CHAPTER I

GENERAL INTRODUCTION

1. Problem Definition

In quantitative analysis researchers have to determine the amount of analyte within samples. It means that they have to detect and report the results of the amount of the analyte. The preparation procedures depend on the method of detection. Samples are prepared in forms that are appropriate to the instruments or methods chosen. There are some detection methods which do not utilize complicated sample preparation, for instance, reagents are added to samples and counted by special instruments such as scintillation counters, or in some cases a radio isotope is introduced to samples and detected it later. On the other hand, for high performance liquid chromatography and gas chromatography, the sample must be isolated in a liquid or gaseous form to be injected. Therefore, sample preparation precedures are required. The types of sample matrix encountered depend on one or more approaches as may be required for a particular sample. Some samples can even be injected directly or require little preparation (Pharmaceutical formulation, beverages, polymer additives are examples of these) (1). However, other sample matrices are complex but of fairly consistent composition such as blood plasma and protein hydrolysates. These require a standard and rugged sample preparation method. Some sample matrices such as urine environmental and food sample, also can be complex and inconsistent often requiring a separate preparation procedure for each sample encountered.

Much of the developmental work in HPLC and in GC has been focused on increasing the speed, sensitivity and efficiency of the techniques. Separation times for both techniques are now measured in minutes and high resolution GC and HPLC columns have been a reality for sometime. However, sample preparation had not been received as much attention as the chromatographic techniques. The preparation procedures of matrix samples can still be troublesome espectially, for the determination of trace analytes in matrix samples. The matrix can strongly interfere with the analytes. Matrix consideration include identification of major matrix components, general matrix effects on the isolate, and other properties relevant to the extraction.

Milk is an essential first food for human. For countless generations, it has formed an important part of the human diet, not only for the infant but, in many societies, throughout life. It is well recognized for its nutritional value, especially as a source of calcium and phosphorus and as a major source of high quality protein. It is also a good source of vitamins (2,3). For these reasons, we chose to examine milk and dairy products. Dairy products commonly move through several steps from producer to consumer and may be transported beyond the regulatory jurisdiction within which they are produced. The ultimate aim of control procedures should be to provide a product in which the original nutritive qualities, flavour and appearance have been preserved and no harmful organisms or substances are present to affect the consumer adversely (4). The total milk solids content ranges from 10.4 % to 15.5 % and is composed of fat and non fat solids. Due to the milk solids, milk is the matrix sample. According to the matrices, the sample preparation procedures of milk are tedious. Moreover, recently the determination of trace substances in milk has become more important and requires development

Phthalate esters (PEs) are significant substances which can contaminate milk due to their lipophilicity. PEs are widely used as plasticizers in plastics and resins. They are readily released to the environment by leaching

from plastics and other sources. Their widespread usage, coupled with their stability, has led to PEs being present as ubiquitous environmental contaminants. Hence, the sample preparation procedures to determine trace PEs in milk need to be studied and developed. Up to the present, sample preparation methods for milk are classical methods and usually involve liquid liquid extraction (LLE). LLE is widely used but it is tedious, lobour-intensive procedure, time consuming and costly. This method not only requires several sample handling steps but may also present the following problems to the analyst: phase emulsion, large solvent volumes, impure and wet extracts, as well as nonquantitative and irreproducible extraction. An additional concern is the disposal of the enormous amount of solvent used in the extraction procetures, creating an environmental problem. In the mid 1970's, a simpler alternative approach, solid phase extraction (SPE), was introduced (5). This concept, similar to low pressure liquid chromatography, involved, the design of small disposable columns filled with a variety of sorbents. It has been used for sample preparation of water for two decades (6-14). Solid phase extraction membrane (SPEM) is the newest medium of SPE and has been widely used for water sample preparation for 5-6 years (15). It provide as advantages over the conventional SPE medium, i.e., allows a much higher flow rate, decreases the chance of plugging and improves the mass transfer. Therefore, it should be introduced as a sample preparation device for the determination of trace PEs in milk.

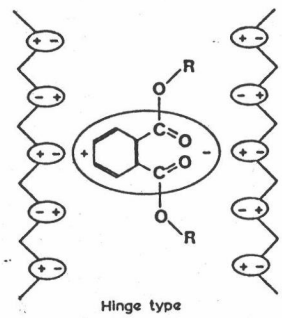
Phthalate Esters

Di-phthalate and tri-phthalate have a high degree of compatibility with resins, ease of fusion with polymers and are chosen based on processing requirements and the intended end use of the plastic. Phthalic acid esters

(usually called phthalate esters; PEs) which are widely used nowadays for plasticizers, are mainly six substances. They are dimethylphthalate (DMP), diethylphthalate (DEP), dibutylphthalate (DBP), butylbenzylphthalate (or benzenebutylphthalate, BBP), di(2-ethylhexyl)phthalate (or bis(2-ethylhexyl) phthalate) (DEHP) and di-n-octylphthalate (DOP). The Environmental Protection Agency of the United State (EPA) includes them in the list of EPA priority toxic pollutants and five of them are in the Resource Conservation and the Recovery Act. (RCRA) Toxic Waste; DMP : number u109, DEP : number u088, DBP : number u069, DEHP : number u028, DOP : number u107. Furthermore, DBP and DEHP are noted that to be a hazardous substance and a carcinogen respectively. The acute toxicity of phthalate esters is very low. High doses may, however, produce somnolence, weight loss, dyphnea and cyanosis. Moreover, recently, there have been many reports about the harmful effect of the PEs, especially DEHP. DEHP and its major metabolite, mono (2-ethylhexyl) phthalate and 2-ethylhexanol as well as other phthalate esters such as DMP, DEP, DBP, BBP and DOP, may have many effects on humans such as testicular atropy, lung hemorrhage, hepatomegaly, cytotoxicity, proliferation of hepatic peroxisomes, carcinogenicity, teratogenicity, thyromimeticity, enzyme function change, metabolism changing and cardiodepressive properties (16-23). Although, a report from China showed that DBP causes selective elimination of cancer cells, it still has many other harmful effects (24).

The PEs are mainly used in commercially as solvents and as plasticizers of synthetic polymers such as polyvinylidene chloride, polyamide, polycarbonate, acrylic, polystyrene, fluoroplastic and thermosetting resin. Some of lower aliphatic phthalates are used in the manufacture of varnishes, dopes and insecticides DMP is used as an insect repellant; DEP is used as a solvent for cellulose esters, as a vehicle in pesticide sprays, as a fixative and solvent in perfumery, as a plasticizer in solid rocket propellants; DBP is used as plasticizer in vinyl acetate and cellulose esters and as an insect repellent). The

PEs is the external plasticizer, while the internal plasticizer is a plasticizer which copolimerized with the monomer to become part of the polymer backbone. Some scientists present the model of the PEs in polymer by the Hinge type (25).



Hinge type

DEHP has been used enormously in the plasticizer market, accounting for one third of the total. Besides, its toxicity, occasional to migration from plastic, EPA and many organizations regulate the distribution of it as an environmental pollutants. Migration will occur when the two materials are in surface contact. The tranfers takes place by diffusion, and thus the main factors governing its rate and final extent will be the chemical make-up and molecular size and shape of the plasticizer, its concentration gradient, etc. DEHP and DBP are the most prominent plasticizers and they are especially important in medical devices such as blood storage bags and tubing. DEHP leaches out of plastics, since it is soluble in lipoproteins. Many medical reports show the presence of phthalate in the spleen and lungs when the patient receives large volumes of blood stored in plastic bags and in neonatal heart and gastrointestinal tissue of infant transfused via umbilical catheters. In food and milk, some reports show the amount of DEHP leaching (17). For the protection of human health, the EPA established the permissible concentration of these PEs in air at 5 mg/m^3 for DMP, DBP and DEHP while the concentration of these PEs in water are 313, 350, 34, 15 mg/L for DMP, DEP,DBP and DEHP respectively (17).

Although the PEs are only slightly soluble in water they can contaminate the environment through such sources as wastewater from plastic and plastic material products factories. Futhermore, pesticides which contain PEs carriers may release them into air, soil and water. Unfortunately, the PEs are compatible with lipophilic substances so they may leach from plastic blood bags, medical tubing and food packaging.

2. Literature Reviews

Literature Review of Solid Phase Extraction (SPE)

Liquid-Liquid Extraction (LLE) is well understood, and most laboratories have the glassware and equipment necessary to perform the technique. This method is labor-intensive, however, and slow. It requires large volumes of solvent and the sample transfer steps and possible emulsion formation can result in sample loss. Another technique, SPE, is growing in popularity, especially because it is convenient, easy to use and requires very small quantities of organic solvents. SPE cartridges are readily available (1, 26-31). SPE is a technique in which the basic principle of liquid chromatography is used to isolate the compound of interest from the sample matrix. SPE has a number of adventages over other sample preparation techniques, such as smaller volumes and greater recoveries.

Polymeric-based SPE cartridge are available from many manufacturers. Many types of sorbent that are used in SPE are similar to LC column packing materials. There are several ways in which SPE can be performed. First, it can be used for trace enrichment, second, SPE can be used for sample isolation, third, it can be used for matrix isolation and finally SPE can be used for sample storage and transport. Furthermore, robot-compatible cartridges are available from several different suppliers.

Although SPE using disposable columns or cartridges has been widely and successfully used, difficulties can arise in their routine application due to the narrow internal diameter limit that necessitates long times for large sample volumes. Moreover, the dirty samples can rapidly plug the cartridges. For this reason, SPE disk are beginning to be used in separation of biological compounds such as the study of drugs in urine and water samples. The disks are available with a diameter (47 mm) and size similar to LC solvent filters. In addition, SPE disks can be used by robots (26, 31, 32).

Hodgeson and his group determined acid herbicides in an aqueous medium using Empore-3M C_{18} and Polystyrene-Divinylbenzene (PS-DVB) resin for sample preparation and detected them by gas chromatography equipped with electron capture detector (GC-ECD) (33). Schmidt and his team studied phenols which were extraction by PS-DVB solid phase extraction membrane (SPEM). This study used 200 - 500 mL of water and sodium chloride for salting out of phenols. Methanol was used as a solvent in this study (34). In some cases, water samples are prepared by SPEM with glass bead, used as an in situ filtration aid to improve flow rate when the sample was particle-containing (35). Furthermore, SPEM is applied to use with sediment sample such as blood (36-37).

Literature Review of Sample Preparation Procedure for Analytical Method in Milk by LLE

LLE is a fair preparation procedure for any samples. Toyoda and his co-workers in Japan(1990) (38), used the LLE as the analytical method for the study of organophosphate pesticides in milk. They used acetonitrile as the solvent and zinc acetate as a fat removal reagent. The last step is gas

chromatography equipped with flame photometric detector (GC-FPD). Recoveries of 6 pesticides spiked in milk at levels of 0.1 and 1 ppm are 82.1 - 93.8 and 79.6 - 96.6 % respectively. This work used 500 mL and 1 L separatory funnels, and a milky sample of 25 mL.

Kroneld and Reunanen from Finland in 1990 use 0.5 mL of n-pentane with a milk sample of 5 mL for extraction and then the n-pentane phase was analysed by gas chromatography equipped with mass selective detector (GC-MSD) (39). Antibiotics or vitamin K_1 in milk were also prepared by LLE. In 1995, Moats and Herik-Khan (40), determined β-lactam antibiotics by LC detection. They use acetonitrile as a solvent to extract and tetraethyl ammonium chloride to deproteinize milk. The last step of sample preparation was passage through polyvinylidene difluoride filter cartridge and clean up by LC. Idyle and his team researchers, determined vitamin K_1 in milk by extraction of hexane (41). Not only milk but also dairy products, such as a cheese sample were prepared by LLE. Bentabol and Jodral determined organochlorine pesticides in cheese using 3 key steps: isolating the fat from the cheese, extracting the chlorinated pesticides from fatty acid treatment with concentration sulfuric acid and hexane, and the last step was extraction of chlorinated pesticides from the fat by basic treatment with 10% KOH/ethanol solution (42). According to the above reports, when LLE is used for sample preparation, a clean up step may be included. A new alternative that may or may not be used with LLE is derivatization. Chin-En Tsai and Kondo determined six sulfonamides in milk by using fluorescamine derivative after extraction by acetonitrile (43). Weiss and co-workers determined sulfodimethoxine in milk by four comparison sample preparations. They used benzoic acid treated with the sample and the results were read on a plate viewer. The second method was radiolabeling. Scintillation fluid was added, and then was detected by a scintillation counter. The third method was prepared by 3:1 methylene chloride: chloroform

extraction and uses LC as a detection instrument. The last method in this study was enzymelinked immunosorbent assay (44).

Literature Review of Milk Sample Preparation by SPE device

When SPE was introduced in 1970's, it was widely used for many samples and substances including milk. For the determination of drugs, herbicides, aflatoxin, pesticides, peptides etc, SPE has been applied to sample preparation. It is not simple to use SPE with milk or matrix samples, so many methods are combined with SPE such as centrifugation, protein precipitation, dialysis etc (45-58).

Clean up methods that are required for milk sample and matrix sample. Florisil is the most popular for clean up of lipid from samples (59-60). Florisil SPE cartridge are avaliable for clean up of the environmental samples (61).

Literature Review of the Determination of Phthalate Esters and their Metabolite

Furtmann (62) in Germany determined phthalate esters in water. DEHP was the interesting compound and the workers used C_8 and C_{18} SPE cartridges to concentrate phthalate esters (PEs). It was unnecessary to clean up when GC-MSD was used as a detector. This preparation was established to be a method for routine trace level analysis. The results of this study showed high recoveries (all phthalate esters have 98± 5%) and good precision. Mono phthalate esters and phthalide extracted were also determined. They were an prepared by SPE and detected by LC. Mono-2 ethylhexyl phthalate, monobutyl

phthalate and phthalide were determined by Snell in 1993. He used acetonitrile as a solvent in this study and showed high precision (63). 2-Ethyl-1-hexanol (2-EH) was studied in drinking water in 1993 by Vitali and Leoni. 2-EH is widely used in industrial to produce plasticizer so it may leach out from the production factories. The researchers used CS_2 as a solvent and was detected by GC (64).

Many reports have shown that phthalate esters used as plasticizer in industry can be leached from plastic packing. Leibowitz, Samiento, and their co-workers determined six common phthalates in grain neutral spirit and vodka. PEs were detected in many real samples in a range of 62 - 495 ppb for DEHP and 22 - 156 ppb for DEP and in trace amounts for DOP (65).

In 1967 DEHP, formerly known on DOP in industry, in milk was first determined. This was done by dialysis and evaporation to dryness. Petroleum ether was used as the solvent, and IR was to detect DEHP (66). In 1994, Sharman and Read determined DEHP in milk cream and butter from variety of source in European countries. Follow by liquid-liquid extraction and size-exclusion chromatography clean up (67).

3. Hypothesis

SPEM is considered to be an attractive technique for sample preparation. The reason is that this technique has a number of advantages over other sample preparation procedures. A high sample volume can be applied, a rapid flow rate can be adjusted and some matrix samples can also be applied. For this reason, in this study, we tried to develop the use of SPEM as a device for sample preparation.

The general character of the matrix indicates how it should be handled. If the matrix is a liquid or a solid, the treatment should reflect both the matrix composition and the desired extraction mechanism. Milk is a sample which is

composed of lipids, carbohydrates and proteins. Lipids and proteins are the main matrix which has to be considered. In solid phase extraction, lipids can adsorb in large amounts as the sorbent surface, reducing the capacity for isolate retention. Proteins and solids often interact with the isolate making it unavailable for sorbent retention.

For sample preparation developing purpose, the determination of phthalate esters in the milk sample is introduced. The sample preparation is divided in two major methods. The first is very simple and less time consuming. The other is the combination of sample pretreatment (for example centrifugation and pH adjustment) and SPEM. For optimizing results, sample clean up is introduced.

4. The Purpose of the Study

Many reports have shown the toxicity and leaching of PEs from plastic containers, so it is important to develop a method to determine in a milk sample.

SPEM is developed for determination of some PEs, i.e., DMP, DEP, DBP, BBP, DEHP and DOP. This work aims at the studies of various sample preparation methods .Two major methods are introduced to procede with SPEM.

1. Without sample pretreatment.
    1.1 Direct method with SPEM
    1.2 Dilution of sample with SPEM
    1.3 Filter Aid with SPEM
    1.4 Dilution of sample with Filter Aid and SPEM
2. With sample pretreatment
    2.1 Centrifugation with SPEM

2.2 Shuffenburg's precipitation method with SPEM

2.3 Solvent extraction before passing through SPEM

2.4 pH adjustment with SPEM

In addition, time requirement, percent recovery, method detection limit and precision of various methods are studied to evaluate each method.

The optimum sample preparative method from this study is chosen to determine PEs in dairy products from supermarkets in Bangkok.