to validate extraction and analysis methodologies for phthalates. Therefore, it is necessary to investigate the extraction efficiency of the procedures before the discussion of the obtained data set since the concentrations of the phthalates are quite low in the milk samples.

Determination of the extraction recovery might be performed by two ways.

- Spike of the Standard Mix with a known concentration
- Addition of the Surrogate Standard Mix

Surrogate Standards are some kind of organic compounds that cannot be found in samples naturally. Nevertheless, they have similar chemical features of the analytes. When both of these ways to measure the extraction recovery are examined, it is observed that Addition of the Surrogate Standard Mix is not always suitable when high

number of analytes are looked for inside the samples. Because surrogate standards are organic compounds that are not naturally found in environmental samples but have similar chemical composition and behavior in the analytical method. If many analytes are to be determined for in the samples, using surrogate standard might not be always appropriate since the chemical properties of surrogate standards are not always similar to the properties of analytes in the same level. However, it is easy to measure the extraction recovery by means of this method due to its simplicity. Because this method concludes only one extraction recovery in a numeric value for each sample. However, Spike of the Standard Mix with a known concentration gives more detailed information about the extraction recovery since it gives numeric value as many as the analytes in the sample.

During the phthalate determination of milk samples and milk packages two different extraction methodologies are used. They are UA-DLLME and UBE, respectively. The reason why two different methods are used is that milk samples have a very complex nature due to the fat and proteins that they contain. The more the complex structure, the more complex the applied extraction method. That's why, although the phthalates inside the milk samples are extracted by UA-DLLME, which is a method containing separation steps of the milk proteins and fat, the phthalates inside the milk packages are extracted by a more simple extraction method, which is UBE.

During the phthalate determination of milk samples, spike of the Standard Mix with a known concentration is performed. Firstly, 25 ppb spiked milk sample was extracted. Then, the same milk sample was spiked with 125 ppb of phthalate ester mix and extracted. After the measurement steps, by the difference of the obtained phthalate values, extraction efficiency of the procedure (UA-DLLME) was determined.

However, during the determination of phthalate content of milk packages, Surrogate Standard Mix was used to decide the extraction efficiency of the procedure (UBE).
The names of the Surrogate Standards that were used in the extraction of phthalates from the milk packages are di-isophenyl phthalate, dibenzyl phthalate, diphenyl phthalate. However, since their retention times are very close to each other and Di-isophenyl Phthalate has thinner and taller peak in the GC-MS chromatograms than the

others, it is decided as the Surrogate Standard to investigate the phthalate extraction recovery after the ultrasonic bath extractions.

Percent recoveries were calculated for UA-DLLME as:

$\%Recovery = [(c_{s2} - c_{s1})_{experimental} / (c_{s2} - c_{s1})_{theoretical}] 100$

where ,

Cs_1. concentration of the lower spiked sample

Cs_2. concentration of the higher spiked sample

Percent recoveries were calculated for UBE as:

$\%Recovery = [(c_{surrogate})_{experimental} / (c_{surrogate})_{theoretical}] 100$

$c_{surrogate}$: concentration of the surrogate standard

When the extraction recoveries of the methods are between 70-130 %, the extraction method is acceptable according to the United States Environmental Protection Agency (USEPA) standards (EPA Method 8000C).

In the Table 15, the calculated extraction recoveries were found in the acceptable range of USEPA except for DOP that are found in the milk samples (66%) due to its low stability. Moreover, the obtained extraction values were used for the correction of the phthalate concentrations that are extracted from the milk and milk packages.

Table 15. Extraction Recoveries of the UA-DLLME for the Milk Samples That are Measured by the Spike of the Standard Mix with a Known Concentration

	Extraction Recovery (%)	**RSD (%)**
DMP	91±2.3	2.5
DEP	100±4.7	4.7
DBP	97±4.0	4.1
BBP	93±2.1	2.3
DEHP	86±2.2	2.5
DOP	66±1.2	1.8

Table 16. Extraction Recoveries of the UBE for the Milk Packages That are Measured by the Addition of the Surrogate Standard Mix

Surrogate Name	Average Extraction Recovery (%)	RSD (%)
Di-isophenyl Phthalate	120	7.6

3.1.4. Quality Assurance (QA) and Quality Control (QC) Tests During the Analysis

In addition to the determination of the extraction recovery of the methods (UA-DLLME & UBE), it is also required to check the stability of the instrument since during the analysis there might be some deviations from the calibration parameters.

To be able to eliminate such kind of instrumental error, internal standard addition method is used in the first part of the experiment which includes the determination of the milk samples. However, in the second part, for the analysis of the milk packages,

any internal standards are not used since the measurements were performed in a very short time.

An internal standard is added to the extracted sample with a known concentration and it is repeated for each sample measurement to eliminate the variations of the analysis system. For the determinations of the phthalates of the milk samples Di-isophenyl Phthalate was used as the internal standard with a fixed concentration of 500 μg/L.

The stability of the GC-MS system during the first part of the experiment, analysis of the phthalates of the milk samples, is shown in the Figure 17. Since the measurements took seven days, the figure shows the fluctuations of all seven days. Every day, three times (in the morning, afternoon and evening) 750 ng/mL of the phthalate ester standard mix was injected to the GC-MS system. As a result, the following figure was obtained.

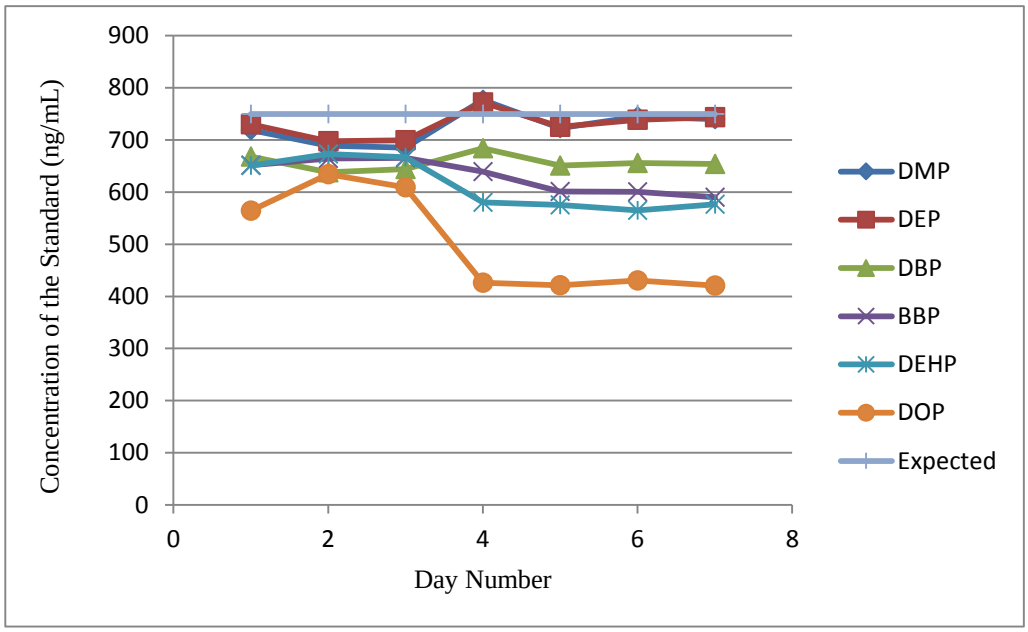


Figure 17: Stability of GC-MS System for Phthalate Determination

As seen from the Figure 17, the readings of the standard solutions were almost constant except for DOP. It has lower stability when it is compared to the other analyte phthalate esters. However, the others are more stable.

As seen from the Figure 18, as the results of the 5 measurement replicates, precision of the method is quite high. It has percent relative standard (%RSD) values between 2-5 % and percent recovery values are greater than 70% except for DOP (66%).

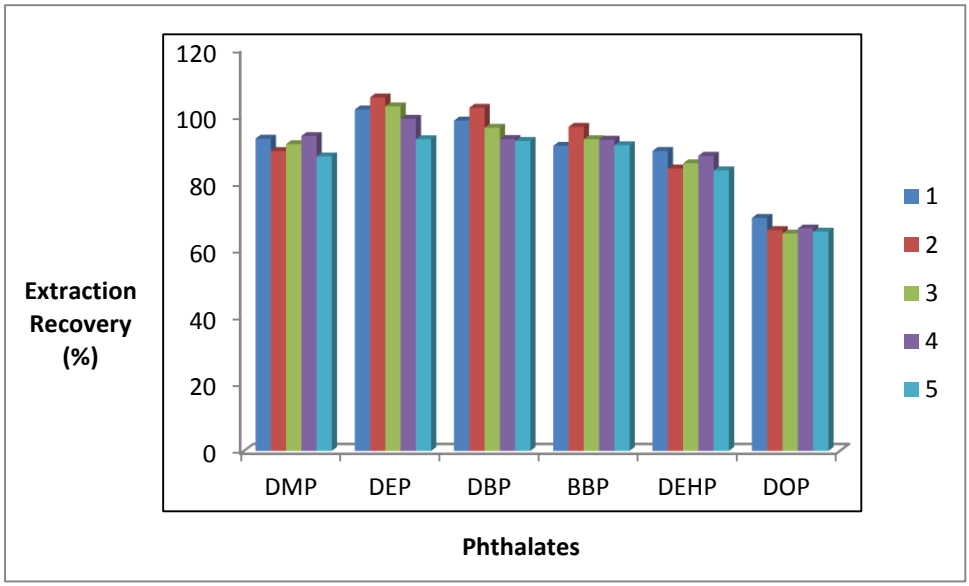


Figure 18. Precision and Extraction Recovery of the UA-DLLME Method

In order to show the data of precision and extraction recovery more clearly, the following table, Table 17, can be seen.

Table 17. Method Performance Results for Phthalate Esters for Pasteurized Milk Samples (n=5)

Compound	Certified Conc. (µg/L)	Found Conc. (µg/L)	RSD (%)	% Recovery
DMP	1.00	0.91±0.03	3	91±3
DEP	1.00	1.01±0.05	5	101±5
DBP	1.00	0.97±0.04	4	97±4
BBP	1.00	0.93±0.02	2	93±2
DEHP	1.00	0.86±0.03	3	86±3
DOP	1.00	0.66±0.01	2	66±1

Another reason of application of replicate measurements is to understand the existence of fluctuations. There might be some undesired fluctuations in the measurements due to the very small injection amount of the sample (1 µL). By the help of the results of replicate measurements some uncertainties can be easily detected.

In such kind of analytical analysis, the analysis system could detect very low concentrations of the analytes. To be able to check the capability of the analysis systems, limit of detection (LOD) and limit of quantification (LOQ) values are needed to be calculated by the help of the instrumental software. LOD can be described as the smallest quantity that can be detected. However, LOQ is described as the smallest concentration that is analyzed with reasonable reliability. The values shown in Table 18 are the LOD and LOQ values for the analysis of the pasteurized milk samples. LOD and LOQ values were calculated as the concentrations of the analytes at which the signal to noise (S/N) ratio is equal to 3 and 10, respectively.

Table 18. LOD and LOQ values for the analysis of the phthalate esters in the pasteurized milk samples

Phthalate Ester	LOD (ng/g)	LOQ (ng/g)
DMP	0.6	2.0
DEP	0.6	2.0
DBP	0.1	0.3
BBP	0.2	0.7
DEHP	0.3	1.0
DOP	0.5	2.0

The second part of the experiment consists of the analysis of the phthalate esters that the milk packages contain by UBE.

Unlike the first part, in this part surrogate standard was used in order to measure phthalate extraction recoveries in milk packages. Extraction recoveries for the samples were changing between %115 and %127. The found values were in the acceptable range. In addition, corrections were done by the found extraction efficiency values. Moreover, five replicate results show that this method has %RSD values changing between 2-6%, which is very high precision for this kind of analysis.

The following table, Table 19, shows the LOD and LOQ values of the measurement results of the phthalate esters that were extracted by UBE from the milk packages.

Table 19. LOD and LOQ values of the measurement results of the phthalate esters that were extracted by UBE from the milk packages

	LOD (ng/g)	LOQ (ng/g)
DMP	0.3	1.0

Table 19. LOD and LOQ values of the measurement results of the phthalate esters that were extracted by UBE from the milk packages (Cont'd)

DEP	0.3	1.0
DBP	0.2	0.7
BBP	3.0	10.0
DEHP	1.0	3.0
DOP	2.0	7.0

When Table 18 and Table 19 are evaluated and they are compared to the analysis results, it is observed that there are some found values which are below limit of detection (BLOD) and below limit of quantification (BLOQ) in the analysis of milk samples. However, there is no found values of BLOD or BLOQ as a result of analysis of milk packages.

3.2. Evaluation of the Data

In this part of the discussion, the produced data for the phthalate esters in pasteurized milk samples and their packages will be presented and discussed.

3.2.1. Concentration of Phthalate Esters in Pasteurized Milk Samples

The data set that is obtained in all the study for phthalate esters in 5 different pasteurized milk samples are shown in Table 20. Some of the values are the concentrations above the Limit of Quantification (LOQ), which is determined by using the response value where S/N value is 10. Injection of these five samples of pasteurized milk samples for phthalate ester content determination was performed as three replicates in order to evaluate the precision of the data set.

Table 20. Concentrations of Phthalates Found in Milk Sample Analysis

Name of Milk Samples (ng/g)	DMP	DEP	DBP	BBP	DEHP	DOP
Milk Sample A	**BLOQ** (0.50±0.02)	**BLOQ** (0.94±0.04)	**4.40±0.20**	**BLOQ** (0.47±0.02)	**BLOQ** (0.41±0.02)	**BLOQ**
Milk Sample B	**BLOQ**	**BLOQ**	**5.00±0.21**	**BLOQ** (0.68±0.13)	**1.00±0.03**	**BLOQ**
Milk Sample C	**BLOQ** (0.46±0.05)	**BLOQ**	**3.10±0.42**	**BLOQ**	**BLOQ** (0.53±0.05)	**BLOQ**
Milk Sample D	**BLOQ**	**BLOQ**	**3.30±0.21**	**BLOQ** (0.18±0.01)	**4.00±0.19**	**BLOQ**
Milk Sample E	**BLOQ** (0.58±0.03)	**BLOQ**	**3.30±0.07**	**BLOQ** (0.24±0.01)	**BLOQ** (0.65±0.08)	**BLOQ**

BLOQ=Below Limit of Quantitation

It can be interpreted from the Table 20 that DBP and DEHP are the most common phthalate esters that are found in pasteurized milk samples as expected. Because when other studies from other countries are examined, it is seen that DBP and DEHP are also commonly observed in their experimental results. In this study, the phthalate esters were analyzed in each of five pasteurized milk samples. Then, BBP and DMP follow DBP and DEHP in terms of their existence in the samples, respectively.

Although DEHP is found around 75 ng/g in Romanian studies (Miclean, 2012) and 25 ng/g in Belgium studies (Fierens, 2012c), in this study it is found at most 4 ng/g. It is understood that DEHP level of Turkish Milk can be contaminated in a lower amount. When it is looked at the other common phthalate level which is DBP, it is seen that it is mostly in the same concentration level when compared with other studies from other countries. It is around 3-5 ng/g. Relative standard deviation (RSD) values of this study are quite lower indicating the high precision of the analytical system.

If we look at the average concentration of each measured phthalates, DBP and DEHP show highest concentration. DBP is approximately 4 ng/g while DEHP is 1.5 ng/g. This data are shown as a bar graph in Figure 19. Next, as seen on the Figure 19, BBP, DMP, and DEP follows them respectively according to their average concentrations in the milk samples. However, DOP cannot be seen in any pasteurized milk samples. These are the expected results. Because when other studies from the other countries are searched it is seen that the most common phthalate esters in the milk samples are DBP and DEHP. Next the others: follow them.

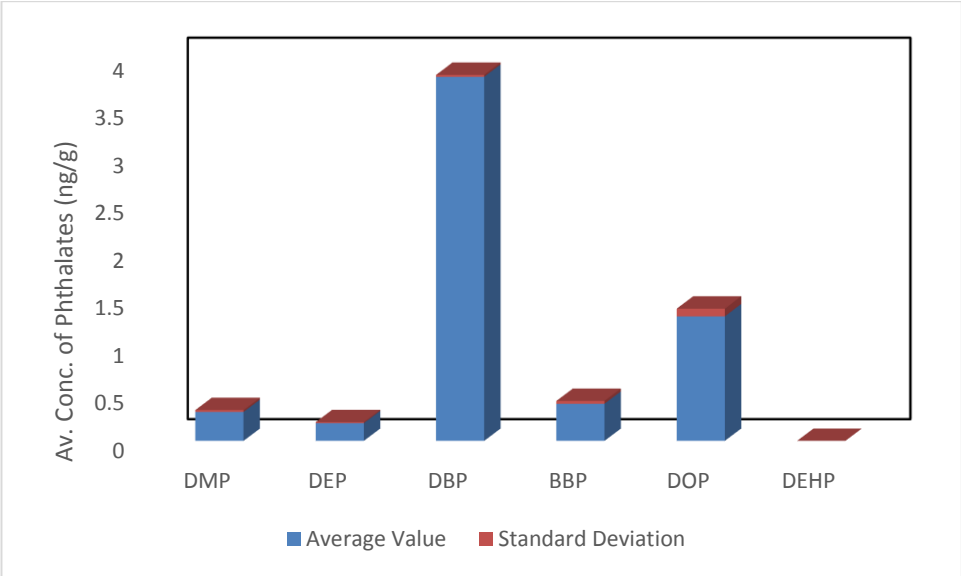


Figure 19: Average Concentrations of Phthalate Esters in Pasteurized Milk Samples

3.2.2. Concentration of Phthalate Esters in Pasteurized Milk Sample Packages

The data set that is obtained in all the study for phthalate esters in 5 different pasteurized milk sample packages are shown in Table 21. The values are the concentrations above the Limit of Quantification (LOQ), which is determined by using the response value where S/N value is 10. Injection of these five samples of pasteurized milk sample packages for phthalate ester content determination was performed as three replicates in order to evaluate the precision of the data set.

Table 21. Pasteurized Milk Sample Packages

	DMP (ng/cm^2)	**DEP** (ng/cm^2)	**DBP** (ng/cm^2)	**BBP** (ng/cm^2)	**DEHP** (ng/cm^2)	**DOP** (ng/cm^2)
Milk Package A	BLOQ	BLOQ	1.8±0.1	BLOQ	34.6±6.4	2.4±0.3
Milk Package B	BLOQ	BLOQ	2.1±0.1	BLOQ	62.6±3.8	BLOQ
Milk Package C	BLOQ	BLOQ	1.1±0.03	BLOQ	47.4±1.2	BLOQ
Milk Package D	BLOQ	BLOQ	1.4±0.1	BLOQ	30.0±4.3	BLOQ
Milk Package E	0.93±0.06	BLOQ	2.0±0.2	5.39±0.64	41.4±3.7	BLOQ

BLOQ: Below Limit of Quantitation

It can be seen from the Table 21 that DBP and DEHP are the most common phthalate esters that are found in pasteurized milk sample packages like milk samples. They were determined in each of five pasteurized milk sample packages. Then, DMP, BBP and DOP follow DBP and DEHP in terms of their concentrations in the samples,

respectively. Relative standard deviation (RSD) values are quite low that shows the high precision of the analytical system.

The average concentration of each measured phthalates, DBP and DEHP show highest concentration. This data are shown as a bar graph in Figure 20. As can be seen from Figure 20, BBP, DMP, and DEP follows DBP and DEHP, respectively according to their average concentrations in the milk sample packages. However, DEP cannot be seen in any pasteurized milk package samples.

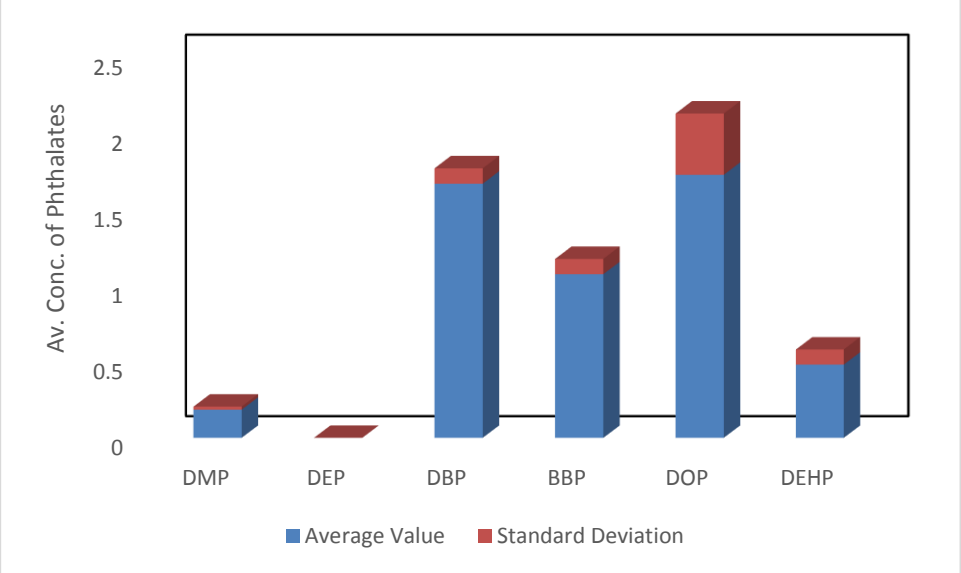


Figure 20: Average Concentrations of Phthalate Esters in Pasteurized Milk Sample Packages

Since one of the aim of this study is showing the phthalate ester profile of the milk samples and their packages and getting an idea whether the phthalate migration from the packages to milk samples are possible, it is necessary to indicate both milk sample and package phthalate content on the same figure. For that purpose Figure 21 is drawn in terms of the phthalate content of milk sample and package C. As it is seen from the Figure 21, only the migration of DEHP from milk package to milk sample is possible since it is in higher amount in milk package than milk sample.

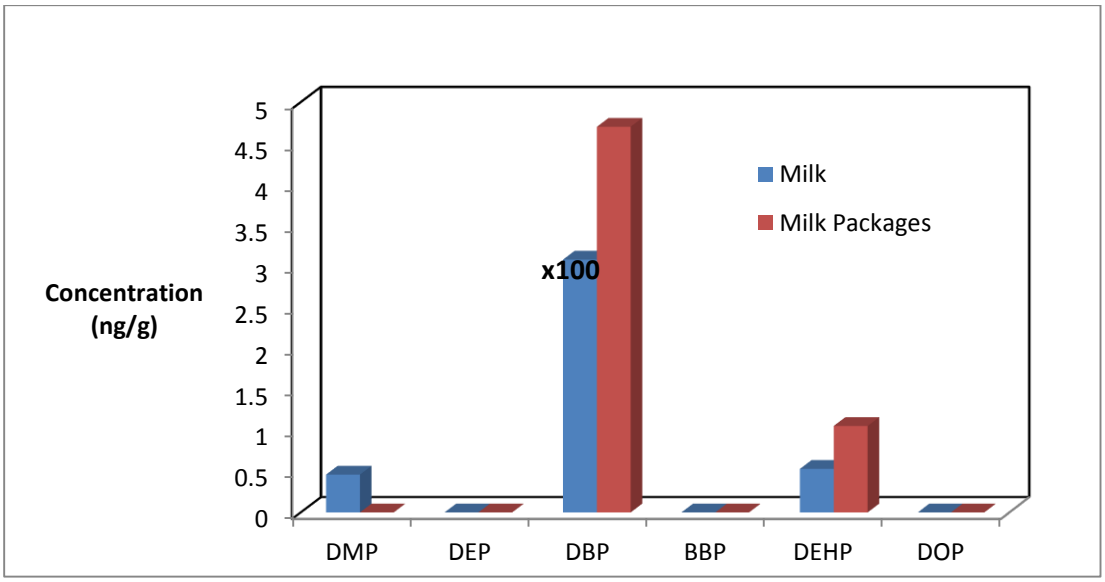


Figure 21. Phthalate Concentration Comparison of Milk Sample D and Package D

3.3. Comparison of Results and Performance Characteristics of the Method with the Literature for Phthalate Esters

3.3.1. Pasteurized Milk Samples

As this is the first study in Turkey concerning phthalates, it is very useful to compare our values with other countries' values. For an analytical study, it is necessary to compare the obtained results as much as performing the other steps of the experiment. Due to that reason, all the data that is obtained for pasteurized milk samples in terms of their phthalate ester content were compared with other three studies using same and different techniques. In this study, combination of UA-DLLME and GC-MS was used as the analytical tool. Obviously, it was difficult to find data set which uses exactly the same extraction and analysis methodologies with us, that is why we made comparison with available data in the literature. There is no study conducted in Turkey. Therefore, Table 22, which compares the phthalate of milk samples found in this study with others does not include a work from Turkey.

In the first method that takes place in China, same this study, UA-DLLME is used as the extraction technique of the phthalate esters from the milk samples. But, GC-FID is used as the analytical instrument instead of using GC-MS (Yan, 2011).

In the second compared method, HS-SPME is used as the extraction technique and instrumentation is GC-MS. The experiment was performed in Romania (Miclean, 2012).

In the third and the last method, Gel Permeation Chromatography (GPC) is used as the separation technique of phthalates from the other organics. Next, GC-EI-MS is used as the analytical method in Belgium (Fierens, 2012c).

These studies with ours are compared in Table 22. When the Table 22 is examined, the results that were obtained after the performance of above methods can be seen. When this study results are compared with the other three studies, the results of phthalate levels of milk samples are mostly lower than the other ones. Especially, when this study is compared with Method 2 and Method 3, some phthalate concentration of milk samples are quite lower than the others.

Table 22. Comparison of Phthalates in the milk samples in this study with the other three studies

	Method 1 (Yan, 2011)	Method 2 (Miclean, 2012c)	Method 3 (Fierens, 2012c)	This Study
DMP max-min (ng/g)	BLOQ-6.4	BLOQ	BLOQ -0.5	BLOQ -0.6
DEP max-min (ng/g)	BLOQ	BLOQ	BLOQ -0.11	BLOQ -0.9
DBP Max-min (ng/g)	BLOQ -5.2	2.1-3.9	BLOQ -54.0	3.1-5.0
BBP max-min (ng/g)	BLOQ	BLOQ	BLOQ -8.2	BLOQ -0.7
DEHP max-min (ng/g)	NI	36.8-77.1	BLOQ -27.5	0.4-4.0
DOP max-min (ng/g)	BLOQ	BLOQ	BLOQ -5.7	BLOQ

BLOQ: Below Limit of Quantitation

NI: No Information

When LOD values of these four studies are compared to each other, it is observed that except for Method 3, all the other ones are compatible with each other. Method 3, which is performed by the combination of GPC and GC-EI-MS, has higher LOD values for phthalates. However, this study has the lowest LOD for the following phthalate esters: DBP, BBP, and DEHP. The details can be seen in the below Figure 22.

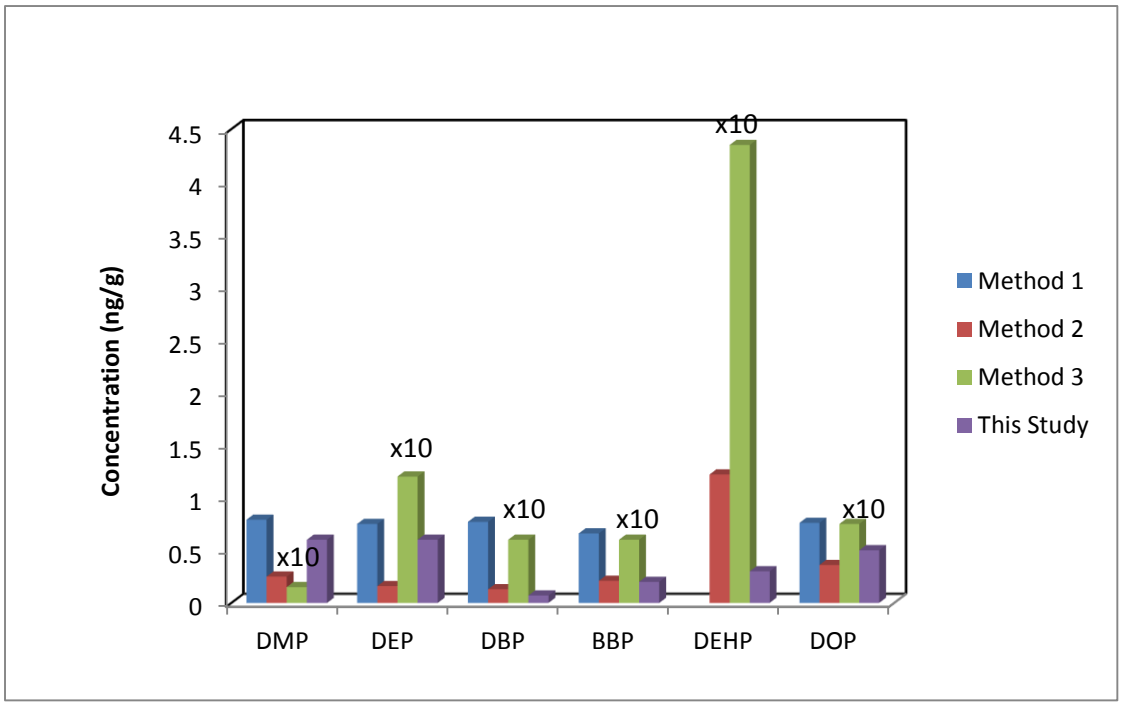


Figure 22. LOD Comparison of This Study with Others from Literature

The same comparison of LOD values is also valid for the LOQ values since only the difference is that LOQ value is calculated where S/N ratio is 10 instead of 3.

After the comparison LOD and LOQ values, it is necessary to evaluate the precision of the experiment. Due to that reason, same experimental method was performed with 5 replicates. Then, % RSD values are calculated to show the precision of the performed experiment. When the found results are compared with the %RSD values of the other studies, as can be seen from the Figure 23, the results of this study are highly compatible with the other ones. The study results show that especially for the DMP, BBP, and DOP, this method has the lowest %RSD values. That means it has the highest precision for these three mentioned phthalate esters.

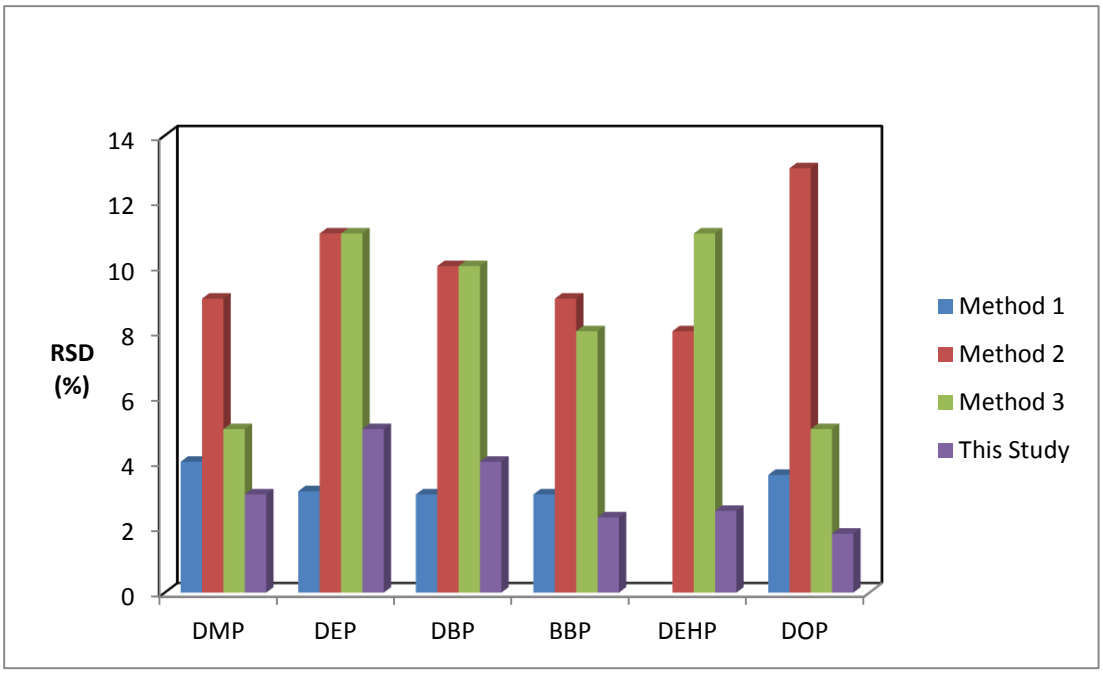


Figure 23. Comparison of Precision

3.3.2. Pasteurized Milk Sample Packages

All the data that is obtained after the analysis of pasteurized milk sample packages for their phthalate ester content were compared with other three studies with same and different techniques of this study. In this study, combination of UBE and GC-MS was used as the analysis pathway.

In the first study, the same procedure that is followed in the concerning study was performed. In other words, UBE is used as the extraction method and GC-EI-MS is used as the analytical instrumentation (Fierens, 2012c).

In the second study, the extraction of phthalates from the milk packages was performed by the help of Soxhlet apparatus. In addition, GC-MS system was used for the analytical instrumentation (Balafas, 1998).

When the Table 23 is examined, the results that were obtained after the performance of above methods can be seen. When it is time to compare the results of this study, a difficulty is faced since there are many different units that cannot be converted to each other. When this study results are compared with the first study, the results of phthalate levels of milk sample packages are lower. However, when the study results are compared to the second study, it can be observed from the Table 23 that, the concentration of the phthalates are higher than the Method 2.

Table 23. Comparison of Method Performance of the Study for Milk Packages with Other Studies

	Method 1 (Fierens, 2012c) (ng/cm^2)	**This Study** (ng/cm^2)	**Method 2** (Balafas, 1998) (ng/g)	**This Study** (ng/g)
DMP max-min	BLOQ-0.4	BLOQ -0.9	BLOQ	BLOQ -4.7
LOQ	0.1	1.2	NI	-
%RSD	5.0	3.0	NI	-
DEP max-min	BLOQ -41.0	BLOQ	BLOQ	BLOQ
LOQ	0.5	1.1	NI	-
%RSD	12.0	2.0	NI	-
DBP max-min	BLOQ -96.0	1.1-2.1	53.0-7.0	5.26-10.2
LOQ	1.5	0,8	NI	-
%RSD	10.0	2.0	NI	--

Table 23. Comparison of Method Performance of the Study for Milk Packages with Other Studies (Cont'd)

BBP max-min **LOQ** **%RSD**	BLOQ -24.0 0.5 8.0	BLOQ -5.4 11.0 4.0	BLOQ -3.0 NI NI	BLOQ -26.9 - -
DEHP max-min **LOQ** **%RSD**	1.1-319.0 0.5 14.0	30.0-47.2 4.2 4.0	20.0-28.0 NI NI	150.0-313.0 - -
DOP max-min **LOQ** **%RSD**	BLOQ -1.5 0.5 6.0	BLOQ -2.4 8.1 6.0	BLOQ NI NI	BLOQ -12.1 - -

BLOQ: Below Limit of Quantitation

NI: No Information

When LOD values of these three studies are compared to each other, it is observed that there is no enough information about the LOD and LOQ values of this method on the paper. However, when this study and Method 1 are compared to each other it is seen that Method 1 has lower LOD/LOQ values than this study as seen on the Table 23.

After the comparison LOD and LOQ values, it is necessary to evaluate the precision of the experiment. Due to that reason, same experimental method was performed with 5 replicates. Then, % RSD values are calculated to show the precision of the performed experiment. When the found results are compared with the %RSD values of the Method 1, as can be seen from the Table 23, the results of this study are better than the

other one. The study results show that %RSD values of this method are lower than 10%. That means it has very high precision. The same argument done before is true for Table 23. None of the compared studies have exactly the same methodology with this study.

3.4. Student's t-Test

In order to explain comparisons, using statistical terms we applied Student's T-Test to our results.

3.4.1. Student's t-Test Comparison of two Means

As it is known, a two-sample student's t-test is used to test if the means of two normally distributed populations are similar or not. When each of the two samples consists of independent and identically distributed observations, and is obtained from its corresponding population separately from the other sample, then the two-sample student's t-test is called an unpaired two-sample student's t-test (McDonald, 2008).

The two means and the corresponding standard deviations are calculated by using the following equations (n_A and n_B are the number of measurements in data set A and data set B, respectively).

$$\bar{x}_A = \sum_{i=1}^{n_A} x_i \Big/ n_A \qquad \bar{x}_B = \sum_{i=1}^{n_B} x_i \Big/ n_B$$

$$s_A = \sqrt{\frac{\sum_{i=1}^{n_A} (\bar{x}_A - x_i)^2}{n_A - 1}} \qquad s_B = \sqrt{\frac{\sum_{i=1}^{n_A} (\bar{x}_B - x_i)^2}{n_B - 1}}$$

Then, the pooled estimate of standard deviation s_{AB} is calculated.

$$s_{AB} = \sqrt{\frac{(n_A - 1)\, {s_A}^2 + (n_B - 1)\, {s_B}^2}{n_A + n_B - 2}}$$

Finally, the statistic t_{exp} (experimental t value) is calculated.

$$t_{exp} = \frac{|\bar{x}_A - \bar{x}_B|}{s_{AB}\sqrt{\frac{1}{n_A} + \frac{1}{n_B}}}$$

t_{exp} value is compared with the critical (theoretical) t_{th} value corresponding to the given degree of freedom N (in the present case $N = n_A + n_B - 2$) and the confidence level chosen. Tables of critical t values can be found in any book of statistical analysis, as well as in many quantitative analysis textbooks. If $t_{exp} > t_{th}$ then H_0 is rejected else H_0 is retained (Efstathiou).

3.4.2. Student's t-Test Results for Milk Samples

In this part of the study, found experimental results for phthalate levels of pasteurized milk samples will be compared to other studies by the help of student's t-test. For that purpose only DBP is chosen as the phthalate that to be compared since its found level is quite close to the ones of other studies. Student's t-test is used to determine whether these small differences of the DBP levels in milk samples are important or not. Due to that reason, this study is compared to other 3 studies that took place in Canada, Romania, and China. Then the following table is obtained.

Table 24. Comparison of This Study Results of DBP Amount in Milk Samples with 3 other Studies with Respect to t-Test Results

Student's t-Test Result of This Study DBP.	**The Country that The Study Took Place**	**Theoretical $t_{critical}$ Value**	**Found t Value**
Study 1 (Feng, 2005) (n=5)	Canada	2.353	23.215
Study 2 (Miclean, 2012) (n=3)	Romania	2.132	2.363
Study 3 (Cheng, 2011) (n=3)	China	2.132	8.367
This Study (n=3)	Turkey (DBP conc.=3.82 ng/g)		

As can be seen from the Table 24 the found experimental t values are higher than the theoretical $t_{critical}$ values that are found from the t-table at 95 % confidence level. This indicates that the difference of DBP level between the milk from Turkey and other countries (Canada, Romania, and China) are high. DBP level in Turkish pasteurized milk samples are very much different than the others in 95% confidence levels.

CHAPTER 4

CONCLUSION

Throughout this study, phthalate ester contamination levels of pasteurized milk samples and their packages were investigated in Turkey for the first time. For the purpose of finding a suitable analytical method, five different pasteurized milk samples and their packages were analyzed and method validation of both extraction and instrumentation were done. During the study, following six commonly used phthalates were the target ones. Dimethyl Phthalate (DMP), Diethyl Phthalate (DEP), Dibutyl Phthalate (DBP), Butyl Benzyl Phthalate (BBP), Diethylhexyl Phthalate (DEHP), and Dioctyl Phthalate (DOP).

The selected phthalate esters were decided to be extracted from pasteurized milk samples with a very new technique which is Head Space Solid Phase Micro Extraction (HS-SPME). But, due to low precision of the method and high fragility of the SPME fibres, Ultrasound Assisted-Dispersive Liquid Liquid Microextraction (UA-DLLME) was used instead.

The phthalate esters in pasteurized milk samples were extracted by UA-DLLME technique. The extraction recoveries of phthalate esters were found in between 66-100% for the pasteurized milk samples. Only the extraction recovery of DOP was below 70%, which is the lowest successful limit of the extraction recovery. These results indicate that the UA-DLLME technique was appropriate for the extraction of phthalate esters in pasteurized milk samples.

The phthalate esters in milk packages were extracted by Ultrasonic Bath Extraction (UBE). The extraction recoveries of phthalate esters from the pasteurized milk sample packages were in between 115-127%. Since all the values were below the 130%, which

is the highest acceptable extraction recovery level, it can be said that UBE technique was a suitable one for the extraction of phthalate esters from the milk packages.

The accuracy of the measurements and the stability of the analysis systems were measured by the phthalate standard mixes. Moreover, the Limit of Detection (LOD) and Limit of Quantitation (LOQ) values were calculated as the concentrations of the analytes at which the signal to noise (S/N) ratios are 3 and 10, respectively. The highest LOQ values were found for DMP and DEP as 2 ng/g while the lowest one was found for DBP as 0.23 ng/g.

For both the pasteurized milk samples and their packages, DBP and DEHP were found as the most common phthalate esters. DBP values that are obtained after the analysis of milk samples were between 3.08-5.03 ng/g while DEHP values were in between 0.41-4.00 ng/g. In addition, DBP values that are obtained after the analysis of milk packages were in between 1.05-2.03 ng/g while DEHP values were in between 30.0-62.6 ng/cm^2. DOP was found as the lowest concentrated phthalate ester in milk packages. It is only found in Milk Package Sample A as 2.42 ng/g while no DOP was found in milk samples.

The results of this study is compared with other studies. Although DEHP in milk samples is found around 75 ng/g in Romanian studies and 25 ng/g in Belgium studies, in this study it is found at most 4 ng/g. Lower DEHP level of Turkish Milk indicates lower contamination of milk by DEHP. When it is looked at the second common phthalate level which is DBP, comparable results were obtained. In this study, DEHP concentration is around 3-5 ng/g. The milk package results are also compared with other studies. DBP and DEHP levels of Turkish milk packages were around 10 times lower than the Belgium milk packages. However, when phthalate levels of Turkish milk packages are compared with Australian ones, it is observed that Australian packages are safer in terms of their phthalate ester content. In addition, since DEHP levels in milk packages are higher than the milk samples, the migration of DEHP from milk package to milk sample is possible. As far as food safety is concerned we did not observe any alarming results. But more extensive research with more number of

samples is required. This study is important as it is the first research on phthalates in food.

As a future work, DEHP migration from milk package to milk sample can be researched.

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