SCREENING OF ENDOCRINE DISRUPTING COMPUNDS IN BIOLOGICAL SAMPLES AND EVALUATION OF THEIR ACCUMULATION PATTERNS

BY

MUHAMMAD SAJID

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This thesis, written by **MUHAMMAD SAJID** under the direction of his thesis advisor and approved by his thesis committee, has been presented and accepted by the Dean of Graduate Studies, in partial fulfillment of the requirements for the degree of **DOCTOR OF PHILOSOPHY IN CHEMISTRY**.

Dr. Basheer Chanbasha
(Advisor)

12/5/2016

Dr. Abdulaziz Alsaadi
Department Chairman

Dr. Abdalla Abulkibash
(Member)

Dr. Salam A. Zummo
Dean of Graduate Studies

Dr. Nisar Ullah
(Member)

1/6/16

Date

Dr. Abdulaziz Alsaadi
(Member)

Dr. Mohammad Riaz
(Member)

Dedicated to my beloved parents who always supported me through their prayers and love. No words can describe the support they provided me throughout my life and educational career. Thank you Dear Parents.

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LIST OF ABBREVIATIONS

EDCs	:	Endocrine disrupting compounds
POPs	:	Persistent organic pollutants
PCBs	:	Polychlorinated biphenyls
OCPs	:	Organochlorine pesticides
PEs	:	Phthalate esters
GC-MS	:	Gas chromatography-mass spectrometry
HPLC	:	High performance liquid chromatography
UV	:	Ultraviolet
SPE	:	Solid phase extraction
LLE	:	Liquid-Liquid extraction
SPME	:	Solid phase microextraction
LPME	:	Liquid phase microextraction
d-SPE	:	Dispersive solid phase extraction
DLLME	:	Dispersive liquid-liquid microextraction
µ-SPE	:	Micro-solid-phase extraction

ABSTRACT

Full Name : Muhammad Sajid

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Endocrine disrupting compounds (EDCs) pose serious health risks to human and wild life due to their toxicity, very long half-lives and bioaccumulation. EDCs induce a variety of carcinogenic and non-carcinogenic disorders in human and they become functional at extremely low concentrations. Efficient and cost-effective analytical methods are always demanded in order to extract and determine such low concentrations of EDCs in environmental and biological samples. In this research work, different analytical methods were developed for extraction and determination of different classes of EDCs in biological samples. Analytical methods were developed by using four famous classes of EDCs i.e. parabens, phthalate esters (PEs), organochlorine pesticides (OCPs) and polychlorinated biphenyls (PCBs). EDCs were also screened in biological samples.

A simple and efficient analytical method was developed for quantification of parabens in cancer tissues. This method involved the simultaneous use of microwave-assisted solvent extraction (MASE) and micro-solid phase extraction (μ-SPE), in tandem with high performance liquid chromatography (HPLC/UV). The parabens studied included methyl, ethyl, propyl and butyl parabens. Optimization of the experimental parameters for MASE and μ-SPE was performed. RSDs ranged from 0.09 – 2.81%. Coefficients of determination (R^2) up to 0.9962 were obtained across a concentration range of 5.0- 200 ng g^{-1}. The method detection limits for parabens ranged from 0.005 - 0.0244 ng g^{-1}. The procedure was initially tested on prawn samples to demonstrate its feasibility on a complex biological matrix. Preliminary studies on human ovarian cancer tissues showed presence of parabens. Higher levels of parabens were detected in malignant ovarian tumor tissues compared to benign tumor tissue samples.

For the first time, an efficient and cost effective natural material (seed powder of *Moringa oleifera*) was employed as sorbent in µ-SPE. The sorbent was found to be naturally enriched with variety of functional groups and having a network of interconnected fibers. This method of extraction integrated different steps such as removal of proteins and fatty stuff, extraction and pre-concentration of target analytes into a single step. Thirteen phthalate esters were selected as target compounds for the development and evaluation of method. Some key parameters affecting the extraction efficiency were optimized, including selection of membrane, selection and amount of sorbent, extraction time, desorption solvent, volume of desorption solvent, desorption time and effect of salt addition. Under the optimum conditions, very good linearity was achieved for all the analytes with coefficient of determinations (R^2) ranging between 0.9768 and 0.9977. The limits of detection ranged from 0.01 to 1.2 µg L^{-1}. Proposed method showed satisfactory reproducibility with relative standard deviations ranging from 3.6% to 10.2% (n=7). Finally, the developed method was applied to tetra pack and bottled milk samples for the determination of phthalate esters.

A novel modification in membrane protected micro-solid phase extraction was introduced for screening of PCBs in serum samples. This new configuration of micro-solid phase extraction was termed as stir-bar supported micro-solid-phase extraction (SB-µ-SPE). A tiny stir-bar was packed inside the porous polypropylene membrane along with sorbent material and the edges of membrane sheet were heat sealed to secure the contents. The packing of stir-bar inside the µ-SPE device, does not allow the device to stick with the wall or any corner or float over the surface of the sample solution. This mode also enhances effective surface area of the sorbent exposed to sample solution. Polychlorinated biphenyls (PCBs) were selected as model compounds and the method performance was evaluated in human serum samples. After extraction, samples were analyzed by gas chromatography mass spectrometry (GC-MS). The factors that affect extraction efficiency of SB-µ-SPE were optimized. Under optimum conditions, a good linearity (0.1 – 100 ng mL^{-1}) with coefficients of determinations ranging from 0.9868 to 0.9992 were obtained. Limits of detections were ranged between 0.003 and 0.047 ng mL^{-1}. Acceptable values for inter-day (3.2 – 9.1%) and intra-day (3.1 – 7.2%) relative standard deviations were obtained. The

optimized method was successfully applied to determine the concentration of PCB congeners in human serum samples.

In another work, ZnO nanoparticles incorporated porous carbon foam was utilized as sorbent for extraction and preconcentration of OCPs in milk samples. This sorbent was prepared by a facile single step reaction between the sucrose and zinc nitrate by slight modification of a reported procedure. The sorbent was characterized by scanning electron microscopy and X-ray diffraction. The sorbent was then packed inside a porous polypropylene membrane sheets by heat sealing of membrane to construct µ-SPE devices. The factors that affect performance of µ-SPE were optimized. This method provided good linearity up to 0.9998 with limit of detections ranging from 0.19 to 1.64 ng/mL. This method also provided satisfactory values for intra and inter-day precision with RSDs ranged between 2.3 to 10.2%.

In the last part of the work, OCPs and PCBs were screened in real human cancer tissues and plasma samples and accumulation patterns were studied to sort out most frequently detected compounds.

ABSTRACT IN ARABIC

ملخص الرسالة

الاسم الكامل: محمد ساجد

عنوان الرسالة: فحص مركبات اضطراب الغدد الصماء في العينات البيولوجية وتقييم أنماط تراكمها

التخصص: كيمياء

تاريخ الدرجة العلمية: مايو 2016

تشكل مركبات اضطراب الغدد الصماء مخاطر صحية خطيرة على حياة الإنسان والحياة البرية نظراً لسميتها، طول نصف عمر تفككها وكذلك تراكمها البيولوجي. هذه المركبات تؤدي لمجموعة متنوعة من الاضطرابات المسببة وغير المسببة للسرطان في الإنسان وهي ذات أثر حتى بتراكيز منخفضة للغاية. هناك حاجة لأساليب تحليلية قليلة التكلفة وذات كفاءة عالية لاستخلاص وتحديد مثل هذه التراكيز المنخفضة من المركبات في العينات البيئية والبيولوجية.

في هذا البحث، تم تطوير طرق تحليل مختلفة لاستخلاص وتحديد الأنواع المختلفة من هذه المركبات في العينات البيولوجية. وقد تم تطوير طرق للتحليل باستخدام أربعة أنواع من هذه المركبات هي البارابينات، استرات الفثالات، مبيدات الكلور العضوية ومركبات ثنائي الفينيل متعدد الكلور، وتم عمل مسح لها في عينات بيولوجية.

تم تطوير طريقة تحليلية بسيطة وفعالة لتقدير البارابينات في الأنسجة السرطانية، تضمنت هذه الطريقة الاستخلاص بالمذيب بمساعدة الميكروويف متزامناً مع الاستخلاص الدقيق باستخدام مادة صلبة، متبوعين بالتحليل باستخدام الكروماتوغرافيا السائلة عالية الاداء مع كاشف الأشعة فوق البنفسجية. وتضمنت البارابينات تحت الدراسة كل من باربين الميثيل، الإيثيل، البروبيل والبوتيل. تم إجراء عملية تحسين للمتغيرات التجريبية، وتراوح الانحراف المعياري النسبي المئوي ما بين 0.09 – 2.81 % بينما وصلت قيمة مربع معامل الإرتباط إلى 0.9962 في مدى من التراكيز تراوح بين 0.5 – 200 نانوغرام لكل جم، وتراوحت الحدود الأدنى الكشف بين 0.005 – 0.0244 نانوغرام لكل جم. تم إجراء اختبار مبدئي للطريقة على عينات من الجمبري لإثبات قابليتها للتطبيق على المصفوفات البيولوجية المعقدة. أظهرت الدراسات الأولية على أنسجة سرطانية للمبيض في الإنسان وجود هذه البارابينات، كما أظهرت التحاليل وجود مستويات أعلى من البارابينات في عينات الأنسجة الخبيثة مقارنة بعينات الأنسجة السرطانية الحميدة.

للمرة الأولى تم استخدام مسحوق بذور البان الزيتوني كمادة مازة طبيعية قليلة التكلفة وفعالة في الاستخلاص الدقيق بالمادة الصلبة، حيث إنها تحتوي على مجموعات وظيفية مختلفة وشبكة من الألياف المترابطة. تعتبر هذه الطريقة في الاستخلاص طريقة متكاملة، حيث تشتمل على إزالة البروتينات والمواد الدهنية، واستخراج و تركيز التحاليل المستهدفة في خطوة واحدة. وقد تم اختيار ثلاثة عشرة من المركبات المستهدفة من استرات الفثالات لتطوير وتقييم الطريقة. تمت

دراسة بعض المعايير الأساسية التي تؤثر على كفاءة الاستخلاص مثل نوع الغشاء، كمية المادة الماصة زمن الاستخلاص. كما تمت دراسة نوع وحجم مذيب الإنحلال والزمن اللازم لذلك، وتأثير إضافة الملح. تحت الظروف الأمثل تم الحصول على مجال خطي جيد جدا لجميع المواد المحللة تحت الدراسة بمعاملات ارتباط تراوحت ما بين 0.9768 و 0.9977، وتراوحت الحدود الأدنى الكشف بين 0.01 – 1.2 ميكروغرام لكل لتر. أظهرت الطريقة المقترحة نتائج تكرار مرضية مع انحرافات معيارية نسبية مئوية تراوحت بين 3.6 – 10.2 % (عدد مرات التكرار = 7). أخيراً، تم تطبيق هذه الطريقة لتقدير استرات الفثالات في مجموعة من عينات الحليب المعبأة.

تم إدخال تعديل جديد في الغشاء للاستخلاص الدقيق بواسطة مادة صلبة للكشف عن مركبات ثنائي الفينيل متعدد الكلور في عينات مصل الدم . في هذا التركيب الجديد تم وضع قضيب تحريك مع المادة الماصة داخل غشاء البولي بروبلين وتم قفله بالحرارة لمنع محتوياته من التسرب للخارج. هذا الترتيب يمنع المادة المازة من الالتصاق بجدار الغشاء كما يمنعها من الطفو فوق سطح المحلول مما يزيد من مساحة سطح المادة المازة. بعد استخلاص مركبات ثنائي الفينيل متعدد الكلور من عينات مصل الإنسان، تم تحليلها بواسطة كروماتوغرافيا الغاز المقترنة بمطياف الكتلة. تم تحسين العوامل التي تؤثر على كفاءة الاستخلاص، وتحت الظروف الأمثل تم الحصول على مجال خطي جيد جدا في مدى من التراكيز تراوح بين 0.1 – 100 نانوغرام لكل مل بمعاملات ارتباط تراوحت ما بين 0.9868 و 0.9992، بينما تراوحت الحدود الأدنى الكشف بين 0.003 – 0.047 نانوغرا لكل مل. أظهرت الطريقة المقترحة نتائج تكرار مقبولة مع انحرافات معيارية نسبية مئوية تراوحت بين 3.2 – 9.1 % في أيام مختلفة و 3.1 – 7.2 % خلال اليوم الواحد.

في عمل آخر، تم دمج أكسيد الزنك ذو الحجم النانوي في رغوة الكربون بغرض تكوين مادة مازة لاستخلاص مبيدات الكلور العضوية من عينات الحليب. تم تحضير المادة المازة بتفاعل بسيط وفي خطوة واحدة بين السكروز و نترات الزنك. وتم تشخيصها بواسطة المسح الإلكتروني المجهري وحيود الأشعة السينية. تمت تعبئة هذه المادة المازة داخل غشاء البولي بروبلين المسامي والذي تم الصاق جوانبه بالحرارة لعمل أداة للاستخلاص الدقيق بواسطة مادة صلبة. تم تحسين العوامل التي تؤثر على كفاءة الاستخلاص، وتحت الظروف الأمثل تم الحصول على مجال خطي جيد جدا وصلت إلى 0.9998، وتراوحت الحدود الأدنى الكشف بين 0.19 – 1.64 نانوغرا لكل مل. أظهرت الطريقة نتائج تكرار مقبولة مع انحرافات معيارية نسبية مئوية تراوحت بين 2.3 – 10.1 % في أيام مختلفة وخلال اليوم الواحد.

في الجزء الأخير من هذا البحث تم فحص مركبات الكلور العضوية ومركبات ثنائي الفينيل متعدد الكلور في عينات حقيقية لأنسجة سرطانية وعينات بلازما بشرية وتمت دراسة أنماط التراكم وذلك لفرز هذه المركبات التي أوضح الكشف عنها عن وجودها في معظم الأحيان.

CHAPTER

INTRODUCTION

1.1. Analysis of endocrine disrupting compounds in biological samples

Endocrine disrupting compounds (EDCs) are the chemicals that interfere with endocrine system in human and wildlife. These chemicals disrupt the endocrine system by interacting with hormonal functions [1]. Exposure to EDCs may induce developmental, reproduction, neurological and immunological disorders. EDCs have also been associated with diseases like cancer, diabetes and decreased fertility. Most of the EDCs are persistent in nature and have ability to accumulate in biological tissues.

It is the matter of fact that health related issues increasing and more and more abnormalities are being associated with the use of EDCs. Some recent reports show that EDCs has resulted in increased rates of testicular and thyroid cancers among adult male population [2][3]. EDCs have very high impact on insulin signaling, reproductive health, functioning of thyroid and bone growth. Some PCBs and pesticide atrazine has been reported to have effect over proteomes of the breast cancer cells. EDCs are present in environment as mixture and they can have synergistic or additive effects. [4].

The man-made EDCs include pesticides, polychlorinated biphenyls, personal care products, plasticizers, heavy metals and other industrial products. Such EDCs are abundant

in the environment. Human exposure to these chemicals may occur through inhalation, dermal contact and digestion. Contaminated air, water and food are major sources of EDCs.

There is the lack of knowledge on the role of EDCs in causing different kind of health related issues in Kingdom of Saudi Arabia. This is leading factor which motivates to screen biological samples for possible presence of EDCs. Since the country is the largest producers of oil based products which might result in the production of toxic by-products released into the environment. Studies in other parts of the world have associated presence of EDCs in the break down products released from oil combustion and they share similar structures to the EDCs [5–8]. Such compounds could enter the system through contaminated air, water and food. Till now, no studies were performed to access the effects of these toxic compounds in triggering different endocrine related cancers in the Kingdom. The study attains more importance considering the drastic increase in the cases of different endocrine related cancers in the Kingdom in the recent years which has been mostly attributed to changes in life style and over dependence on processed food products which might be exposed at some stage to these harmful chemicals [9].

EDCs become functional at very low concentrations. The presence of EDCs in biological samples such as tissues, milk, blood and urine can give an insight to their exposure. Thus, accurate measurements of EDCs in biological samples are needed. As biological samples represent very complex matrix, sample preparation is an unavoidable step before instrumental analysis of EDCs in biological samples. This sample preparation typically involves removal of interferences, preconcentration of target compounds and extract clean up. Moreover, an ideal sample preparation method for biological samples should utilize minimum amount of biological samples.

Traditional sample preparation methods such as solid phase extraction (SPE) and liquid-liquid extraction require large amount of sample. In addition, they utilize extremely high volumes of hazardous organic solvents and other chemicals. Thus, the area of sample preparation is now shifting toward development of miniaturized extraction techniques. In last few years, number of new microextraction techniques have been developed. These techniques include solid phase microextraction (SPME), liquid phase microextraction (LPME), dispersive solid phase extraction (d-SPE), dispersive liquid-liquid microextraction (DLLME) and many of their modified forms. These techniques utilize are either solventless or utilize small volume of organic solvents (up to few hundred microliters). However, for analysis of complex biological samples, these techniques have some limitations such as SPME fiber cannot be directly immersed in biological samples because fats can adsorb over it. Similarly, DLLME require pretreatment of the sample, as it best suits for clean samples. The major objective of this work is to develop the sample preparation methods that can be utilized for screening of EDCs in biological samples.

1.2. Thesis Summary

This section provides brief summary of each chapter. Each chapter is written in manuscript format.

1.2.1. Chapter-2

Chapter-2 provides a comprehensive literature survey on advancements and challenges in analysis, and health effects of endocrine disrupting and persistent organic pollutants. Persistent and endocrine disrupting organic pollutants pose serious health risks to humans

and wild life due to their long half-lives, bioaccumulation and toxicity. These compounds have negative impact on human health and induce a variety of carcinogenic and non-carcinogenic disorders. Extremely low concentrations of these compounds can induce adverse health effects in human. Efficient analytical methods are always demanded in order to extract and determine such low concentrations in environmental and biological samples. In the last decade, studies mostly focused on profiling and identification of persistent and endocrine disrupting pollutants in tissues and body fluids using various analytical methods. However, investigation of trace level pollutants associated with various disorders requires complex analytical protocols. Biological matrix presents serious challenge in extraction of these pollutants mainly because of its complexity and inherent limitations of extraction procedures. The advantages and pitfalls of recent extraction approaches are highlighted. Analysis of these compounds is carried out by using gas and liquid chromatographic methods coupled with different detectors. The current status of advancements in area of analytical chromatography and accomplishments and weaknesses have been pointed out. At the end, some health effects and clinical correlates of persistent and endocrine disrupting organic pollutants are encapsulated.

1.2.2. Chapter 3

This chapter describes an application of microwave assisted micro-solid-phase extraction for screening of parabens in human ovarian cancer tissues. Parabens (alkyl esters of p-hydroxybenzoic acid) are widely used as preservatives in food, cosmetics and pharmaceutical products. However, weak estrogenicity of some parabens has been reported in several studies, which provided the impetus for this work. Here, a simple and efficient analytical method for quantifying parabens in cancer tissues has been developed. This

technique involves the simultaneous use of microwave-assisted solvent extraction (MASE) and micro-solid phase extraction (μ-SPE), in tandem with high performance liquid chromatography (HPLC/UV) analysis for the determination of parabens. The pollutants studied included four parabens (methyl, ethyl, propyl and butyl parabens). Optimization of the experimental parameters for MASE and μ-SPE was performed. Good relative standard deviation (%R.S.D) ranged from 0.09 – 2.81% and high enrichment factors (27 – 314) were obtained. Coefficients of determination (r^2) up to 0.9962 were obtained across a concentration range of 5.0- 200 ng g^{-1}. The method detection limits for parabens ranged from 0.005 - 0.0244 ng g^{-1}. The procedure was initially tested on prawn samples to demonstrate its feasibility on a complex biological matrix. Preliminary studies on human ovarian cancer (OC) tissues showed presence of parabens. Higher levels of parabens were detected in malignant ovarian tumor tissues compared to benign tumor tissue samples.

1.2.3. Chapter 4

This chapter describes method development for screening of phthalate esters in milk samples. In the present study, a natural sorbent based micro-solid phase extraction (μ-SPE) was developed for determination of phthalate esters in milk samples. For the first time, an efficient and cost effective natural material (seed powder of *Moringa oleifera*) was employed as sorbent in μ-SPE. The sorbent was found to be naturally enriched with variety of functional groups and having a network of interconnected fibers. This method of extraction integrates different steps such as removal of proteins and fatty stuff, extraction and pre-concentration of target analytes into a single step. Thirteen phthalate esters were selected as target compounds for the development and evaluation of method. Some key

parameters affecting the extraction efficiency were optimized, including selection of membrane, selection and amount of sorbent, extraction time, desorption solvent, volume of desorption solvent, desorption time and effect of salt addition. Under the optimum conditions, very good linearity was achieved for all the analytes with coefficient of determinations (R^2) ranging between 0.9768 and 0.9977. The limits of detection ranged from 0.01 to 1.2 µg L^{-1}. Proposed method showed satisfactory reproducibility with relative standard deviations ranging from 3.6% to 10.2% (n=7). Finally, the developed method was applied to tetra pack and bottled milk samples for the determination of phthalate esters. The performance of natural sorbent based µ-SPE was better or comparable to the methods reported in the literature.

1.2.4. Chapter 5

This chapter describes a novel modification in membrane protected micro-solid phase extraction for screening of polychlorinated biphenyls in serum samples. This new configuration of micro-solid phase extraction was termed as stir-bar supported micro-solid-phase extraction (SB-µ-SPE). A tiny stir-bar was packed inside the porous polypropylene membrane along with sorbent material and the edges of membrane sheet were heat sealed to secure the contents. The packing of stir-bar inside the µ-SPE device, does not allow the device to stick with the wall or any corner of the sample vial during extraction, which is, however, a frequent observation in routine µ-SPE. Moreover, it enhances effective surface area of the sorbent exposed to sample solution through continuous agitation (motion and rotation). It also completely immerses the SB-µ-SPE device in the sample solution even for non-polar sorbents. Polychlorinated biphenyls (PCBs) were selected as model compounds and the method performance was evaluated in human serum samples. After

extraction, samples were analyzed by gas chromatography mass spectrometry (GC-MS). The factors that affect extraction efficiency of SB-µ-SPE were optimized. Under optimum conditions, a good linearity (0.1 – 100 ng mL^{-1}) with coefficients of determinations ranging from 0.9868 to 0.9992 were obtained. Limits of detections were ranged between 0.003 and 0.047 ng mL^{-1}. Acceptable values for inter-day (3.2 – 9.1%) and intra-day (3.1 – 7.2%) relative standard deviations were obtained. The optimized method was successfully applied to determine the concentration of PCB congeners in human serum samples.

1.2.5. Chapter 6

Organochlorine pesticides belong to famous persistent organic pollutants and are well known for their harmful effects on human health and wildlife. The milk stands among highly consumed foods and it can get contaminated with OCPs through agricultural food that is provided to animals. This demands development of highly efficient and precise analytical methods for determination of OCPs in milk samples. In this work, ZnO nanoparticles incorporated porous carbon foam was utilized as sorbent for extraction and preconcentration of OCPs in milk samples. This sorbent was prepared by a single step and fast reaction between the sucrose and zinc nitrate that was accomplished by heating the mixture for 10 minutes. The resulting sorbent was characterized by scanning electron microscopy and X-ray diffraction. The sorbent was then packed inside a porous polypropylene membrane sheets by heat sealing of membrane to construct µ-SPE devices. The factors that affect performance of µ-SPE were optimized. This method provided good linearity up to 0.9998 with limit of detections ranging from 0.19 to 1.64 ng/mL. This

method also provided satisfactory values for intra and inter-day precision with RSDs ranged between 2.3 to 10.2%.

1.2.6. Chapter 7

This chapter describes screening of endocrine disrupting compounds in different types of human cancer tissues. Endocrine disrupting compounds are well known for interfering with normal hormonal activity in human and thus triggering different kind of carcinogenic and non-carcinogenic disorders. Organochlorine pesticides (OCPs) and polychlorinated biphenyls (PCBs) have negative impact on the human health. They are persistent in nature and accumulate in cells and tissues and induce different type of cancers as a result of endocrine disruption. We have evaluated the burdens of OCPs and PCBs in breast, colorectal, thyroid and kidney cancer tissues collected from Saudi Arabia. The compounds were extracted using ultrasonic assisted solvent extraction and determined by gas chromatography mass spectrometry. ΣOCPs were highest in colorectal (3951.8 ng/g) and breast (3921.3 ng/g) tissues while significant concentrations were also found in thyroid and kidney tissues. ΣPCBs were highest in thyroid tissues followed by colorectal, breast and kidney tissues. ΣOCPs were several times higher than ΣPCBs in all types of cancer tissues. In order to get more deep insight into concentrations and bioaccumulation trends in cancer tissues, OCPs were divided into five groups of closely related compounds. Concentrations of ΣBHCs were higher than ΣCHLs, ΣDDs, Σdrins and Σendosulfan in all type of tissues except the kidney where ΣCHLs were slightly higher than ΣBHCs. These high concentrations of OCPs and PCBs in cancer tissues suggest that the environment of Saudi Arabia is heavily polluted with these pollutants.

1.2.7. Chapter 8

This chapter describes screening of organochlorine pesticides in the plasma samples of the patients suffering from different type of cancer. In this study, plasma samples were collected from 73 patients suffering from colorectal or breast cancers. Fifteen organochlorine pesticides (OCPs) were determined in all samples. The volume of the plasma sample that was used for extraction of target compounds was 500 µL. The extraction was carried out by adding 1.0 mL of toluene into plasma samples in Eppendorf vial and then sonicating for 5 minutes. The vials were placed in refrigerator for 1 hour to freeze the plasma and water content inside the vial. Then, 1 µL from the upper layer was injected into GC-MS. Out of fifteen OCPs analyzed, only three compounds (heptachlor, endrin and endrin ketone) were detected in plasma samples. Data analysis was carried out by dividing plasma samples based on malignancy, malignancy type and stage. The data analysis indicated that heptachlor and endrin were only present in the plasma samples of the patients with malignancies while they were absent in the samples of patient with non-malignant tumors. This might be an indication that exposure to heptachlor and endrin can be a cause of malignancy. However, this needs to be confirmed by the cell line studies. Heptachlor and endrin were only found in the plasma samples of colorectal cancer patients while they were not detected in any of the samples collected from breast cancer patients. It indicates that these two compounds may have a dominant role in triggering colorectal cancer tissues. Endrin ketone was detected in the samples of colorectal as well as breast cancer patients with high mean concentrations of 154.57 and 83.84 (ng/mL) respectively. Moreover, endrin ketone was found in samples of all stages with high mean concentrations in stage I and III.

1.2.8. Chapter 9

A short summary of conclusions and recommendations is presented in this chapter.

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2. CHAPTER

PERSISTENT AND ENDOCRINE DISRUPTING ORGANIC POLLUTANTS: ADVANCEMENTS AND CHALLAENGES IN ANALYSIS, HEALATH CONCERNS AND CLINICAL CORRELATES

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Abstract

Persistent and endocrine disrupting organic pollutants pose serious health risks to humans and wild life due to their long half-lives, bioaccumulation and toxicity. These compounds have negative impact on human health and induce a variety of carcinogenic and non-carcinogenic disorders. Extremely low concentrations of these compounds can induce adverse health effects in human. Efficient analytical methods are always demanded in order to extract and determine such low concentrations in environmental and biological samples. In the last decade, studies mostly focused on profiling and identification of persistent and endocrine disrupting pollutants in tissues and body fluids using various analytical methods. However, investigation of trace level pollutants associated with various disorders requires complex analytical protocols. Biological matrix presents serious challenge in extraction of these pollutants mainly because of its complexity and inherent limitations of extraction procedures. We outline advantages and pitfalls of recent extraction approaches. Analysis of these compounds is carried out by using gas and liquid chromatographic methods coupled with different detectors. We have reviewed current status of advancements in area of analytical chromatography and accomplishments and weaknesses have been pointed out. At the end, we encapsulate some health effects and clinical correlates of persistent and endocrine disrupting organic pollutants.

Keywords

Persistent organic pollutants, endocrine disrupting compounds, micro extraction, biological matrix, gas and liquid chromatography, mass spectrometry, health effect

2.1. Introduction

Persistent organic pollutants (POPs) represent a toxic class of organic chemicals which have potential to travel in lipids, accumulate in tissues and induce variety of health complications. Because of very long half-lives, they manage to sustain in environment for years and that is the reason why some POPs can be still found in the environment although their use was banned in early 1970s. The causalities caused by these chemicals are blowout throughout the history, but the more terrible aspect arises from the fact that this series of events have not stopped yet. A more recent story is published about excessive use of endosulfan based pesticides in an Indian state, Kerala, where almost 45000 people have been reported suffering from different diseases being victim of haphazard usage of such chemicals in pesticides [1].

More chemicals are being added to the famous “dirty dozen” which were confirmed as POPs by Stockholm Convention and now this number is preceded to 23. This is a significant increase over a period of one decade. Moreover, number of other chemicals have been reported to possess persistent nature and ability to accumulate within biological matrix and have not been regulated yet. Stockholm convention categorizes these 23 POPs under different annexes like A, B and C. POPs in annex A are banned for production, use and applications. POPs belonging to Annex B are also banned for production and use but there exist certain exemptions. POPs in annex C are produced as a consequence of some unintended processes. It is pretty important to note that these 23 compounds have been regulated but there are hundreds of other organic compounds which show similar characteristics but have not been paid that much attention with regard of regulations. For

examples some organo-metals such as methylmercury and other derivatives are highly persistent and tend to bioaccumulate in food chain and human body [2].

POPs present a global challenge and hundreds of reports are published every year describing current status, concentrations in different matrix and health effects. Primary sources of POPs, although, have been banned but they had heavily contributed in developing secondary sources which include seawater, sediments, aquatic organisms, other water bodies, and vegetation soils. These secondary sources are expected to release POPs for next hundred years or even more. POPs have been extensively reviewed from different aspects in past few years.

Many organic pollutants including POPs are well-known to interfere with endocrine system by imitating, hindering and prompting normal activity of hormones and thus effect the health and reproductive system of humans and wildlife [3]. These compounds are named as endocrine disrupting compounds (EDCs). Number of these compounds is increasing day by day due to continuous consumption and applications in the industrial sector. These compounds are basically of xenobiotic and exogenous origins which are considered to have adverse effect on the normal action of endocrine system and disturb all the functions i.e. synthesis, secretion, transport, and binding of hormones.

EDCs are broadly classified into four categories (i) naturally occurring androgens and estrogens (ii) artificially synthesized androgens and estrogens (iii) phytoestrogens (iv) other industrial compounds [4]. Synthesized or industrial EDCs are members of different classes of chemical compounds and they have been identified in all industrial products including pesticides, alkyl phenols, personal care products, polychlorinated biphenyls,

heavy metals and so on. Synthesized EDCs were basically designed to perform a certain kind of action such as plasticizer, solvent or pesticide but later on it was realized that they have functional properties which can result in disruption of endocrine systems.

The studies describing adverse effects of such compounds on human health have been increased in recent years [5]. As a result of rapid industrialization throughout the world, the production of such chemicals and their introduction into environment has massively increased. Prolonged exposure to trace level concentrations of these compounds can induce very serious health complications in human body and wild life [6]. Figure 2-1 summarizes sources, exposure pathways, health effects and analytical strategies for analysis of POPs and EDCs. Table. 2-1 lists some organic pollutants which show different kind of endocrine related activities. In the earlier years, it was thought that the EDCs act through nuclear hormone receptors (genomically) but now it has been recognized that mechanism of interaction of EDCs with body involves many other pathways (non-genomically) as well [3]. Endocrine disrupting mechanism of chemicals is described in a recent review article [85].

It is matter of fact that POPs and EDCs occur at trace levels in various environmental and biological samples and thus their detection and removal presents a great challenge to analytical chemists. It remained a major focus of researchers to extract and detect extremely low concentrations of these toxic pollutants. This has been accomplished by developing microextraction procedures for sample preparation in combination with advances in analytical instrumentation. This review is aimed to probe current status and challenges in extraction and detection approaches for analysis of minute concentrations of POPs and EDCs in environmental and biological matrices. Chromatographic methods have

been considered in detail and accomplishments, weaknesses and challenges in dealing with such methods will remain a major focus of this review. We have also listed some studies investigating health effects of POPs and EDCs to human and wildlife. A brief detail of clinical correlates of EDCs and database resources used to identify perturbations in gene and pathways by environmental endocrine disrupters is also included.

2.2. Specific Classes of Persistent and endocrine disrupting organic pollutants

We describe three major classes of persistent and endocrine disrupting compounds; polychlorinated organic compounds represent a very well established class of persistent and endocrine disrupting organic pollutants while other two classes mentioned in section 2.2.2 and 2.2.3 represent some emerging persistent chemicals.

2.2.1. Polychlorinated organic compounds

This class of compounds mainly includes organochlorine pesticides (OCPs), polychlorinated biphenyls (PCBs), polychlorinated dibenzofurans (PCDFs), and polychlorinated dibenzo-p-dioxins (PCDDs). Such compounds are defined by aromatic structures, low water solubility and high chemical stability. PCDFs and PCDDs are obtained as a product of some chemical, photochemical, thermal and enzymatic reactions. These compounds are discharged into environment via some incineration plants which include clinical, municipal and industrial waste incinerators[7].

PCBs are persistent synthetic lipophilic compounds, except few of them. In past, PCBs had been used in variety of consumer products including plastics, lubricants, capacitors, vacuum pumps, paints, transformers, flame retardants, adhesives, gas transition tubes,

hydraulic fluids, sealants, and heat transfer fluids but their manufacturing was stopped in late 1970s. Recent reports reveal their measureable levels in the serum [8]. PCB concentrations vary from place to place depending on geographical conditions. Higher concentrations are found in urban areas relative to rural areas which show substantial contribution from the local sources situated in these areas [9]. These compounds have ability to bio accumulate in food chain and finally reach to human body. PCBs have also been reported to induce diabetes and also have strong effect on thyroid signaling [10]. Not only had the PCBs, all classes of polychlorinated compounds were studied to determine their possible character as endocrine disrupters and various correlations were observed. Tetrachlorodibenzo-p-dioxin (TCDD) has been reported as an inhibitor of estrogen mediated activity but their exact mechanism is not known yet [11]. Another complication about PCBs is that they do not show antiestrogenic activity but in reality they are estrogenic.

Classification of pesticides is done based on their action with different substances. They are generally classified as insecticides, germicides, fungicides, herbicides, rodenticides, avicides, larvicides, and acaricides. They are composed of organic compounds which are volatile, semi-volatile or non-volatile in nature. Pesticides have widespread applications in the agriculture sector to grow crops and different food stuff. Through food chain, these pesticides find their way to human body and wild animals. Volatile pesticides may be present in outdoor environment and thus accumulate with various materials. Pesticides are added to carpets, paints and building materials, this makes indoor environment suspected for their presence and accumulation on stuffs like toys, carpets and any other material [8].

Although persistent organic compounds like OCPs were banned to use as pesticides but still they can be detected in environmental samples. Stockholm convention has declared so for 23 POPs and among them 13 belongs to OCPs. These include aldrin, dieldrin, endrin, chlordane, heptachlor, DDT, hexachlorobenzene, toxaphene, mirex, lindane, chlordecone, α-hexachlorocyclohexane and β-hexachlorocyclohexane [12].

Generalizing health effects of OCPs is a really difficult task because they comprise a diversity of chemicals. Pesticides are reported to have very severe neurotoxicity, effects on developing reproductive system, ability to induce various forms of cancers. They also damage the normal function of thyroid hormones in humans and animals [13].

2.2.2. Pharmaceuticals and personal care products

Although pharmaceuticals and personal care products (PPCPs) have not been declared POPs but they are thought to be potential endocrine disruptors and there are a lot of questions about their persistence in environment. The presence of pharmaceuticals and their bioactive metabolites in environment has been studied very extensively in last decade. The part of environment which is focused in this regard is water and particularly drinking water. Most comprehensive study for emerging organic pharmaceuticals in water resources was carried out by U.S Geological Survey. A wide range of pollutants such as antibiotics, prescription and non-prescription pharmaceutical drugs were detected [14].

It has been reported by many researchers that many bacterial strain has developed resistance against existing antibiotic drugs and there is a need to find new antibacterial agents. Keeping in view this aspect of resistance, natural synthetic chemists are searching and preparing new drugs to overcome resistance offered by bacterial strains [15]. Their

number is increasing and through wastewater or sewage discharges, they absorb into soil or sediments and remain persistent. Persistence depends on photo stability, degradation capacity and leaching to water. Concentrations higher than estrogenic or proestrogenic end point are found in environment samples. Most of veterinary drugs go with manure which is spread in agricultural fields and enter into food and water chain. Some reports indicated their improper removal from wastewater that leads to contaminate other water resources [16].

Personal care products (PCPs) are being used by all communities in the world as disinfectants, conservation agents, fragrances and sunscreens. Fate of PPCPs in any environment is governed by chemical, environmental, retention and transport, and accumulation factors [17]. Chemical compounds which are added to sunscreen cosmetics to save the consumers from the harm effects of solar UV radiation are known as Organic UV filters. But these compounds are not only being added to sunscreen cosmetics but all type of cosmetic products. Although they are applied on the outer skin but they have ability to absorb through skin and take part in metabolism resulting in bioaccumulation or excretion. This absorption may also trigger estrogenic [18] and carcinogenic activity [19] . Biotransformation pattern decides about the fate of an exogenous compound in the body and it has been evaluated for only few compounds only in serum and plasma and hence there is a pressing need to investigate biotransformation patterns of all other compounds in variety of tissues (liver, brain, heart, kidney, intestine, skin) [20]. Parabens are also widely used in cosmetics, pharmaceutical and preservatives in food. They have been widely adopted because of their low toxicity, inertness, broad activity, low cost and long history of safe use. However, they have now been reported to show weak estrogenicity which

encourages to develop analytical methods for their quantification at low level concentrations [21].

2.2.3. Phthalates and food contact materials

Phthalates have very wide spread applications in cosmetics, plasticizers, insecticide carriers, chloride resins, adhesives and cellulose film coatings. The worldwide production of these compounds is more than 2.7 million metric tons per year and they are released to environment via direct or indirect corridors. Direct emissions take place during the production of plastics while indirect emissions take place through leaching and volatilization from plastic products and bottles particularly when they are subjected to disposal and incineration. Large number of phthalates is included in “priority pollutants”. Although these substances have low acute and chronic toxicity but they are suspected EDCs [22].

Food contact materials (FCMs) are the chemicals which leach from the packing of food and contaminate it. They are underestimated source of chemical but humans are exposed to these EDCs on regular basis. Population is exposed to low level concentration of FCMs throughout their lives. Exposure of general public to contaminants by FCMs is quantified based on amount of food consumed and FCMs leached into food. Food simulants are used for this purpose but they don’t predict leaching with desired precision. A famous FCM substance, Bisphenol A (BPA), its leaching is always misjudged based on conventional methods. Exposures of these compounds are normally analyzed from mutagenicity and geno-toxicity. In this approach all other toxicological effects are neglected including endocrine disruption, development toxicology and toxicity due to combined effects of

several FCM chemicals. Keeping in view these new toxicology parameters, the more sensitive population group comprises children, women of children bearing age and women with pregnancy. Any innovative method for assessment of toxicology due to FCM should incorporate all toxicological parameters and routine analysis of EDCs, with special reference to sensitive age groups [23].

Plastics are the most famous materials used for packaging of food and thus their polymeric layers are always in contact with food materials. Plastics, coatings and polymeric layers are combination of complex chemical substances. Understanding the nature of all these chemicals would be helpful to calculate risk assessment related to FCMs. Chemistry involved in formation of polymeric plastics is very complex and some unknown chemicals are added to the final products which can migrate into food. It is difficult to identify all the substances in the plastics even if starting chemicals and additives are well known particularly due to non-intentionally added substances. Monomers and additives are considered while assessing the toxicity effects, while rest of the leachate is ignored. It is pertinent to mention that in final packaging of food, adhesives, printing inks and labels may introduce some supplementary compounds into the food [24]. This department of food contamination should be focused more because all the population is unceasingly exposed to these products.

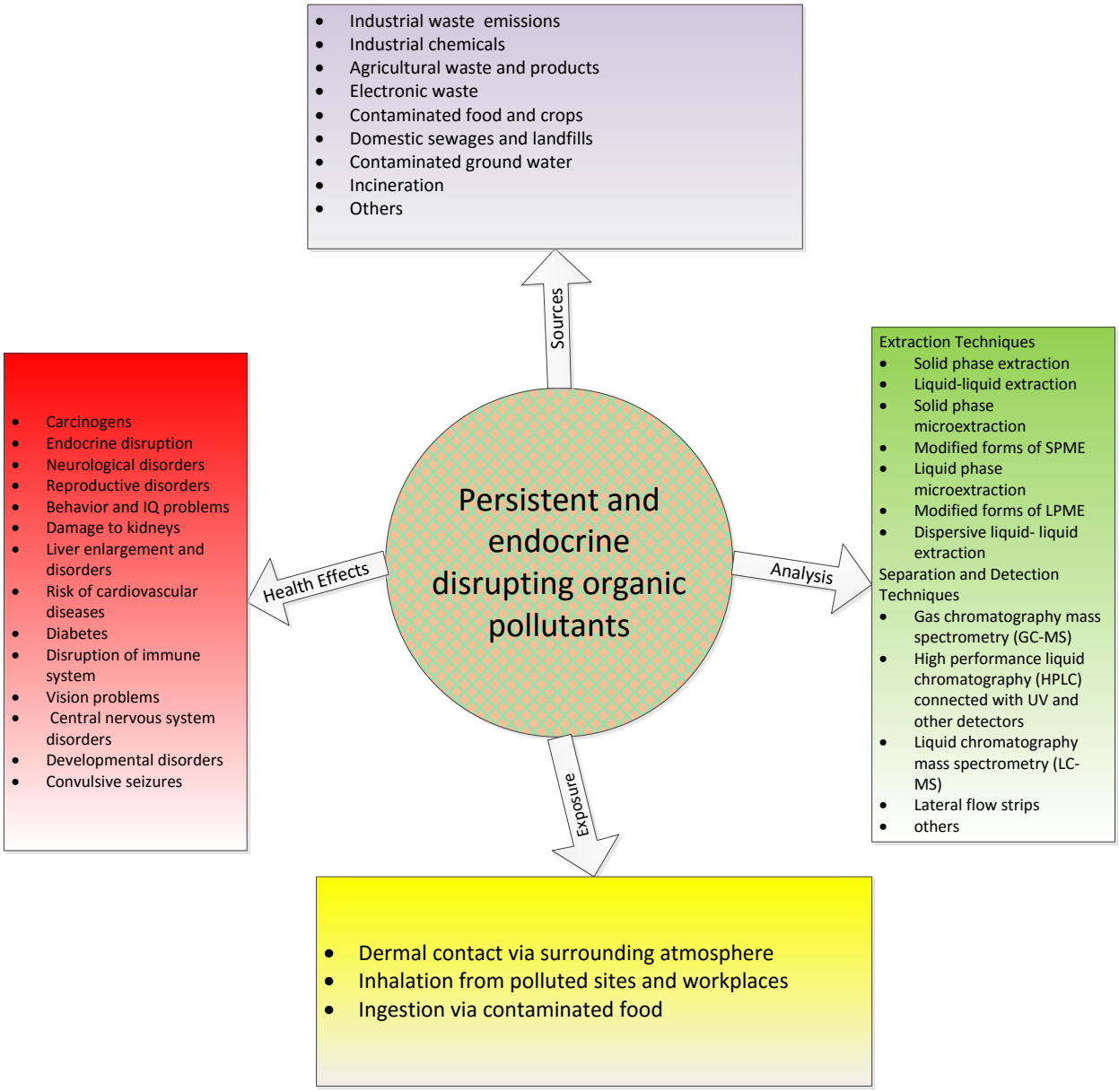

Figure 2- 1: Summary of sources, health effects, and routes of exposure to human and analysis of persistent and endocrine disrupting organic pollutants

Table 2- 1: List of Some common organic compounds which show endocrine disrupting properties

Class of compounds	**Chemicals**	**Uses**	**Problems induced in human**	**Activity**	**Ref.**
Pesticides	DDT, Chlordane, Methoxychlor	Insecticides, fungicides etc.	Neurotoxicity, Disorders in reproductive systems, cancers including breast cancer	Estrogenic, anti-estrogenic, anti-androgenic	[86,87] [8]
Polybrominated compounds	Polybrominated diphenyl ethers (PBDEs)	Flame Retardants	Harm to developing brain and reproductive organs	Effect on thyroid hormones	[88]
Plolychlorinated compounds	Polychlorinated biphenyls (PCBs), Dioxins	Electrical equipment, by product of incineration	Endometriosis	Anti-estrogenic	[3]
Phthalates	Dibutyl phthalate, Benzylbutyl phthalate	Plasticizers, detergents, resins	Infertility, effects on developing male reproductive tract	Estrogenic	[89]
Alkylphenols	Octylphenol, nonylphenol	surfactants	Reproductive disorders in	Estrogenic	[90]

			human, carcinogenic		
Parabens	Para-substituted hydroxybenzoate	Preservatives and anti-microbial agents	Decreased sperm number and motile activity	Estrogenic	[91]
Natural and synthetic hormones	Estradiol, 17β-Estradiol, Estrone, Estriol Ethinylestradiol		Reproductive disorders	Estrogenic	[45]

2.3. Methods for analysis of POPs and EDCs and problems associated with them

2.3.1. Extraction procedures

Efficient extraction and sample preparation methods are required to extract extremely low level concentrations of POPs and other potential emerging pollutants present in complex environmental and biological matrices. Classical extraction techniques are time consuming and labor extensive because several clean-up cycles are needed after extraction which may take several hours to few days [25].

Solid phase extraction (SPE) and liquid-liquid extraction (LLE) are among most commonly employed extraction procedures for POPs in complex environmental and biological samples. SPE uses a solid sorbent which captures a particular analyte through adsorption when liquid or gas sample is passed through it. A washing solution is used to remove any unwanted constituents captured with target analyte and finally target analyte is eluted by using a suitable organic solvent or mixture of organic solvents. Selection of suitable combination of sorbent material and solvents for extraction of particular class of target compounds is critical. The sorbent is normally packed into small tubes or cartridges that are available in different shapes and prepared by many companies with specific housing for suction or pressure generation to make the elution through the column a faster process.

Conventional SPE and LLE require large volume of organic solvents and thought to be environment unfriendly. Moreover, requirement of large volumes of samples, huge time consumption and multiple clean-up procedures make them rather difficult extraction techniques. In current years, there remained a trend in development of miniaturized

extraction procedures which can be easily automated and configured according to analysis requirement and more importantly they are environment friendly. The main idea behind miniaturized extraction techniques is development of methods which can easily extract target compounds at trace level concentration in different matrices by employing minimum amount of sorbents, samples and solvents. But still they are accurate, precise, and reproducible.

Solid phase microextraction (SPME) uses fibers coated with different organic materials and these fibers are either dipped in the aqueous samples or placed in headspace in case of complex matrix. Target compounds are adsorbed or absorbed on the fiber which is then injected to GC column where analytes are desorbed by providing high temperature for a certain period of time. Very high enrichment factors are achieved by using SPME. SPME can be fully automated with GC systems. SPME was used for extraction of polybrominated diphenyl ethers (PBDEs) in sediments [26]. Another recent publication describes use of headspace SPME (HS-SPME) for extraction of POPs in serum samples followed by GC-MS analysis. As this HS-SPME was coupled with instrument, so the steps like sample handling, removal and transfer of solvents, separate extraction steps and sample transferring to instrument station were reduced. High enrichment factors are obtainable because analyte transfers from relatively large volume of liquid sample to solid fiber. SPME has some limitations which include expensive and fragile fibers, lesser affinity and selectivity of commercial fibers towards target compounds [27].

Liquid phase microextraction (LPME) has been attempted in various ways. Earlier in 1997, static and dynamic LPME was developed using a single drop of organic liquid at the tip of conventional micro syringe. In static mode, tip of syringe is immersed in aqueous sample

and then a drop of organic liquid is formed at the tip and exposed for certain time to the sample and retract back into syringe and injected to the instrument for analysis. In dynamic mode, organic solvent is taken in a micro syringe and syringe is immersed in the sample solution. Few microliters of samples are withdrawn into syringe and then re-injected into sample vial. Procedure is repeated for several times and finally organic drop from the syringe is injected into instrument [28]. Single drop LPME (SD-LPME) has also been tried as headspace technique, but the stability of the drop is main drawback because most of the organic solvents have high vapor pressures and they are easily evaporated. So in such case, selection of suitable solvent is crucial. Another modification in LPME procedure was done by putting a hollow fiber at the tip of the syringe. Fiber is filled with organic solvent which activates the pores of the fiber. Then this is exposed to liquid samples. Small pore size of the fiber does not allow large sized molecules and hence this can be an ideal method for extraction of organic pollutants from complex biological matrix because it can easily reject any unwanted fatty stuff [29]. Hollow fiber protected LPME (HF-LPME) can easily be used for headspace because it provides a support for the organic solvent to be held for longer time [30].

Dispersive liquid-liquid microextraction was developed in 2006 for extraction of PAHs in water samples. This involves a rapid injection of proper mixture of extraction and disperser solvent in water samples which changes to cloudy and extraction solvent is dispersed throughout the sample and is recollected by centrifugation. Method has been extremely used for extraction of organic pollutants from aqueous samples [31]. More recently, DLLME was used for extraction of organochlorine pesticides (OCPs) in honey [32]. The major advantages of DLLME lie in less extraction time, simple operation, lesser volumes

of organic solvents and high recoveries and enrichment factors [31]. DLLME has been applied for extraction of variety of organic pollutants in variety of matrices [33]. Area of LPME has gone through a rapid progression and new advancements have been included in the procedure, these review articles can be helpful in understanding advancement in LPME with time [34–36] .

Micro solid phase extraction (µ-SPE) uses small amount of sorbent compared to SPE. It works in two ways (1) Solid is directly immersed in liquid samples to extract target compounds and then separated by centrifugation and desorbed in small volume of organic solvent (2) solid is packed inside a porous membrane and thrown into liquid sample and then this membrane packed solid is taken out and desorbed with small amount of organic solvent. Nature of sorbent is critical in such extractions and choice is dictated by nature of target compounds. Main advantages are requirement of lesser amount of sorbents and organic solvents, higher enrichment factors and freedom in choice of sorbents. µ-SPE was used for extraction of persistent organic pollutants from biological tissue samples [37] . As sorbent is effectively secured inside the porous membrane in µ-SPE, it does not allow extraneous matter or fats to be adsorbed over the sorbent. Advantages and disadvantages of commonly used extraction techniques for POPs and EDCs from environmental and biological samples have been described in Table 2.

Table 2- 2: Advantages and pitfalls of extraction techniques

Extraction	**Advantages**	**Pitfalls**
Solid phase extraction	• Selective sorbents have high affinity towards target POPs • Molecularly imprinted polymers are sorbents which can be designed according to the structure of target analytes. • Method is suitable for large volume of samples.	• Consumption of large volumes of organic solvents. • Huge time consumption • Multi clean-up procedures
Liquid-liquid extraction	• Simplicity of operation • Rapid analysis • Reasonable selectivity	• Large volumes of organic solvents are needed. • Environment unfriendly • Formation of emulsions which prevents proper separation of two phases. • Co-extraction of some interferences
Solid phase microextraction	• Solvent less technique • Works on adsorption equilibrium between SPME fiber and sample containing analytes. • It can be easily automated with GC and HPLC systems. • SPME can be performed in normal as well as headspace mode for more complex samples. • Due to small size of device, equally suitable for in field applications. • Rapid, simple and sensitive method for variety of target compounds.	• Fibers are highly expensive and fragile. • Carry over effects even after longer period of thermal desorption. • Low recommended operating temperature ranges. • Fibers are highly sensitive to organic solvents and easily deshaped on contact.
Micro solid phase extraction	• Sorbent required is in range of milligrams • Small volume of organic solvents are needed for desorption.	• In case of porous membranes packed µ-SPE, pores may get blocked by the

	• It has variety of formats like immersing a solid directly into sample solution or packing the solid in a membrane. • Same sorbent can be used for various cycles of extraction. • Extraction can be assisted by high temperature or microwave radiation.	contents of real samples. • Selection of sorbent is critical in order to increase the performance of extraction process.
Liquid phase microextraction	• Few µL of organic solvents are needed. • Huge reduction in acceptor to donor phase ratios. • It has lot of operational flexibility. • Number of well-known LPME techniques has been developed by variation in extraction procedure. • Some examples are single drop liquid micro extraction, hollow fiber liquid microextraction, dispersive liquid-liquid microextraction and many more.	• Like SPME, no preconditioning is needed. • In case of single drop LPME, solvent with low vapor pressure are employed. • Difficult to maintain a single drop. • SD LPME, not suitable for complex samples. • In case of hollow fiber membranes, extraction time is enhanced because membrane acts as a barrier between donor and acceptor phase. • Fiber pores can be blocked in case of fatty or dirty samples.

2.3.2. Detection procedures

POPs and EDCs are very diverse classes of chemical compounds and hence large numbers of instrumental methods are employed to analyze these compounds. Gas chromatography and liquid chromatography with MS and tandem MS detectors are among few best instrumental techniques because of their inherent ability to analyze certain classes of analytes present within very complex matrices providing high sensitivity and selectivity [38]. Main problem concerned with chromatographic methods is their limitation of separating single class of compounds in one run under specified set of operating conditions. Developing a method which would be capable of detecting multi classes of POPs will be a great achievement.

Because of their tendency to induce serious health problems in humans and wild life at very low concentration and inherently low concentrations in environmental and biological samples, researchers intend to improve sensitivity of analytical methods for detection of POPs that can go down to parts per billion (ppb) or even low.

Gas chromatography coupled with electron capture detector (GC-ECD) is most commonly employed when compounds of interest are halogenated such as OCPs and PCBs. This is so for least expensive instrumental approach for chlorinated organic pollutants. GC-MS has now arrived in variety of MS detectors which perform a specific job. Problem of co-elution of similar compounds which is experienced in GC-ECD is resolved by application of MS detectors. Low resolution MS is now a days most commonly employed detector with GC systems and it can go up to pg levels detection of PCBs and OCPs when operated under selective ion monitoring (SIM) mode under electron impact ionization. High resolution MS

can give more specificity for closely related chlorinated compounds and it can go up to ultra-trace levels [12].

High pressure liquid chromatography (HPLC) is also another chromatographic method used for analysis of EDCs. Hyphenated liquid chromatography with mass spectrometry has resulted in increased sensitivity and has become a unique method for analysis of organic pollutants in environmental and biological samples. Analytical methods are judged based on their ability of performing high-throughput analysis and time required to complete the analysis. Three approaches have been adopted to achieve these goals in HPLC without affecting resolution and separation efficiency. These approaches involve use of monolith-sorbent bases columns [39], performing separation at higher temperatures [40] and use of ultra-high pressures [41]. Mainly for bioanalysis, monolith columns are promising and they allow very high flow rates of solvent nearly 10 mL/min without producing significant back pressures and compromising characteristic performance of HPLC. High temperature liquid chromatography is the one where high temperatures are used to perform the separation process in normal length columns. High temperature reduces viscosity of mobile phase which makes analysis faster and minimizes total run time. But the main problem with high temperature liquid chromatography is availability of column materials which would be stable under high temperature conditions. Also, any thermally sensitive compounds may degrade at high temperatures. Ultra-high pressure liquid chromatography (UHPLC) is the one in which very short columns with narrow diameters are employed. This allows using very high pressures which results in very well peak separation and reduces analysis time significantly [42].

Recent advancements in chromatographic systems have led to highly efficient separations. This is mainly attributed to fast GC and LC methods by using narrow bored, short columns, high mobile phase flow rates with the help of ultra-high pressures. With these advanced systems, total run time for analysis has greatly reduced from tens of minutes to few minutes. Shortening of analysis time is very promising step towards high throughput analysis which is generally required by research and analysis laboratories [43]. These highly efficient separation systems must be accompanied by suitable sample preparation and after all a good detection system for ultra-trace level analysis of EDCs.

Advancement in detection area is coupling of GC or LC with Mass spectrometry (MS) detector. This detector can determine molecular structure with high sensitivity and selectivity. Compounds are separated by chromatographic methods and then identified qualitatively and quantitatively by the MS. Use of selective ionization mode, coupling of quadruple with time of flight (TOF) enables the method to analyze and quantify target compounds from very complex matrices [44].

Whole organism assay is also used for monitoring of EDC in some aquatic organisms and it gives indication of total estrogenic activity. But these assays suffer from lack of specificity of organism response towards certain classes of EDCs. Cellular based assays are another attractive alternative to mass based analytical techniques but the results are not consistent and repeatable. Non-cellular assays which don’t require whole cells make the analysis simple and more quantitative with much better detection limits [45].

Among non-cellular assays, enzyme linked imuunosorbent assay (ELISA) is most famous method but it has several disadvantages including longer analysis time, washing and

addition of reagents, multi-step procedures, and requirement for experienced personnel. Another Immunochromatographic assay combines unique detection capabilities of molecular recognition probes like antibodies, aptamers with separation advantages chromatography. Strips based on Immunochromatographic principle are known as lateral flow test strips (LFTS). These strips are being used in detection of EDCs such as pesticides [46]. But the major problem related with these strips is their selectivity for only one or two compounds at a time and cannot detect number of EDCs at a time. Also they suffer from the problems in the selection of molecular recognition probes, conjugation with label molecules and less sensitivity. From this detail, it can be concluded that gas and liquid chromatography coupled with mass spectrometric detector are best techniques to analyze number of EDCs in single sample in a single run.

2.4. Challenges in Analysis of POPs and EDCs in biological samples

Despite of all advancements in analytical instrumentation, pretreatment of sample to remove complex matrix effects is considered as the bottleneck in all analytical methods. In this way, sample pretreatment plays a vital role in enhancing the sample amount and removing interferences due to matrix.

Biological samples present various challenges prior to their analysis by using chromatographic methods. These challenges arise because of the complex nature of biological samples which sometimes contain huge protein content. This protein content can adsorb on the column in an irreversible way and thus reduce column efficiency and produce significant backpressure. This complex biological matrix when combined with trace level concentrations of EDCs emphasis to treat the sample before analysis by any instrumental

technique. This pretreatment helps in enriching the analyte and removing interferences from biological matrices. But these sample pretreatment methods are most time consuming parts of analytical process. In multiresidue analysis, the biggest challenge is optimization of experimental conditions. Some compromises are to be done in this step.

Biological samples include urine, tissues, plasma or serum, breast milk, faeces, and semen .One recent study reveals that organic UV filters which are used in cosmetics (which are potential EDCs) have been mostly studied in urine (57%) and then plasma or serum (23%) and rest of matrices have not been focused much [20].

For the extraction of organic pollutants from urine samples different extraction methodologies are applied such as solid phase extraction (SPE), liquid-liquid extraction (LLE), membrane assisted liquid-liquid extraction (MALLE). But the big disadvantage with these extraction methods is that they consume too much organic. These organic solvents are not only toxic and expensive but they are also considered as environmental pollutants. In order to overcome this problem some other extraction techniques such as solid phase micro extraction (SPME) [47], liquid phase micro extraction (LPME), hollow fiber liquid phase micro extraction (HF-LPME) [48], stir bar sportive extraction (SBSE)[49] were employed and these methods consume organic solvents in microliters.

In case of blood, it is treated to get plasma or serum. Plasma is obtained by removing cellular components of the blood and retaining proteins that are responsible for the coagulation by centrifuging the blood sample in heparinized tubes while serum is obtained by removing both cellular components and proteins and this is attained by doing centrifugation in non-heparinized tubes [50]. Once the serum or plasma sample is obtained,

hydrolysis is carried out to determined bonded or conjugated target analyte. When anlayte is attached to proteins, acidic hydrolysis helps to determine it. On the other hand, proteins are the materials which are thought to be biggest hurdle in analytical detection of organic compounds as they represent a very complex class of matrix. These proteins are precipitated and then removed from the samples by the use of organic solvents to avoid interferences. Further purification could be achieved by LLE or SPE.

In case of semen, breast milk and tissues hydrolysis as well as removal of proteins is a mandatory step before detection of target analye in the sample through any instrumental techniques. Selection of suitable solvent is crucial. Tissues are normally homogenized with water or acetonitrile and LLE is done to remove any protein content. Soxhlet extraction or LLE is used for pretreatment of faeces [50,51].

Data summarized in Table 2 shows some of the recent studies which were carried out in different parts of the world for detection of EDCs in environmental and biological samples. It can be noted that EDCs pose serious environmental and health challenges to the human all around the globe and all the components of the environment (air, water, soil and biota) are being contaminated with EDCs.

2.5. Health Effects of POPs and EDCs

EDCs are associated with changed endocrine functions both in animals and humans. They create long lasting adverse effects on the metabolism, neurological function, reproduction, and other physiologically important processes. Health concerns are being raised with every passing day and more and more abnormalities are being associated with the use of these toxic compounds. Some recent reports show that EDCs has resulted in high incidences of

thyroid and testicular cancers in males [52,53]. EDCs disturb hormonal signaling. Those which block or interrupt the function of sex hormones have been paid much attention but it is recognized fact that EDCs have very high impact on insulin signaling, functioning of thyroid and bone growth. Some PCBs and pesticide atrazine has been reported to have effect over proteomes of the breast cancer cells. Literature reveals that there is a great debate on the classification and effects of EDCs on humans. EDCs are present in environment as mixture and they can have synergistic or additive effects. So determining the effects from individual EDCs will be really difficult without considering all other affecting parameters [54].

Human exposure to EDCs have gained too plentiful consideration in recent years due to experimental results indicating endocrine-related effects on reproduction, development, cancer, and metabolism, and observations for increasing tendencies (as well as geographic trends) in endocrine-related ailments among populations [55]. There are thousands of persistent organic pollutants for which toxicity information either does not exist or insufficient[56]. The main targets of EDC effects are homeostasis of sex steroids and the thyroid ; hence, reproductive health, is also concerned with endocrine disruption [57]. However, studies carried out to find the impact of environmental EDCs in relation to endocrine associated cancers and outcomes in human are limited in number.

A significant increase in breast cancers in last 50 years has developed a perception that hormonally active industrial chemicals which are result of industrial revolution could be possible reason for these cancers. Same half century witnessed an increased incidence of testicular cancers and disorders in quantity and quality of human sperm [58,59]. EDCs are involved in disruption of reproductive system in human and other animals. They are

thought to be responsible for incidences of cancers and transgenerational effects [60,61]. Glyphosate which is used in pesticides was thought to be safe but a new study states that it has endocrine disrupting properties and promotes breast cancer on human cells through estrogen receptors at very low concentrations of 10^{-12} to 10^{-16} M [62]. When human peripheral blood cells were exposed to micro molar concentrations of EDCs, it resulted in change of their gene expressions. This change in gene expression was independent of gender [63]. Luteinizing hormone receptor (Lhcgr) of zebrafish follicle cells showed very high response to estrogenic EDCs [64]. Human MCF-7 cells were exposed to PCB-153 and atrazine concentrations for a period of 36 hours and proteins isolated from cytosol and membrane were tested for presence of biomarker as a result of this exposure. It resulted in an altered protein expression which is an indication that these compounds can have potential impacts on human health [65] A study was carried out to see the effects of mixture of five pesticides when exposed to rats, developmental changes in gestation length and increased number of nipples and genital disfigurements were observed in male offsprings [66]. It is an indication that how these compounds can effect on sexual developments in human and other animals. A comprehensive review was done which included 91 studies dealing with BPA and human health and it showed that BPA exposure has adverse effects on human of all age groups and also early exposure in children leads to asthma and changed behavior [67]. A study which was designed to determine endocrine disrupting activity of some compounds in bottled flavored and mineral drinking water showed hormonal activity in 78 % of samples [68]. EDCs have great impact on reproductive system particularly disorders in female fertility [69]. Estrogenic activity of fruits and vegetables was

determined and it showed positive results. This activity was attributed to presence of pesticide residues in fruits and vegetables [70].

Studies suggest a link between exposure to low doses of POPs and incidence of type 2 diabetes. This risk is boosted when exposed to POP mixtures instead of few individual POPs. But results are somehow inconsistent in this regard because of unequal distribution of POPs in environment [71]. High levels of POPs concentrations were found in biological and environmental matrices around the globe. A recent report indicates frequent presence of POPs in the serum of most of Tunisian women [72]. POPs and EDCs are linked with malfunctions in ovarian function and the women exposed to such compounds are more likely to get menopausal at earlier ages than non- exposed women [73]. Similarly, an association between concentrations of POPs in breast milk of mothers and faeces of infants was found [74]. Subject of health effects of POPs and EDCs is highly diversified and it covers wide range of ailments in human and wildlife and reports covering this aspect are increasing by every coming day. Covering every single report which describes negative impact of POPs is beyond the scope of this review. Some review articles covering health effects of POPs and EDCs can be studied to get more comprehensive overview of the subject [75–79].

2.6. Conclusive Remarks

It needs to develop systematic criteria in order to assess toxicity and risk assessment related to new organic pollutants being added into environment. Most scarce aspect of risk assessment studies is that researchers focus on particular aspect of their interest. Compiling

such reports is very difficult and it cannot lead to straightforward conclusions about a compound or group of compounds.

Consumable foods in different localities need to be explored for presence of POPs and EDCs levels. Studies in this regard are highly scattered. For example, a review article was conducted to compile the studies carried out in Spain to find the relationship between human diet and its effect on POP concentrations in humans. Results reveal that only few compounds were consistently studied in fish and human serum but most of the foods such as cereals, vegetables and fruits have not been evaluated for POP concentrations [80]. All food stuff has to be studied in systematic pattern at all geographical locations in order to reach some conclusive outcomes.

There exists sufficient evidence that POPs have considerable concentrations in fats and other body compartments of the people in all around the globe which have been linked with serious health implications [81–84]. These POPs come from environment and contaminated food and thus accumulate in fatty tissues. There should be some minimum allowable limits of POPs in serum, adipose tissues and other body compartments, so that above levels can be treated as health risk. POPs concentrations in body fluids can be used as a biomarkers for near or far future health implications. Although hundreds of reports have been published describing toxicity and health effects of POPs but standard procedures and limits to be regularized in clinical applications to determine and establish a relationship between POP levels and disease stages.

2.7. Conclusion

In this review article we have comprehensively looked into the diverse types of POPs and EDCs prevalent in human environment. We have also described the various methods that could be used to enrich these compounds from tissues, body fluids and environmental samples, which could enable their detections using advanced mass spectrometry based chromatographic techniques. We have very clearly highlighted the advantages and disadvantages of more commonly used extraction methods. Advancements in analytical instrumentation has made the analysis of low levels of organic pollutants a doable job within a time span of few minutes but still there exists some challenges while dealing with complex biological samples. Accomplishments, weaknesses and future challenges in area of analytical chromatography are critically discussed. Gene-protein-metabolite relationships play a key role in the incidence of diseases associated with exposure to toxic chemicals in the environment. We have summarized those reports associated with role of POPs and EDCs in triggering health related problems. Studies in this regard could be extended to the development of novel sensors and biosensors that could be used to monitor the range of toxicity associated with newly identified toxic chemicals.

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3. CHAPTER

APPLICATION OF MICROWAVE ASSISTED MICRO-SOLID-PHASE EXTRACTION FOR DETERMINATION OF PARABENS IN HUMAN OVARAIN CANCER TISSUES

This chapter has been published in “Journal of Chromatography B”.

Abstract

Parabens (alkyl esters of p-hydroxybenzoic acid) are widely used as preservatives in food, cosmetics and pharmaceutical products. However, weak estrogenicity of some parabens has been reported in several studies, which provided the impetus for this work. Here, a simple and efficient analytical method for quantifying parabens in cancer tissues has been developed. This technique involves the simultaneous use of microwave-assisted solvent extraction (MASE) and micro-solid phase extraction (μ-SPE), in tandem with high performance liquid chromatography (HPLC/UV) analysis for the determination of parabens. The pollutants studied included four parabens (methyl, ethyl, propyl and butyl parabens). Optimization of the experimental parameters for MASE and μ-SPE was performed. Good relative standard deviation (%R.S.D) ranged from 0.09 – 2.81% and high enrichment factors (27 – 314) were obtained. Coefficients of determination (r^2) up to 0.9962 were obtained across a concentration range of 5.0- 200 ng g^{-1}. The method detection limits for parabens ranged from 0.005 - 0.0244 ng g^{-1}. The procedure was initially tested on prawn samples to demonstrate its feasibility on a complex biological matrix. Preliminary studies on human ovarian cancer (OC) tissues showed presence of parabens. Higher levels of parabens were detected in malignant ovarian tumor tissues compared to benign tumor tissue samples.

3.1. Introduction

Chemical compounds which possess endocrine disrupting properties represent a wide and ever growing class of chemicals. Endocrine disrupting compounds (EDCs) interfere with normal hormonal function in humans and wildlife by interacting with estrogen receptors and induce adverse effects in developmental [1,2], reproductive [3,4] and neurological systems [5]. EDCs have been associated with increasing susceptibility to different kind of cancers [6–8] . They become functional at very low concentrations [9]. Alkyl esters of 4-hydroxybenzoic acid (methyl paraben, ethyl paraben, n-propyl paraben, and n-butyl paraben) are also found to exhibit estrogenic activity [10,11].

The properties which dictate widespread applications and worldwide regulatory acceptance of parabens include broad activity, a long history of safe applications, low cost, wide working pH range, reasonable solubility in water, no significant odor or taste and inertness [12]. Because of their high antimicrobial potential, parabens are used as preservatives in food, cosmetics and pharmaceuticals and personal care products [13]. However, current studies report parabens as endocrine disruptors because of their estrogenic activity [14], the magnitude of which is reliant on their structure [15,16]; though, much lower than that of estradiol [17]. In recent years, more research focused on finding out role of parabens in inducing infertility [18] and different cancers in human. Measureable concentrations of parabens have been found in human breast cancer tissues and linked as one possible cause for the disease [19,20]. Presence of parabens in urine can serve as a biomarker to assess human exposure [21]. Concentrations of parabens in urine may have effect on ovarian age and reproductive health [22] .

Several analytical methods have been reported for the sample clean-up of parabens in different complex matrices. Specifically, parabens have been pre-concentrated by using molecularly imprinted polymer solid phase extraction in milk samples[23], liquid-liquid extraction (LLE) in human placental tissue samples [24], solid-phase extraction (SPE) in human urine [25] and breast cancer tissues [26], and solid phase microextraction (SPME) in pharmaceutical formulations [27] and cosmetics [28].

Since parabens are relatively lipid soluble compounds, they would tend to bioaccumulate in the lipid fraction of the biological tissues. Hence, it is necessary to perform sample clean-up to extract parabens from fatty contents which otherwise can interfere with the experimental results. When it comes to extract organic pollutants from real biological samples, above mentioned techniques suffer from some inherent limitations such as SPME fibers have been reported to absorb fatty contents and exhibit carryover effects due to incomplete desorption by gas chromatography [29]. Similarly, SPE and LLE require large volumes of solvents which are by itself a drawback.

The key advantages of microwave assisted solvent extraction (MASE) lie in reduced solvent usage and less time consumption. Furthermore, multi-vessel systems can be used for simultaneous MASE. Moreover, lesser amounts of samples are required which significantly reduce waste generation. Temperature can also be effectively controlled and the entire process is not labor intensive. MASE is carried out by high frequency microwave energy which lies in between RF and the IR regions of electromagnetic spectrum [30–32]. In contrast to the conventional heating such as in normal solvent extraction heat is provided from outside and it gradually transfers to middle parts of solid sample, but microwave energy starts from core of sample and spreads from inside to outside. We have

already developed MASE for analysis of persistent organic pollutants in marine sediments [33]. Due to increased extraction efficiency, less interferences from complex matrices, no need for additional clean ups, membrane protected μ-SPE has been used for extraction of halo acetic acids in swimming pool waters [34], acidic drugs and organophosphorus pesticides in wastewater [35,36], persistent organic pollutants in tissue samples [37].

Combination of MASE and μ-SPE provides a single step extraction and clean up procedure for analysis of target analytes present in complex matrices [38]. Extraction of analytes can be performed by throwing number of μ-SPE devices in a microwave vessel which contains sample in NaOH solution. Extraction of analytes from tissues to NaOH solution and then adsorption on μ-SPE devices take place simultaneously. The porous membrane of the μ-SPE device allows the diffusion of parabens onto the sorbents packed within the μ-SPE device, while eliminates the entrance of fatty contents. μ-SPE does not require many experimental steps, as the device can be easily made and is readily available for usage by simply conditioning it via ultrasonication in methanol. Another advantage of μ-SPE is that it requires as little as 200 μL of organic solvent for desorption of parabens. This volume of solvent consumption is significantly much lower compared to the volume of organic solvent required by SPE, which is in the range of tens of milliliters. Thus, this makes it an environmental-friendly technique. Furthermore, the length of desorption time required is short, which means that considerable amounts of time are reduced when dealing with multiple samples. Hence, the new μ-SPE technique can be a better alternative to SPE.

To our knowledge, this is the first study to report the concentrations of individual parabens in human ovarian cancer tissues using single step extraction and clean up procedure involving MASE and μ-SPE. This method could be used for rapid, precise, and cost-

effective analyses of large numbers of samples in epidemiologic studies to assess human exposure to parabens. Structures of target anayltes are given in Table3-1.

3.2. Experimental

3.2.1. Chemicals

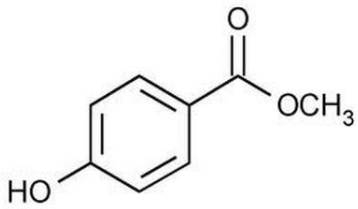
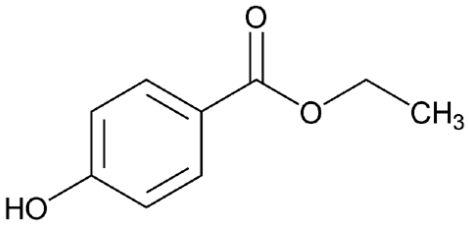
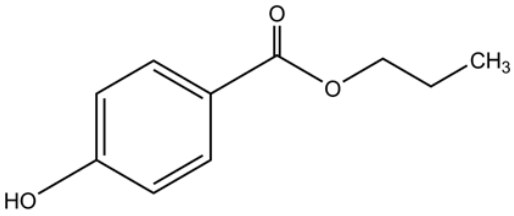
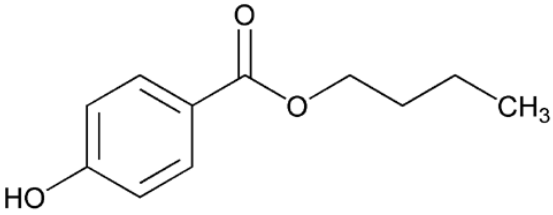
HPLC-grade methanol, acetone, hexane and acetonitrile were bought from Tedia Company, Inc. (Fairfield, OH, USA). Sodium hydroxide (NaOH) of analytical grade was obtained from J.T. Baker (Philipsburg, NJ, USA). Nanopure water obtained from a Nanopure water purification system (Barnstead, USA) was used. The four parabens studied were methyl paraben (purity 99%), ethyl paraben (purity 99%), propyl paraben (purity 99%) and butyl paraben (purity 98%). They were purchased from Alfa Aesar (A Johnson Mattey Company). Stock solutions containing 1mg ml^{-1} of each parabens were prepared in methanol and diluted with methanol to obtain working solutions at various concentrations. All solutions were filtered and stored at $4^{o}C$. C_2, C_8, C_{18}, HayeSepA and HayeSepB were obtained from Alltech (Deerfield, IL, USA). Q3/2 Accurel 2E HF (R/P) polypropylene sheet (157 µm thickness, 0.2 µm pore size) were purchased from Membrana (Wuppertal, Germany). 0.6 mL microcentrifuge tubes used for chemical desorption by ultrasonication and pipet tips from Axygen Scientific (California, USA) were used. 0.45 µm syringe filters from Titan (New York) were also used. A MARS (CEM, Matthews, NC, USA) microwave extraction system (maximum power: 1200W) was used for parabens extraction from tissues. Uncontaminated grey prawns were bought from a supermarket. Ovarian tumor (malignant and benign) samples were obtained from Department of Obsterics and Gynaecology, Yong Loo Lin School of Medicine, National University of

Singapore with approval from the Domain Specific Review Board (DSRB). Safety considerations were put in place in the handling of tissues. All tissues and solvents used in this project were discarded according to the safety procedures. The apparatus used were decontaminated in a proper manner.

3.2.2. Preparation of µ-SPE device

The µ-SPE device was made by packing the sorbent in a polypropylene envelope. The procedure for making µ-SPE device was described earlier [35]. Briefly, the envelope was fabricated by folding the equal lengths of membrane over each other and open ends were heat sealed except the one end which was left for packing of the sorbent. 25 mg of sorbent was packed via the remaining open end, which was then heat-sealed to produce the extraction device (2.0 cm x 0.5 cm). The dimensions of the µ-SPE device ensured that it could fit into a 600 µl plastic vial during the desorption process. Each device was first conditioned by ultrasonication in nanopure water for 5 minutes followed by 10 minutes ultrasonication in methanol. The µ-SPE device was then stored in methanol until use. C_2 (Si-C_2H_5), C_8 (Si-C_8H_{17}), C_{18} (Si-$C_{18}H_{37}$), HayeSepA (divenylbenzene-ethyleneglycoldimethylacrylate) and HayeSepB (divinylbenzene-polyethyleneimine) were the sorbent materials that were used in the making of µ-SPE device. Both HayeSepA and HayeSepB are polar polymer based sorbents. The former is of intermediate polarity while the latter is of high polarity.

Table 3- 1: Structures of analytes

Analyte	Structure	Elution order
Methyl paraben		1
Ethyl paraben		2
Propyl paraben		3
Butyl paraben		4

3.2.3. MASE-μ-SPE

5 g of prawn tissues were accurately weighed and taken in a microwave vessel. These tissues were spiked with known concentrations of parabens and vessel was covered and kept overnight so that analytes can be properly entrapped within the matrix. The sample was then subjected to microwave heating after addition of 10 mL of 5 mol L^{-1} NaOH solution in same microwave vessel (600W, 90°C, 20 minutes). Two μ-SPE devices were introduced into the microwave vessel prior to microwave heating and allowed to tumble freely in the stirred sample during extraction (Figure 3-1). After the completion of MASE, the μ-SPE devices were removed with a pair of tweezers. The analytes were desorbed by ultrasonication in 200 μl of acetonitrile for 20 minutes. No further clean-up was required. Lastly, 80 μL of extracts were injected into the HPLC system for analysis.

3.2.4. MASE-SPE

SPE was performed using a 12-cartridge vacuum extraction manifold (Supelco) with commercially available SPE cartridge with 200 mg (6 cc) WATERS Oasis HLB sorbent. The cartridge used was first conditioned with 1 mL of ultrapure water, followed by 1 mL of methanol. Upon MASE digestion (10 mL of NaOH and 10 mL of hexane, 600W, 90ºC, 20 minutes), the digested spiked prawn tissue was first filtered using SPE filtering cartridge (Waters Oasis HLB Vac RC 60 mg Extraction cartridge) and washed with 10 mL of hexane. The filtered sample solution was collected and SPE was then carried out at a flow rate of approximately 5 mL min^{-1}. The cartridge was then washed with 15 mL of a mixture of acetone and n-hexane 1: 1 volume ratio. The extracts were subsequently evaporated to near

dryness by rotatory evaporator and then reconstituted in 1 mL of acetonitrile, followed by HPLC/UV analysis.

3.2.5. HPLC conditions

Analysis of samples was performed using a Waters Analytical liquid chromatography system made up of Waters 600E quaternary pump, Waters 486 UV detector with a 100 µl loop and EMPOWER software. In this study, a Shimpack C18 VP-ODS column (250 mm x 4.6 mm column, 5 µm particle size) from Shimadzu was used. The detector wavelength was set at 254 nm. The mobile phase ratio was acetonitrile – water (65: 35) at a flow rate of 0.5 mL min^{-1}.

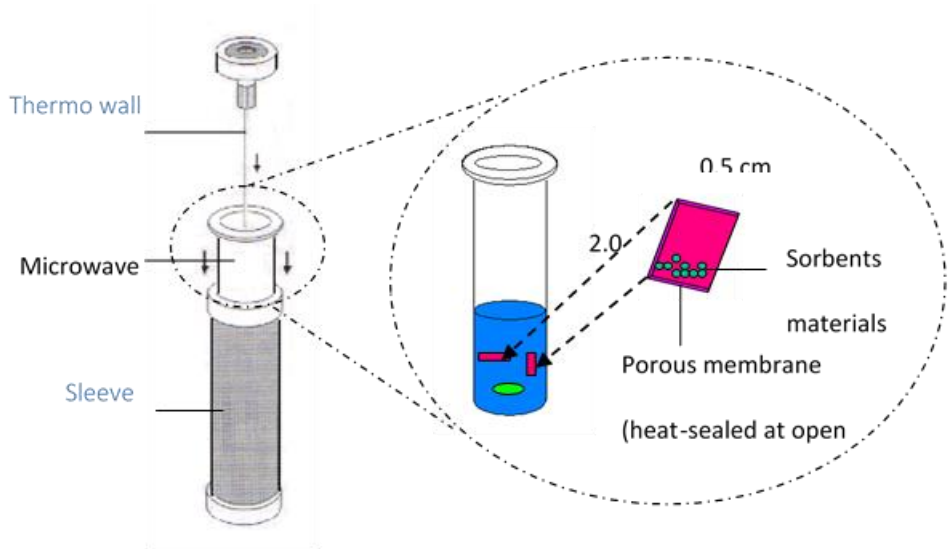


Figure 3- 1: (a) MASE - μ-SPE setup (b) μ-SPE system (c) enlarge image of extraction device (not drawn to scale

3.3. Results and discussion

3.3.1. Optimization of MASE-μ-SPE procedure

Optimization was carried out by considering triplicate uncontaminated grey prawn sample analysis spiked with known mass fractions of parabens. The parameters investigated include: MASE duration and temperature, membrane selection, suitable sorbent materials, multiple μ-SPE devices, desorption solvent and desorption time.

The minimum amount of solvent recommended by the manufacturer of the microwave extraction system was 10 mL. This volume of NaOH solution was sufficient for the complete immersion and digestion of tissue samples. Hence, 10 mL of NaOH was used for digestion of all tissues because higher volumes would lead to dilution of analytes.

Here it is important to describe that method development and optimization cannot be performed on real samples particularly when you are dealing with very expensive and rare human cancer tissues. Following are the major reasons that we adopted prawn tissues for method development and optimization

(1) Among other tissues (fish, chicken etc.), prawn tissues represent very complex matrix because they contain lot of fatty stuff.

(2) Number of values are needed to be optimized for every parameter. For example, for optimization of type of sorbent, five different sorbents were tried. Each sorbent type was tested for n=3. In this way 15 samples are needed only for the optimization of sorbent type. Similarly, each affecting parameter will require at least 15 samples. For optimization of all factors this number may reach to100.

(3) This much number of cancer tissues is difficult to obtain and that is why methods are developed on closely related matrices and then their applicability is further confirmed by recovery experiments. Good recovery values are supportive to apply the method for tested matrix.

3.3.1.1. MASE duration and temperature

The time required for MASE was short compared to conventional Soxhlet extraction. Digestion time was evaluated between 10 and 30 minutes at 5-minute intervals. It required 15 minutes for the complete digestion of prawn tissues into solution and 20 minutes was found to be optimal. A longer digestion time would result in the bursting of μ-SPE devices. Therefore, 20 minutes was selected as MASE time for further optimization of the method.

The effect of temperature was investigated over a range of 50ºC to 90ºC. The optimum extraction efficiency was found to be at 90ºC. This could be attributed to the fact that an increase in temperature weaken the interactions between parabens and tissue, thereby increase the desorption rate of parabens from tissue to solution phase. The temperature was sufficient to digest the tissues and release the analytes into solution. The vials were cooled to room temperature at rate of 5°C $minute^{-1}$ before collection of μ-SPE devices for desorption.

3.3.1.2. Membrane selection

There were many different types of commercially available membranes, which could be used to contain sorbent materials. Porous polypropylene membrane was chosen as the most

suitable membrane in the making of μ–SPE device. This was based on the earlier experimental results obtained from our research group. The high temperature resistance nature and wide chemical compatibility of polypropylene membrane makes it an appropriate material for μ–SPE device. In addition, membranes such as polycarbonate, cellulose acetate and PTFE were deemed unsuitable. Polycarbonate membrane was flimsy whereby vigorous stirring during extraction would tear the device even before the completion of extraction. Cellulose acetate membrane was brittle and could snap upon folding during the preparation of μ –SPE device. PTFE membrane was not heat sealable.

3.3.1.3. Suitable sorbent materials and number of μ –SPE devices

The selection of suitable sorbent materials is crucial as the target analytes (parabens) have different affinities for the functional groups of the different sorbent materials. Different sorbent materials were tested in the extraction of parabens. Five different sorbents materials (C_2, C_8, C_{18}, HayeSepA and HayeSepB) were investigated. It was found that HayeSepA, which is of intermediate polarity, showed higher extraction efficiency towards parabens (Figure 3-2). This is probably because parabens are relatively polar and thus higher electrostatic interaction occurs between the polar paraben and polar porous polymer base sorbents.

The number of μ –SPE devices added into the microwave vessels for extraction was investigated as well. Experiments were performed by adding in one or two μ–SPE devices. For the studied parabens, the addition of two μ–SPE devices gave greater peak areas compared to just adding in one μ–SPE device. This could be due to the increase in surface

areas, which facilitate the adsorption of parabens onto the sorbent materials packed within the polypropylene membrane.

3.3.1.4. Desorption solvent and desorption time

The analytes were desorbed in a suitable organic solvent after extraction. Since parabens are generally polar compounds, they tend to be more favorably desorbed into a polar solvent during the desorption process. Thus, various polar organic solvents such as methanol, water, acetone and acetonitrile were tested. Acetonitrile was chosen as the suitable desorption solvent as it gave the greatest peak area.

The effect of desorption time is investigated over a range of 5 to 30 minutes. Parabens were completely desorbed within 20 minutes of ultrasonication. Desorption was incomplete when shorter periods of time were used. Above 20 minutes, no considerable increase in desorption efficiency was observed (Figure 3-3).

After the first desorption round, the μ-SPE device was re-desorbed in methanol for the second time to investigate for carryover effects. No analytes were detected in the second desorption round. However, protein from the prawn sample tends to clog the pore of the μ-SPE device and compromise its extraction efficiency. Thus, we decide not to re-use the μ-SPE device.

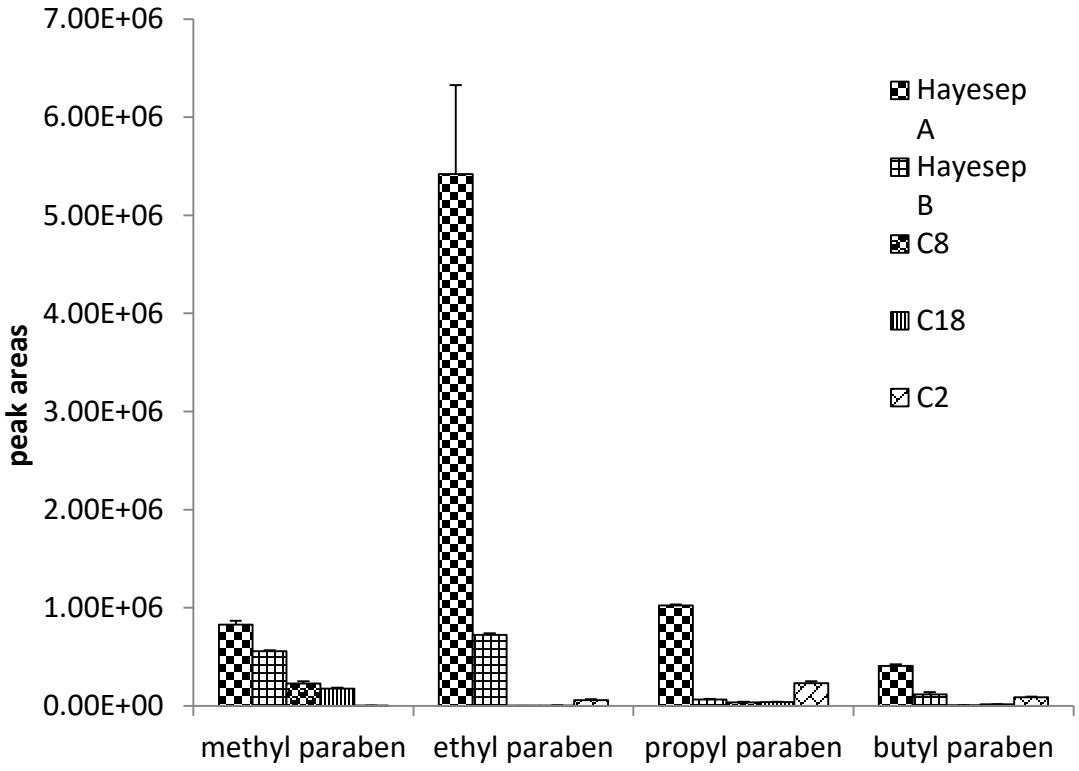


Figure 3- 2: Effect of various sorbents on the extraction efficiency of MASE-µ-SPE of parabens in spiked prawn samples. Prawn samples were spiked with 25 ng g^{-1} of parabens.

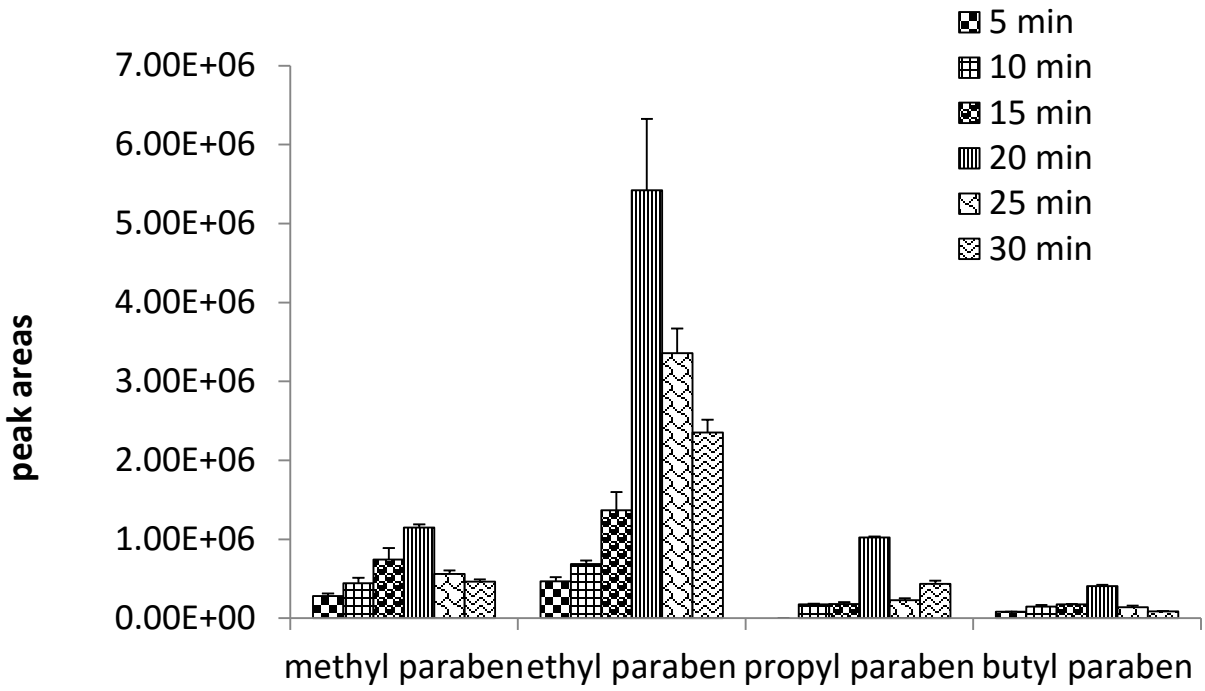


Figure 3- 3: Effect of desorption time on the extraction efficiency of MASE-µ-SPE of parabens in spiked prawn sample. Prawn samples were spiked with 25 ng g^{-1} of parabens

3.3.1.5. μ-SPE after digestion

The effects of conducting μ-SPE only after MASE were also investigated. This was based on the assumption that microwave heating of the sample in absence of μ-SPE devices can desorb the analytes more favorably from the biological tissues. Thus, conducting μ-SPE separately might give better analysis. After conducting MASE, the microwave vessels were allowed to cool down to room temperature before adding two μ-SPE devices for extraction. Different extraction times were investigated from 10 to 25 minutes at 5- minute interval. The digested samples were stirred at 1000 rpm using a magnetic stirrer. After μ-SPE extraction, the subsequent steps and conditions were kept constant as mentioned earlier. It was found that 20 minutes of extraction time gave the optimized results for this sequential approach. After 20 minutes, there was no significant increase in the adsorption efficiency.

Experimental results of simultaneous MASE-μ-SPE were compared with MASE followed by μ-SPE, no significant differences in peak areas of analytes were observed. Hence, simultaneous mode which was less time consuming was selected as an optimum mode.

3.3.2. Method validation

To access the practical applicability of the proposed MASE-μ-SPE method, the optimized conditions for MASE and μ-SPE were adopted in the evaluation of the method's linearity, reproducibility, limits of detection (LODs) and enrichment factor (Table 3-2). Calibration plots were obtained by plotting the HPLC peak area of the analytes against their corresponding concentrations in the uncontaminated grey prawn samples spiked with known mass fractions of parabens ranging from 5-200 ng g^{-1} (seven calibration levels). The linearity of the calibration plot was evaluated using the least-squares linear regression

analysis. All the parabens exhibited good linearity with coefficients of determinations (r^2) up to 0.9962. The relative standard deviations (RSDs) corresponding to peak areas were calculated from analysis of five replicates of uncontaminated grey prawn samples spiked at 5 ng g^{-1} which were subjected to same extraction procedure and good values ranging from 0.09 – 2.8% were obtained. LODs were determined by progressively decreasing the mass fractions of parabens spiked in the uncontaminated grey prawn tissues until distinct responses were still clearly observed at a signal to noise (S/N) ratio of 3. The LODs for parabens were found to be between 0.005 – 0.0244 ng g^{-1}. Enrichment factor (EF) was calculated as the ratio of extracted analyte concentration to its initial concentration in the prawn tissues. EFs were calculated by using three replicates of uncontaminated grey prawn samples spiked at 25 ng g^{-1} and their values ranged from 27 to 314.

In order to study the applicability of the proposed method for the determination of parabens in real cancer tissues, ovarian cancer tissues (with predetermined mass fractions of parabens) were spiked with 25 ng g^{-1} of each compound and recoveries were calculated after subjecting to the proposed procedure. Recoveries were calculated as the percentage ratio of mass fraction of the analyte found to mass fraction of the analyte added.

Table 3-3. Compares current method with some methods reported in literature for extraction of parabens in different biological matrix.

3.3.3. Comparison of µ-SPE with SPE

The extraction efficiency of µ-SPE was compared with the commercial SPE using Waters Oasis HLB SPE-sorbent. Real ovarian cancer tissues were spiked with 25 ng $g^{-1}$of parabens and standard addition method was used for calculation of percentage recoveries. The

recoveries of the parabens using MASE-SPE and MASE-μ-SPE methods were reported in Table 3-4. Theoretically, recoveries of SPE are expected to be higher than any micro-extraction technique because it is an exhaustive extraction technique. Micro extraction techniques are non-exhaustive and extraction is based on concentration equilibrium between extraction phase and sample solution, in turn, their recoveries are expected to be lower relative to SPE. However, from Table 3, we observed that the recoveries for MASE-μ–SPE are slightly higher than MASE-SPE.

One possible explanation could be that the type of packing sorbents used in the μ-SPE device was more suitable for extracting the targeted parabens. The packing sorbents used in the μ-SPE device was HayeSepA, which was of intermediate polarity. The SPE cartridges utilized in this experiment were non-polar silica-based C_{18} as the packing sorbent. The targeted parabens were relatively polar thus; HayesepA provided better affinity for the parabens. Very good recoveries (82-100%) were obtained for all of the parabens using MASE-μ-SPE.

Table 3- 2: Analytical features of MASE-μ-SPE method

Analytes	**Coefficients of determination [a]**	**RSDs [b] (%, n = 5)**	**LOD [c] (ng g^{-1})**	**Enrichment Factors**
Methyl paraben	0.9939	0.0919	0.022	222
Ethyl paraben	0.9905	0.7733	0.005	314
Propyl paraben	0.9963	0.2054	0.024	70
Butyl paraben	0.9923	2.813	0.015	27

[a] Coefficients of determination for linear range of 5-200 ng g^{-1}

[b] Relative standard deviation evaluated at 5 ng g^{-1}

[c] Limit of detection evaluated at 5 ng g^{-1}

Table 3- 3: Comparison of analytical performance data of the proposed method with reported methods for determination of parabens in different biological samples

METHOD	**Matrix**	**LODs**	**RSDs (%)**	**REF.**
SM-SLLME-UHPLC-MS/MS	Human breast milk	0.1-0.2 ng mL^{-1}	<8	[40]
LLE-SPE-GCMS	Human breast cancerous tissues	1.05-3.75 ng g^{-1}	4.6-13	[26]
SPE-LC-ESI-MS/MS	Human urine	1-3 pg mL^{-1}	1.2-4.5	[25]
SPE-HPLC-MS/MS	Human Urine	0.2 ng mL^{-1}	5.3-10.6	[41]
MSPD-UHPLC-MS/MS	Human placental tissues	0.1 ng g^{-1}	5.4-12.8	[42]
MASE-μ-SPE-UPLC-UV	Human Ovarian cancerous tissues	0.005 - 0.0244 ng g^{-1}	0.09 - 2.81	Current work

Table 3- 4: Comparison of paraben recoveries from ovarian cancer tissue achieved by MASE-SPE and MASE-µ-SPE (n=3)

Analytes	**Recovery* (%) for MASE-SPE**	**RSD (%)**	**Recovery* (%) for MASE-µ-SPE**	**RSD (%)**
Methyl paraben	81	2.6	82	1.2
Ethyl paraben	83	4.2	85	2.7
Propyl paraben	102	3.5	92	1.0
Butyl paraben	76	5.6	100	2.1

*evaluated at 25 ng g^{-1}

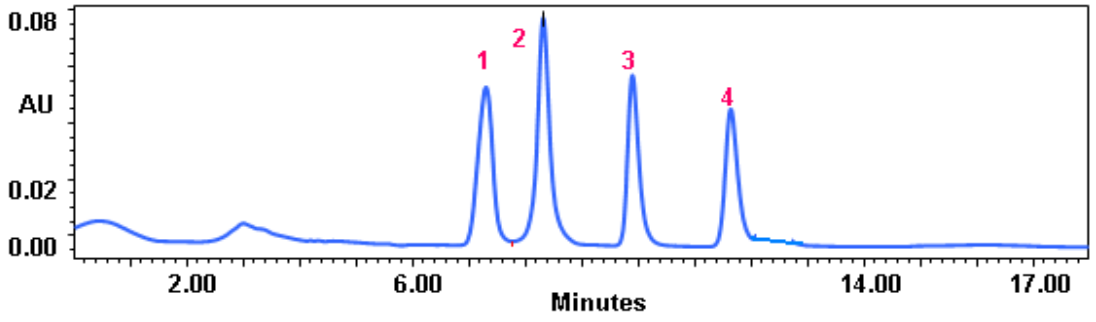


Figure 3- 4: HPLC/UV chromatogram of parabens standards peaks: (1) methyl paraben, (2) ethyl paraben, (3) propyl paraben & (4) butyl paraben

3.3.4. Studies of human ovarian tissues

We applied MASE–μ–SPE method to determine the concentrations of the four parabens that might be present in 30 human ovarian tissues (15 ovarian cancer tissues and 15 benign tissues). These ovarian tissues were not spiked with parabens. The mass fractions of parabens present in human ovarian tissues were then calculated as shown in Table 3-5 and 3-6. The tissue mass fractions of methyl paraben and propyl parabens were the highest among the four parabens examined. This finding is in agreement with another work which reports higher concentrations of methyl paraben in human breast tumors compared to concentrations of other parabens [19]. In addition, the higher tissue mass fractions of methyl and propyl parabens could be due to the fact that these two parabens are the most widely used [39]. From our studies, the tissue mass fractions of four parabens in all the ovarian cancer tissues are at least twice as much as those present in the benign tissues. The HPLC/UV chromatograms of the OC and benign tissues studied were shown in Figure 3-5.

Table 3- 5: Mass fractions of parabens found in human ovarian benign tissues from 15 patients

Analytes	**Mass fractions of parabens (ng g^{-1})**														
Benign tissues	T11	T12	T15	T19	T20	1099	1055	1045	1124	1003	1169	1138	1164	1144	1120
Methyl paraben	2.4	2.38	2.76	3.58	3.3	1.15	3.33	2.86	1.15	3.08	0.1	1.77	5.94	5.98	0.17
Ethyl paraben	0.16	0.11	0.3	0.29	0.5	1.65	0.35	0.52	0.06	0.31	0.43	0.61	2.02	0.74	0.35
Propyl paraben	1.44	1.18	1.13	1.67	0.7	1.28	0.54	1.04	0.18	0.64	0.51	0.71	0.18	0.99	1.29
Butyl paraben	0.16	0.55	0.04	0.1	0.1	0.19	0.02	2.16	0.01	0.02	0.11	0.02	n.d	0.37	0.8

Table 3- 6: Mass fractions of parabens found in human ovarian cancer tissues from 15 patients

Analytes	**Mass fractions of parabens in (ng g^{-1})**													
Ovarian cancer tissues	T10	T16	T35	T39	T40	1086	1060	1072	1080	1101	1161	1089	1022	109
Methyl paraben	6.26	10.86	14.91	17.06	6.03	12.13	5.65	2.38	2.23	4.45	2.78	1.77	2.66	2.8
Ethyl paraben	1.2	0.23	0.14	0.24	1.1	0.61	1.01	1.56	1.03	1.57	1.85	0.61	0.86	0.5
Propyl paraben	1.09	3.35	1.64	3.08	1.53	1.22	0.63	3.13	0.67	0.82	0.71	0.71	0.85	0.6
Butyl paraben	0.08	4.12	3.24	3.75	2.45	0.84	0.17	1.67	0.24	0.23	0.36	0.02	0.02	0.

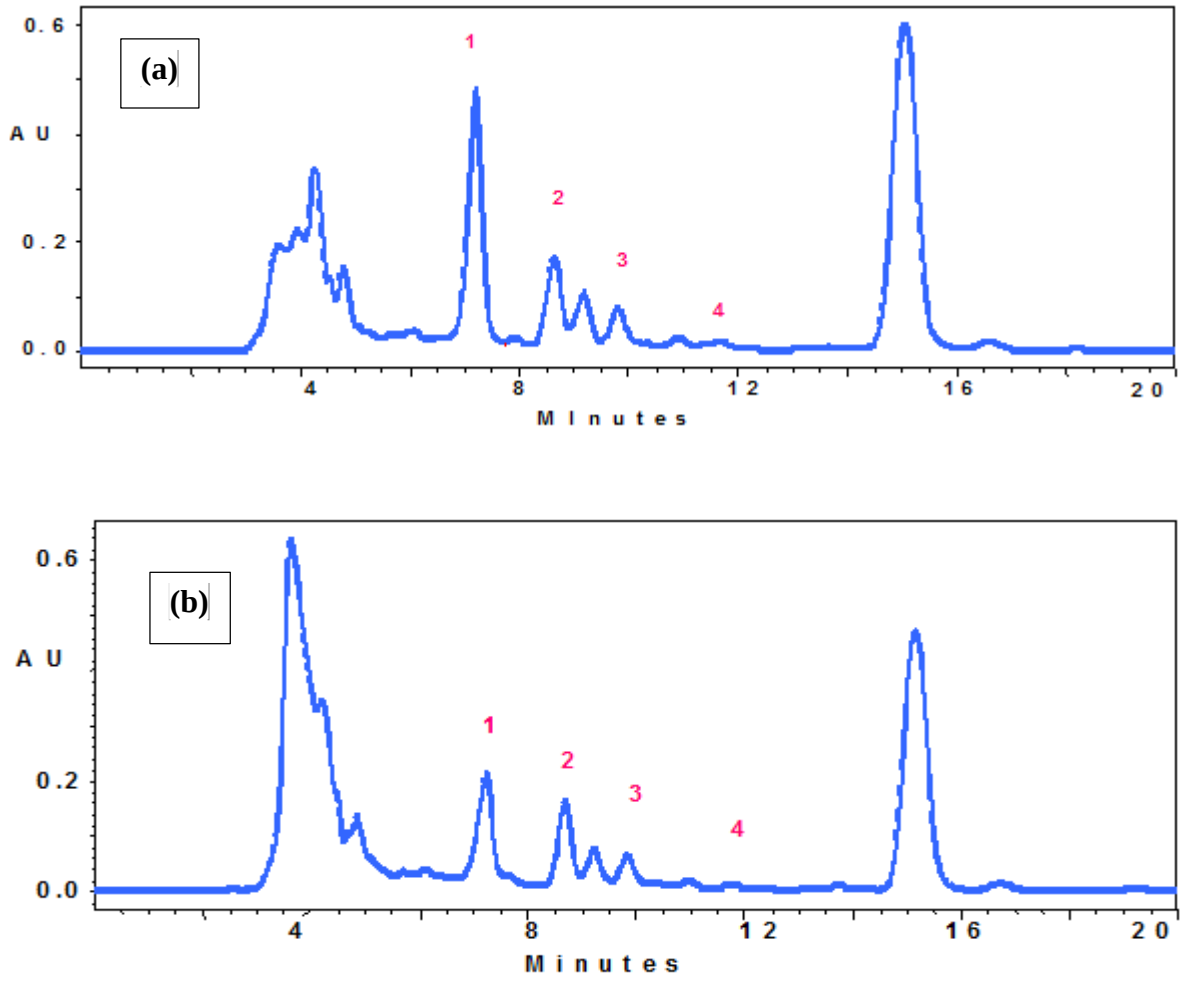


Figure 3- 5: HPLC/UV chromatogram of parabens extracted from (a) ovarian cancer tissues and (b) ovarian benign tissues. Peaks: (1) methyl paraben (2) ethyl paraben (3) propyl paraben and (4) butyl paraben.

3.4. Conclusion

The optimized MASE μ-SPE technique used in tandem with HPLC/ UV as a novel and efficient method in identifying and quantifying parabens present in biological tissues was reported. The porous polypropylene membrane of the μ-SPE device, which eliminates the entrance of fatty contents and interferences, removes the necessity of conducting additional sample-cleanup. Reduced solvent consumption and time usage are major advantages of μ-SPE compared to SPE. Furthermore, μ-SPE device is inexpensive and can be prepared easily. Our research suggests that human ovarian cancer tissue contains significant mass fractions of parabens, although a direct causative effect cannot yet be attributed to these compounds. This work is continuing in our laboratory.

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4. CHAPTER

DEVELOPMENT OF NATURAL SORBENT BASED MICRO-SOLID-PHASE EXTRACTION FOR DETERMINATION OF PHTHALATE ESTERS IN MILK SAMPLES

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Abstract

In the present study, a natural sorbent based micro-solid phase extraction (µ-SPE) was developed for determination of phthalate esters in milk samples. For the first time, an efficient and cost effective natural material (seed powder of *Moringa oleifera*) was employed as sorbent in µ-SPE. The sorbent was found to be naturally enriched with variety of functional groups and having a network of interconnected fibers. This method of extraction integrates different steps such as removal of proteins and fatty stuff, extraction and pre-concentration of target analytes into a single step. Thirteen phthalate esters were selected as target compounds for the development and evaluation of method. Some key parameters affecting the extraction efficiency were optimized, including selection of membrane, selection and amount of sorbent, extraction time, desorption solvent, volume of desorption solvent, desorption time and effect of salt addition. Under the optimum conditions, very good linearity was achieved for all the analytes with coefficient of determinations (R^2) ranging between 0.9768 and 0.9977. The limits of detection ranged from 0.01 to 1.2 µg L^{-1}. Proposed method showed satisfactory reproducibility with relative standard deviations ranging from 3.6% to 10.2% (n=7). Finally, the developed method was applied to tetra pack and bottled milk samples for the determination of phthalate esters. The performance of natural sorbent based µ-SPE was better or comparable to the methods reported in the literature.

Key words: Natural sorbent, food analysis, microextraction, gas chromatography/mass spectrometry

4.1. Introduction

Phthalate esters (PEs) are famous class of polymer additives, which are used to introduce plasticity and durability to polyvinyl chloride (PVC) and other plastic materials. Since, they are not chemically bound to the structure of the polymer chains in plastics, they can migrate from plastics to the substances they are in contact with. The considerable environmental concern arises from their migration to food materials and water [1].

PEs have been specially considered from the perspective of their health implications on the human and wildlife. They are reported to induce reproductive, developmental and neurological disorders and thus considered as endocrine disrupters (EDCs) [2–4]. Due to their potential risks to human health and environment, PEs have been placed in priority pollutant list issued by United States Environmental Protection Agency (USEPA). Ingestion of contaminated food is major route of human exposure to PEs. Therefore, it is highly desired to develop sensitive analytical methods for trace level monitoring of PEs in food.

Due to its nutritional value and immunological benefits to infants and aged people, milk stands among imperious and highly consumed foods. Thus, monitoring of potential pollutants such as PEs in milk samples has great significance from human health perspective [5]. Milk can get contaminated with PEs through different sources including environmental diffusion, direct uptake by the animals through air or food, and processing by contact with different plastics, pipes, containers etc. [6]. In dairy forms and milk processing units, bulk transfer of milk between tankers and storage tanks is carried out through plastic pipes which can introduce PEs into milk [7]. Machine milking, where milk

comes in contact with rubber parts of the machine, was also reported to enhance PE contents in milk [8].

Since milk represents a very complex matrix which is enriched with fats, lipids, carbohydrates, vitamins and minerals. Extraction of target compounds from milk samples is a rather challenging task. Conventional extraction methods such as liquid-liquid extraction and solid phase extraction require large amounts of solvents, samples and adsorbent materials. Furthermore, they are labor and time extensive and materials have lesser possibility to be reused. Thus, sample preparation scientists are nowadays dedicated to develop efficient, green, inexpensive and miniaturized extraction procedures in order to extract and pre-concentrate the analytes of interest prior to instrumental analysis [5]. Hence, over a period of time, number of microextraction methods have been developed for extraction of PEs in milk samples. In this regard, most commonly used methods are dispersive liquid-liquid extraction (DLLME) [5,9] and headspace solid phase microextraction (HS-SPME) [10]. For DLLME, clean samples are preferred for proper phase separation. Additionally, complex samples like milk cannot be used directly without pre-treatment. In order to remove proteins and fatty stuff from the samples, DLLME involves some additional steps such as use of number of organic solvents, which are not desired when analyzing PEs because PE residues can be found even in organic solvents and they are abundant in laboratory environment [11]. Moreover, pretreatment of proteins and fatty stuff using organic solvents such as methanol and acetonitrile may lead to loss or dilution of the target analytes. HS-SPME also has several limitations when dealing with PEs, particularly most of PEs show low volatility and reasonably high temperatures are required to vaporize PEs. In addition, SPME fibers are expensive and highly fragile [12].

In the current study, we applied porous membrane protected micro-solid phase extraction (μ-SPE) for extraction of PEs in milk samples. This technique was first introduced in 2006 [13] and is increasingly popular sample preparation method for different classes of organic compounds [14–16] present in food [17–19], environmental [20,21], and biological [22,23] matrices. Technique continued to evolve over the period of time and it was assisted by vortex [21], microwave [24], and by combining with other extraction approaches [17,25].

In most of the previous studies, synthetic sorbents were used in μ-SPE. Although these sorbents offer unique advantages such as high affinity and selectivity toward target compounds but their preparation is labor and time extensive due to extended synthesis procedures. Any small variations in synthesis conditions may affect the activity of sorbent to a large extent. Likewise, the consumption of large amount of chemicals for synthesis of selective sorbents is hazardous both for workers and environment and contradicts basic rules of green chemistry approaches. Hence, there is a pressing need to explore green and readily available sorbent materials for extraction applications. Thus, low cost, easily assessable and disposable natural sorbents derived from plants can be an alternative.

We tested different natural sorbents for extraction of PEs in milk samples. Inspired from its better extraction efficiency and highly fibrous, heterogeneous and naturally functionalized surface, seed powder of *Moringa oleifera* (*M. oleifera*) was selected as sorbent. *M. oleifera* belongs Moringaceae family and it grows in many countries. It is an environment friendly, non-toxic and biodegradable sorbent [26]. A recent review article describes potential of *M. oleifera* for treatment of water and wastewater [27]. In addition, it has been widely used for removal of metals [28,29] and dyes [30] in aqueous solutions. However, in few studies, it has been employed for analytical extractions [31]. To best of

our knowledge, this is the first report where seed powder of *M. oleifera* is used as sorbent for extraction of PEs.

4.2. Experimental

4.2.1. Chemicals and materials

The mixture of PE standards was purchased from Restek (Bellefonte, PA, USA). Following 13 compounds were considered for analysis from the mixture: dimethylphthalate (DMP), diethylphthalate (DEP), phthalic acid diisobutyl ester (DIBP), di-n-butylphthalate (DNBP), bis(2-methoxyethyl)phthalate (BMEP), bis(4-methyl-2-pentyl)phthalate (BMPP), bis(2-ethoxyethyl)phthalate (BEEP), dipentylphthalate (DPP), di-n-hexyl phthalate (DNHP), benzyl butyl phthalate (BBP), bis(2-n-butoxyethyl)phthalate (BBEP), phthalic acid dicyclohexyl ester (DCHP), bis(2-ethylhexyl)phthalate (BEHP). HPLC-grade toluene, acetone, acetonitrile, carbon tetrachloride and n-hexane were purchased from Tedia Company (Fairfield, OH, USA). Ultrapure water was produced on a Siemens Ultra Clear water purification system. Q3/2 Accurel 2E HF (R/P) polypropylene (PP) membrane sheet (157 μm thickness, 0.2 μm pore size) was purchased from Membrana (Wuppertal, Germany) and used for fabricating envelopes for the μ-SPE device. Cellulose acetate (0.2 μm pore size, 47 mm diameter), polytetrafluoroethylene (PTFE) (0.2 μm pore size, 47 mm diameter) and polycarbonate (0.2 μm pore size, 47 mm diameter) were purchased from Advantec (Toyo Roshi Kaisha, Japan). C_{18} was obtained from Alltech (Deerfield, USA). Dried *M. oleifera* seeds were purchased from local market (Alkhobar, Saudi Arabia) and ground to powder before using as sorbent. Banana peels were properly dried and powdered to use as sorbent and rice husk was obtained by a rice mill in India.

4.2.2. GC-MS

Analysis was carried out using Shimadzu QP2010 GC–MS system equipped with a Shimadzu AOC-20i autosampler and Rxi -5 Sil MS (Restek) fused silica capillary column (30 mm × 0.25 mm internal diameter, 0.25 μm film thickness). High purity helium (99.9999%) was used as carrier gas at a flow rate of 1.01 mL/min. Samples were injected in splitless mode. The injector temperature was set at 250 °C and the interface temperature maintained at 220 °C. The GC oven was initiated from 40 °C, and was then increased to 200 °C at 8 °C/min, and held for 1 min, after that it was increased to 220°C at 5 °C/min, and held for 1 min, and finally it was increased to 250 °C at 3 °C/min . First, a standard solution containing high concentrations of all analytes was run in scan mode for qualitative analysis and analyte peaks were detected and confirmed using MS library. The analytes were analyzed in selective ion monitoring mode for quantitative determination. The monitored ions were selected based on good selectivity and high sensitivity, selected m/z values are listed in Table 4-1.

4.2.3. Preparation of natural sorbent and its characterization

Natural sorbent was obtained by grinding the seeds of *M. oleifera* to fine powder. This powder was then passed through 75 μm sieve and washed with deionized water for several times to remove any exterior contamination. After washing, sorbent was dried in oven at 60°C for 8 hours. Field emission-scanning electron microscopy (FE-SEM), Fourier-transform infrared spectroscopy (FTIR), X-ray diffraction (XRD), thermogravimetric analysis (TGA) and differential scanning calorimetry (DSC) were used to characterize the sorbent.

Table 4- 1: List of target compounds, their CAS numbers and chromatographic parameters

Analyte	**CAS Number**	**Retention time**	**Target ions m/z for SIM mode**	**Elution order**
DMP	131-11-3	15.875	163, 77	1
DEP	84-66-2	18.050	149, 177	2
DIBP	84-69-5	22.145	149,57,223	3
DNBP	84-74-2	23.735	149,104	4
BMEP	117-82-8	24.340	59,149,207	5
BMPP	146-50-9	25.575	149,85, 167	6
BEEP	605-54-9	26.385	45, 72, 149, 104	7
DPP	131-18-0	27.185	149,237	8
DNHP	84-75-3	31.260	149,251	9
BBP	85-68-7	31.440	149, 91, 206	10
BBEP	117-83-9	34.120	57, 149, 193	11
DCHP	84-61-7	35.140	149, 167	12
BEHP	117-81-7	35.645	149, 167, 279	13

4.2.4. Sample preparation

A stock solution (1000 µg L^{-1}) was prepared by diluting the standards mixture of PEs with n-hexane, and was stored in the refrigerator at 4 °C. Working standard solutions (50 µg L^{-1}) were freshly prepared by diluting the stock solution appropriately. Milk samples used for studying the extraction performance were prepared by spiking PEs free milk at known concentrations. Different brands of bottled and tetra-pack milk were purchased from the local market and stored at 4 °C till the analysis.

4.2.5. µ-SPE

The µ-SPE device was constructed by packing natural sorbent in a PP envelope. Fabrication of PP envelope to serve as µ-SPE device was based on very simple procedure. Briefly, the envelope was fabricated by folding the equal lengths of membrane over each other and open ends were heat sealed except the one end which was left for packing of the sorbent. 30 mg of natural sorbent was packed via the remaining open end, which was then heat-sealed to produce the extraction device (2.0 cm × 0.7 cm). The dimensions of the µ-SPE device ensured that it could fit into a 600 µL glass vial during the desorption process. Fabrication of a single µ-SPE device took 3 to 4 mins and about 15 to 20 devices could be fabricated within one hour. Each device was first conditioned and cleaned by dipping in acetone and ultrasonicating for 10 mins. After conditioning µ-SPE devices were air dried.

For extraction, the µ-SPE device and magnetic stirrer were placed in a 5 mL sample solution and vortexed for 10 mins. After extraction, using a pair of tweezers, the µ-SPE device was removed, dried with lint free tissue, and then fitted into a glass vial for desorption. Analytes were desorbed by adding 100 µL of acetonitrile into glass vial.

Desorption was assisted by ultra-sonication for 5 mins. After that, μ-SPE device was taken out of the glass vial and 1 μL of extracted liquid was injected into GC-MS. The schematic of extraction procedure is shown in Figure 4-1.

4.2.6. Optimization of extraction conditions, calibration plots and analysis of real samples

Factors that affect extraction efficiency of μ-SPE were optimized. These factors included membrane selection, selection of sorbent and its amount, desorption solvent and its volume, extraction and desorption times, effect of salt addition. Carryover effects and reusability of the μ-SPE device were also tested. All the optimization experiments were performed using 5 mL sample solution containing 50 μg L^{-1} of PEs.

6-point calibration plots were drawn under optimum extraction conditions by using PEs free milk spiked at 1, 5, 10, 25, 50 and 100 μg L^{-1}. Finally, the method was adopted for extraction and quantitation of PEs in three different brands of milk samples.

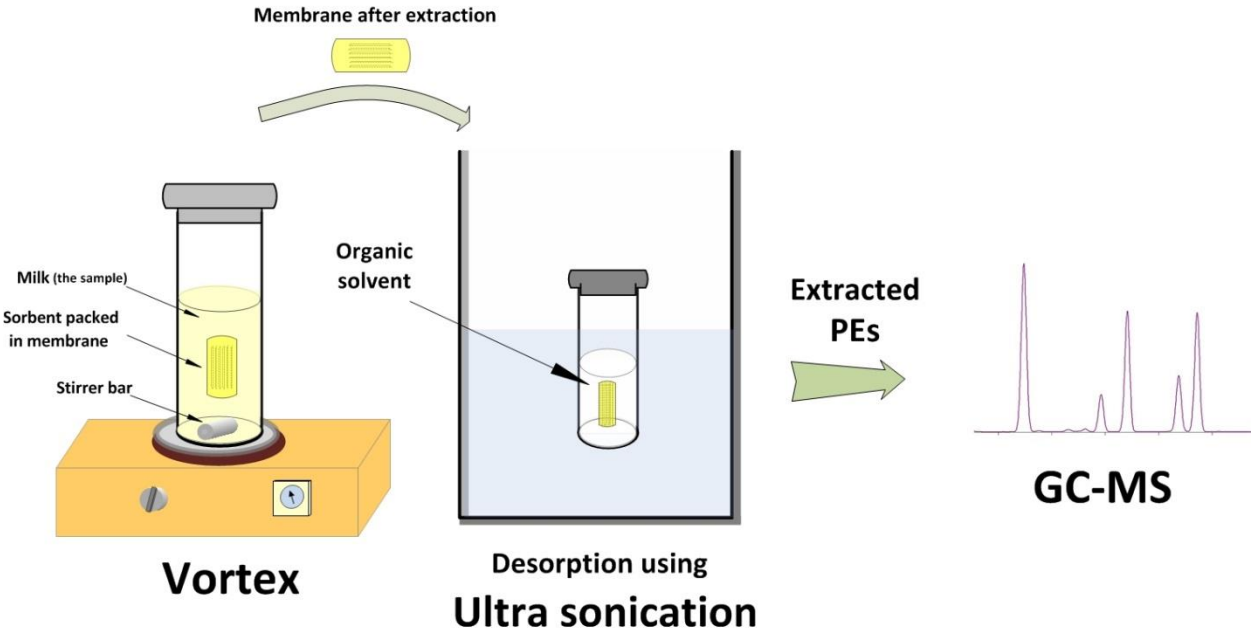

Figure 4- 1: Schematic of extraction method using µ-SPE. For clarity, schematic not drawn to scale.

4.3. Results and discussion

4.3.1. Characterization of natural sorbent

Surface morphology is critical in determination of the sorption properties of the sorbents. FE-SEM images were used to inspect surface morphology. Highly porous framework of interconnected fibers was observed by SEM images which reveals the potential of material as sorbent (Figure 4-2). Presence of various functional moieties was confirmed by FTIR spectra of the sorbent (Figure 4-3). A peak of O—H stretching found at 3429 cm^{-1}corresponds to proteins, fatty acids, carbohydrates and lignin units. Because of high protein content, same region is also attributed to N—H stretching of amides. Peaks at 2924 and 2853 cm^{-1} correspond respectively to asymmetric and symmetric stretching of C—H in CH_2 group. Peaks at 1710 and 1653 cm^{-1} are related to C=O stretching, which is present in proteins and fatty acid structures. Hence, the functional groups enriched bio-sorbent is supposed to interact with target compounds through above said functional moieties.

The XRD patterns of the sorbent showed a single and poorly resolved broad peak indicating the amorphous nature of the material (figure not shown). TGA) and DSC were performed using SDT Q600 analyzer (TA instruments, USA) to characterize the stages of decomposition and the thermal stability of the sorbent. Both techniques present the mass loss curve of the bio-sorbent material. TGA curve (Figure 4-4a) indicates that the sorbent consist of a mixture of several components confirming the heterogeneous nature. The three main stages in the mass loss curve are: (I) from 30-128^{o}C, 8% of mass loss related to water desorption, (II) from 128-268^{o}C, 32% of mass loss occurs due to decomposition of organic matter and protein components; (III) from 268-541^{o}C the greater decomposition part of the

seed constituents was observed, most probably including fatty acids such as oleic acid. At 950^{o}C, decomposition occurred due to the presence of some inorganic oxides and ash. These facts were also supported by DSC curve (Figure 4-4b).

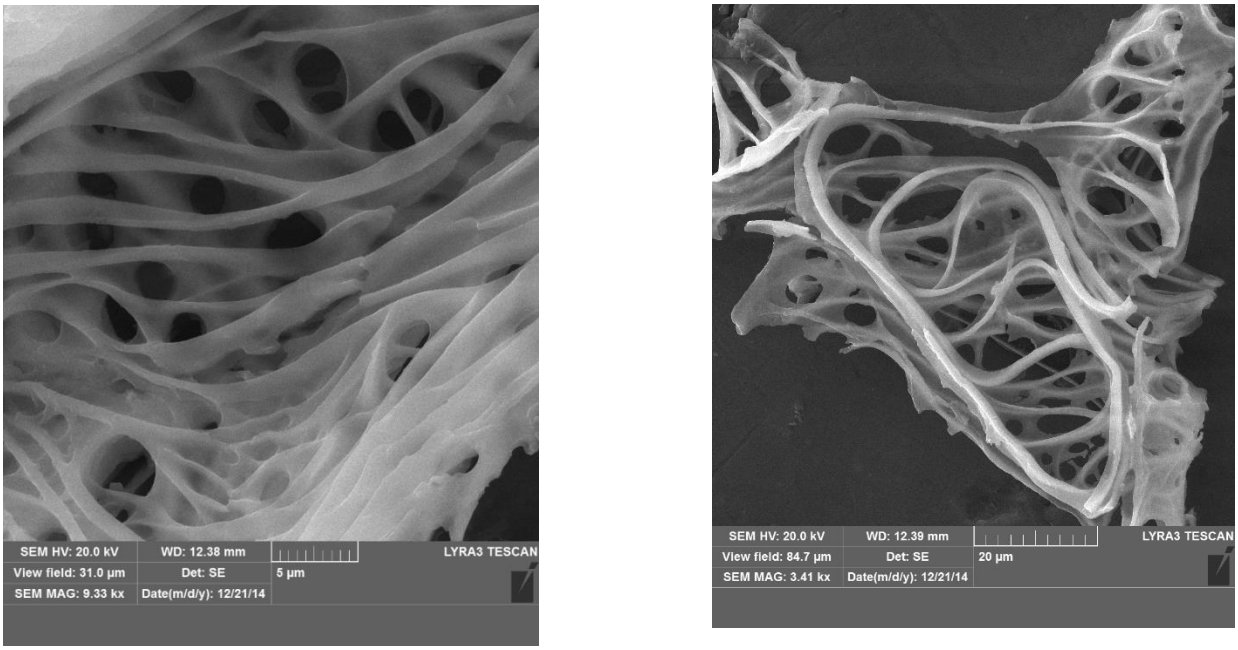

Figure 4- 2: SEM images of natural sorbent (Seed powder of M. oleifera) at different magnifications

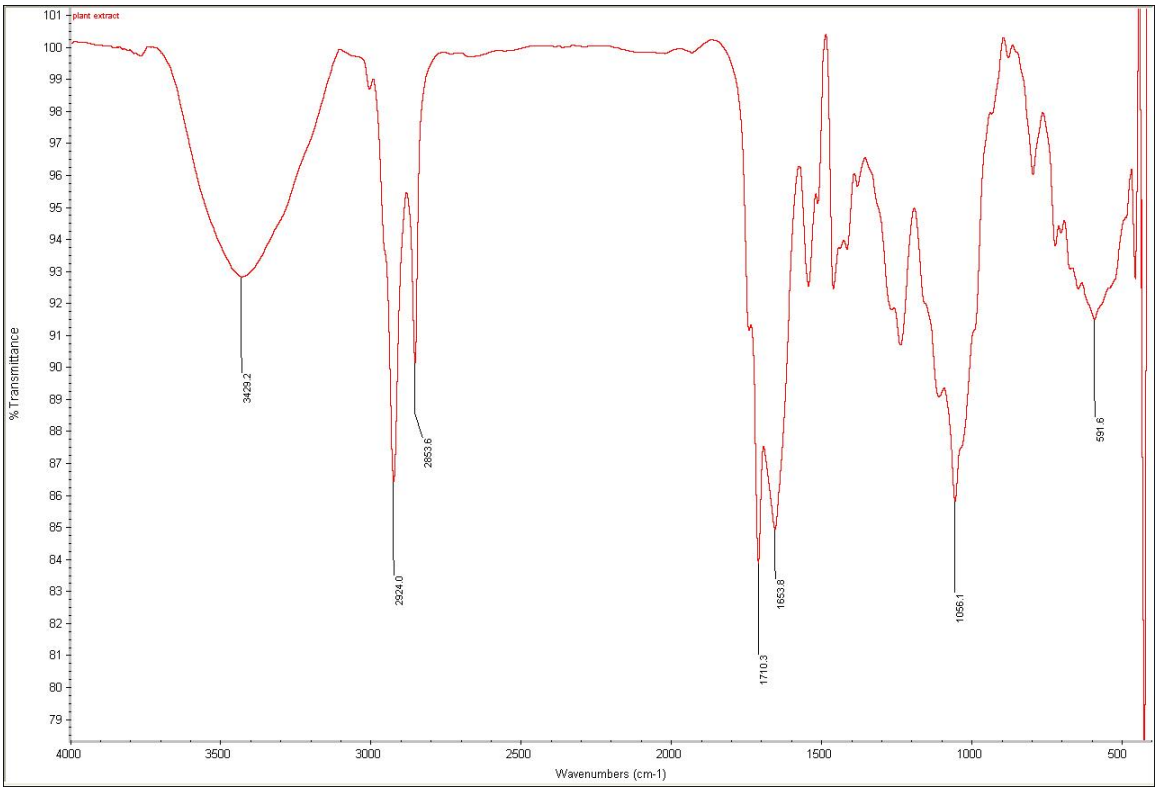

Figure 4- 3: FTIR spectrum of seed powder of M. oleifera

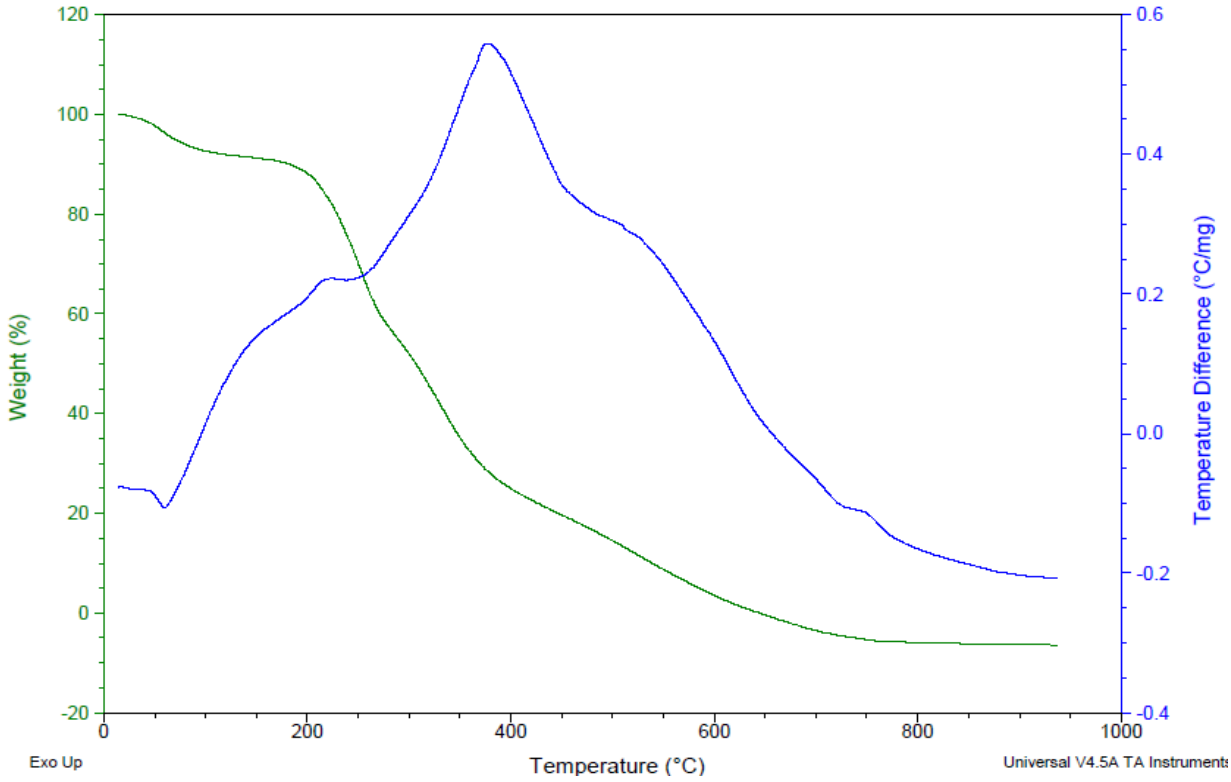

Figure 4- 4: TGA (a) and DSC (b) of seed powder of M. oleifera

4.3.2. Optimization: Factors affecting extraction efficiency

The most frequent problem that has been reported to encounter while analysis of PEs is contamination of PEs from the glassware, reagents and plastic materials in the lab. There is a probability of contamination during any step of analysis. Hence, in order to avoid the contamination of PEs, glassware was washed with ultrapure water, acetone and methanol and then dried at 120°C overnight. This glassware was then stored in a thermally treated aluminum foil. The ultrapure water was extracted by proposed method and then desorbed by toluene. The resulting chromatogram showed some PE peaks. In order to find the source of these peaks, blank toluene was also injected into GC-MS. The concentrations of PEs in toluene was same as found in ultrapure water. Hence, the source was toluene.

Other solvents employed during this extraction were also tested for contamination and PE traces were also found in carbon tetrachloride. No traces of PEs were found in acetonitrile and n-hexane. The solvents toluene and carbon tetrachloride were also investigated during solvent optimization step, however, the initial contamination was taken into account while quantitation of PEs. The best extraction performance was obtained with acetonitrile. As initial screening showed that it was free of PE traces, hence, no additional calculations were needed for all upcoming optimization procedures.

Optimization was carried out by analysis of triplicate uncontaminated milk samples spiked with 50 μg L^{-1} of PEs.

4.3.2.1. Membrane selection

We tested different types of commercially available porous membranes (PP, polycarbonate, cellulose acetate and PTFE in order to contain sorbent materials for μ–SPE. Porous polypropylene membrane was chosen as the most suitable membrane for making μ–SPE devices. The wide chemical compatibility of PP membrane makes it an appropriate membrane for μ–SPE device. Other membranes such as polycarbonate, cellulose acetate and PTFE were deemed unsuitable. The polycarbonate membrane was flimsy whereby vigorous stirring during extraction would tear the device even before the completion of extraction. Cellulose acetate membrane was brittle and could snap upon folding during the preparation of μ –SPE device. PTFE membrane was not heat sealable.

4.3.2.2. Sorbent selection and amount

The selection of the sorbent is of vital importance for μ-SPE because it determines selectivity towards analytes. Extraction efficiency of seed powder of *M. oleifera* was compared with C_{18}, banana peel and rice husk. C_{18} is commonly used conventional sorbent in μ-SPE for extraction of organic analytes [32] while banana peel and rice husk are among other commonly used bio-sorbents. The constant mass of each sorbent (50 mg) was used for selection of optimum sorbent. Seed powder of *M. oleifera* was found to be most effective sorbent and gave highest peak areas compared to other sorbents (Figure 4-5). *M. oleifera* seeds are reported to contain significant fiber, protein and ash content [33]. All these properties are advantageous for adsorption of target compounds. Hence, better extraction performance of *M. oleifera* may be attributed to

(i) Its fibrous structure which comprise a highly porous network of interconnected fibers. In general, fibrous structures have good adsorption capabilities.

(ii) Presence of proteins and other naturally functionalized moieties that may interact with target compounds. Larger the amount of proteins in a bio-sorbent corresponds to abundance of functional groups. The overall protein content of *M. oleifera* seeds is reported up to 38% [34] and it is much higher than the protein content of banana peel (0.90%) [35] and rice husk (2.5%) [36].

(iii) Electrostatic interactions between carbon content of ash and target compounds.

Hence, *M. oleifera* was selected as sorbent for further experiments.

Different amounts (20, 30, 60, 90, 120 mg) of natural sorbent were packed into polypropylene membrane and 30 mg was found as an optimum sorbent amount. Peak areas increased up to 30 mg and then became constant. This amount can easily fit into a desorption vial and desorption can be done using smaller volume of the organic solvent.

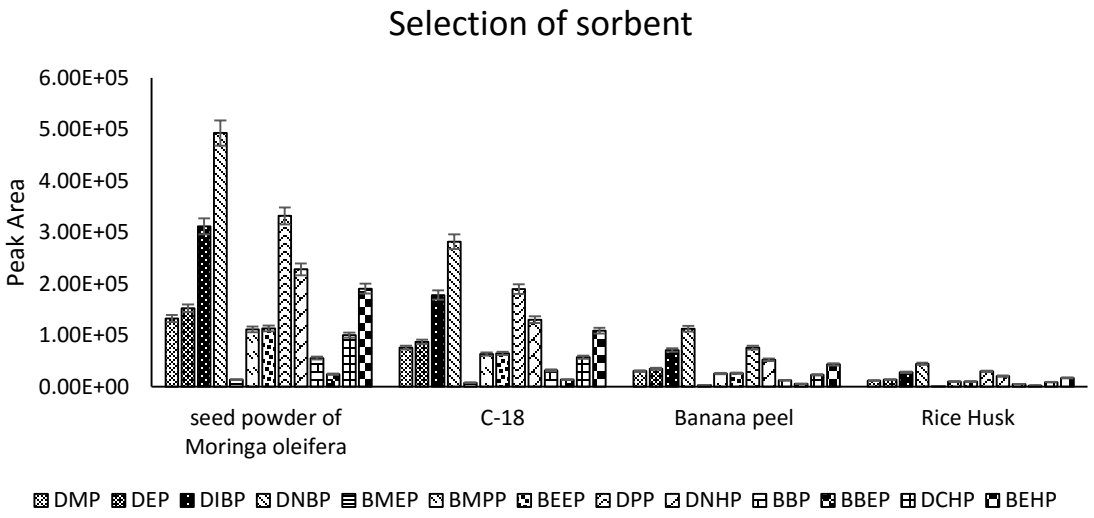

Figure 4- 5: Influence of sorbent type on extraction of PEs. Conditions: PEs concentration: 50 µg L^{-1}; extraction time: 10 mins; desorption solvent: toluene; desorption volume: 250µL; desorption time: 5 mins; amount of salt: 0%.

4.3.2.3. Desorption solvent and its volume

After extraction, the µ-SPE device was transferred into a glass vial. After that, desorption solvent was added to the glass insert and desorption was carried out with aid of ultrasonication. Desorption solvents with varying polarity index were investigated to get the maximum desorption of the analyte from sorbent to desorbing solvent. The solvents toluene, acetonitrile, n-hexane and carbon tetrachloride were tested. Figure 4-6 shows that highest peak areas were obtained by using acetonitrile and it was selected as an optimum desorption solvent for further experiments.

A series of experiments was performed to find out the optimum volume of the desorption solvent (acetonitrile). Different volumes of acetonitrile ranging from 100 to 400 µL were investigated. The highest peak areas were obtained with 100 µL of desorption solvent. This can be attributed to the high enrichment of analytes in lower volumes. The volumes below 100 µL were not considered in order to have sufficient volume for immersion of µ-SPE device and complete desorption thereafter. Hence, 100 µL acetonitrile was adopted as an optimum value of desorption solvent (Figure 4-7).

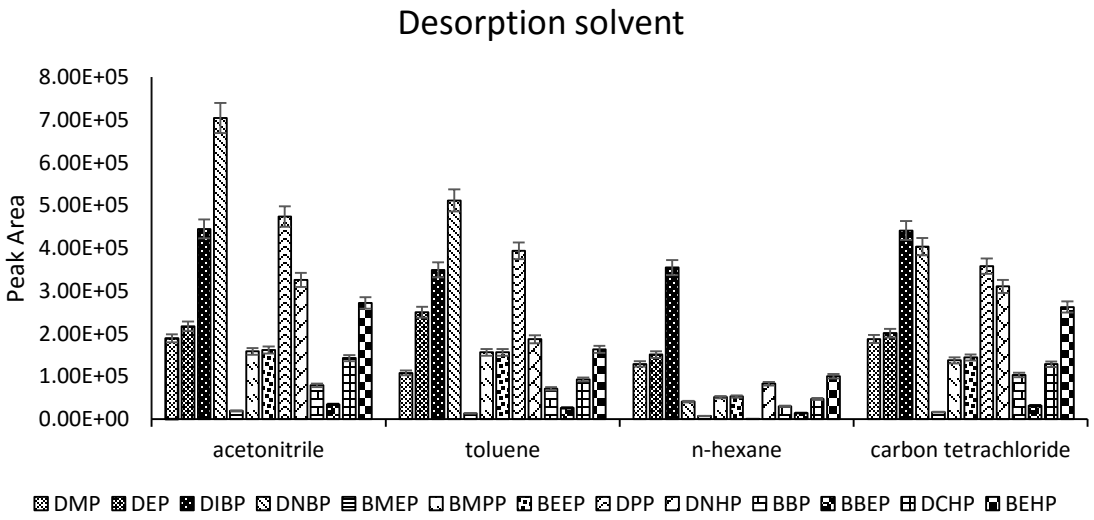

Figure 4- 6: Influence of desorption solvent on extraction of PEs. Conditions: PEs concentration: 50 µg L^{-1}; extraction time: 10 mins; desorption volume: 200µL; desorption time: 5 mins; amount of salt: 0%.

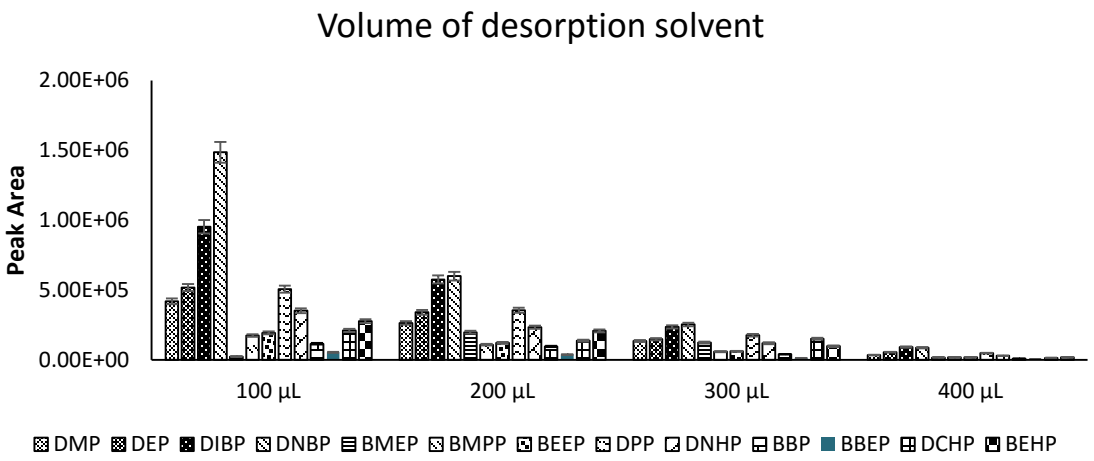

Figure 4- 7: Influence of volume of desorption solvent on extraction of PEs. Conditions: PEs concentration: 50 µg L^{-1}; extraction time: 10 minutes; desorption solvent: acetonitrile; desorption time: 5 minutes; amount of salt: 0%.

4.3.2.4. Extraction and desorption times

The extraction efficiency of µ-SPE device depends on the rate of mass transfer of analytes from the donor phase (sample solution) to the sorbent phase. Sample agitation improves extraction efficiency by enhancing contact between the analytes and sorbent phase. Hence, extraction (agitation) time is an important parameter to consider. The extraction time was evaluated in range of 5–20 mins. Figure 4-8 shows that peak areas of analytes increased with extraction up to 10 mins with no significant increase thereafter. After 10 mins, peak areas reached to a steady state. As in µ-SPE, the analytes are dynamically distributed between the sorbent and solution phase. Establishing a steady state after 10 mins is indicative to mass transfer equilibrium between sorbent and solution phase. Hence, extraction time of 10 mins was selected as an optimum time for next experiments.

The effect of desorption time was investigated over a range of 5 to 20 mins. Maximum extraction efficiency was observed at desorption time of 5 mins. After 5 mins, extraction efficiency was gradually decreased which can be attributed to increased temperature as a result of longer periods of ultra-sonication. PEs may evaporate at increased temperatures (Figure 4-9).

4.3.2.5. Effect of salt addition

In most of the microextraction studies, experiments are performed to evaluate the effect of salt addition on extraction efficiency. Upon salt addition, solubility of the target analytes decreases in the aqueous phase and it leads to their migration to extractant phase. Salting-out effect was studied by adding various amounts of NaCl to milk samples (ranging from

0 to 20%, w/v). The highest peak areas were obtained at 0% (without salt addition) (Figure 4-10).

Upon addition of 5% salt, we observed that peak areas of some analytes were significantly decreased while for other compounds they remained constant. Further addition of salt up to 20% did not affect peak areas. This decrease in extraction efficiency upon salt addition can be due to enhanced viscosity of the sample solution which hinders movement of target analytes from donor to acceptor phase [37]. Moreover, salt addition will precipitate proteins which may hinder adsorption of target compounds into μ-SPE device.

4.3.2.6. Carry over effects and reusability of μ-SPE device

After the first desorption round, the μ-SPE device was re-desorbed in acetonitrile to investigate carryover effects. No analyte peaks were detected which meant that device can be re-used. Hence, we investigated the repeated use of μ-SPE device and results confirmed that it could be used for 20 to 25 times. RSD values for reusability data ranged between 3.8 to 9.2 %. The reusability of μ-SPE device is, however, highly dependent on the durability of membrane material itself.

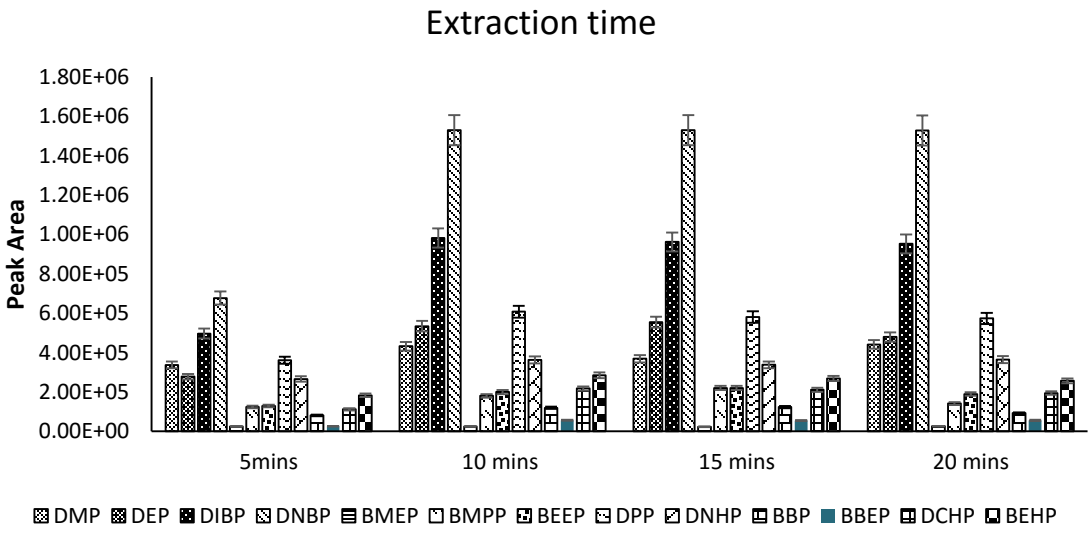

Figure 4- 8: Influence of extraction time on extraction of PEs. Conditions: PEs concentration: 50 µg L^{-1}; desorption solvent: acetonitrile; desorption volume: 100µL; desorption time: 5 mins; amount of salt: 0%.

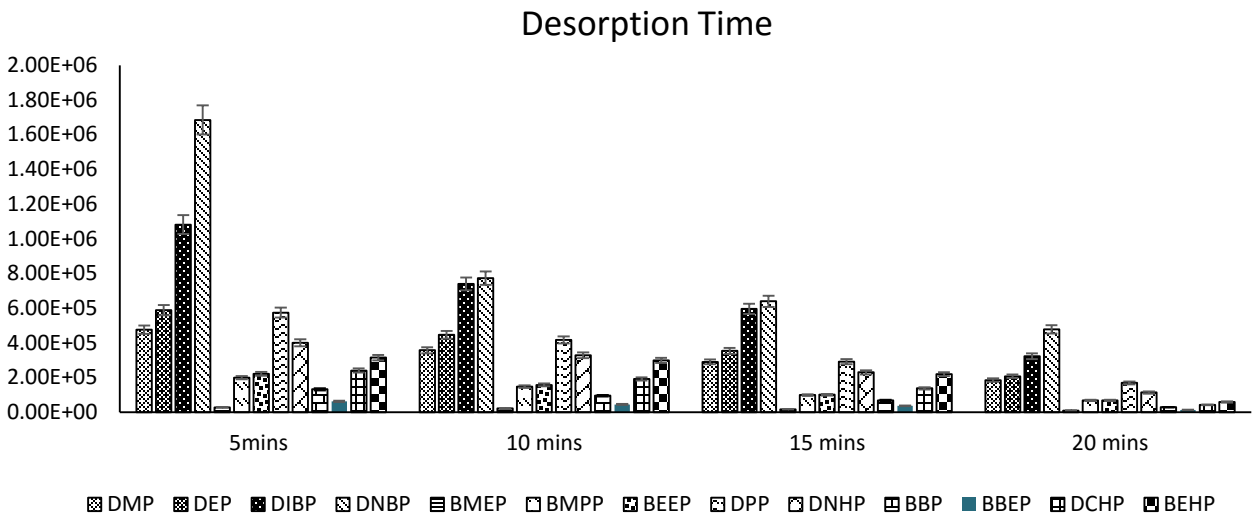

Figure 4- 9: Influence of desorption time on extraction of PEs. Conditions: PEs concentration: 50 µg L^{-1}; desorption solvent: acetonitrile; desorption volume: 100µL; extraction time: 10 minutes; amount of salt: 0%.

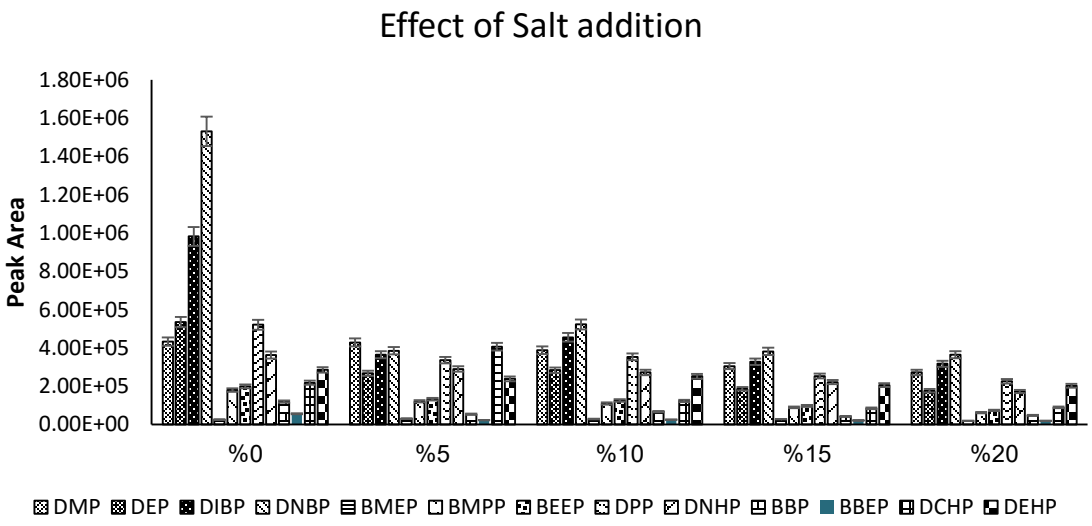

Figure 4- 10: Influence of salt addition on extraction of PEs. Conditions: PEs concentration: 50 µg L^{-1}; desorption solvent: acetonitrile; desorption volume: 100µL; extraction time: 10 minutes; desorption time: 5 minutes.

4.3.3. Method validation

To evaluate analytical performance of the proposed natural sorbent based µ-SPE-GC-MS method, parameters like linearity, repeatability, limits of detection and enrichment factors were investigated under optimum experimental conditions. The results are summarized in Table 4-2.

Calibration curves were plotted under most favorable extraction conditions using milk samples that were originally free from PEs and spiked with different concentrations of PEs (1, 5, 10, 25, 50, 100 µg L^{-1}) . Each point of the calibration curve corresponded to the average value obtained from seven measurements. Good linearity was observed over a concentration range of 1-100 µg L^{-1} with coefficient of determination (R^2) ranging from 0.9768 to 0.9977. LODs calculated based on signal-to-noise (S/N) ratio of 3, were in the range of 0.01 to 1.2 µg L^{-1}. Precision of any developed method is determined by reproducibility data. Intra-day and inter-day reproducibility was accessed by extracting the spiked milk samples (n=7 for each) under the same extraction conditions and the values of relative standard deviations (RSDs) were in between 3.6% and 10.2%. This reflects an acceptable precision. Similarly, repeatability of extraction was studied with different µ-SPE devices and satisfactory RSD values ranging between 2.5% to 7.2% were obtained.

Table 4- 2: Analytical features of the proposed method

Compound	**DLR[a] (µg L^{-1})**	**Coefficient of determination (r^2)**	**LOD[b] (µg L^{-1})**	**LOQ[c] (µg L^{-1})**	**RSD[d](%) (n=7)**	
					Intra-day	**Inter-day**
DMP	1-100	0.9977	0.07	0.22	5.4	6.7
DEP	1-100	0.9966	0.06	0.18	4.1	4.5
DIBP	1-100	0.9973	0.04	0.12	8.7	9.1
DNBP	1-100	0.9928	0.01	0.4	6.0	5.7
BMEP	5-100	0.9908	1.2	3.7	7.6	8.3
BMPP	1-100	0.9943	0.07	0.21	3.6	5.8
BEEP	1-100	0.99	0.09	0.27	5.9	7.2
DPP	1-100	0.9914	0.03	0.10	7.0	6.7
DNHP	1-100	0.9768	0.06	0.18	4.6	4.3
BBP	1-100	0.9949	0.06	0.19	4.7	5.9
BBEP	1-100	0.9898	0.11	0.34	8.4	7.8
DCHP	1-100	0.9926	0.08	0.24	6.2	4.7
BEHP	1-100	0.9843	0.07	0.21	9.4	10.2

[a]Dynamic linear range; [b]Limit of detection: calculated as three time of the baseline noise ;
[c]Limit of quantitation: 3×LOD; [d]reproducibility.

4.3.4. Real Samples

The proposed method was applied for determination of PEs in the milk samples. For this purpose, three different brands of milk samples (2 bottled and 1 tetra pack) each with fat content of 3% were obtained. In order to access the matrix effect, the relative recoveries of the method were calculated by extracting the spiked milk samples and comparing with extraction of the same concentrations spiked in water samples. The mean recoveries for all analytes in three different brands of milk samples are listed in Table 4-3. Some differences in the recoveries were observed such as long alkyl chain containing PEs e.g. BBEP and BEHP showed relatively low recoveries. It can be explained by two ways

(i) As the sorbent is naturally functionalized with polar moieties, it will interact strongly with relatively polar PEs. As the length of alkyl chain increases in PEs, their polarity will decreases, which in turn leads to their relatively poor extraction by the sorbent.

(ii) Secondly, it is probably an indication that matrix effect is less effective in case of relatively polar PEs but as the length of alkyl chain in PE molecules increases, the interactions between PEs and proteins in the milk become more prominent. Similar findings have also been reported in another study [5].

Only seven compounds DMP, DNBP, BMEP, DPP, BBP, DCHP and BEHP were detected in some of the real milk samples (Table 4-4). Maximum contamination levels (MCLs) of PEs in milk have not been regulated yet, but the MCLs for DEHP in water is 6 μg L^{-1} [38]. The concentrations found in milk samples were higher than this level.

The performance of the proposed method was compared with those reported in the literature (Table 4-5). The natural sorbent based μ-SPE-GC-MS method has several

advantages on the methods reported in literature. It requires small volume of sample (5 mL). In addition, it utilizes very small amount of the natural sorbent (30 mg) compared to conventional SPE. The proteins and fats from the milk samples cannot deposit over the sorbent as it is protected inside the membrane by heat sealing. No additional steps are needed for removal of proteins from the milk prior to extraction, which is usually time extensive. Moreover, very small volume (100 µL) of the organic solvent is required for desorption of the target analytes from the sorbent, which is a unique advantage in terms of green methods. A single µ-SPE device can be reused for 20-25 times before any signs of wear and tear appears on it. Similarly, very good values of LODs and linear range make this method comparable or superior than the reported methods. All this discussion shows that proposed method is rapid, and efficient for determination of PEs in the milk samples.

Table 4- 3: Study of matrix effect on extraction of PEs from different brands of milk samples

Mean relative recovery ± standard deviation (n=3)						
Analyte	**Spiked at 25µg L^{-1}**			**Spiked at 50 µg L^{-1}**		
	Brand 1	**Brand 2**	**Brand 3**	**Brand 1**	**Brand 2**	**Brand 3**
DMP	102±3	99±4	101±4	101±5	99±2	100±1
DEP	98±2	100±3	99±6	102±2	98±5	99±4
DIBP	98±3	99±6	94±4	100±2	99±3	96±3
DNBP	97±2	98±5	100±4	99±2	96±6	101±1
BMEP	99±5	102±4	100±3	97±1	103±4	101±2
BMPP	96±5	96±7	93±5	98±4	97±6	98±5
BEEP	89±8	92±7	95±4	90±9	92±6	97±5
DPP	91±7	88±8	94±3	93±9	91±8	96±4
DNHP	86±11	89±9	85±4	88±5	92±5	88±3
BBP	89±9	98±3	82±8	91±8	101±2	83±4
BBEP	78±5	79±4	82±5	79±6	81±4	85±7
DCHP	81±6	79±5	85±2	82±7	78±4	83±5
BEHP	79±5	77±9	83±6	80±4	78±5	81±2

Table 4- 4: Concentrations of PEs in real milk samples

Analyte	**Concentrations of PEs (μg L^{-1}) in real milk samples**		
	Brand 1	**Brand 2**	**Brand 3**
DMP	ND	25.1	ND
DEP	ND	ND	ND
DIBP	ND	ND	ND
DNBP	32.3	6.3	19.2
BMEP	ND	ND	12.5
BMPP	ND	ND	ND
BEEP	ND	ND	ND
DPP	ND	15.2	ND
DNHP	ND	ND	ND
BBP	15.8	ND	ND
BBEP	ND	ND	ND
DCHP	18.8	ND	ND
BEHP	8.9	10.2	ND

ND= not detected

Table 4- 5: Comparison of the proposed method with others reported in the literature.

Method	**Number of PEs studied**	**LODs (µg L^{-1})**	**Linear range (µg L^{-1})**	**Extraction Time (min)**	**Sample volume (mL)**	**Reference**
SPE-GC-MS	5	0.06-0.12	-----	--------	----	[6]
DLLME-GC-FID	5	0.5-3	50-800	15	5	[5]
LE-SPE-LC-MS/MS	6	0.01-0.5	-----	100	3	[39]
LLE-SPE-LC-MS/MS	5	5-9		LLE and SPE needs extraction, drying, reconstitution and clean up. Huge time consumption	Large volumes of organic solvents utilized.	[40]
µ-SPE-GC-MS	13	0.01-0.20	1-100	15	5	Present work

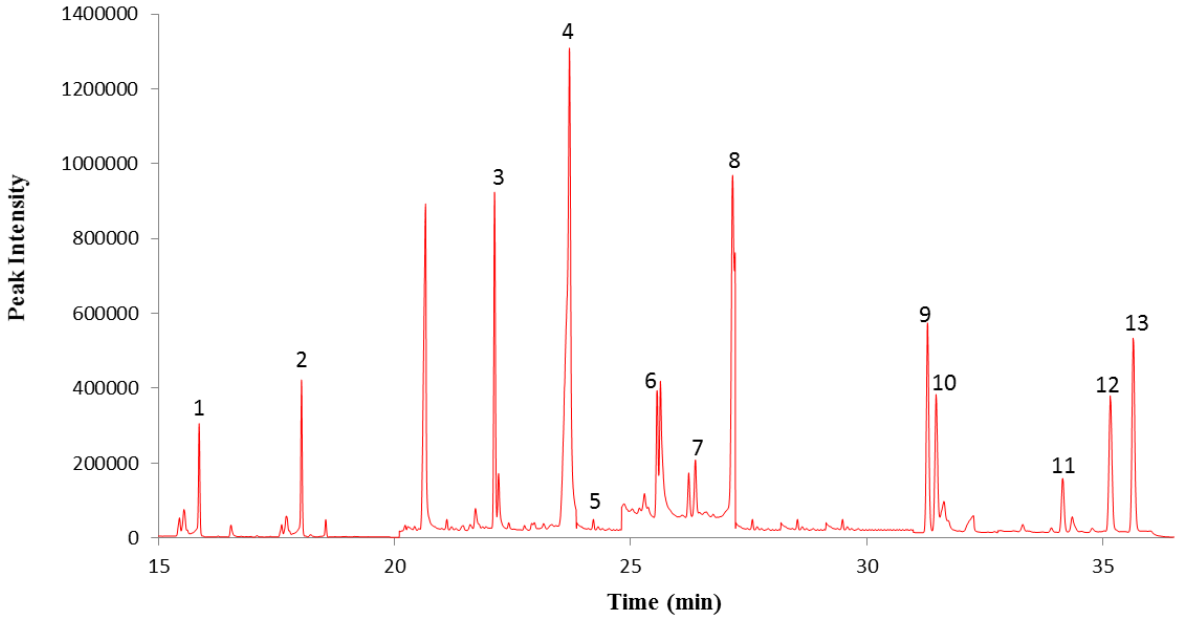

Figure 4- 11: GC-MS trace of a spiked real milk sample extracted under most favorable µ-SPE conditions. (1) DMP, (2) DEP, (3) DIBP, (4) DNBP, (5) BMEP, (6) BMPP, (7) BEEP, (8) DPP, (9) DNHP, (10) BBP, (11) BBEP, (12) DCHP, (13) BEHP.

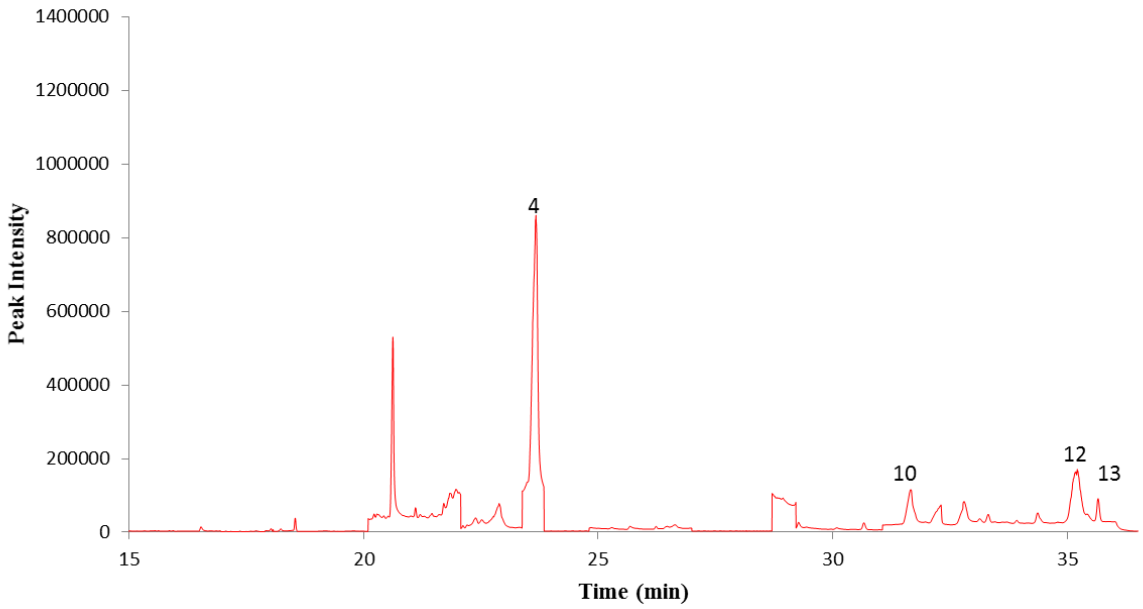

Figure 4- 12: GC-MS trace of a real milk sample extracted under most favorable µ-SPE conditions. Detected compounds are: (4) DNBP, (10) BBP, (12) DCHP, (13) BEHP.

4.4. Conclusion

In the present study, a natural sorbent based µ-SPE was utilized for the extraction of PEs in the milk samples. Efficient extraction of PEs was originated from the unique properties of the natural sorbent such as porous and fibrous structure and natural enrichment with functional moieties. *M. oleifera* as sorbent is easy to use, reuse and dispose of. It is green, low cost and readily available sorbent. The proposed natural sorbent based µ-SPE method gave reasonable reproducibility and good linearity with LODs lower or comparable to the methods reported in the literature.

4.5. References

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5. CHAPTER

STIR-BAR SUPPORTED MICRO-SOLID-PHASE EXTRACTION FOR DETERMINATION OF POLYCHLORINATED BIPHENYLS IN SERUM SAMPLES

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Abstract

In present work, a new configuration of micro-solid phase extraction was introduced and termed as stir-bar supported micro-solid-phase extraction (SB-µ-SPE). A tiny stir-bar was packed inside the porous polypropylene membrane along with sorbent material and the edges of membrane sheet were heat sealed to secure the contents. The packing of stir-bar inside the µ-SPE device, does not allow the device to stick with the wall or any corner of the sample vial during extraction, which is, however, a frequent observation in routine µ-SPE. Moreover, it enhances effective surface area of the sorbent exposed to sample solution through continuous agitation (motion and rotation). It also completely immerses the SB-µ-SPE device in the sample solution even for non-polar sorbents. Polychlorinated biphenyls (PCBs) were selected as model compounds and the method performance was evaluated in human serum samples. After extraction, samples were analyzed by gas chromatography mass spectrometry (GC-MS). The factors that affect extraction efficiency of SB-µ-SPE were optimized. Under optimum conditions, a good linearity (0.1 – 100 ng mL^{-1}) with coefficients of determinations ranging from 0.9868 to 0.9992 were obtained. Limits of detections were ranged between 0.003 and 0.047 ng mL^{-1}. Acceptable values for inter-day (3.2 – 9.1%) and intra-day (3.1 – 7.2%) relative standard deviations were obtained. The optimized method was successfully applied to determine the concentration of PCB congeners in human serum samples.

5.1. Introduction

Despite of all major advancements in analytical instrumentation, sample preparation is an unavoidable step prior to instrumental analysis. It is because original samples are sometimes composed of matrix which is not compatible with the components of the instrument or too dilute that target compounds cannot be detected directly by the instrument or too dirty that matrix components can interfere with analysis. Sample preparation, generally involves sample clean-up, removal of interferences and preconcentration of target compounds [1]. Solid-phase extraction (SPE) and liquid-liquid extraction (LLE) are most commonly used sample preparation methods but they consume large amounts of chemicals and hazardous organic solvents. Moreover, they are laborious because of extended multistep extraction procedures [2]. The trend has been shifting toward miniaturized sample preparation methods. Over the last two decades, number of microextraction methods have been introduced including solid phase microextraction [3], liquid-phase microextarction [4,5] , dispersive solid phase extraction [6], dispersive liquid-liquid microextraction [7] and many of their modifications. These methods overcome disadvantages of conventional SPE and LLE. In addition, these methods are comparatively simple, cost effective, efficient, green, and easy to perform.

Micro-solid phase extraction (µ-SPE) was introduced by Basheer et al., in 2006 [8]. Since then, µ-SPE has been extensively used in environmental [9,10], biological [11,12] and food [13] analysis. This approach involves packing of small quantity of sorbent material inside a porous membrane, whose ends are heat sealed. This sorbent containing bag is then placed inside the sample solution and allowed to tumble freely using magnetic stirrer. After

extraction, the analytes are desorbed from the packed sorbent in suitable desorption solvent by ultrasonication. Although, in µ-SPE, a stir-bar is placed inside the sample solution to agitate the solution by magnetic stirring but it has been frequently observed that µ-SPE device sticks to the one wall of the sample vial or floats over the sample solution, which allows the µ-SPE device to extract only on the surface of the sample solution. Polypropylene (PP) is commonly used membrane for fabrication of µ-SPE devices. It is hydrophobic in nature [14]. Even after conditioning in organic solvent, µ-SPE device does not get enough wettability that it can totally sink inside the sample solutions. This is only possible when water penetrates inside the membrane and adsorbed by the sorbent [15]. However, the hydrophobic nature of PP membrane and sorbent does not allow that much water to be adsorbed, thus it keeps floating near the surface. This can be seen in a figure of µ-SPE device given in a published work [16]. Hence, we have proposed a simple modification in µ-SPE device to solve this problem by packing a stir-bar inside polypropylene envelope along with sorbent. In this way, packed stir-bar does not allow the membrane to float over surface or stick to the wall of the glass vial. Moreover, it keeps the µ-SPE device in continuous state of motion and rotation, which in turn increases effective surface area of the sorbent exposed to the target compounds in the sample solution.

Although stir-bar sorptive extraction (SBSE), is a well-established micro-extraction technique but it has limitation that only few materials can be coated over the stir-bar. Thickness and stability of the coating is very critical in order to evaluate reproducibility of SBSE. Most commonly used PDMS coated stir-bars can only be employed for extraction of non-polar or weekly polar target compounds [17]. However, our proposed stir-bar supported micro-solid phase extraction (SB-µ-SPE), has flexibility for selection of sorbent.

Sorbent can be selected according to the nature of target compounds and then an accurate amount can easily be packed inside the membrane along with stir-bar.

To evaluate the performance of SB-μ-SPE, determination of PCBs in serum samples were investigated. PCBs were widely used in industrial applications before 1970. Although, they were banned in 1970s [18] but still their measurable concentrations have been reported in the environment. They are persistent in nature and have ability to accumulate in tissues and biological matrix [19]. Their presence in body fluids such as blood has been linked with different carcinogenic[20] and non-carcinogenic [21] disorders. Hence, it is highly desirable to develop sensitive analytical methods to monitor PCBs in environmental and biological matrices.

5.2. Experimental

5.2.1. Chemicals and reagents

Accruel polypropylene sheet membrane (pore size of 0.2 μm, 157 μm thickness) was purchased from Membrana (Wuppertal, Germany). HPLC-grade solvents (methanol, toluene, acetonitrile and n-hexane) were obtained from Fisher (Loughborough, UK). The PCB congeners mixture (100 μg mL^{-1}) was purchased from Restek (Bellefonte, US). Stock solution of PCBs mixture (10 μg mL^{-1}) was prepared in methanol and stored at -4°C and working solutions (0.1 – 100.0 ng mL^{-1}) were freshly prepared before experiments. C_{18} column packing material (average particle size: 12 μm; surface area; 180 m^2/g) was obtained from Sigma-Aldrich. Muti-wall carbon nanotubes (MWCNTs) (outer diameter: 20 – 30 nm, length 10 – 30 μm and purity > 95%) were purchased from Cheap Tubes Inc.

(Cambridgeport, USA). Coconut activated carbon was purchased from Cenapro Chemical Corporation (Mandauae City, Philippines).

5.2.2. Serum samples and standard solutions

Real pooled human serum was used for method development and it was obtained from KFUPM laboratory, Alkhobar. Research ethics regarding use of human biological samples were taken into account while collection of the samples. The blood samples were extracted from volunteers by venipuncture in sterilized blood tubes. Serum was separated by centrifugation at 6000 rpm for 12 min and collected together in 50 mL glass bottles equipped with Teflon caps. Collected serum was stored at -20°C prior to use. The serum samples used for method optimization were free of PCBs . Optimization experiments were carried out with 10 times diluted serum samples spiked at 25 ng mL^{-1}. Serum samples were also collected from individual patients to test the applicability of the method. Standard safety protocols were adopted for handling and disposing of biological samples.

5.2.3. Gas chromatography-mass spectrometric (GC-MS) analysis

The separation and quantitation of target compounds were performed on a Shimadzu (Kyoto, Japan) QP2010 GC-MS. The system was equipped with a Shimadzu AOC-20s auto sampler and AOC-20 auto injector. Rxi-5 Sil MS column with thickness of 0.25 µm, length of 30.0 m and diameter of 0.25 mm (Restek, Bellefonte, US) was used for separation of target compounds. The high purity helium gas was employed as carrier gas at flow rate of 1.01 mL min^{-1}. The GC injection port temperature was kept 250°C and all the samples were injected in splitless mode. The opening time of split vent was 1.0 min. The GC-MS interface temperature was 220°C and ion source temperature was 200°C.The oven temperature was programmed as follows: initial temperature was 40°C and held for 1 min;

then increased to 100°C at 10°C/min and held for 2 min; then increased to 165°C at 10°C/min and held for 0 min; then increased to 190°C at 6°C/min and held for 3 min; after that it was increased to 220°C at 3°C/min and held for 3 min; and finally increased to 240°C at 2°C/min and held for 1 min. Total run time was 46.67 min. For qualitative analysis, data acquisition was performed in scan mode to confirm the retention times of target compounds. For quantitative analysis, selective ion monitoring (SIM) mode was employed. Selected target ions are listed in Table 5-1.

5.2.4. SB-µ-SPE procedure

SB-µ-SPE device was fabricated by packing a tiny stir-bar (7 mm x 2 mm) and accurately weighed amount of sorbent into a porous polypropylene (PP) membrane envelope whose edges were heat sealed. Briefly, PP flat membrane sheet was cut into rectangular pieces with dimensions of 1.6 cm by 2.0 cm. The shorter length was folded and heat sealed with an electrical heat sealer. One of the two remaining open edges was heat sealed to give it a shape of an envelope with one open edge. The sorbent and tiny stir bar were added from the last open edge that was finally heat sealed to give SB-µ-SPE device.

Before extraction, SB-µ-SPE device was conditioned in toluene for 1 min. For extraction, SB-µ-SPE device was placed inside a glass vial containing 5 mL of sample or spiked matrix solution. This vial was placed on a magnetic stirrer. The SB-µ-SPE device was stirred in the sample solution for an optimum extraction time .SB-µ-SPE device was taken out of the sample solution, and dried with the help of lint free tissue. The device was then inserted into an Eppendorf vial and 250 µL of desorption solvent were added. Analytes were then effectively desorbed or eluted by using ultra-sonication for an optimum desorption time. After desorption, 1 µL of extract was injected into GC-MS.

Table 5- 1: List of target compounds, their CAS numbers, m/z of selected target ions and chromatographic retention times

Compound name	Congener	CAS number	m/z of target ions	Retention Time
2-Chlorobiphenyl	PCB-1	2051-60-7	188, 152, 126	16.90
2,3-Dichlorobiphenyl	PCB-5	16605-91-7	222, 152, 186	20.02
2,2',5-Trichlorobiphenyl	PCB-18	37680-65-2	256, 221, 186	21.50
2,4',5-Trichlorobiphenyl	PCB-31	16606-02-3	256, 186, 93	23.62
2,2',5,5'-Tetrachlorobiphenyl	PCB-52	35693-99-3	292, 220, 255	25.31
2,2',3,5'-Tetrachlorobiphenyl	PCB-44	41464-39-5	292, 220, 255	26.27
2,3',4,4'-Tetrachlorobiphenyl	PCB-66	32598-10-0	292, 220, 184	28.63
2,2',4,5,5'-Pentachlorobiphenyl	PCB-101	37680-73-2	326, 324, 256	29.92
2,2',3,4,5'-Pentachlorobiphenyl	PCB-87	38380-02-8	326, 324, 291	31.31
2,3,3',4',6-Pentachlorobiphenyl	PCB-110	38380-03-9	326, 324, 254	31.82
2,2',3,5,5',6-Hexachlorobiphenyl	PCB-151	52663-63-5	360, 325, 290	32.47
2,2',4,4',5,5'-Hexachlorobiphenyl	PCB-153	35065-27-1	360, 290, 218	34.87
2,2',3,4,5,5'-Hexachlorobiphenyl	PCB-141	52712-04-6	360, 325, 327	35.70
2,2',3,4,4',5'-Hexachlorobiphenyl	PCB-138	35065-28-2	360, 290, 325	36.71

2,2',3,4',5,5',6-Heptachlorobiphenyl	PCB-187	52663-68-0	394, 396, 359	37.84
2,2',3,4,4',5',6-Heptachlorobiphenyl	PCB-183	52663-69-1	394, 396, 359	38.26
2,2',3,4,4',5,5'-Heptachlorobiphenyl	PCB-180	35065-29-3	394, 396, 324	41.94
2,2',3,3',4,4',5-Heptachlorobiphenyl	PCB-170	35065-30-6	394, 396, 359	44.01

5.3. Results and discussion

5.3.1. Optimization of SB-µ-SPE

The factors that affect the extraction efficiency of SB-µ-SPE were optimized by using real human serum samples spiked at 25 ng mL^{-1}.

An ideal sorbent for µ-SPE should have good solid state flow properties. It should be "non-sticky" so that it can be accurately weighed and transferred into PP bag. It should also be "non-adhesive" to the membrane material so that interference with heat sealing of PP sheet during sorbent packing can be avoided [22]. The presence of solid particles in heat sealed area of µ-SPE device tend to weaken the seal during sonication for desorption process. In addition, sorbent should allow target compounds to be easily desorbed under mild desorption conditions. Application of sonication for extensively longer period of time may result re-adsorption of target compounds into sorbent and tearing of µ-SPE device. Similarly, use of very high temperatures during desorption may result in evaporation of target compounds.

The selection of the suitable sorbent is very critical in all types of solid phase extractions and it is mainly dictated by the nature of target compounds to be extracted. Hence, three commonly used sorbents; multiwall carbon nanotubes (MWCNTs), C_{18} and activated carbon were investigated. 25 mg of each sorbent were used to fabricate SB-µ-SPE. Figure 5-1 shows that best extraction results were obtained with C_{18} and this might be due strong hydrophobic interactions of PCBs toward C_{18} [23]. Although, carbon based materials such as MWCNTs and activated carbon show strong interactions toward hydrophobic compounds, their low extraction efficiency might be attributed to different factors. When the sorbent has strong hydrophobic interactions with target compounds, desorption

becomes a more difficult task. Thus low extraction efficiencies of CNTs might be due to poor desorption of target compounds by sonication. Increase in sonication time might be helpful. Thus sonication time was evaluated between 5 – 45 min. However, no significant increase in chromatographic peak areas was observed after 15 min. Desorption times above 35 min resulted in decrease of peak areas. This is attributed to temperature rise by continuous sonication that transfers the target compounds to headspace above the desorption solvent in desorption vial and they may escape when vial is opened. Moreover, longer sonication time may also result in re-adsorption of target compounds into sorption sites by diffusion through pores [10,12,24]. Third factor is related to packing of sorbent above than a certain amount into µ-SPE envelope that makes the device too large to fit in desorption vial. Such device will not immerse completely in solvent and results in poor desorption [12]. Since, 25 mg of C_{18} occupies very less space compared to 25 mg of CNTs. CNTs are light weight and occupy more space. Thus, 25 mg of CNTs will result in a large sized µ-SPE device and it will require extra desorption volume to desorb the analytes. Hence, C_{18} was selected as an optimum sorbent for the next optimization experiments as it fulfills aforementioned criterion for an ideal sorbent. Moreover, it can be facilely handled for µ-SPE procedures compared to CNTs and activated carbon [22,25].

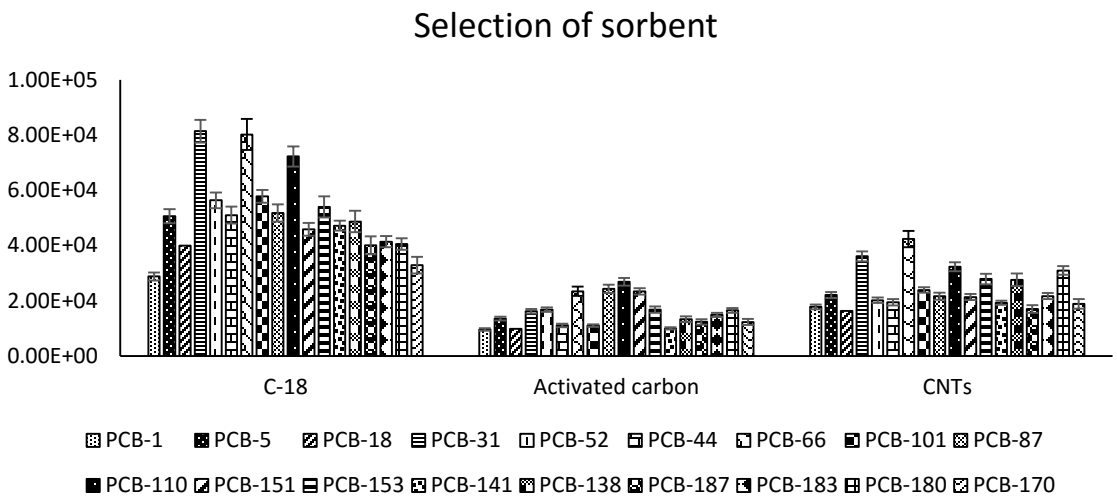

Figure 5- 1: Influence of sorbent type on extraction of PCBs. Conditions: PCBs concentration: 25 ng mL^{-1}; amount of sorbent: 25mg; extraction time: 15 min; desorption solvent: toluene; desorption volume: 250µL; stirring speed: 800 rpm; desorption time: 10 min.

The amount of sorbent and volume of desorption solvent should have a good compromise between them. The amount of sorbent should be sufficient enough to reproducibly extract target compounds with selected volume of desorption solvent. The volume of desorption solvent should be sufficient enough to completely immerse the SB-µ-SPE device for effective desorption. Thus amount of sorbent was investigated in range of 15 – 75 mg and desorption volume was tested in range of 50 – 350 µL. Desorption volume (250 µL) was sufficient to completely immerse the device for all range of amount of sorbent. Under the same extraction and desorption conditions, chromatographic peak areas of all the analytes were increased up to 45 mg. Further increase in the amount of sorbent had not resulted in increase of peak areas (Figure 5-2). Hence, 45 mg of C_{18} was used as optimum amount of sorbent. For all the optimization experiments, volume of desorption solvent was fixed as 250 µL, because it is minimum volume that can effectively desorb or elute the target compounds from SB-µ-SPE device. Volumes of desorption solvent lesser than 250 µL gave poor reproducibility because of ineffective desorption while volumes higher than 250 µL resulted in decrease of chromatographic peak areas because of dilution.

Desorption solvent is of prime importance for effective desorption or elution of analytes from SB-µ-SPE device. Hence, the solvents with varying polarity index were investigated. Figure 5-3 shows that best extraction results were obtained with toluene followed by n-hexane, methanol and acetonitrile. PCBs are non-polar and hydrophobic in nature and it is expected that n-hexane will give best desorption. The reason for better desorption with toluene, might be attributed to its role in effectively dilating the pores of porous PP membrane than n-hexane. Acetonitrile and methanol are polar solvents and thus lesser desorption or elution was observed with these solvents.

The amount of analytes that is transferred from donor phase (sample solution) to acceptor phase (sorbent) is dependent on rate of mass transfer. This mass transfer is expected to increase with the time of extraction before reaching to a steady state. Hence, the time of extraction is an important parameter to consider. Extraction times were evaluated in range of 5 – 25 min. The higher peak areas for all the analytes were obtained at extraction time of 20 min (Figure 5-4). After 20 min, almost a steady state was achieved. Hence, a time of 20 min was selected as an optimum extraction time.

Salting out effect is another important parameter to consider in microextractions. Addition of salt into sample solution decreases the solubility of target compounds in the donor phase. In current optimization, a slight increase was observed in peak areas upon addition of 5% (w/v) of NaCl. Afterwards, any further increase of salt into sample solution resulted in significant decrease in peak areas of all target compounds. This might be attributed to increased viscosity of the solution which may hinder mass transfer of target compounds from donor to acceptor phase. Such observations are also very common in microextraction methods [26,27]. Because there were no significant enhancements in peak areas of target analytes, thus for the simplicity of sample preparation, no salt was added into the sample solution.

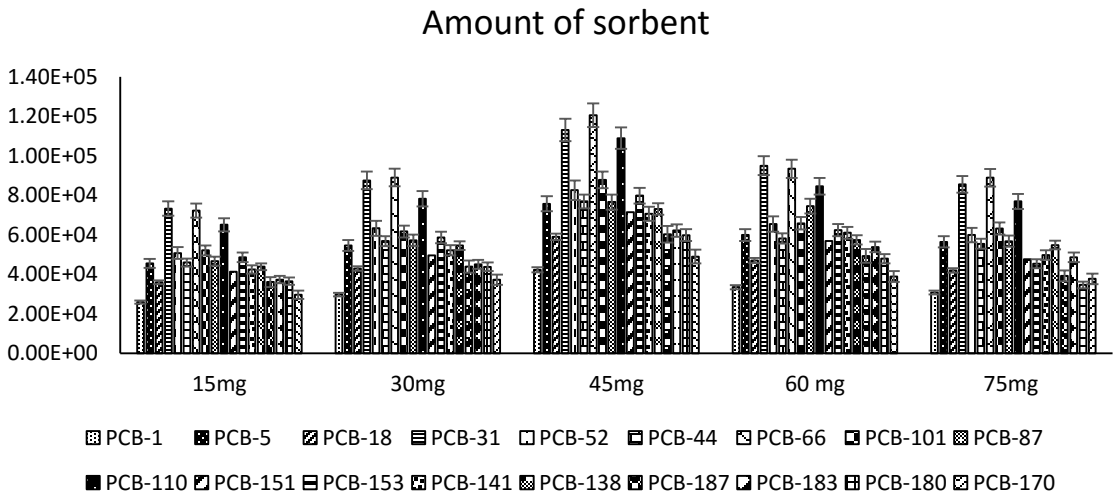

Figure 5- 2: Influence of sorbent amount on extraction of PCBs. Conditions: PCBs concentration: 25 ng mL^{-1}; sorbent: C_{18}, extraction time: 15 min; desorption solvent: toluene; desorption volume: 250µL; stirring speed: 800 rpm; desorption time: 10 min.

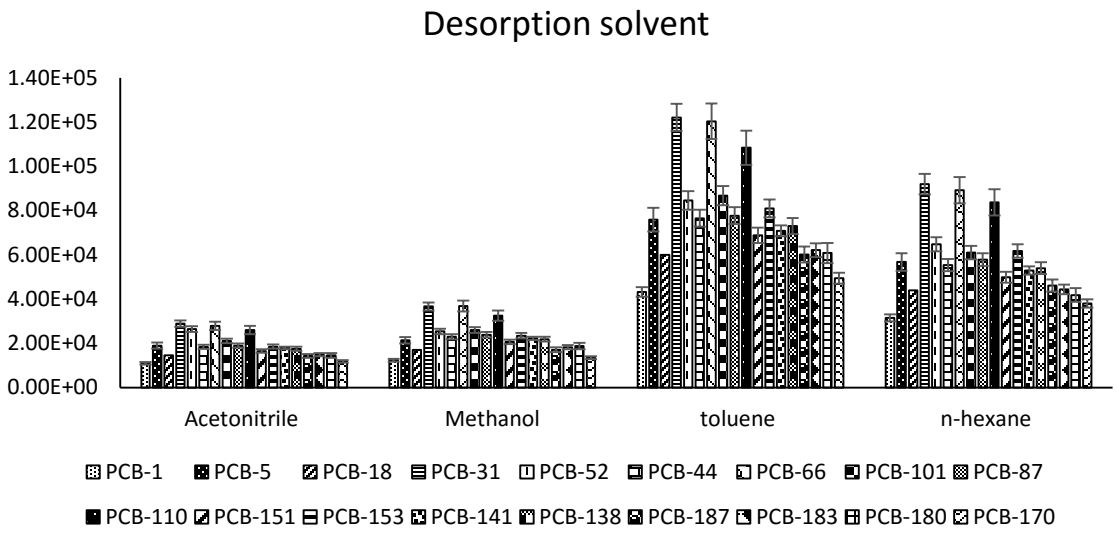

Figure 5- 3: Influence of desorption solvent on extraction of PCBs. Conditions: PCBs concentration: 25 ng mL^{-1}; sorbent: C_{18}, amount of sorbent: 45mg; extraction time: 15 min; desorption solvent: toluene; desorption volume: 250µL; stirring speed: 800 rpm; desorption time: 10 min.

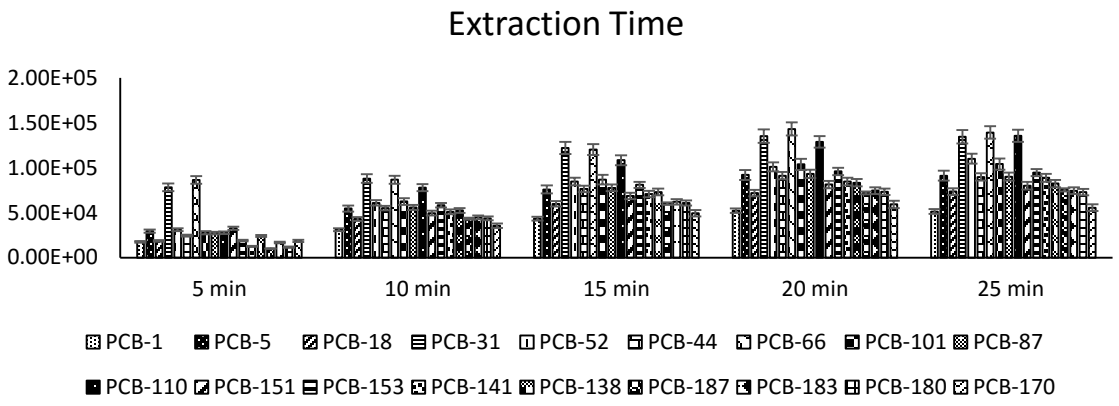

Figure 5- 4: Influence of extraction time on extraction of PCBs. Conditions: PCBs concentration: 25 ng mL^{-1}; sorbent: C_{18}, amount of sorbent: 45mg; desorption solvent: toluene; desorption volume: 250µL; stirring speed: 800 rpm; desorption time: 10 min.

As stir-bar was packed inside the membrane along with sorbent, stirring speed may influence the extraction process. Stirring the sample solution permits effective contact between the target compounds and SB-µ-SPE device by bringing fresh solution to the surface. Hence, the effect of stirring speed on extraction process was examined in the range of 200 – 1000 rpm. The highest chromatographic signals for all the analytes were obtained at 800 rpm and afterwards, no significant increase was observed. Thus, 800 rpm was adopted as optimum stirring speed (Figure 5-5). SB-µ-SPE gives two unique advantages compared to µ-SPE from stirring perspective (i) it does not allow the extraction device to stick in any corner of the glass vial (ii) the packed stir bar keeps the extraction device positioned inside the solution in addition to normal stirring of sample solution.

As it was a previously investigated parameter, the effect of desorption time for C_{18} based µ-SPE device was investigated in range of 5 to 20 min. An increase in peak areas was recorded up to 15 min. Afterwards, a slight decrease in chromatographic signals was noted. This may be due to increase of the temperature of sonication bath with the time. In case of higher temperature, analytes may evaporate and distribute into headspace of desorption vial leading to decreased concentrations in desorption solvent. Thus, 15 min was selected as an optimum desorption time (Figure 5-6).

The results of µ-SPE and SB-µ-SPE were compared under optimum extraction and desorption conditions. Figure 5-7 clearly indicates that the latter is more effective extraction approach. The reasons for high extraction efficiency of SB-µ-SPE can be explained by (i) enhanced contact between sorbent containing device and the target compounds in the sample solution after packing stir-bar inside the device (ii) complete immersion of the device inside the sample solution.

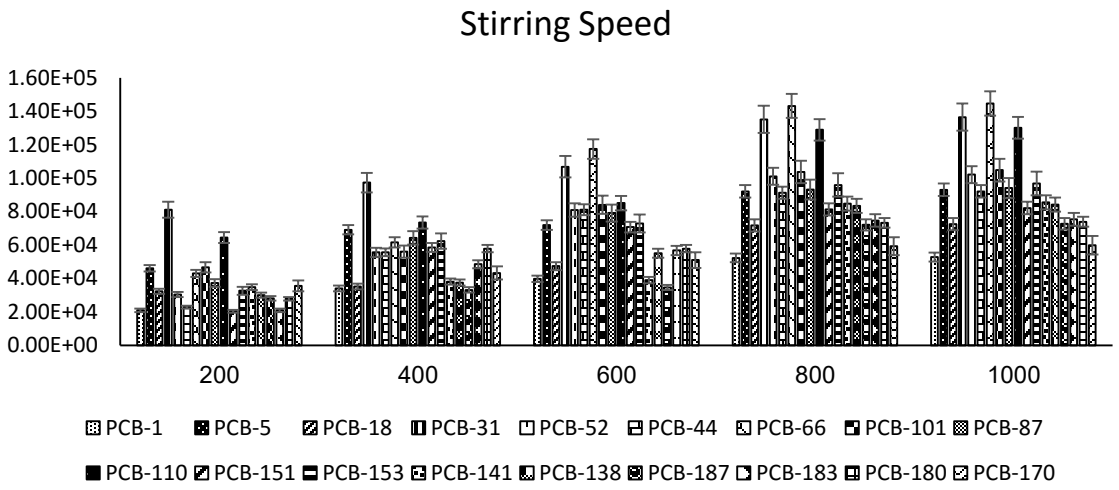

Figure 5- 5: Influence of stirring speed on extraction of PCBs. Conditions: PCBs concentration: 25 ng mL^{-1}; sorbent: C_{18}, amount of sorbent: 45mg; desorption solvent; extraction time: 20 min, toluene; desorption volume: 250µL; desorption time: 10 min.

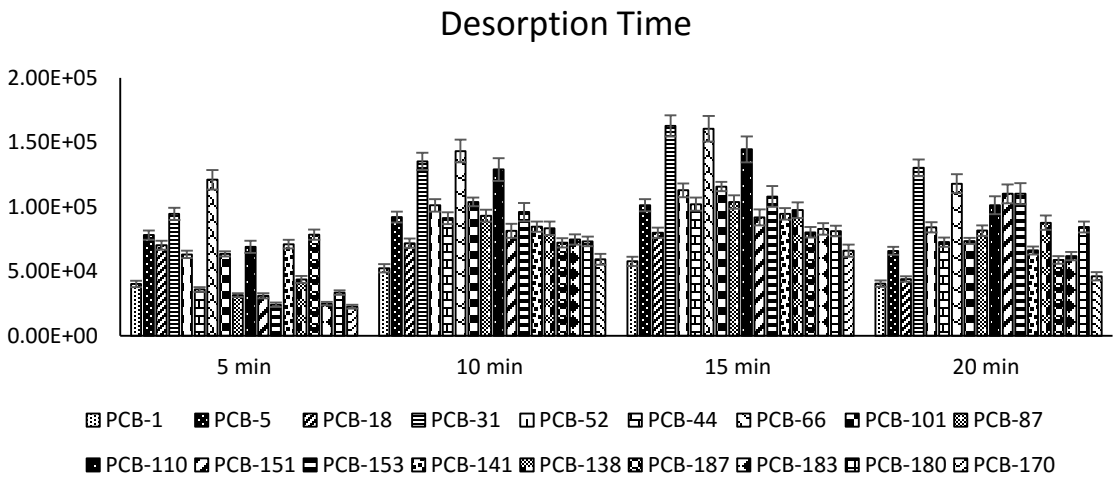

Figure 5- 6: Influence of desorption time on extraction of PCBs. Conditions: PCBs concentration: 25 ng mL^{-1}; sorbent: C_{18}, amount of sorbent: 45mg; desorption solvent: toluene; extraction time: 20 min, desorption volume: 250µL; stirring speed: 800 rpm.

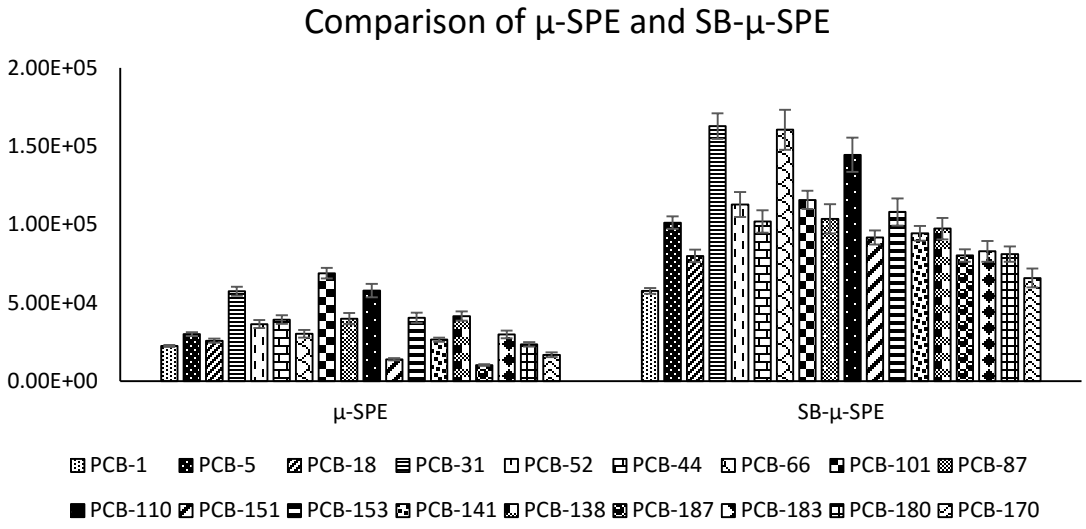

Figure 5- 7: Comparison of µ-SPE and SB-µ-SPE under optimum extraction conditions. PCBs concentration: 25 ng mL^{-1}; sorbent: C_{18}, amount of sorbent: 45mg; desorption solvent: toluene; extraction time: 20 min, desorption volume: 250µL; stirring speed: 800 rpm; desorption time: 15 min.

5.3.2. Method validation, real sample analysis and comparison with reported methods

To access the practical applicability of proposed SB-µ-SPE method, most favorable extraction conditions were adopted to evaluate method's linearity, limit of detections (LODs) and reproducibility. 6-point calibration lines were plotted for all target compounds using real human serum samples spiked with known concentrations of PCBs ranging from 0.1 to 100 ng mL $^{-1}$. Good linearity for all tested PCBs was obtained with coefficients of determination (r^2) between 0.9868 and 0.9992. LODs were calculated based on signal to noise ratio of 3. LODs were ranged in between 0.003 and 0.047 ng mL $^{-1}$. Method's precision was assessed based on inter-day and intra-day reproducibility by using real human serum samples spiked at 25 ng mL $^{-1}$ (n=7). RSDs were in range of 3.1 – 7.2 % and 3.2 – 9.1% for intra-day and inter-day precision respectively, indicating good precision of the method. Relative recoveries for all target compounds were calculated by spiking to real serum sample at 1.0, 25 and 50 ng mL $^{-1}$ (n=5). The relative recovery, in this case, will be defined as the ratio of the peak areas of the analytes in serum to ultrapure water sample extracts, when both samples spiked at the same concentration levels of PCBs. Relative recoveries of all target compounds were ranged between 85.2 to 103.6%. Absolute recoveries were ranged from 59.0 to 86.7. µ-SPE is not an exhaustive technique like SPE rather it is based on equilibrium. Hence low absolute recoveries are justified. Enrichment factors were calculated based on ratio of concentration of analytes in sample solution to concentration of analytes in final extract. Enrichment factors in range of 11.7– 18.4 were obtained. Analytical features of proposed method are given in Table 5-2.

The developed SB-µ-SPE method was used to determine PCB concentrations in serum samples collected from 6 patients visited the clinic for routine checkup. Some PCB congeners were detected in real samples. Real concentrations of PCBs (only for congeners which were detected) are given in Table 5-3. Figure 5-8 compares chromatograms of unspiked and spiked (at 25 ng mL^{-1}) real samples.

Table 5-4 compares the performance of SB-µ-SPE with microextraction methods reported in the literature for extraction of PCBs in blood or serum. It can be noted that analytical parameters for proposed SB- µ-SPE are either better or comparable with methods reported in literature.

Table 5- 2: Analytical features of proposed method

Compound	DLR[a] (ng mL^{-1})	r^2	LODs[b] (ng mL^{-1})	RSD[c](%) (n=7)		Relative Recoveries (%) (n=5)			Absolute recovery (%) (n=5)	Enrichment factors (n=3)
				Intra-day	Inter-day	1 ng mL^{-1}	25 ng mL^{-1}	50 ng mL^{-1}	25 ng mL^{-1}	
PCB-1	0.1 – 100.0	0.9969:	0.017	3.1	4.1	96.4	97.3	98.0	78.4	15.7
PCB-5	0.1 – 100.0	0.9897	0.030	3.9	4.5	86.1	88.3	88.5	59.0	11.8
PCB-18	0.1 – 100.0	0.9992	0.047	2.5	3.2	98.5	99.1	100.0	75.1	15.0
PCB-31	0.1 – 100.0	0.9863	0.026	5.3	5.7	99.7	101.2	99.5	67.3	13.5
PCB-52	0.1 – 100.0	0.9908	0.013	2.9	3.6	100.0	103.6	102.7	63.9	12.8
PCB-44	0.1 – 100.0	0.9972	0.027	3.6	6.2	97.6	99.0	98.5	62.3	12.5
PCB-66	0.1 – 100.0	0.9901	0.024	6.1	5.9	94.0	93.8	95.1	60.5	12.1
PCB-101	0.1 – 100.0	0.9914	0.043	5.2	6.7	94.2	98.5	99.3	60.3	12.1
PCB-87	0.1 – 100.0	0.9889	0.025	3.8	4.3	98.5	102.3	98.4	59.0	11.8
PCB-110	0.1 – 100.0	0.9992	0.041	4.6	4.5	97.6	99.4	101.2	58.5	11.7
PCB-151	0.1 – 100.0	0.9898	0.028	2.9	3.5	95.0	97.2	97.0	62.3	12.5
PCB-153	0.1 – 100.0	0.9976	0.036	6.5	5.8	92.1	95.0	98.2	62.5	12.5
PCB-141	0.1 – 100.0	0.9886	0.004	2.3	3.7	88.4	91.8	92.7	61.4	12.3
PCB-138	0.1 – 100.0	0.9986	0.003	3.1	5.3	94.0	94.5	95.1	61.9	12.4
PCB-187	0.1 – 100.0	0.9893	0.019	4.6	4.2	86.3	89.6	92.6	65.1	13.0
PCB-183	0.1 – 100.0	0.9977	0.046	3.5	5.9	96.9	99.1	99.0	58.8	11.8
PCB-180	0.1 – 100.0	0.9967	0.042	4.9	5.2	85.2	88.4	88.7	65.0	13.0
PCB-170	0.1 – 100.0	0.9979	0.042	7.2	9.1	96.4	100.0	99.4	86.7	17.3

[a]Dynamic linear range; [b]Limit of detection: calculated as three time of the baseline noise ; [c]reproducibility; r^2 (coefficient of determination)

Table 5- 3: Concentrations of PCBs in real serum samples

Sample No.	**PCBs Detected**	**Concentration (ng mL^{-1})**
1	PCB-1	0.24
	PCB-5	0.32
	PCB-18	0.65
	PCB-31	0.34
2	PCB-31	0.36
3	PCB-52	0.17
4	PCB-5	0.19
	PCB-31	0.45
	PCB-101	0.23
	PCB-87	0.43
5	PCB-31	0.13
6	PCB-5	0.15
	PCB-18	0.27
	PCB-31	0.24

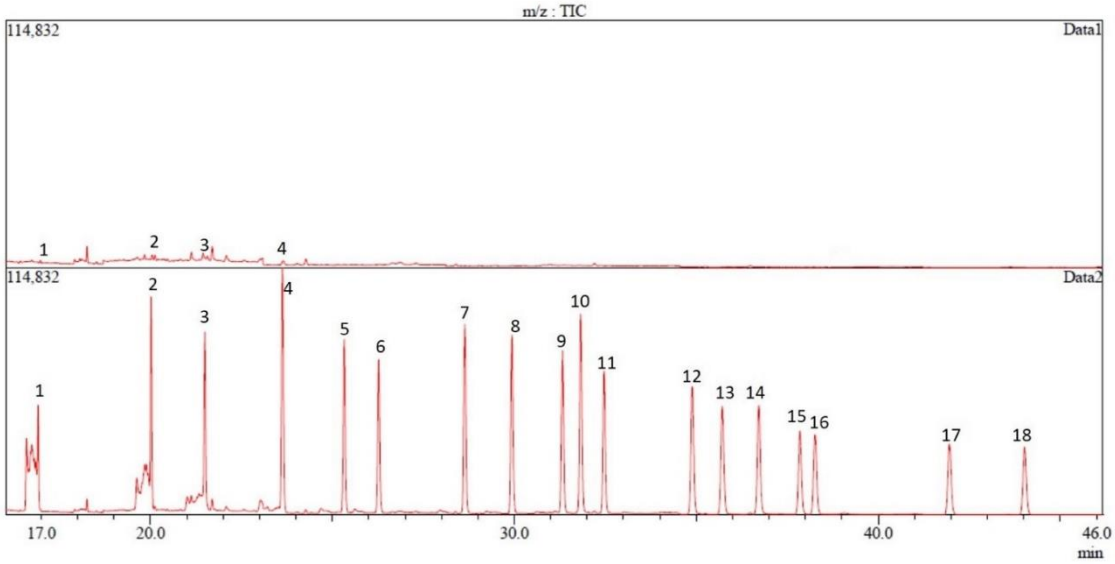

Figure 5- 8: GC-MS trace of a real serum sample (a) and spiked real sample at 25 ng mL^{-1} (b) both extracted under most favorable SB-µ-SPE conditions. (1) PCB-1 (2) PCB-5 (3) PCB-18 (4) PCB-31 (5) PCB-52 (6) PCB-44 (7) PCB-66 (8) PCB-101 (9) PCB-87 (10) PCB-110 (11) PCB-151 (12) PCB-153 (13) PCB-141 (14) PCB-138 (15) PCB-187 (16) PCB-183 (17) PCB-180 (18) PCB-170.

Table 5- 4: Comparison of SB-µ-SPE with other methods reported in literature

Method	**Number of PCB congeners studied**	**Matrix**	**Linear Range**	**LODs**	**Enrichment factors**	**RSDs (%)**	**Ref**
LPME-GC/MS	8	Blood	2.5 – 200	0.07 – 0.93	67 – 241	2.5 – 10.8	[19]
HS-SPME-GC/ECD	18	Serum	0.051 – 0.207	0.001 – 0.012		3.0 – 12.0	[28]
SPE-GC/µECD	15	Serum	Different ranges for different compounds	0.0012 – 0.0051		2.5 – 26.4	[29]
SB-µ-SPE	18	Serum	0.1 – 100	0.003 – 0.047	11.7 – 18.4	3.1 – 9.1	Present work

5.4. Conclusion

In this work, a new mode of membrane protected µ-SPE was described as stir-bar supported µ-SPE. In this mode, a magnetic stir-bar and an accurately weighed amount of sorbent was packed inside the porous polypropylene membrane. This mode of extraction improved the extraction performance considerably. PCBs were employed as model compounds to access the extraction performance of the proposed method in human serum samples. The extracted samples were analyzed by GC-MS. The performance of this method in extraction of PCBs from serum samples was excellent. This method provided low LODs, good linearity and satisfactory intra-day and inter-day reproducibility for all tested PCBs. SB- µ-SPE can be extended to analysis of other types of organic and inorganic pollutants in complex sample matrices.

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6. CHAPTER

APPLICATION OF ZINC OXIDE INCORPORATED CARBON FOAM AS SORBENT IN MICRO-SOLID-PHASE EXTRACTION OF ORGANOCHLORINE PESTICIDES IN MILK SAMPLES

Abstract

Organochlorine pesticides belong to famous persistent organic pollutants and are well known for their harmful effects on human health and wildlife. The milk stands among highly consumed foods and it can get contaminated with OCPs through agricultural food that is provided to animals. This demands development of highly efficient and precise analytical methods for determination of OCPs in milk samples. In this work, ZnO nanoparticles incorporated porous carbon foam was utilized as sorbent for extraction and preconcentration of OCPs in milk samples. This sorbent was prepared by a single step and fast reaction between the sucrose and zinc nitrate that was accomplished by heating the mixture for 10 minutes. The resulting sorbent was characterized by scanning electron microscopy and X-ray diffraction. The sorbent was then packed inside a porous polypropylene membrane sheets by heat sealing of membrane to construct µ-SPE devices. The factors that affect performance of µ-SPE were optimized. This method provided good linearity up to 0.9998 with limit of detections ranging from 0.19 to 1.64 ng/mL. This method also provided satisfactory values for intra and inter-day precision with RSDs ranged between 2.3 to 10.2%.

6.1. Introduction

Organochlorine pesticides (OCPs) are famous class of persistent organic pollutants (POPs). OCPs are hazardous and persistent environmental pollutants that are also susceptible to long range atmospheric transport because of their volatility. They can accumulate in the tissues of living organism through food web or polluted environment. OCPs have been linked with several carcinogenic and non-carcinogenic disorders in the human. Although, their industrial production was banned in 1970s but still their measurable concentrations have been reported in various matrices.

Milk has high nutritional and immunological value to infants and aged people and it stands among imperious and highly consumed foods. Milk can get contaminated with OCPs through different sources including environmental diffusion and direct uptake of contaminated agricultural food by the animals. It is therefore highly desired to monitor OCPs in the milk samples.

Milk is highly complex matrix because of presence of variety of biomolecules, salts, proteins, fats and vitamins. This high fat and protein content can interfere during analytical determination of target compounds. Hence, pretreatment or/ and extraction is unavoidable step prior to instrumental analysis. This process of pretreatment or extraction may involve several clean-up steps to remove any extraneous material from the matrix. Thus, routine procedures for extraction of OCPs in milk samples such as LLE and SPE [1] are time and labor intensive, consume large volumes of organic solvents and produce considerable quantity of waste. Over the last two decades, area of sample preparation is moved toward development of miniaturization extraction techniques. These techniques are featured by

minimum or no consumption of organic solvents, small sample size and shorter extraction time. Various methodologies such as QuEChERS [2], SPME [3,4] and MSPE [5] were used for determination of OCPs in milk samples. Although, these methods provide adequate efficiency, but when it comes to complex samples like milk, some limitations are associated with them. For example, SPME fibers cannot be directly exposed to milk samples because fats and other large biomolecules will adsorb on the fiber. Hence, two approaches are adopted either sample is diluted by several folds or SPME fiber is exposed only in headspace.

Membrane protected micro-solid-phase extraction (μ-SPE) was introduced by Basheer et al., as an alternative to multistep SPE. In this technique, a small amount of solid sorbent is secured inside a porous polypropylene (PP) bag through heat-sealing. In this way, sorbent does not come directly in contact with sample solution and target compounds are transferred to the sorbent through porous PP sheet. After extraction, the sorbent containing PP bag is taken out of the sample solution, washed with water and dried with lint free tissues. Then analytes are then desorbed into suitable desorption solvent by aid of ultrasonication. This technique particularly suits for samples with complex biological matrices because PP membrane effectively secures the sorbent from fats, proteins and other large biomolecules. Hence, μ-SPE is widely used for extraction of variety of organic pollutants from tissues and biological fluids. Thus, it obviates the aforementioned steps required for SPME.

In recent years, novel carbon based materials have shown exciting potential for analytical extraction of target compounds in variety of matrices. These novel materials include pure and functionalized graphene, carbon nanotubes, graphite fiber, carbon soot and their

modified forms. Extremely high surface area and excellent chemical, thermal and mechanical stabilities and ability to form **Π-Π** interactions with target compounds, make carbon based materials as an attractive choice for extraction applications[6]. Notably, there has also been seen a growing trend in use of metal and metal oxide nanoparticles in combination with carbon materials as sorbent for analytical extractions [7]. Functionalization or incorporation of other materials into carbon nanotubes or graphene is accomplished by extended synthesis protocols. Recently, carbon foam has shown some exciting applications in different areas. ZnO incorporated carbon foam was synthesized through a facile one step procedure. To the best of our knowledge, carbon foam has not been utilized in analytical extractions. For the first time, we have explored the potential of ZnO incorporated carbon foam as a sorbent for µ-SPE of OCPs in milk samples.

6.2. Experimental

6.2.1. Chemicals and materials

A mixture of OCPs standard was purchased from Restek (Bellefonte, US) and 15 compounds listed in Table 6-1 were considered for analysis. $Zn(NO_3)_2.6H_2O$ was purchased from Sigma Aldrich. Crystalline sucrose was purchased from local market. Accruel polypropylene sheet membrane (pore size of 0.2 µm, 157 µm thickness) was obtained from Membrana (Wuppertal, Germany). HPLC-grade solvents (methanol, toluene, acetone, acetonitrile and n-hexane) were purchased from Fisher (Loughborough, UK). C_{18} column packing material (average particle size: 12 µm; surface area; 180 m^2/g) was provided by Sigma-Aldrich. Muti-wall carbon nanotubes (MWCNTs) (outer diameter:

20 – 30 nm, length 10 – 30 µm and purity > 95%) were obtained from Cheap Tubes Inc. (Cambridgeport, USA).

6.2.2. Synthesis of ZnO incorporated carbon foam and its characterization

ZnO incorporated carbon foam was synthesized with certain modifications in the procedure already reported in the literature for synthesis of carbon foam [8]. Briefly, 3 grams of sucrose and 1.5 g of $Zn(NO_3)_2.6H_2O$ were taken in a beaker and heated on a hot plate whose temperature was programmed to reach at 110°C. As the temperature increased, mixture started melting. During melting, mixture was continuously stirred with a glass rod. At 105°C, a yellow colored, foam like swelled material, started expanding in size to upward and after heating for approximately 10 minutes at 110°C, the color of the foam started to change to black and within next few seconds it burned in presence of O_2 and changed into carbon foam.

The reaction can be represented by the following chemical equation

$$C_{12}H_{22}O_{11} + Zn(NO_3)_2.6H_2O \xrightarrow[\text{10 minutes}]{\text{110°C}} C_{\text{foam}} + ZnO + N_2 + H_2O + NOx + CO_2$$

$Zn(NO_3)_2.6H_2O$ is a strong oxidizing agent, after its thermal decomposition and reaction with sucrose, different gases are generated (N_2, NO_x, CO_2, H_2O vapours etc.). These gases cause melted mixture to blow and swell. This swelling increase the surface area and change the material into foam. After, complete emission of all the gases, material (which is now

combination of C and ZnO) changes to black color and ultimately burns and splashes into ash consisting of very fine particles of carbon and ZnO.

The synthesized material was characterized by scanning electron microscope and x-ray diffraction.

6.2.3. µ-SPE procedure

µ-SPE device consisted of sorbent enclosed in an envelope made from porous polypropylene (PP) membrane sheet whose edges were heat sealed. The dimensions of µ-SPE device were 2.2 cm × 1.0 cm. Before use, the sorbent inside the device was conditioned by putting it in the toluene and sonication for 3 mins.

A 10 mL aliquot of milk sample was taken in a sample vial (15 mL). A magnetic stir bar (15mm × 5mm) was added to sample solution. The µ-SPE device was then added to sample solution and stirred at 1000 rpm. The extraction was allowed to take place for 30 min. The µ-SPE device was then removed from the sample vial, rinsed with water, dried with lint free tissue and transferred into a 600 µL desorption vial. Then toluene (300 µL) was added into the desorption vial and analytes were desorbed by ultrasonication for a period of 10 min. After desorption, extract was injected to GC-MS system for analysis.

6.2.4. GC-MS

GC-MS (QP2010 Shimadzu (Kyoto, Japan)) was used for separation and quantitation of target OCPs. The system was equipped with a Shimadzu AOC-20s auto sampler and AOC-20 auto injector. Rxi-5 Sil MS column with thickness of 0.25 µm, length of 30.0 m and diameter of 0.25 mm (Restek) was used for separation of target compounds. The high

purity helium gas was used as carrier gas at flow rate of 1.00 mL min^{-1}. The GC injection port temperature was maintained 200°C while GC-MS interface temperature was kept at 220°C. The ion source temperature was fixed as 200°C.The oven temperature was programmed as follows: initial temperature was 50°C that was increased to 180°C at 10°C/min and held for 4 min; then it was further increased to 210°C at 2°C/min and held for another 4 min; and finally it was increased to 231°C at 3°C/min and held for 5 min. Total run time was 48 min. For qualitative analysis, data acquisition was performed in scan mode to confirm the retention times of target compounds. For quantitative analysis, selective ion monitoring (SIM) mode was employed. Selected target ions are listed in Table 6-1.

Table 6- 1: Target compounds, their retention time and selected target ions for SIM mode

Retention time	**Target compounds**	**Selected target ions**
16.20	Alpha Lindane	219, 181, 109
17.68	Lindane	109, 111, 181
19.35	Delta Lindane	109, 111, 219
21.36	Heptachlor	100, 65, 272
26.19	Heptachlor epoxide	81, 353, 355
27.91	cis-Chlordane	373, 375, 237
28.85	trans-Chlordane	375, 373, 377
30.85	4,4'-DDE	246, 318, 248
32.31	Endrin	81, 67, 79
33.41	Endosulfan	195, 160, 197
34.46	4,4'-DDD	235, 237, 165
37.14	Endosulfan sulfate	272, 274, 229
38.02	4,4'-DDT	235, 237, 165
41.12	Endrin ketone	67, 317, 315
43.24	Methoxychlor	227, 228, 113

6.3. Results and discussion

6.3.1. Characterization of zinc oxide incorporated carbon foam.

The synthesized material was characterized by scanning electron microscope and x-ray diffraction. SEM images revealed foam like structure of the sorbent. Figure 6-1 shows overall morphology of the material surface at different magnifications. Figure 6-2 shows SEM images indicating uniformly distributed ZnO NPs over the surface of carbon foam.

Energy dispersive x-ray (EDX) analysis can be used to find out the semi-quantitative composition of a specimen in SEM images. EDX spectra of different positions on SEM image also supported uniform distribution of ZnO NPs over the surface of the carbon foam (Figure 6-3).

XRD spectrum shows characteristic peaks of ZnO but resolution is indicative to poorly crystalline material (Figure 6-4). However, when carbon foam was synthesized under controlled nitrogen environment and heated up to 180°C, it does not burn. When this material was calcined in tube furnace at 900°C for 2 hours under nitrogen environment, material became more crystalline and sharp XRD peaks were obtained (Figure 6-5). However, this calcined carbon foam had relatively low extraction efficiency than ZnO incorporated carbon foam.

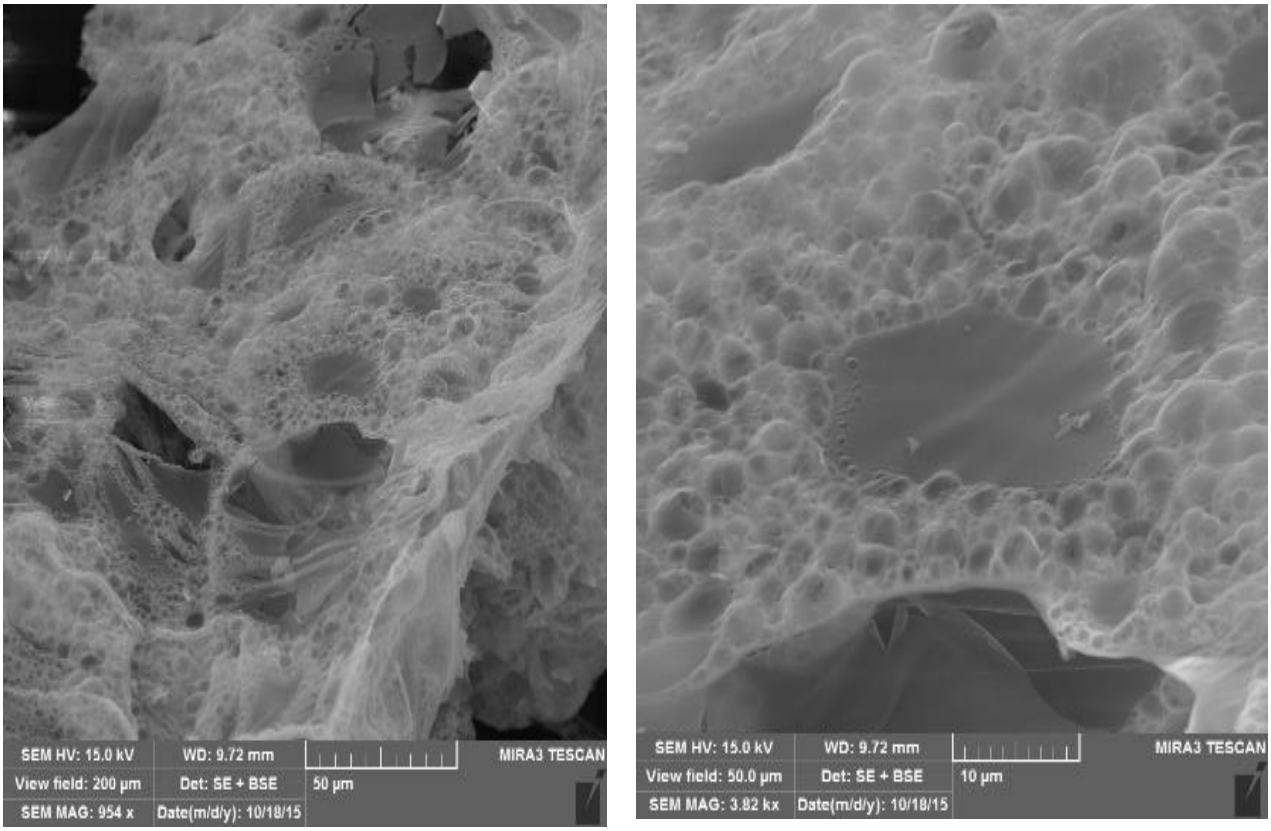


Figure 6- 1: SEM images of ZnO incorporated carbon foam showing overall surface morphology at different magnification levels

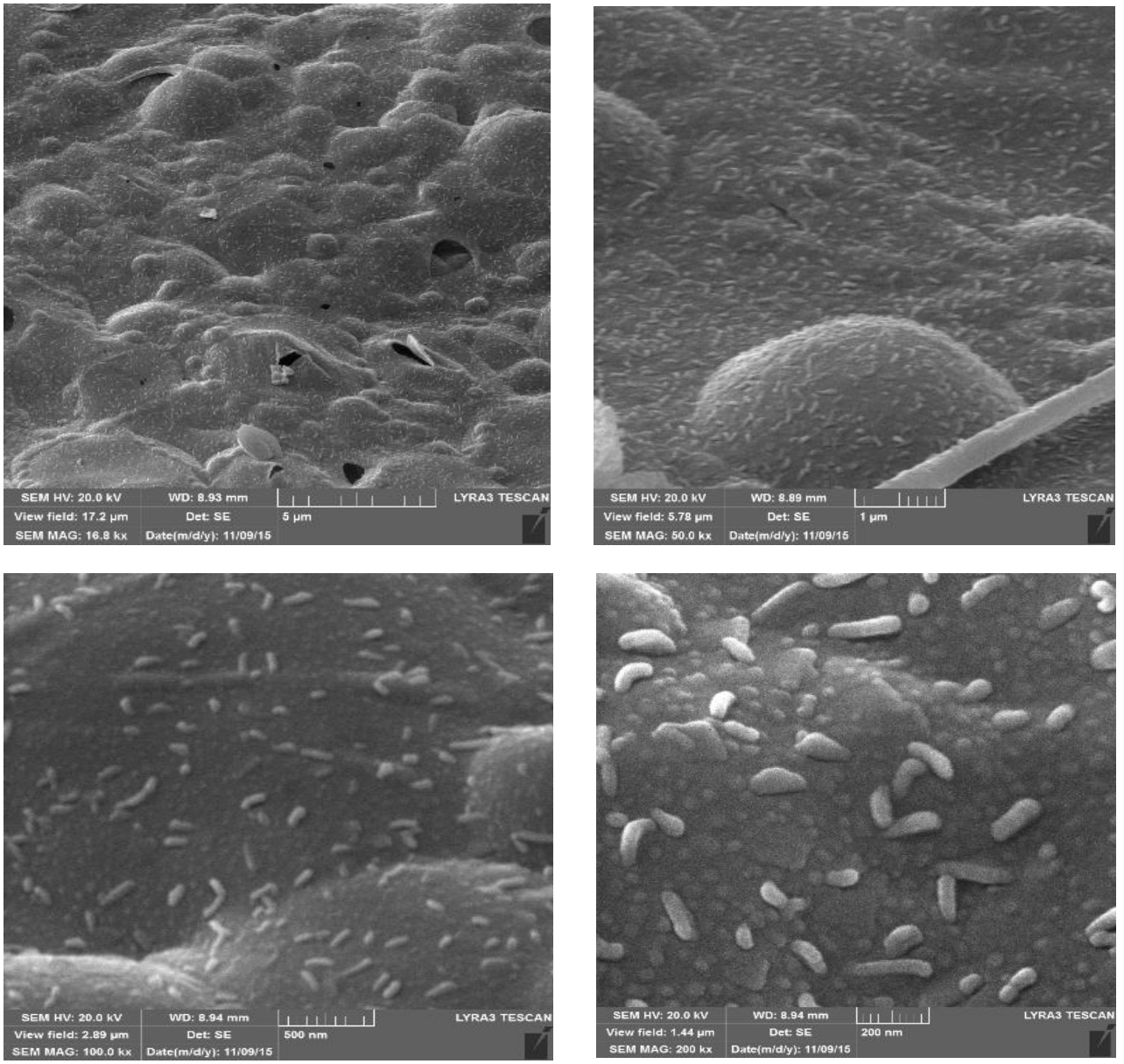
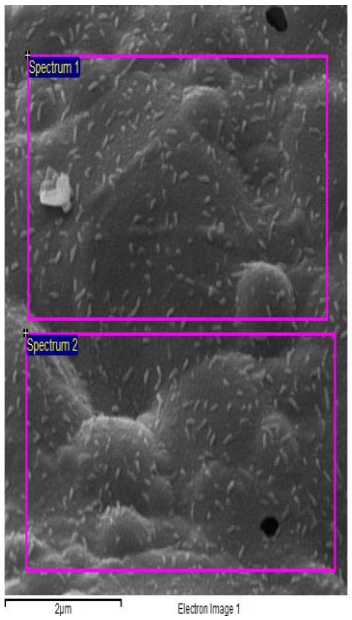


Figure 6- 2: SEM images at different magnifications showing distribution of ZnO nanoparticles on the surface of carbon foam



Spectrum	In stats.	C	O	Zn
Spectrum 1	Yes	60.56	33.71	5.74
Spectrum 2	Yes	61.47	33.15	5.37

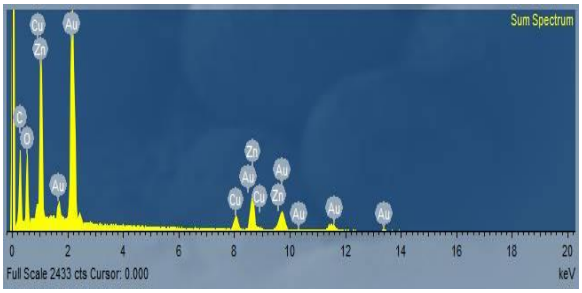


Figure 6- 3: EDX of ZnO incorporated carbon foam

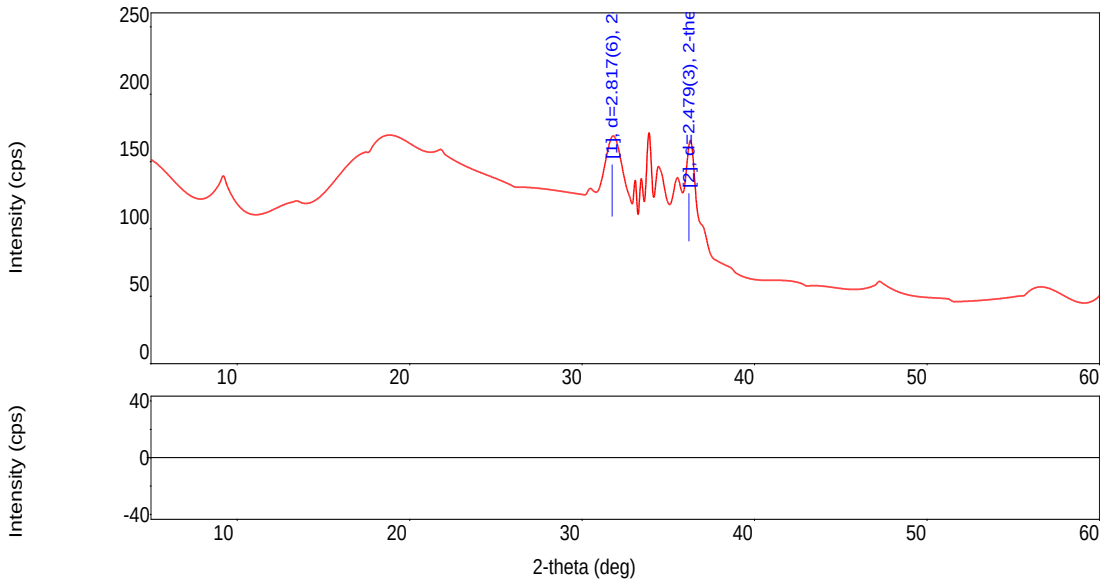


Figure 6- 4: XRD pattern of ZnO incorporated carbon foam

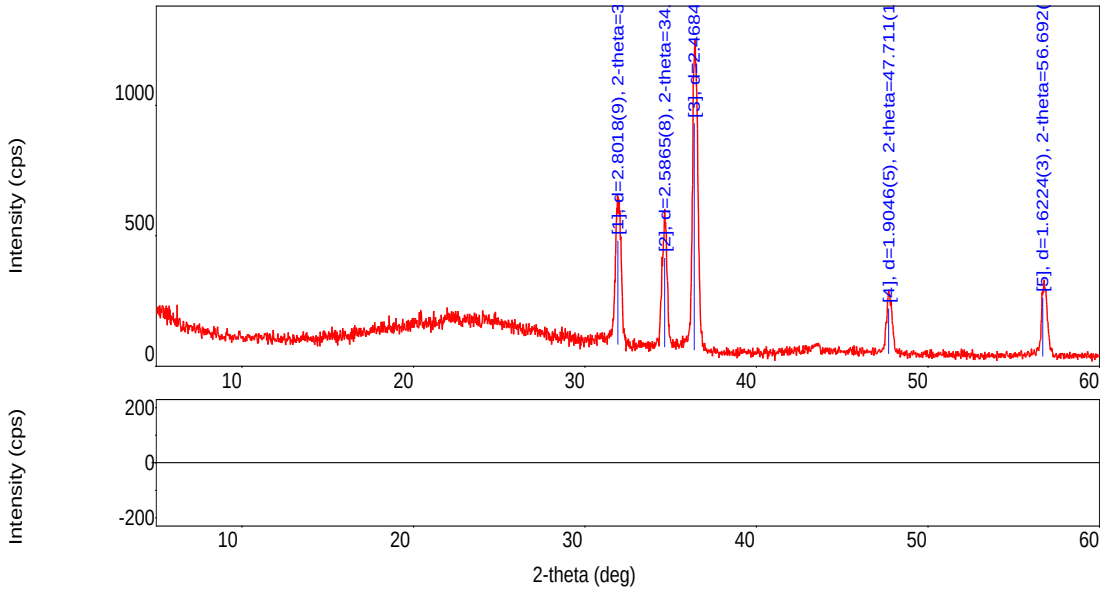


Figure 6- 5: XRD pattern of calcined ZnO incorporated carbon foam

6.3.2. Optimization of µ-SPE

Optimization experiments were conducted using OCPs free milk samples spiked at 25 ng mL^{-1}. Each optimization experiment was conducted in triplicate. The parameters that affect the efficiency of µ-SPE including sorbent type, amount of sorbent, desorption solvent, desorption volume, extraction time, stirring speed and desorption time were suitably optimized. Extraction efficiency was evaluated based on chromatographic peak areas.

Like SPE, selection of suitable sorbent material is of prime importance in µ-SPE. The affinity toward target compounds is decisive factor in selection of suitable sorbent. The materials such as MWCNTs, activated carbon and C_{18} are known for their excellent affinity toward hydrophobic analytes. In order to evaluate the extraction potential of ZnO-CF, its extraction efficiency was compared with MWCNTs, ZnO-CNTs hybrid and C_{18}. For this purpose, 20 mg of each sorbent was packed inside the µ-SPE device. The highest extraction efficiency was provided by ZnO-CF (Figure 6-6). This high extraction efficiency was attributed to **Π-Π** electrostatic interaction between CF and target analytes. The uniformly distributed ZnO NPs were considered to add an extra value to the extraction because there are several examples where metal oxide NPs functionalized over different materials showed better extraction efficiency [9].

The amount of sorbent was investigated in range of 5 – 25 mg. The extraction efficiency was increased up to 15 mg and then became constant. Hence 15 mg was selected as an optimum amount of sorbent (Figure 6-7).

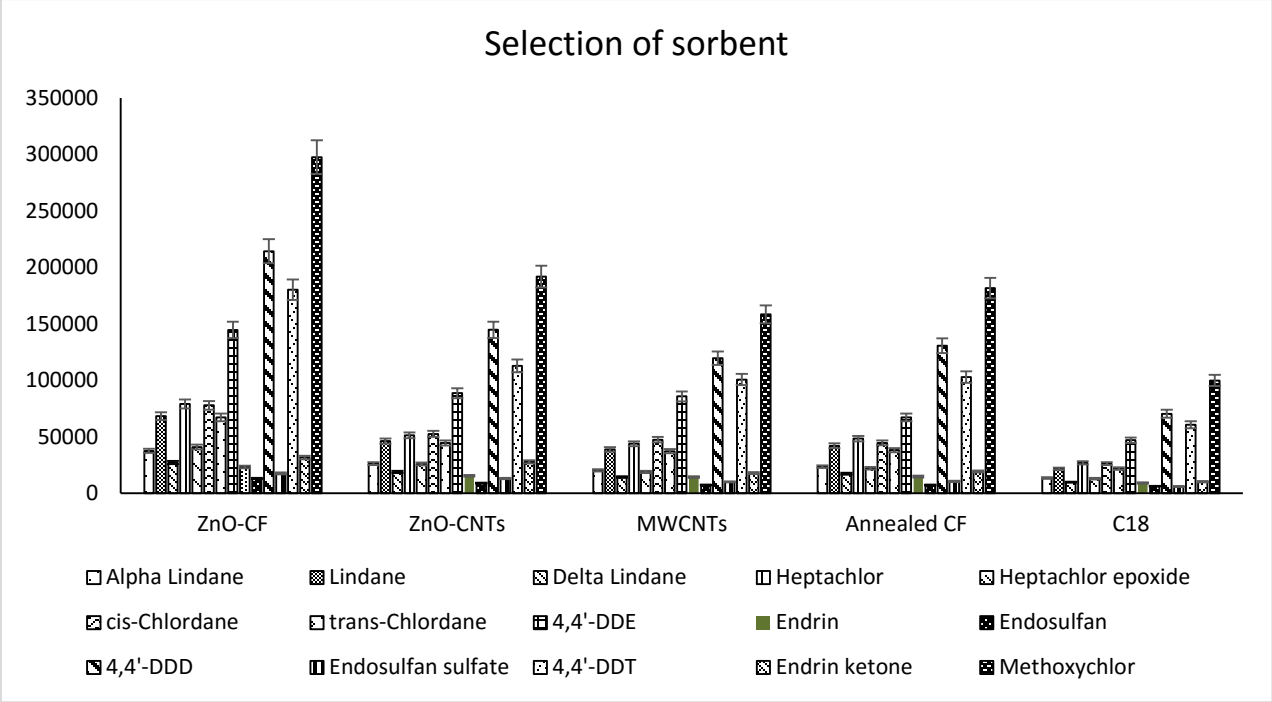


Figure 6- 6: Influence of different sorbents on extraction efficiency of µ-SPE.

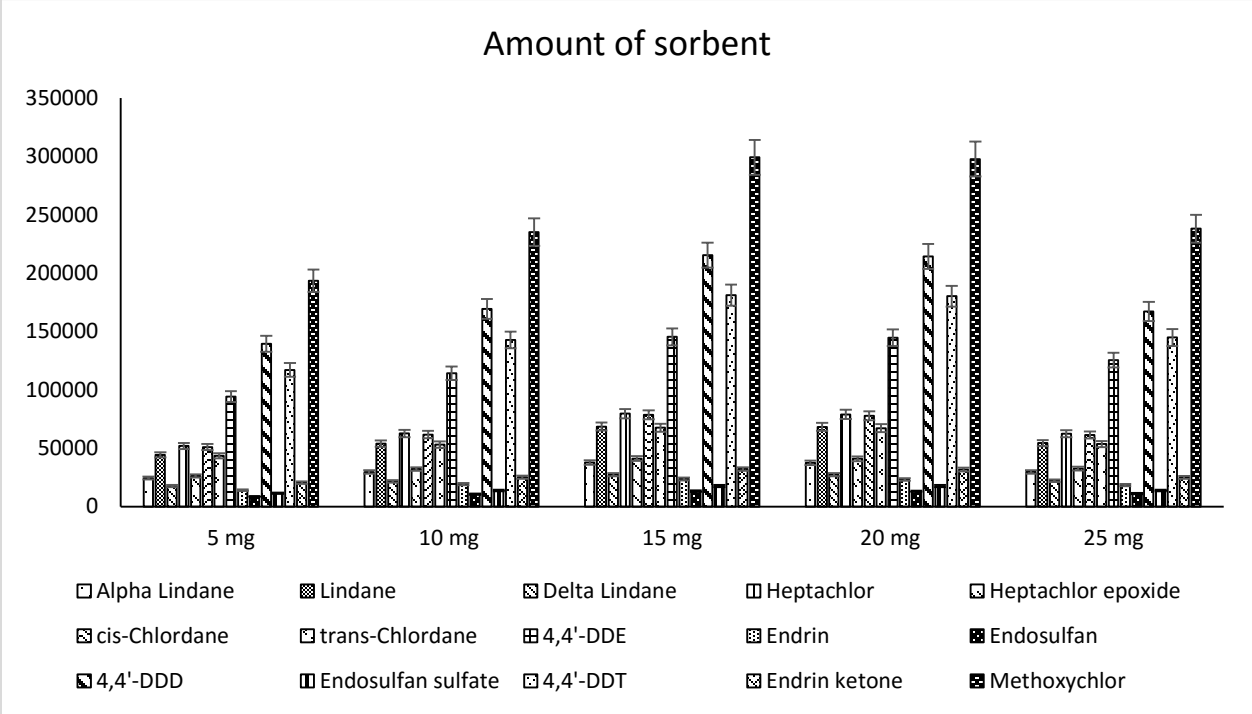


Figure 6- 7: Influence of amount of sorbent on extraction efficiency of µ-SPE.

The desorption solvent or eluent should be carefully selected as it has significant importance in desorption process. Affinity between desorption solvent and target compounds in terms of polarity is an important parameter to consider. In µ-SPE, desorption solvent should not only effectively desorb the target compounds from the sorbent but it should also properly dilate the pores of PP membrane for effective transfer of target compounds from sorbent to desorption solvent. A wide range of organic solvents with varying polarity index was employed as desorption solvent. Toluene was found most effective desorption solvent compared to methanol, n-hexane and acetonitrile. Toluene was observed to effectively dilate the pores of PP membrane compared to other solvents. Hence, it was selected as an optimum desorption solvent (Figure 6-8).

In order to elute the target compounds from µ-SPE device in a reproducible manner, the volume of desorption solvent should be sufficient enough to completely immerse the device. 300 µL was minimum volume of the desorption solvent to completely immerse µ-SPE device. Desorption volumes lesser than 300 µL provided low reproducibility because of ineffective desorption of target compounds while volumes higher than 300 µL gave low chromatographic peak areas because of dilution. Hence, 300 µL of desorption solvent was used throughout optimization experiments (Figure 6-9).

µ-SPE is time dependent equilibrium based procedure. The mass transfer of target compounds increases with extraction time until an equilibrium is attained. The extraction time was investigated in range of 10 – 50 min. After 30 min, no further increase in peak areas of target compounds was observed. Thus, extraction time of 30 min was opted as optimum extraction time (Figure 6-10).

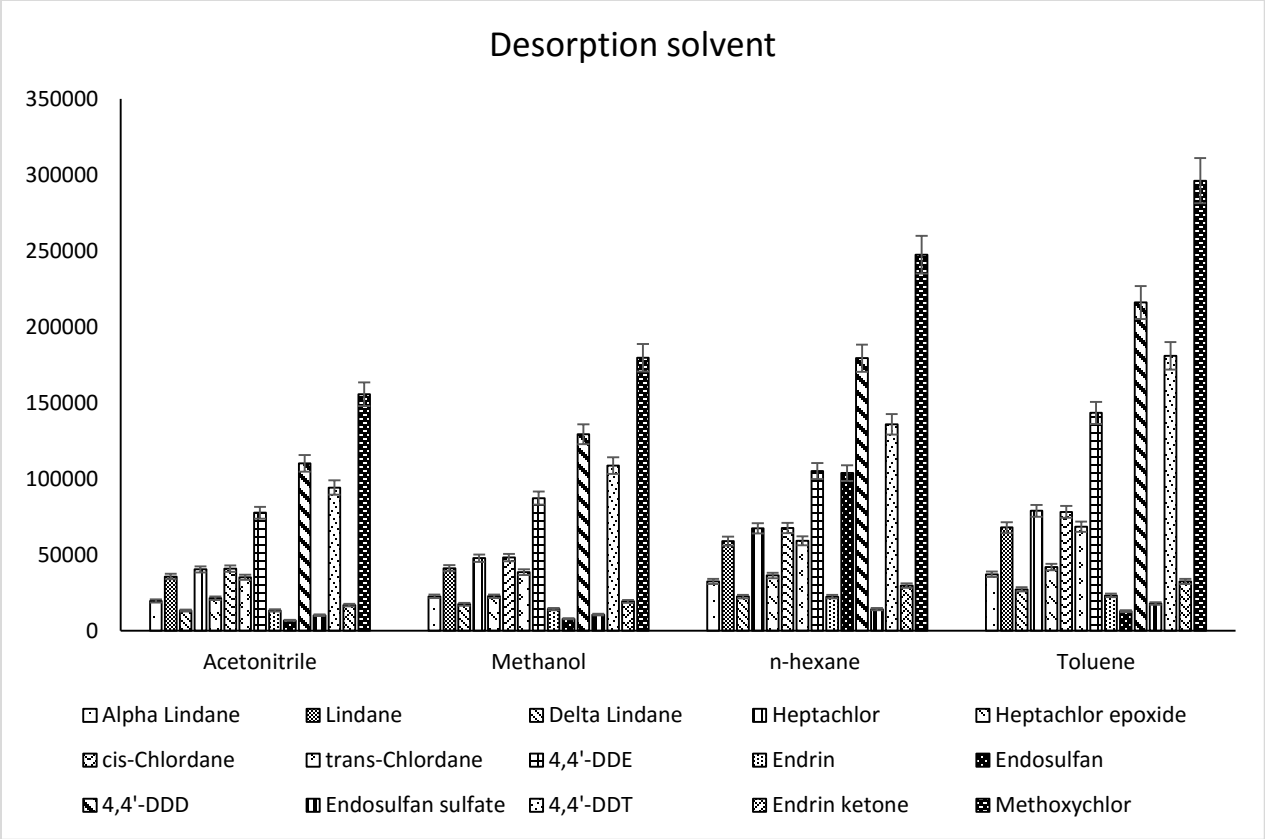


Figure 6- 8: Influence of desorption solvent on extraction efficiency of µ-SPE

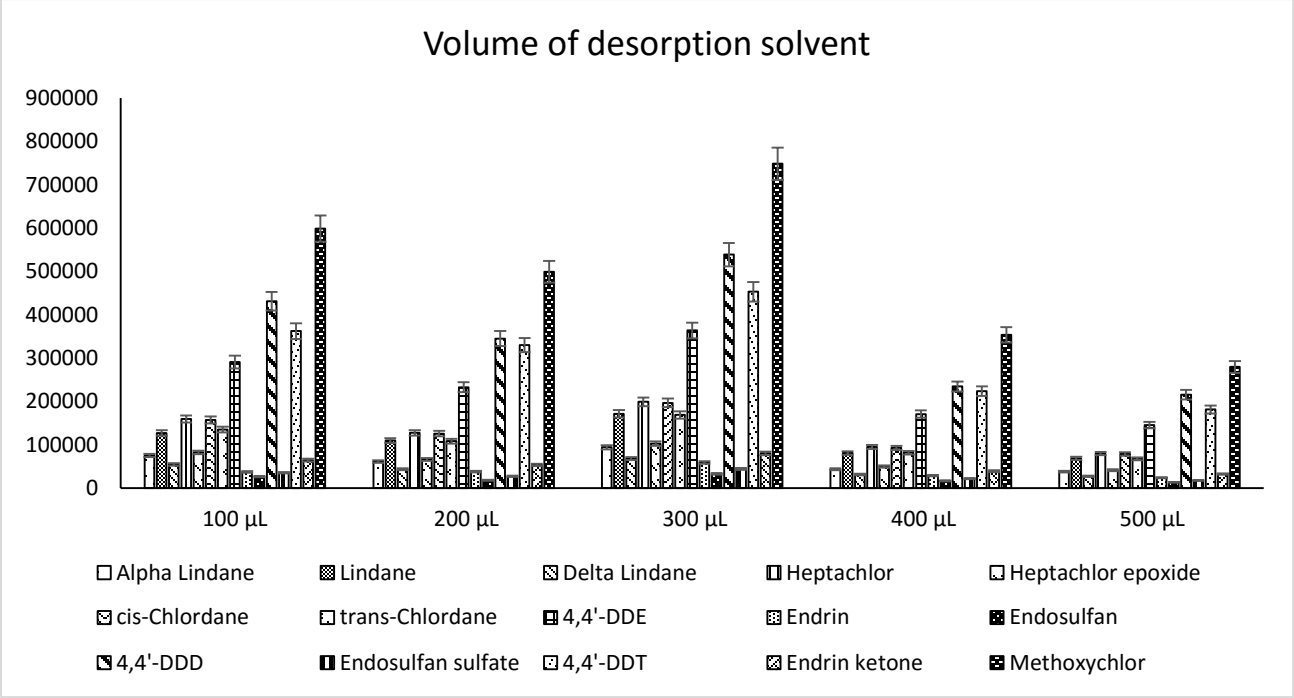


Figure 6- 9: Influence of volume of desorption solvent on extraction efficiency of µ-SPE

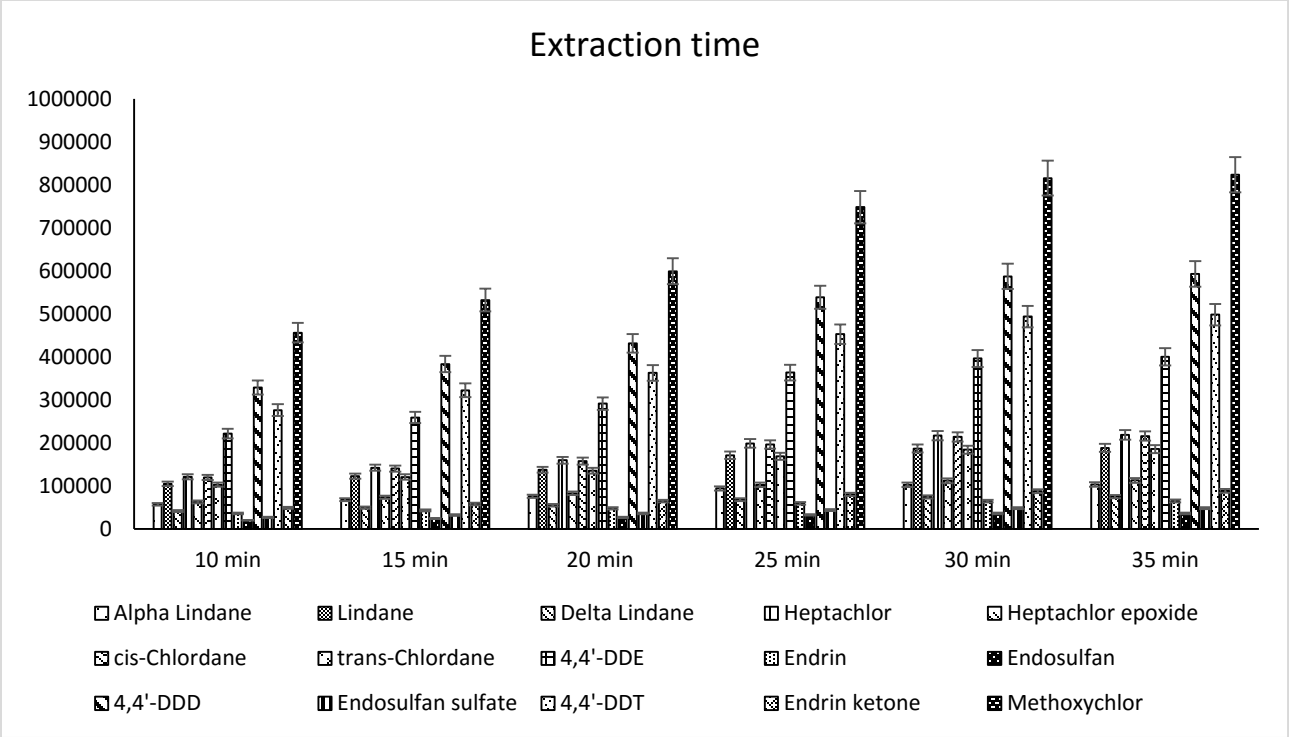


Figure 6- 10: Influence of extraction time on extraction efficiency of µ-SPE

It is very common to evaluate the effect of salt addition on extraction efficiency of microextraction techniques. Basically, addition of salt into sample solution decreases solubility of target compounds in the donor phase by enhancing salt strength (salting out effect). However, in this case, addition of salt resulted in decreased extraction efficiency. This is probably due to increase of viscosity of the solution which can hinder the diffusion of target compounds into the acceptor phase.

Partitioning of target compounds from donor phase to acceptor phase (µ-SPE device) is based on dynamic equilibrium. Hence, increase in stirring speed can assist in rapid attainment of equilibrium. The stirring speed was investigated in the range of 300 – 1250 rpm. As expected, extraction efficiency was increased with increase in stirring speed up to 1000 rpm. At higher stirring speeds, formation of bubbles on µ-SPE device was observed. These bubbles may slow down the diffusion of target compounds in µ-SPE device. Hence, 1000 rpm was selected as an optimum stirring speed (Figure 6-11).

Desorption was carried out immersing the µ-SPE device in organic solvent containing Eppendorf vial. Desorption was performed by the aid of sonication. Sonication times were evaluated in the range of 5 – 25 min. Peak areas were increased up to 15 min and then a slight decrease was observed. This attributed to rise of temperature by longer sonication times. This rise in temperature may evaporate target compounds in the headspace over the organic solvent in desorption vial. These compounds may escape when vial is opened. The second reason for low extraction efficiency at higher sonication periods may be due to re-adsorption of target compounds (Figure 6-12).

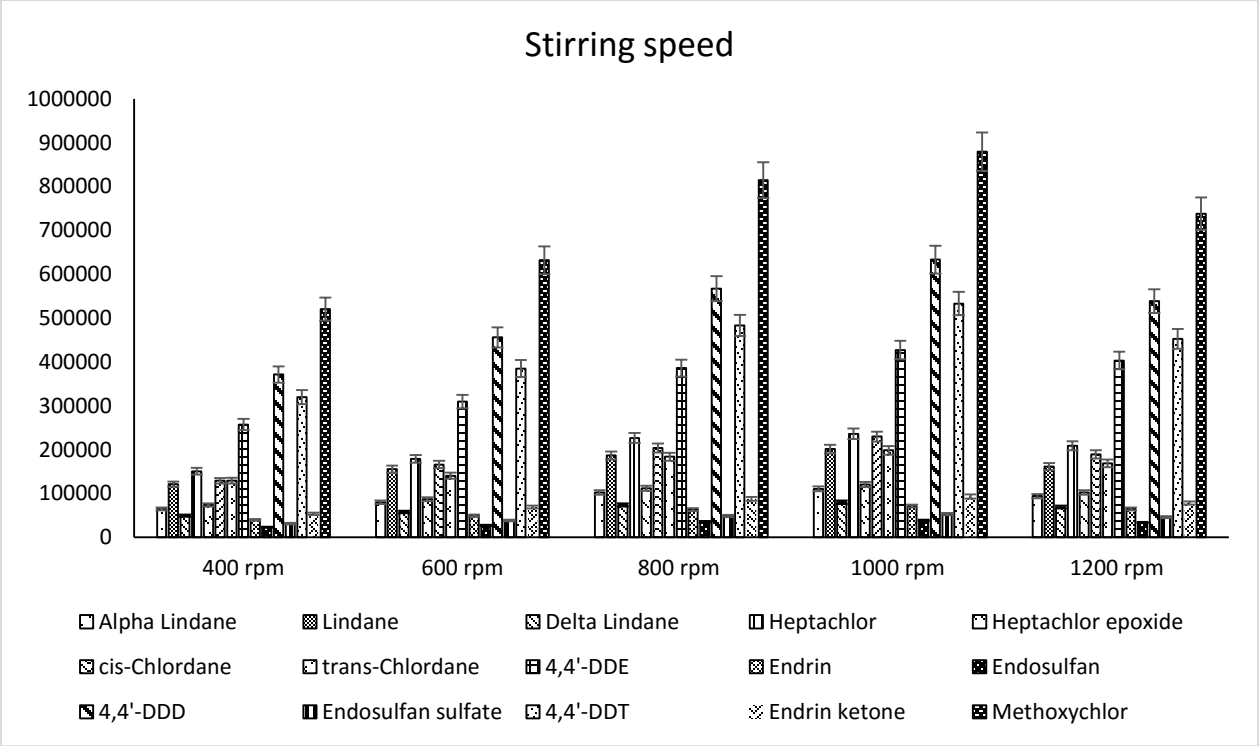


Figure 6- 11: Influence of stirring speed on extraction efficiency of µ-SPE.

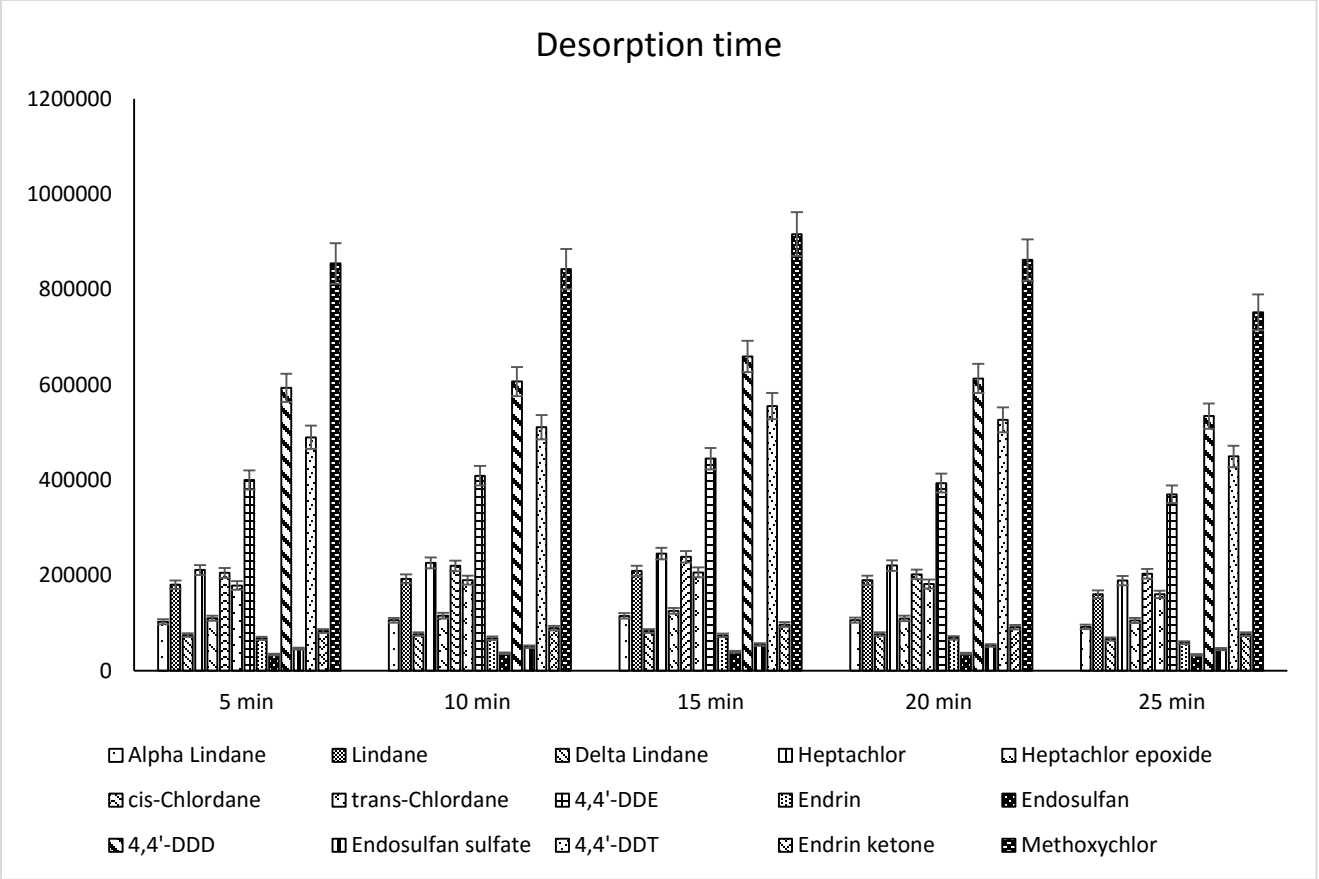


Figure 6- 12: Influence of desorption time on extraction efficiency of µ-SPE.

6.3.3. Method validation

Method validation parameters such as linearity, limit of detections (LODs) and reproducibility were studied under most optimum extraction conditions. 6-point calibration curves were plotted for all target analytes using uncontaminated milk samples spiked with known concentrations of OCPs. Good linearity for all target compounds was obtained with coefficients of determination (r^2) between 0.9926 and 0.9998. LODs were calculated based on signal to noise ratio of 3. LODs were ranged in between 0.19 and 1.64 ng mL^{-1}. Method's precision was evaluated based on inter-day and intra-day reproducibility by using uncontaminated milk samples spiked at 25 ng mL^{-1} (n=7). RSDs were in range of 2.3 – 9.7 % and 2.8 – 10.2 % for intra-day and inter-day precision respectively, indicating good precision of the method. Relative recoveries were calculated in order to access the matrix effect by spiking milk samples at 1.0, 25 and 50 ng mL^{-1} (n=5). Relative recoveries of all tested OCPs were ranged between 85.1 to 100.7%. Analytical features of proposed method are given in Table 6-2.

The developed µ-SPE method was applied to determine OCP concentrations in different brands of milk samples. Figure 6-13 compares chromatograms of unspiked and spiked (at 25 ng mL^{-1}) samples.

Table 6-3 compares the performance of SPE with microextraction methods reported in the literature for extraction of OCPs in milk samples.

Table 6- 2: Analytical features of the method

Compound	Linear range (ng/mL)	Coefficient of determination (r^2)	LOD (ng/mL)	Matrix effect (Mean relative recoveries n=3)			RSDs (%)	
				5 ng/mL	10 ng/mL	25 ng/mL	Intra-day	Inter-day
Alpha lindane	1 – 250	0.9973	0.27	95.3	96.8	97.2	4.5	5.2
lindane	1 – 250	0.9964	0.26	98.2	98.1	99.0	2.3	4.7
Delta lindane	3 – 250	0.9983	0.80	97.2	96.8	98.5	3.7	3.9
Heptachlor	1 – 250	0.9978	0.19	89.3	92.1	95.4	5.1	6.9
Heptachlor epoxide	3 – 250	0.9956	0.90	100.0	101.6	100.7	2.4	3.5
Cis-chlordane	5 – 250	0.9945	1.24	87.5	91.7	92.2	6.2	7.3
Trans-chlordane	5 – 250	0.9966	1.43	94.0	93.2	96.1	5.1	7.6
4,4’-DDE	5 – 250	0.9991	1.64	92.5	92.0	94.1	2.5	2.8
Endrin	5 – 250	0.9968	1.57	85.1	87.2	88.8	9.2	8.5
Endosulfan	5 – 250	0.9949	1.07	88.7	90.2	91.0	4.7	6.2
4,4’-DDD	3 – 250	0.9969	0.69	95.0	97.2	97.0	6.3	8.9
Endosulfan sulfate	3 – 250	0.9926	0.47	95.1	95.0	97.2	3.8	5.3
4,4’-DDT	1 – 250	0.9943	0.35	85.4	85.8	91.7	9.7	10.2
Endrin ketone	5 – 250	0.9998	1.06	94.0	96.2	98.0	2.8	5.3
Methoxychlor	3 – 250	0.9965	0.57	86.3	89.6	90.6	4.5	5.1

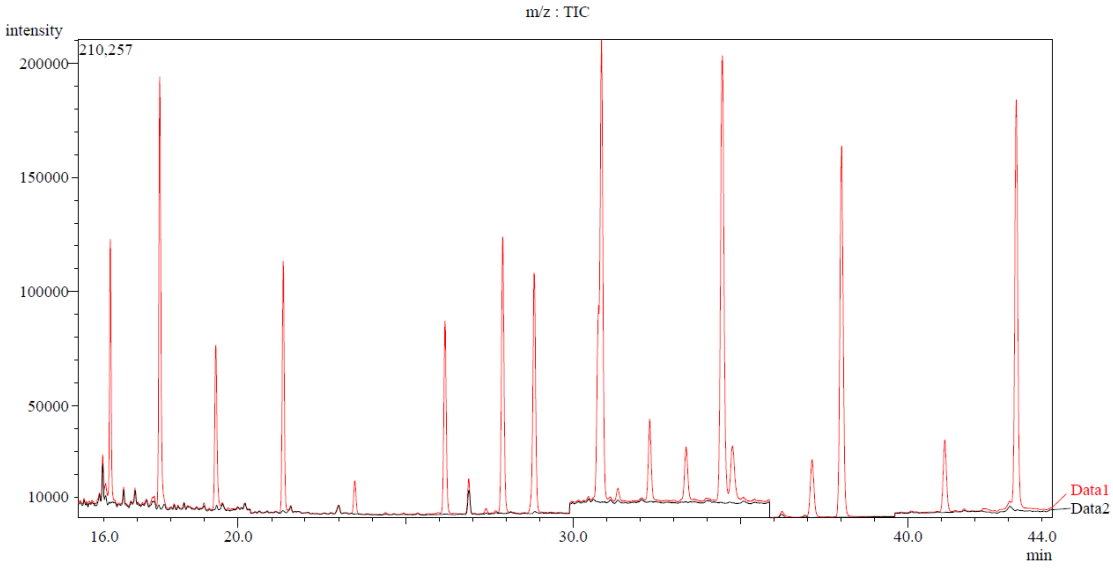


Figure 6- 13: GC-MS chromatogram of non-spiked and spiked (25 ng/mL) milk samples

Table 6- 3: Comparison with methods reported in literature

Method	**Number of OCPs studied**	**Linear Range**	**LODs**	**RSDs (%)**	**Ref**
SPE-GC/ECD	6	--	0.5 – 1.8 ng/mL	1.7 – 5.8	[10]
QuEchERS-GC/ECD	12	0.01 – 0.1 µg/mL	--	0.28 – 7.55	[11]
SPE-GC/ECD	9		0.01mg/Kg		[1]
PLE-GC/MS/MS	4		0.03 – 0.800 µg/Kg	7.0 – 17.0	[12]
ZNO-CF-µ-SPE	15	1, 3, 5 – 250 ng/mL	0.19 – 1.64 ng/mL	2.3 – 10.2	Present work

6.4. Conclusion

In this work, a simple and facile method was adopted for synthesis of zinc oxide incorporated carbon foam. For the first time, this synthesized material characterized using SEM and XRD analysis. Later, it was utilized as sorbent in μ-SPE of OCPs in milk samples. The method provided good linearity and LODs for tested target compounds.

6.5. References

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7. CHAPTER

INVESTIGATION OF RELATIVE ACCUMULATION PROFILES OF ENDOCRINE DISRUPTING COMPOUNDS IN DIFFERENT TYPES OF HUMAN CANCER TISSUES

Abstract

Endocrine disrupting compounds are well known for interfering with normal hormonal activity in human and thus triggering different kind of carcinogenic and non-carcinogenic disorders. Organochlorine pesticides (OCPs) and polychlorinated biphenyls (PCBs) have negative impact on the human health. They are persistent in nature and accumulate in cells and tissues and induce different type of cancers as a result of endocrine disruption. We have evaluated the burdens of OCPs and PCBs in breast, colorectal, thyroid and kidney cancer tissues collected from Saudi Arabia. The compounds were extracted using ultrasonic assisted solvent extraction and determined by gas chromatography mass spectrometry. ΣOCPs were highest in colorectal (3951.8 ng/g) and breast (3921.3 ng/g) tissues while significant concentrations were also found in thyroid and kidney tissues. ΣPCBs were highest in thyroid tissues followed by colorectal, breast and kidney tissues. ΣOCPs were several times higher than ΣPCBs in all types of cancer tissues. In order to get more deep insight into concentrations and bioaccumulation trends in cancer tissues, OCPs were divided into five groups of closely related compounds. Concentrations of ΣBHCs were higher than ΣCHLs, ΣDDs, Σdrins and Σendosulfan in all type of tissues except the kidney where ΣCHLs were slightly higher than ΣBHCs. These high concentrations of OCPs and PCBs in cancer tissues suggest that the environment of Saudi Arabia is heavily polluted with these pollutants.

7.1. Introduction

Endocrine disrupting compounds (EDCs) are known to interfere with normal hormonal activities in humans and wildlife [1]. They affect synthesis, transportation and binding of natural hormones which leads to variety of ailments in human body. EDCs represent a wide class of chemicals including organochlorine pesticides, polychlorinated biphenyls, phthalates, heavy metals, natural and synthetic hormones etc. Exposure to EDCs is associated with problems in cognitive behavior, disorders in neurological and reproductive systems [2] and induction of variety of cancers [3] in humans including breast, prostate and thyroid.

EDCs become functional at very low concentration levels such as part per billion and part per trillion [1]. Human exposure to EDCs occurs through inhalation, dermal penetration and digestion of contaminated food [4]. Major concern about EDCs arises from the studies where their measurable concentrations have been reported in wastewater, drinking water[5,6], food chain[7], indoor and outdoor environment[8].

EDCs are persistent in their nature and have potential to accumulate in tissues [9,10]. Exposure of cells to EDCs may result in variations in structure and gene expressions, leading to initiation of cancers in body [11].Concentrations of EDCs in biological fluids have been associated with high risk of breast cancer [12]. Malignancies related to female reproductive tract are also reported to have direct link with EDC exposures [13]. Indeed, hundreds of studies that report a link between EDCs accumulation and different disorders in human, have been published within past few years. After compiling the reports published worldwide, a recent document issued by UNEP and WHO establishes a

relationship between exposure of EDCs and occurrence of variety of cancers. Moreover, past 40-50 years witnessed unmatchable increase in rates of endocrine related cancers mainly because of large scale production and distribution of EDCs in the environment [14].

Two broad groups of EDCs, organochlorine pesticides (OCPs) and polychlorinated biphenyls (PCBs) have been particularly considered in most of the reports because of their wide abundance in environment and potential to induce serious health implications. They also fall among persistent organic pollutants (POPs). Although their sources are local but they disperse around the globe due to relatively moderate vapor pressures and commerce. Consistent use of these chemicals in a particular place and long range atmospheric transfer, both contribute to their dispersion in environment. Literature reveals that number of studies has been carried out across the globe to find out concentrations of POPs in different kind of biological samples [15–17] and their measureable concentrations were found in human[18] and animal tissues [19]. High concentrations of PCBs and OCPs in developing countries are due to extensive use of these chemicals in the past and poor regulations to control their current use in industry and agrochemical sector [20].

To access the exposure and sources of EDCs in a particular region, environmental pollutants are monitored in biological samples collected from local human and animal population. Cancer incidence rates have been dramatically increased in the kingdom of Saudi Arabia during last few years. According to a report published by Saudi cancer registry for year 2004, endocrine related cancers position among top commonly occurring cancers [21]. The objectives of this study were to investigate burdens of OCPs and PCBs in human cancer tissues collected from Saudi Arabia. We investigated breast, thyroid, colorectal and kidney cancer tissues for screening of OCPs and PCBs to get their relative

bioaccumulation patterns. To best of our knowledge, for the first time in Saudi Arabia, human cancer tissues were screened out to find out bioaccumulation of EDCs.

7.2. Materials and Methods

7.2.1. Sampling site and information about samples

Jeddah is second largest city of Saudi Arabia and it is located on the coast of the Red Sea. It represents the largest seaport on Red Sea. According to 2007 estimations, its population was 3.4 million. It has fourth largest industrial district of Saudi Arabia.

Human cancer tissues were collected from King Fahd Center for Medical Research at King Abdulaziz University, Jeddah. Samples were packed in sterilized plastic tubes and labelled properly. 73 cancer tissue samples were collected in all, which included breast (n=25), colorectal (n=11), thyroid (n=26) and kidney (n=11) tissues. All the samples were stored at temperature of -20°C prior to analysis.

7.2.2. Chemicals and materials

A standard containing mixture of PCBs was purchased from the Restek . 11 compounds which include 2,2′,4,5,5′-Pentachlorobiphenyl (PCB-101), 2,2′,3,4,5′-Pentachlorobiphenyl (PCB-87), 2,3,3′,4′,6-Pentachlorobiphenyl (PCB-110), 2,2′,3,5,5′,6-Hexachlorobiphenyl (PCB-151), 2,2′,4,4′,5,5′-Hexachlorobiphenyl (PCB-153), 2,2′,3,4,5,5′-Hexachlorobiphenyl (PCB-141), 2,2′,3,4,4′,5′-Hexachlorobiphenyl (PCB-138), 2,2′,3,4′,5,5′,6-Heptachlorobiphenyl (PCB-187), 2,2′,3,4,4′,5′,6-Heptachlorobiphenyl (PCB-183), 2,2′,3,4,4′,5,5′-Heptachlorobiphenyl (PCB-180), 2,2′,3,3′,4,4′,5-Heptachlorobiphenyl (PCB-170) were selected as target compounds. A standard

containing mixture of OCPs was purchased from the Restek and 16 compounds (alpha-BHC, gama-BHC, beta-BHC, delta-BHC, Heptachlor, Aldrin, Heptachlor epoxide, cis-Chlordane, trans-Chlodane, 4,4'-DDE, Endrin, Endosulfan, 4,4'-DDD, Endosulfan sulfate' 4,4'-DDT, Methoxychlor) were considered for this study. All the solvents used were of HPLC garde.

7.2.3. Sample preparation, extraction and analysis

Sample preparation is an important step for analysis of trace levels of organic pollutants in any matrix. The quantity of the real cancer tissues we obtained from a local hospital was very small (each sample less than a gram). Hence it was needed to develop an efficient extraction method for pre-concentration of target analytes without losing any sample. Thus, a simple ultra-sonication assisted solvent extraction method was developed for pre-concentration of target analytes. Initially the method was developed on fish tissues and its applicability to real cancer tissues was confirmed by recovery experiments. Good recoveries are supportive to apply the method on real tissues.

The weights of real samples were measured before extraction which were in range of few milligrams to several hundred milligrams. 500 µL of toluene was added to each sample vial and it was properly closed. Then, sample vials were placed in ultrasonication bath for a specified period of time which was operated at certain power. All the samples were then transferred to refrigerator for 1 hour. Purpose of this cooling was to settle and freeze any fatty stuff at the bottom. After 1 hour of cooling, upper layer was taken into GC vials and 1 µL was injected to GC-MS.

Different parameters that may affect the extraction efficiency of ultra-sonication assisted solvent extraction were optimized. These parameters include extraction solvent, power of ultra-sonication and extraction time. As a result of optimization, toluene was selected as extraction solvent. The optimum power at which ultra-sonication device was operated was 75% and the optimum extraction time was found as 25 minutes.

7.2.4. Gas chromatography-mass spectrometry

All the samples were analyzed using gas chromatograph mass spectrometer (Shimadzu, QP2010 ultra equipped with autosampler AOC-20i, column Rxi-5 Sil MS 30.0 m x 0.25 µm x 0.25 µm). Different temperature programs were developed for both PCBs and OCPs.

For OCPs injection temperature was 200°C and the oven temperature was programmed as: initial temperature was 50 °C and then it was increased to 180°C at rate of 10°C/min and held for 4 minutes and then increased to 210°C at rate of 2 °C/min and held for 4 minutes and finally increased to 238 at rate of 3°C/min. Total run time was 45.33 minutes.

For PCBs initial temperature was kept 100°C which was held for 1 minute and then increased to 190°C at the rate of 20°C /min and held for 2 minutes and then increased to 260°C at rate of 3°C /min and held for 3 minutes. Total run time was 30.50 minutes. For qualitative measurements, mass spectrometer detector was operated in scan mode from m/z of 50 to 500. For quantitative determination, mass spectrometer detector was operated in selective ion monitoring mode.

7.2.5. Quality assurance (QA) and quality control (QC)

Quality assurance and quality control were given due importance during whole process of analysis. Glassware was properly washed and oven dried before using for preparation of any solution. No target compounds were found in solvent or procedural blank. Different standard concentrations were extracted using the proposed extraction methods and a linear calibration graph was obtained for each compound (R^2>0.99).

7.2.6. Statistical Analysis

Results of analyzed samples were categorized based on the type of the tissues i.e. breast (n=25), colorectal (n=11), thyroid (n=26) and kidney (n=11) tissues. 11 PCBs and 16 OCPs were screened in all categories of cancer tissues. Mean, standard deviation and range of detected concentrations were calculated and results were compared to get useful conclusions.

16 OCPs were further classified into 5 groups of closely related compounds which were: BHCs (alpha, beta, gamma and delta BHCs), CHLs (heptachlor, heptachlor epioxide, cis-chlordane and trans-chlordane), drins (aldrin and endrin), DDs (4.4'-DDT, 4,4'-DDE and 4,4'-DDD) and endosulfans (endosulfan and endosulfan sulfate). The total concentration (as a sum) of each group was determined in all categories of cancer tissues and represented by ΣBHCs, ΣCHLs, Σdrins, ΣDDs, Σendosulfans. In order to find overall burden of PCBs and OCPs on different type of cancer tissues ΣPCBs and ΣOCPs were also calculated and compared.

7.3. Results and discussion

Optimized conditions were used for the extraction of OCPs and PCBs to enhance their recoveries in biological tissues. The extraction yields were evaluated by peak area analysis using GCMS. Optimization was carried out by considering triplicate uncontaminated fish tissue sample analysis spiked with known concentrations. The parameters optimized were: extraction solvent, extraction time and sonication power. Higher peak areas corresponded to higher extraction efficiencies.

7.3.1. Optimization of extraction parameters

7.3.1.1. Extraction solvent

Solvents with boiling point higher than 60°C were selected for extraction. This is because of heating effect which is produced as a result of ultrasonication and temperature sometimes may rise upto 50°C. Hence, the solvents with low boiling point may evaporate. The Four organic solvents toluene, acetonitrile, propanol and carbon tetrachloride were tested for extraction. Based upon larger peak areas for all extracted compounds (OCPs and PCBs), toluene was found most suitable extraction solvent.

7.3.1.2. Extraction time

Different extraction times (5 min, 15 min, 25 min, 30 min) were evaluated to find the optimum extraction time. Time of 25 min was selected because high peak areas for target compounds. There was no significant increase in peak areas after 25 minutes. The reason for such long time of ultrasonication assisted extraction can be attributed to strong interactions of target compounds within the tissues due to their bio accumulative nature.

7.3.1.3. Intensity of ultra-sonication

Percentage of ultrasonication power can affect extraction of organic pollutants in biological tissues. The higher power can accelerate the mass transport of analytes from tissues to organic solvent. The maximum peak areas for analytes were obtained at ultra-sonicaion power of 75% and then a steady state was reached. Further increase in power of ultra-sonication did not affect extraction efficiency. Hence 75% power was chosen as optimum.

7.3.2. Concentrations of OCPs in human cancer tissues

Mean concentration of OCPs in all type of cancer tissues, the range of detected concentrations, and percentage of the samples in which a particular target compound was detected, are summarized in Table 7-1.

Concentration of OCPs in the tissues have been widely linked with breast cancer. For this purpose, number of studies have been carried out throughout the world. Although, most of the reports describe high concentrations of OCPs in cancer patients than the controls [22,23] but there are some reports where no significant differences in OCP concentrations among cancer patients and controls were observed [24,25]. Without being critical into the debate, one fact is understood that bioaccumulation of OCPs in human and animal tissues may lead to serious consequences and it indicates the nature of the exposure for a certain period of time to contaminated environment. In the current study, Figure 7-1 (a) highlights relative mean concentrations of OCPs in breast cancer tissues (n=25). The highest mean concentration was recorded for 4,4'-DDE which was 888.0 ng/g. 4,4'-DDE was detected in 92% of breast cancer tissues. The other compounds with subsequent higher mean concentrations were: beta-BHC (769.0 ng/g) and heptachlor (733.8 ng/g). Heptachlor and

4,4'-DDE stand among most frequently detected compounds in breast cancer tissues (92% samples). The lowest mean concentration was found 1.8 ng/g for cis-chlordane. The lowest concentration of any individual compound in a single tissue was 0.4 ng/g for 4,4'-DDE and the highest was 2600 ng/g for heptachlor.

Thyroid cancer is rare kind of cancers in human and it accounts less than 2 % of all cancers. However, it is the most common endocrine related malignancy in the world. During past decades, incidence rates of thyroid cancer have been increased significantly[26]. Although some studies linked exposure of OCPs with thyroid cancer [27] but it is difficult to find any reports where OCPs were measured directly in thyroid tissues. In this study we analyzed OCPs in malignant thyroid tissues (n=26), the highest mean concentration was recorded for beta-BHC which was 279.8 ng/g. beta-BHC was detected in 84.6% of thyroid cancer tissues. The other compounds with subsequent higher mean concentrations were: heptachlor (204.0 ng/g) and 4,4'-DDE (189.1 ng/g). 4,4'-DDE was detected in 92.3% of thyroid cancer tissue samples. The lowest mean concentration was found 1.3 ng/g for methoxychlor. The lowest concentration of any individual compound in a single tissue was 18.2 ng/g for cis-chlordane and the highest was 597.4 ng/g for beta-BHC. Overall we observed very high concentrations of BHCs, heptachlor, 4,4'-DDE, gamma-BHC and aldrin. Figure 7-1(b) shows relative mean concentration of OCPs in thyroid cancer tissues.

Colorectal cancer is third most common cancer in the world. Etiology of colorectal cancer is not clearly understood, however, diet is thought to play an important role in determining the risk. Consumption of polluted diet is major source of exposure to organic pollutants and longer contact of these pollutants with colon may lead to development of malignancies. Although, EDCs mainly develop hormone dependent cancers and colorectal cannot be

placed in this category. However, still there is some evidence of role of hormones [28]. Hence, we screened colorectal malignant tissues for presence of OCPs. The highest mean concentration was recorded for 4,4'-DDE which was 1064.8 ng/g. 4,4'-DDE was detected in 90.9% of colorectal cancer tissues. The other compounds with subsequent higher mean concentrations were: beta-BHC (951.5 ng/g) and alpha-BHC (462.6 ng/g). Alpha-BHC, beta-BHC and 4,4'-DDE were detected in 90.9 of the colorectal cancer tissue samples. The lowest mean concentration was found 4.7 ng/g for heptachlor epoxide and it was detected in 27.3% of the samples. Such lowest concentrations were recorded in fish tissues in China by Nakata et al.[23]. Trans-chlordane was not detected in any colorectal cancer tissue samples. The lowest concentration of any individual compound in a single tissue was 0.7 ng/g for 4,4'-DDD and the highest was 2786.0 ng/g for beta-BHC. Figure 7-1(c) gives an overview of relative mean concentrations of OCPs in colorectal malignancies.

The kidney performs many important functions in body such as balance of fluids, removal of waste from blood, formation of urine etc. There is a significant evidence of accumulation of OCPs in kidneys of different animals [29–31], however, literature regarding the humans is lacking this aspect. In our study, 11 samples of kidney cancer tissues were evaluated for presence of OCPs. The highest mean concentration was recorded for heptachlor which was 533.3 ng/g. Heptachlor was detected in 90.9% of kidney cancer tissues. The other compounds with subsequent higher mean concentrations were: beta-BHC (314.0 ng/g), 4,4'-DDE (179.4 ng/g) and endosulfan sulfate (106.7 ng/g). Beta-BHC detected in all kidney cancer tissues. The lowest mean concentration was found 21.5 ng/g for endrin. Methoxychlor was not detected in any samples. Figure 7-1(d) shows mean concentrations of OCPs in different types of cancer tissues.

Beta-BHC was abundantly recorded in all type of cancer tissues standing among the frequently detected compounds with high concentrations. This frequent detection of beta-BHC may be due to its excessive and non-stop use in the past being a relatively cheaper and major contributor in group of OCPs. These observations are in agreement with some other studies where high concentrations of BHCs were recorded in vegetable and food stuff [32,33]. Although all OCPs showed significantly high mean concentrations in tissues samples, but compared to other compounds concentrations of 4,4'-DDE and heptachlor were very high in all type of cancer tissues. These results are not very strange from the perspective of previously reported literature where very high concentrations of these compounds were detected in vegetables and fruits and authors stated that continuous consumption of contaminated food may lead to accumulation and serious chronic effects [34].

OCPs were further divided into groups of closely related compounds in order to find impact of each kind of compounds on different type of cancer tissues i.e. ΣBHC (sum of mean concentration of alpha, beta, gamma and delta BHCs), ΣCHLs(sum of mean concentration of heptachlor, heptachlor epioxide, cis-chlordane and trans-chlordane), Σdrins (sum of mean concentration of aldrin and endrin), ΣDDs (sum of of mean concentration 4.4'-DDT, 4,4'-DDE and 4,4'-DDD) and Σendosulfan (sum of e of mean concentration endosulfan and endosulfan sulfate).Figure 2 shows relative mean concentrations of ΣBHCs , ΣCHLs, Σdrins, ΣDDs and Σendosulfan in cancer tissues. ΣBHCs were highest in colorectal tissues while lowest in kidney cancer tissues. ΣCHLs were highest in breast tissues while lowest in thyroid tissues. ΣDDs showed highest concentrations in colorectal cancer tissues followed by breast cancer tissues. This agrees with another report, where high

concentrations of DDs (DDT and DDD) were observed in breast cancer patients compared to the patients with benign breast disease [35]. Concentrations of different groups of OCPs in all type of cancer tissues are listed in Table 7-2 and Figure 7-2.

ΣOCPs which denotes sum of mean concentrations of all analyzed compounds in one type of cancer tissues, is an important parameter to analyze the overall burden of OCPs in particular type of environmental or biological samples. Σ OCPs were found in following order: colorectal (3951.8 ng/g) > breast (3921.3 ng/g) > kidney (1426.5 ng/g) > thyroid (1135.3 ng/g). Mean concentration of ΣOCPs were almost same in breast and colorectal tissues.

The concentration of OCPs in human tissues recorded in different parts of the world are listed in Table 7-3. Comparison shows that the concentrations of beta-BHC and aldrin were much higher in the current study while the concentrations of 4,4'-DDE were comparable or higher than reported in other parts of the world. The concentartions of 4,4'-DDT were lower or comparable with other studies. This comparison suggests that the environment of Saudi Arabia is highly polluted with beta-BHC, aldrin and 4,4'-DDE.

Table 7- 1: Mean concentrations of OCPs, range of detected concentrations, standard deviation and %age of the samples above LOD

Compounds	Breast tissues (n=25)				Thyroid tissues (n=26)				Colorectal tissues (n=11)				Kidney tissues (n=11)			
	Mean	**Range**	**SD**	**a***	**Mean**	**Range**	**SD**	**a***	**Mean**	**Range**	**SD**	**a***	**Mean**	**Range**	**SD**	**a***
alpha-BHC	267.5	3.2 – 909.4	303.6	84.0	146.8	33.3 – 459.9	165.0	65.4	462.6	31.5 – 1484.0	466.8	90.9	19.4	34.4 – 71.5	28.2	36.4
beta-BHC	769.0	52.8 – 920.0	851.1	80.0	279.8	28.6 – 597.4	213.1	84.6	951.5	51.7 – 2786.0	924.5	90.9	314.0	56.0 – 1012.0	305.1	100.0
gamma-BHC	167.6	0.6 – 1589.8	347.3	80.0	116.0	28.9 – 504.4	139.2	73.1	183.4	4.2 – 1244.0	367.7	100	60.5	28.2 – 143.0	52.1	72.7
delta-BHC	4.5	45.0 – 68.5	16.1	8.0	4.5	22.6 – 69.2	14.8	11.5	25.8	1.3 – 195.0	62.0	27.3	28.8	26.6 – 245.3	73.3	27.3
Heptachlor	733.8	2.1 – 2600.0	855.7	92.0	204.0	46.7 – 567.8	177.6	80.8	195.8	9.2 – 1065.5	321.3	72.8	533.3	23.1 – 981.0	368.5	90.9
Aldrin	286.3	1.6 – 1909.1	534.0	88.0	60.1	24.1 – 449.7	102.5	53.8	131.5	3.7 – 662.0	193.4	81.8	99.9	24.1 – 864.0	255.7	45.5
Heptachlor epoxide	2.5	5.6 – 34.9	7.5	16.0	0.0	N.D	0.0	0.0	4.3	7.3 – 28.9	9.0	27.3	N.D	N.D	0.0	0.0
trans-Chlordane	5.9	3.3 – 83.5	18.6	16.0	1.5	38.7 – 38.7	7.6	3.8	0.0	0.0 – 0.0	0.0	0	14.1	51.3 – 102.6	33.1	27.3
cis-Chlordane	1.8	13.3 – 17.7	5.0	12.0	3.8	18.2 – 54.7	12.0	11.5	14.6	1.8 – 159.4	48.0	18.2	N.D	N.D	0.0	0.0
4,4-DDD	60.1	0.9 – 621.8	127.2	84.0	7.7	21.4 – 72.2	18.2	19.2	81.4	0.7 – 650.0	196.3	72.7	6.6	28.9 – 44.1	15.1	18.2
4,4-DDE	888.0	0.4 – 291	859.2	92.0	189.1	20.2 – 536.6	139.7	92.3	1064.8	32.6 – 2694.0	967.4	90.9	179.4	28.3 – 675.0	275.2	45.5
Endrin	202.3	47.2 – 2570.1	540.6	32.0	43.9	47.0 – 457.6	111.6	23.1	414.5	109.0 – 2192	656.5	54.5	31.1	21.5 – 228.0	71.0	27.3
Endosulfan	63.1	2.3 – 598.7	123.5	80.0	29.8	20.6 – 275.7	70.1	26.9	49.8	8.7 – 143.9	57.1	81.8	30.6	31.8 – 182.9	56.2	36.4
Endosulfan sulfate	200.8	3.6 – 1150.0	338.2	68.0	35.0	26.6 – 172.9	53.0	42.3	195.4	24.6 – 1350.6	391.6	72.7	106.7	85.9 – 858.9	259.3	27.3
4,4-DDT	38.9	1.4 – 223.8	63.0	56.0	12.1	21.2 – 126.9	31.8	19.2	76.6	39.5 – 645.4	194.5	27.3	2.1	23.5 – 23.5	7.1	9.1
Methoxychlor	229.1	6.1 – 2531.8	608.0	48.0	1.3	34.1 – 34.1	6.7	3.8	99.8	11.4 – 937.0	280.4	36.4	N.D	N.D	0	0.0
Σ_{16}***OCPs***	3921.3				1135.3				3951.8				1426.5			

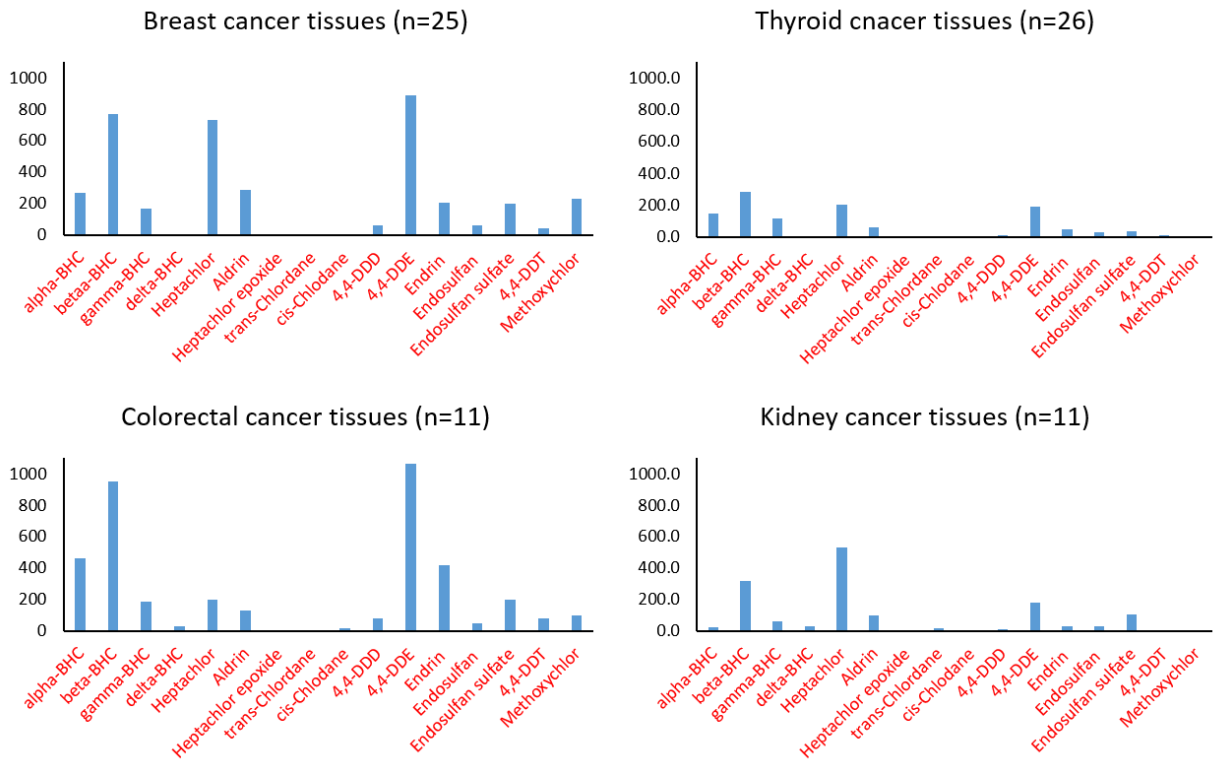

Figure 7- 1: Mean concentrations of OCPs in different types of human cancer tissues

Table 7- 2: Sum of mean concentrations of OCPs in cancer tissues

Groups of OCPs	**Breast tissues (n=25)**	**Thyroid tissues (n=26)**	**Colorectal tissues (n=11)**	**Kidney tissues (n=11)**
BHCs	1208.6	547.1	1623.4	422.6
CHLs	744.0	209.2	214.8	547.5
drins	488.6	104.0	546.0	131.0
endosulfans	263.9	64.8	245.2	137.3
DDs	987.0	208.9	1222.8	188.2

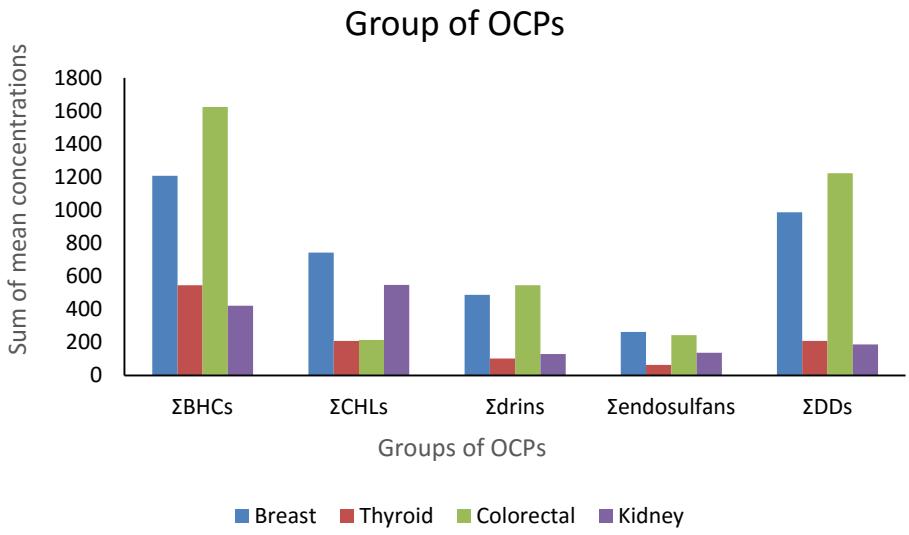

Figure 7- 2: Sum of mean concentrations of different groups of OCPs in different types of human cancer tissues

Table 7- 3: Mean concentrations of some OCPs in tissues samples reported in the literature.

Country/City	Nature and number of samples	***beta-BHC***	***Aldrin***	***4,4-DDE***	***4,4-DDD***	***4,4-DDT***	***Ref***
New York	Controls (n=5)	10.1		160		4.1	[22]
	Breast cancer patients (n=5)	12.5		379		10.7	
Germany	Tissues of breast cancer patients (n=45)	84		838		30	[23]
	Tissues of breast of controls (n=20)	72		450		24	
Connecticut	Adipose tissues of breast cancer patients (n=304)			736.5		51.8	[24]
	Adipose tissues of controls (n=186)			784.1		55.6	
Finland	Adipose breast tissues of	130		960		70	[36]

	patients (n=44)						
	Adipose breast tissues of controls (n=33)	80		980		60	
Spain	Adipose tissues of breast cancer patients (n=198)		2.84			326.86	[25]
	Adipose tissues of controls (n=260)		2.37			307.34	
Woodland Hills, CA	Adipose tissues of breast cancer patients (n=73)			693.6	9.2	231.4	[37]
	Adipose tissues of controls (n=73)			642.1	21.7	197.6	
Canada	Biopsy tissues of breast of cancer patients (n=217)	43.1		693		22	[38]
	Biopsy tissues of breast of	41.5		596		19.3	

	controls (n=217)						
Saudi Arabia	Breast cancer tissues (n=25)	769.0	268.3	888	60.1	38.9	Current study
	Thyroid cancer tissues (n=26)	279.8	60.1	189.1	7.7	12.1	
	Colorectal cancer tissues (n=11)	951.5	131.5	1064.8	81.4	78.6	
	Kidney cancer tissues (n=11)	314.0	99.9	179.4	6.6	2.1	

7.3.3. Concentrations of PCBs in human cancer tissues

Mean concentration of PCBs in each type of cancer tissues, the range of detected concentrations, and percentage of the samples in which a particular target compound was detected, are summarized in Table 7-4.

Exposure to PCBs has been associated with breast cancer risk. We measured 11 PCB congeners in breast cancer tissues. For breast cancer tissues (n=25), the highest mean concentration was recorded for PCB-87 which was 107.6 ng/g. PCB-87 was detected in 56% of breast cancer tissues. The other compounds with subsequent higher mean concentrations were: PCB-138 (84.9 ng/g), PCB-151 (59.8 ng/g) and PCB-183 (51.8 ng/g). The lowest mean concentration was found 0.1 ng/g for PCB-180 and it was detected in only one sample. The highest concentration in any individual breast cancer tissue was 2451.0 ng/g for PCB-187. Figure 7-3 (a) shows mean concentrations of PCBs in breast cancer tissues.

Thyroid cancer is endocrine related malignancy. The incidence rates of thyroid cancers have been increased in last few decades. In addition to several other factors, thyroid malignancies have been linked with exposure to environmental pollutants such as polyhalogenated aromatic hydrocarbons[39] and life style changes [40]. Therefore, we screened PCBs in thyroid cancer tissues (n=26). The highest mean concentration thyroid tissue was recorded for PCB-138 which was 429.4 ng/g. PCB-138 was detected in 84.6% of thyroid cancer tissues. The other compounds with subsequent higher mean concentrations were: PCB-110 (305.3 ng/g) and PCB-151 (48.1 ng/g) It was observed that the mean concentrations of PCB-110 and PCB-138 were relatively much higher in thyroid

tissues than breast tissues. Figure 7-3 (b) shows mean concentrations of PCBs in thyroid cancer tissues.

PCB burdens in the body may initiate several disorders in human body. Exposure to PCBs may also contribute in development of colorectal cancer [28]. In this work, we screened 11 colorectal cancer tissues for presence of PCBs. Our results revealed that PCB-138 had maximum mean concentration (295.9 ng/g) in colorectal tissues. PCB-138 was detected in 63.6% of colorectal cancer tissues. The other compounds with subsequent higher mean concentrations were: PCB-110 (170.8 ng/g) and PCB-87 (90.6 ng/g). PCB-87, PCB-110, PCB-141 and PCB-187 were detected in 90.9% of the samples. The lowest mean concentration was found 1.4 ng/g for PCB-180 and it was detected in only one sample. The mean concentrations of PCBs for most of the compounds in colorectal tissues were higher than both breast and thyroid tissues. Figure 7-3(c) shows mean concentrations of PCBs in colorectal cancer tissues.

The relationship between exposure to PCBs and incidence of kidney cancer is not that much explored. However, occupational exposure to PCBs was suspected to cause kidney cancer in few cases [41]. In our study, for kidney cancer tissues (n=11), the highest mean concentration was recorded as 278.7 ng/g for PCB-87. PCB-87 was detected in 90.9% of kidney cancer tissues. The lowest mean concentration was found 1.0 ng/g for PCB-101. The lowest concentration of any individual compound in a single tissue was 0.1 ng/g for PCB-101 and the highest was 2839.9 ng/g PCB-87. The overall mean concentrations of all the PCBs in kidney tissues were lesser than what were observed in breast, thyroid and colorectal cancer tissues except the PCB-87 which was higher than all other type of tissues. Figure 7-3 (d) shows mean concentrations of PCBs in kidney cancer tissues. Mean

concentrations of some PCBs in tissue samples reported in the literature are given in Table 5 for comparison.

ΣPCBs which denotes sum of mean concentrations of all analyzed compounds in one type of cancer tissues, was also calculated to analyze the overall burden of PCBs in particular type cancer tissues. ΣPCBs were found in following order: thyroid (920.0 ng/g) > colorectal (812.4 ng/g) > breast (398.3 ng/g) > kidney (349.6 ng/g). A comparison with PCB concentrations recorded in other parts of the world is given in Table 7-5.

Table 7- 4: Mean concentrations of PCBs, range of detected concentrations, standard deviation and %age of the samples above LOD

Compounds	Breast tissues (n=25)				Thyroid tissues (n=26)				Colorectal tissues (n=11)				Kidney tissues (n=11)			
	Mean	Range	SD	a*	Mean	Range	SD	a*	Mean	Range	SD	a*	Mean	Range	SD	a*
PCB-101	4.9	0.2 – 101.8	20.5	20.0	6.1	2.9 – 52.3	14.2	30.8	73.2	2.9 – 537.4	166.4	45.5	1.0	0.1 – 8.3	2.5	27.3
PCB-87	107.6	0.9 – 2451.0	489.4	56.0	20.6	0.6 – 186.5	43.3	53.8	97.6	0.4 – 549.4	164.5	90.9	278.7	0.1 – 2839.9	850.8	90.9
PCB-110	42.1	0.7 – 609.6	121.5	72.0	305.3	0.6 – 3244.0	688.6	92.3	170.8	1.8 – 1202.5	371.6	90.9	7.1	0.4 – 31.3	10.4	63.6
PCB-151	59.8	2.9 – 1481.2	296.1	16.0	48.1	8.1 – 1146.9	224.3	23.1	77.8	1.9 – 749.5	223.7	45.5	3.3	0.4 – 23.5	7.6	27.3
PCB-153	8.7	0.4 – 134.8	27.8	36.0	9.1	2.3 – 59.9	18.3	34.6	6.7	0.8 – 44.3	14.0	36.4	1.9	1.2 – 5.9	2.2	54.5
PCB-141	24.7	0.2 – 410.0	82.0	76.0	32.6	0.5 – 50.1	42.1	76.9	41.5	1.7 – 181.0	68.5	90.9	4.0	0.4 – 33.0	9.8	54.5
PCB-138	84.9	2.7 – 1062.7	216.2	52.0	429.4	19.7 – 2268.2	695.0	84.6	295.9	13.4 – 1500.0	495.3	63.6	26.4	10.0 – 190.2	57.6	36.4
PCB-187	13.0	0.4 – 144.9	29.3	96.0	37.5	0.6 – 224.5	57.5	92.3	23.3	0.4 – 106.0	30.5	90.9	5.5	0.7 – 35.1	10.2	72.7
PCB-183	51.8	0.3 – 1114.0	222.3	44.0	11.8	1.1 – 120.1	28.4	42.3	15.0	1.2 – 79.0	25.6	63.6	6.8	0.5 – 45.6	13.9	54.5
PCB-180	0.1	2.7 – 2.7	0.5	4.0	1.4	17.5 – 18.8	4.9	7.7	1.4	15.7 – 15.7	4.7	9.1	1.9	2.4 – 18.1	5.4	18.2
PCB-170	0.8	2.3 – 12.5	2.6	16.0	18.2	9.1 – 253.2	52.7	26.9	9.4	4.1 – 93.8	28.1	36.4	13.1	0.5 – 93.5	29.2	45.5
Σ11 PCBs	398.3				920.0				812.4				349.6			

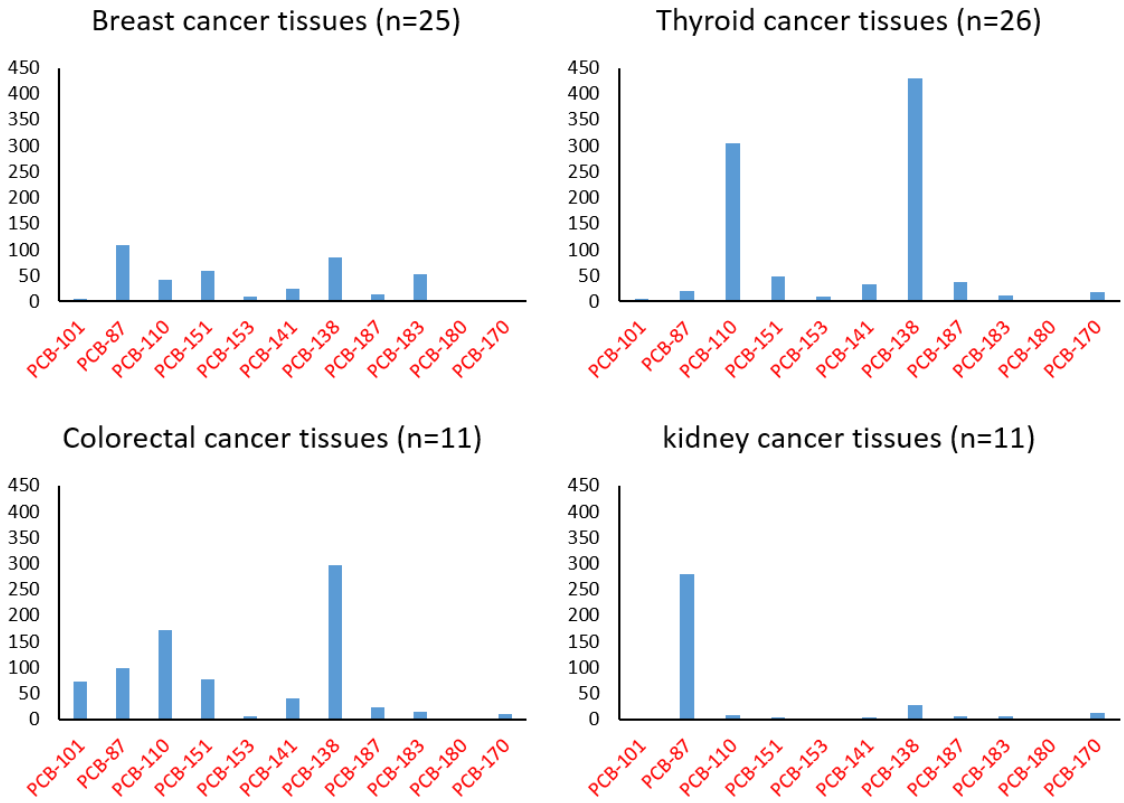

Figure 7- 3: Mean concentrations of PCBs in different types of human cancer tissues

Table 7- 5: Mean concentrations of some PCBs in tissue samples reported in the literature.

Country/City	Nature and number of samples	***PCB-153***	***PCB-138***	***PCB-187***	***PCB-183***	***PCB-180***	***PCB-170***	***Ref***
New York	Breast adipose tissues of controls (n=213)	98.3	66.8	24.2	9.5	65.7	32	[38]
	Breast adipose tissues of cancer patients (n=217)	105.2	73.8	25.7	10.3	71.9	34.3	
Bolivia	Adipose tissues of general population (n=112)	52.7	84.0	--	--	32.8		[42]
China	Adipose tissues of general population of e-waste recycling sites (n=64)	14.5	12.8	2.0	1.03	5.18	3.30	[43]
Saudi Arabia	Breast cancer tissues (n=25)	8.7	84.9	13.0	51.8	0.1	0.8	Current study

	Thyroid cancer tissues (n=26)	9.1	429.4	37.5	11.8	1.4	18.2	
	Colorectal cancer tissues (n=11)	6.7	295.9	23.3	15.0	1.4	9.4	
	Kidney cancer tissues (n=11)	1.9	26.4	5.5	6.8	1.9	13.1	

7.3.4. Comparison of ΣOCPs and ΣPCBs

Figure 4 gives a comparison of total concentrations of OCPs and PCBs in all types of cancer tissues. It can be clearly observed that ΣOCPs were much higher than ΣPCBs in all type of cancer tissues. ΣOCPs to ΣPCBs ratios were 9.83, 6.31, 4.86 and 8.69 in breast, thyroid , colorectal and kidney cancer tissues respectively. This observed scenario supports that the sources of OCPs in the environment of Jeddah are widespread. Such high concentrations may have routes in the contaminated food such as vegetables and fruits.

This is the first work carried out in Saudi Arabia to find out the concentrations of EDCs in cancer tissue samples and it can be considered as a preliminary step towards the investigation of relationship between the environmental pollutants and risk of cancer induction. However, this work lacks in two aspects (i) the sample size (n=73) is very small and it cannot be subjected to statistical analysis to establish a relationship between the causes and effects (ii) we screen the concentrations only in cancer patients and healthy people have not been considered. However, these concentrations are in some cases much higher than what reported in literature for healthy controls.

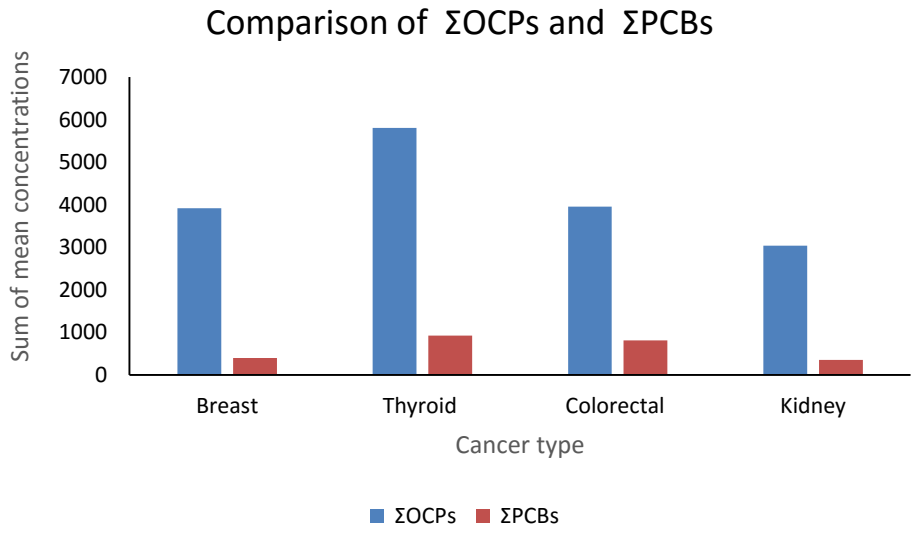

Figure 7- 4: Sum of mean concentrations of OCPs and PCBs in different types of human cancer tissues

7.4. Conclusion

In this work, we have investigated concentrations of OCPs and PCBs in different types of human cancer tissues. Samples were extracted using ultrasonication assisted solvent extraction. . ΣOCPs were highest in colorectal (3951.8 ng/g) and breast (3921.3 ng/g) tissues while significant concentrations were also found in thyroid and kidney tissues. ΣPCBs were highest in thyroid tissues followed by colorectal, breast and kidney tissues. Notably, ΣOCPs were several times higher than ΣPCBs in all types of cancer tissues. OCPs were further classified into five groups of closely related compounds. Concentrations of ΣBHCs were higher than ΣCHLs, ΣDDs, Σdrins and Σendosulfan in all type of tissues except the kidney where ΣCHLs were slightly higher than ΣBHCs. This study suggests sorting out the sources of these compounds in the Saudi Arabian environment for complete correlation among the disease and cause.

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8. CHAPTER

SCREENING OF ORGANOCHLORINE PESTICIDES IN PLASMA SAMPLES OF CANCER PATIENTS

Abstract

In this study, plasma samples were collected from 73 patients suffering from colorectal or breast cancers. Fifteen organochlorine pesticides (OCPs) were determined in all samples. The volume of the plasma sample that was used for extraction of target compounds was 500 µL. The extraction was carried out by adding 1.0 mL of toluene into plasma samples in Eppendorf vial and then sonicating for 5 minutes. The vials were placed in refrigerator for 1 hour to freeze the plasma and water content inside the vial. Then, 1 µL from the upper layer was injected into GC-MS. Out of fifteen OCPs analyzed, only three compounds (heptachlor, endrin and endrin ketone) were detected in plasma samples. Data analysis was carried out by dividing plasma samples based on malignancy, malignancy type and stage. The data analysis indicated that heptachlor and endrin were only present in the plasma samples of the patients with malignancies while they were absent in the samples of patient with non-malignant tumors. This might be an indication that exposure to heptachlor and endrin can be a cause of malignancy. However, this needs to be confirmed by the cell line studies. Heptachlor and endrin were only found in the plasma samples of colorectal cancer patients while they were not detected in any of the samples collected from breast cancer patients. It indicates that these two compounds may have a dominant role in triggering colorectal cancer tissues. Endrin ketone was detected in the samples of colorectal as well as breast cancer patients with high mean concentrations of 154.57 and 83.84 (ng/mL) respectively. Moreover, endrin ketone was found in samples of all stages with high mean concentrations in stage I and III.

8.1. Introduction

Persistent organic pollutants (POPs) exemplify a toxic class of organic chemicals which have budding to travel in lipids, store in tissues and induce variety of health problems. Since POPs very long half-lives, they manage to withstand in environment for years and that is the cause why some POPs can be found in environment although their use was banned in early 1970s.

More chemicals are being added to the famous “dirty dozen” which were confirmed as POPs by Stockholm Convention and now this number is preceded to 23. This is a significant increase over a period of one decade but the story actually does not stop here because many other chemicals have been reported to possess persistent nature and ability to accumulate within biological matrix and have not been regulated yet. Stockholm convention categorizes these 23 POPs under different annexes like A, B and C. POPs in annex A are banned for any production, use and applications. Annex B POPs are also banned for production and use but there exist certain exemptions while POPs in annex C are produced as a consequence of some unintended processes. It is pretty important to note that these 23 compounds have been regulated but there are hundreds of other organic compounds which show similar characteristics but have not been paid that much attention with regard of regulations. For examples some organo-metals such as methylmercury and other derivatives are highly persistent and tend to bioaccumulate in food chain and human body [2].

POPs present a global challenge and hundreds of reports are published every year describing current status, concentrations in different matrix and health effects. Primary

sources of POPs, although, have been banned but they had heavily contributed in developing secondary sources which include seawater, sediments, aquatic organisms, other water bodies, and vegetation soils. These secondary sources have been expected to release POPs for next hundred years or even more. POPs have been extensively reviewed from different aspects in past few years.

Many organic pollutants including POPs are well-known to interfere with endocrine system by imitating, hindering and prompting normal activity of hormones and thus effect the health and reproductive system of humans and wildlife [3]. These compounds are named as endocrine disrupting compounds (EDCs). Number of these compounds is increasing day by day due to continuous consumption and applications in the industrial sector. These compounds are basically of xenobiotic and exogenous origins which are considered to have adverse effect on the normal action of endocrine system and disturb all the functions i.e. synthesis, secretion, transport, and binding of hormones.

EDCs are broadly classified into four categories (i) naturally occurring androgens and estrogens (ii) artificially synthesized androgens and estrogens (iii) phytoestrogens (iv) other industrial compounds [4]. Synthesized or industrial EDCs are members of different classes of chemical compounds and they have been identified in all industrial products including pesticides, alkyl phenols, personal care products, polychlorinated biphenyls, heavy metals and so on. Synthesized EDCs were basically designed to perform a certain kind of action such as plasticizer, solvent or pesticide but later on it was realized that they have functional properties which can result in disruption of endocrine systems.

Studies describing adverse effects of such compounds on human health have been increased in recent years [5]. Because of rapid industrialization throughout the world, the production of such chemicals and their introduction into environment has massively increased. Prolonged exposure to trace level concentrations of these compounds can induce very serious health complications in human body and wild life [6]. It is matter of fact that POPs and EDCs occur at trace levels in various environmental and biological samples and thus their detection and removal presents a great challenge to analytical chemists. It remained a major focus of researchers to extract and detect extremely low concentrations of these toxic pollutants. This has been accomplished by developing microextraction procedures for sample preparation in combination with advances in analytical instrumentation.

Although persistent organic compounds like OCPs were banned to use as pesticides but still they can be detected in environmental samples. Stockholm convention has declared so for 23 POPs and among them 13 belongs to OCPs. These include aldrin, dieldrin, endrin, chlordane, heptachlor, DDT, hexachlorobenzene, toxaphene, mirex, lindane, chlordecone, α-hexachlorocyclohexane and β-hexachlorocyclohexane [7]. Generalizing health effects of OCPs is a really difficult task because they comprise a diversity of chemicals. Pesticides are reported to have very severe neurotoxicity, effects on developing reproductive system, ability to induce various forms of cancers. They also damage the normal function of thyroid hormones in humans and animals [8].

In this part of the work, we have screened OCPs in plasma samples of the cancer patients. The samples were first extracted by sonication assisted solvent extraction and then analyzed by GC-MS.

8.2. Experimental

8.2.1. Sample information

In total, 73 plasma samples were collected from the patients suffering from colorectal and breast malignancies. The samples were further distributed on the nature, type and stage of malignancy. Information about samples is listed in detail in Table 8-1.

8.2.2. Chemicals and reagents

A mixture of OCP standards was obtained from Restek (Bellefonte, USA). Fifteen OCPs considered for analysis are listed in Table 2. HPLC grade toluene was obtained from Sigma Aldrich.

8.2.3. Extraction Procedure

The plasma samples (500 µL) were taken in Eppendorf vial and 1.0 mL of toluene was added into each sample vial. Eppendorf vial was properly closed and samples were placed in a sonication bath for a period of 10 min. All the samples were, then, placed in a refrigerator for 1 hour. This resulted in freezing of water and plasma samples while toluene layer remained in liquid state. 1 µL of toluene layer was injected into GC-MS for determination of OCPs.

8.2.4. GC-MS

GC-MS (QP2010 Shimadzu (Kyoto, Japan)) was used for separation and quantitation of target OCPs. The system was equipped with a Shimadzu AOC-20s auto sampler and AOC-20 auto injector. Rxi-5 Sil MS column with thickness of 0.25 µm, length of 30.0 m and

diameter of 0.25 mm (Restek) was used for separation of target compounds. The high purity helium gas was used as carrier gas at flow rate of 1.00 mL min-1. The GC injection port temperature was maintained 200°C while GC-MS interface temperature was kept at 220°C. The ion source temperature was fixed as 200°C.The oven temperature was programmed as follows: initial temperature was 50°C that was increased to 180°C at 10°C/min and held for 4 min; then it was further increased to 210°C at 2°C/min and held for another 4 min; and finally it was increased to 231°C at 3°C/min and held for 5 min. Total run time was 48 min. For qualitative analysis, data acquisition was performed in scan mode to confirm the retention times of target compounds. For quantitative analysis, selective ion monitoring (SIM) mode was employed. Selected target ions are listed in Table 8-2.

Table 8- 1: Information about plasma samples

Total number of plasma samples	73
Samples based on malignancy	
Samples from patients with non-malignant tumors	8
Samples from patients with malignant tumors	65
Samples based on type of malignancy	
Samples from the patients with colorectal cancer	22
Samples from the patients with breast cancer	43
Samples from the patients with	
Stage I	8
Stage II	15
Stage III	20
Stage IV	12
Stage not known	10

Table 8- 2: Target compounds, their retention times and selected and m/z of selected target ions

Retention time	**Target compounds**	**Selected target ions**
16.20	Alpha Lindane	219, 181, 109
17.68	Lindane	109, 111, 181
19.35	Delta Lindane	109, 111, 219
21.36	Heptachlor	100, 65, 272
26.19	Heptachlor epoxide	81, 353, 355
27.91	cis-Chlordane	373, 375, 237
28.85	trans-Chlordane	375, 373, 377
30.85	4,4'-DDE	246, 318, 248
32.31	Endrin	81, 67, 79
33.41	Endosulfan	195, 160, 197
34.46	4,4'-DDD	235, 237, 165
37.14	Endosulfan sulfate	272, 274, 229
38.02	4,4'-DDT	235, 237, 165
41.12	Endrin ketone	67, 317, 315
43.24	Methoxychlor	227, 228, 113

8.3. Results and discussion

The main objective of this work was to determine the concentrations of OCPs in plasma samples of cancer patients. In total, 73 plasma samples were collected from colorectal and breast cancer patients. The samples were further divided based on nature, type and stage of malignancies.

The plasma samples were extracted using sonication assisted solvent extraction prior to GC-MS analysis. Out of 15 OCPs that were selected as target compounds, only 3 (heptachlor, endrin and edrin ketone) were detected in plasma samples. In order to get a more clear idea about accumulation trends of these 3 OCPs, acquired data was analyzed based on nature, type and stage of malignancies.

8.3.1. OCPs in plasma samples of patients with non-malignant and malignant tumors

Out of 73 samples, 8 belonged to category of non-malignant and rest of 65 belonged to patients with malignant tumors. Heptachlor and endrin were not detected in any of plasma samples of patients with non-malignant tumors while endrin ketone was detected in half of the samples. The highest concentration of endrin ketone that was detected in non-malignant samples was 3519.4 (ng/mL) while the mean concentration of endrin ketone was 464.05 (ng/mL). On the other hand, all 3 compounds were detected in the plasma samples of the patients with malignant samples. The mean concentrations of heptachlor, endrin and endrin ketone were 0.43, 9.68, and 107.78 (ng/mL) respectively. The detection frequency of heptachlor was very low compared to endrin and endrin ketone, as it was detected in only

one sample out of 65 (1.54%). Endrin and endrin ketone were detected in 10.77% and 43.08% of the samples respectively.

The above data indicates that heptachlor and endrin were only present in the plasma samples of the patients with malignancies while they were absent in the samples of patient with non-malignant tumors. This might be an indication that exposure to heptachlor and endrin can be a cause of malignancy. Endrin ketone was detected in both type of samples. However, its mean concentration was much higher in non-malignant type than malignant. This can be inferred that concentration of endrin ketone decreases as the stage develops from non-malignant to malignant (This data is presented in the Table 8-3 and Figure 8-1).

Table 8- 3: Mean concentrations (ng/mL) of OCPs in plasma samples of the patients with non-malignant and malignant tumors

OCPs	**Non-Malignant samples (n=8)**			**Malignant samples (n=65)**		
	Mean	**Range**	**% above LOD**	**Mean**	**Range**	**% above LOD**
Heptachlor	0.0	ND – 0.0	0.0	0.43	ND – 28.22	1.54
Endrin	0.0	ND – 0.0	0.0	9.68	ND – 192.17	10.77
Endrin Ketone	464.05	ND – 3519.4	50.0	107.78	ND – 3052.49	43.08

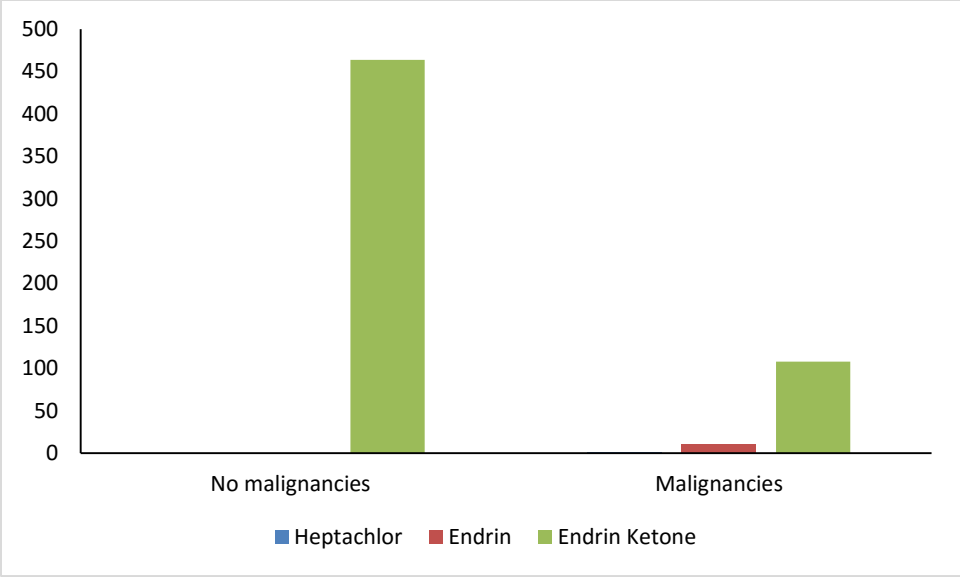

Figure 8- 1: Mean concentrations of OCPs (ng/mL) in plasma samples of the patients with non-malignant and malignant tumors

8.3.2. OCPs in plasma samples of patients suffering from colorectal and breast cancer

The plasma samples were further divided based on the type of malignancy. The samples were collected only from the patients suffering from colorectal or breast cancers. Out of 65 samples collected from the patients with malignancies, 22 belong to colorectal cancer patients while 43 belong to breast cancer patients. It was observed that heptachlor and endrin were only found in the plasma samples of colorectal cancer patients while they were not detected in any of the samples collected from breast cancer patients. It indicates that these two compounds may have a dominant role in triggering colorectal cancer tissues. But this conclusion cannot be supported strongly in favor of heptachlor because it was detected in only one sample of breast cancer patient. Endin was detected in 10.77% of the samples of breast cancer patients with mean concentration of 9.68 (ng/mL).

Endrin ketone was detected in the samples of colorectal as well as breast cancer patients with high mean concentrations of 154.57 and 83.84 (ng/mL) respectively. The concentrations as high as 1849.65 and 3052.49 (ng/mL) were detected in any individual plasma sample collected from colorectal and breast cancer patients respectively (This data is presented in the Table 8-4 and Figure 8-2).

Table 8- 4: Mean concentrations (ng/mL) of OCPs in plasma samples of the patients with different types of cancer

OCPs	**Samples from colorectal cancer patients (n=22)**			**Samples from breast cancer patients (n=43)**		
	Mean	**Range**	**% above LOD**	**Mean**	**Range**	**% above LOD**
Heptachlor	1.28	ND – 28.22	4.54	0.0	ND	0.0
Endrin	28.59	ND – 192.17	36.4	0.0	ND	0.0
Endrin Ketone	154.57	ND – 1849.65	45.5	83.84	ND – 3052.49	44.2

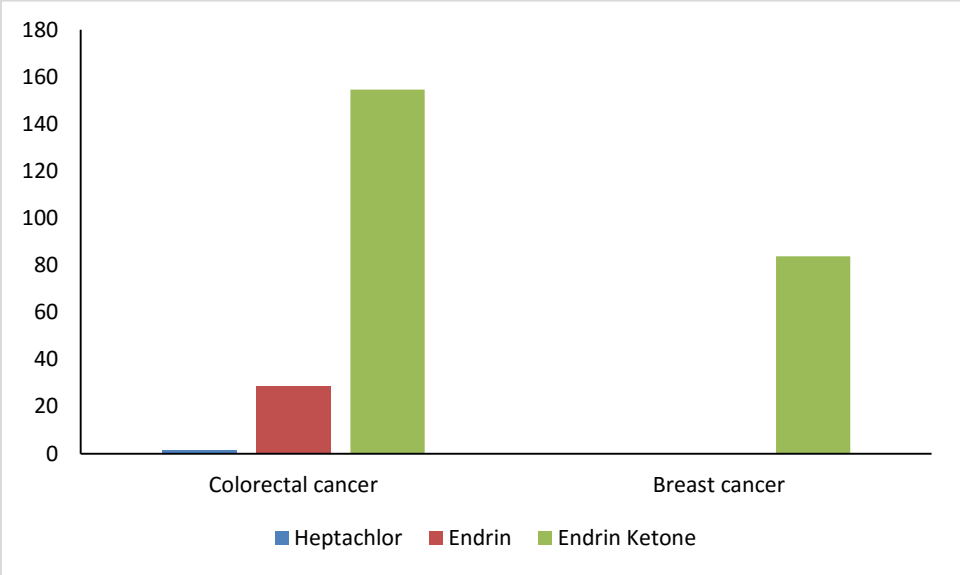

Figure 8- 2: Mean concentrations of OCPs (ng/mL) in plasma samples of the patients with different types of cancer

8.3.3. OCPs in plasma samples of patients at different stages of cancers

In this study, we had 65 plasma samples belonging to patients with malignancies. Out of these 65 plasma samples, only 55 were known for the stage of the malignancy.

The plasma samples collected from the stage I patients were 8 in number. Heptachlor and endrin were not detected in any of the stage I samples. However, endrin ketone was detected in 37.5% of the samples with a mean value of 387.71 (ng/mL). The highest concentration of endrin ketone that was detected in any individual sample was 3052.49 (ng/mL).

The plasma samples that belonged to stage II category were fifteen in number. All 3 compounds heptachlor, endrin and endrin ketone were detected in this category of samples. Both heptachlor and endrin were detected in 6.7% of the samples while endrin ketone was detected in 46.7% of samples. The mean concentrations of heptachlor, endrin and endrin ketone were 1.88, 3.76 and 35.99 ng/mL respectively. In terms of number of samples, heptachlor and endrin were detected only in one sample.

The samples that belonged to stage III were 20 in number. Heptachlor was not detected in any of these samples. Endin was detected in 5% of the samples while endrin ketone was detected in 50% of the samples. The mean concentrations of endrin and endin ketone were 2.68 and 150.96 ng/mL respectively. The highest concentration of endrin ketone in an individual sample was 1849.65 ng/mL.

The samples collected from the patients with stage IV of malignancies were 12 in number. Results of analysis revealed that heptachlor was not detected in these samples. However,

endrin and endrin ketone were detected in 33.4 and 25% of the samples with mean concentrations of 38.72 and 17.36 ng/mL respectively (This data is presented in the Table 8-5 and Figure 8-3).

Table 8- 5: Mean concentrations (ng/mL) of OCPs in plasma samples of the patients at different stages of cancer

OCPs	**Stage I (n=8)**			**Stage II (n=15)**			**Stage III (n=20)**			**Stage IV (n=12)**		
	Mean	**Range**	**% above LOD**	**Mean**	**Range**	**% above LOD**	**Mean**	**Range**	**% above LOD**	**Mean**	**Range**	**% above LOD**
Heptachlor	0.0	ND	0.0	1.88	ND – 28.22	6.7	0.0	ND	0.0	0.0	ND	0.0
Endrin	0.0	ND	0.0	3.76	ND – 56.42	6.7	2.68	ND – 53.56	5.0	38.72	ND – 192.17	33.4
Endrin Ketone	387.71	21.45 – 3052.49	37.5	35.99	ND – 253.58	46.7	150.96	ND – 1849.65	50.0	17.36	ND – 98.30	25.0

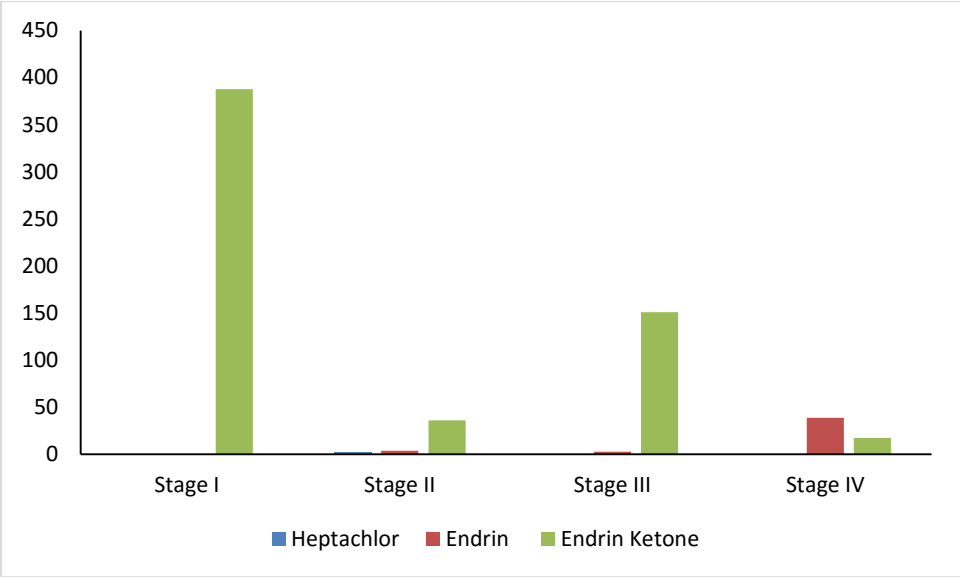

Figure 8- 3: Mean concentrations of OCPs (ng/mL) in plasma samples of the patients with different stages of disease

8.4. Conclusion

In this work, plasma samples belonging to cancer patients were screened for presence of OCPs. Out of 15 OCPs studied, only 3 were detected in plasma samples. Data analysis was carried out by dividing the samples into different categories such as nature, type and stage of cancer. The data analysis indicated that heptachlor and endrin were only present in the plasma samples of the patients with malignancies while they were absent in the samples of patient with non-malignant tumors. This might be an indication that exposure to heptachlor and endrin can be a cause of malignancy. However, this needs to be confirmed by the cell line studies. Heptachlor and endrin were only found in the plasma samples of colorectal cancer patients while they were not detected in any of the samples collected from breast cancer patients. It indicates that these two compounds may have a dominant role in triggering colorectal cancer tissues. Endrin ketone was detected in the samples of colorectal as well as breast cancer patients with high mean concentrations of 154.57 and 83.84 (ng/mL) respectively. Moreover, endrin ketone was found in samples of all stages with high mean concentrations in stage I and III.

8.5. References

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9. CHAPTER

CONCLUSIONS

In this work, different micro-extraction methods were developed for quantification of EDCs in biological samples. A MASE-μ-SPE technique used in tandem with HPLC/ UV as a novel and efficient method in identifying and quantifying parabens present in biological tissues. The porous polypropylene membrane used for construction of μ-SPE device, eliminates the entrance of fatty contents and interferences and removes the necessity of conducting additional sample-cleanup. Reduced solvent consumption and time period are major advantages of μ-SPE compared to SPE. Furthermore, μ-SPE device is inexpensive and can be prepared easily. Our research suggests that human ovarian cancer tissues contains significant mass fractions of parabens, although a direct causative effect cannot yet be attributed to these compounds.

In the second method, a natural sorbent based μ-SPE was utilized for the extraction of PEs in the milk samples. Efficient extraction of PEs was originated from the unique properties of the natural sorbent such as porous and fibrous structure and natural enrichment with functional moieties. *M. oleifera* as sorbent is easy to use, reuse and dispose of. It is green, low cost and readily available sorbent. The proposed natural sorbent based μ-SPE method gave reasonable reproducibility and good linearity with LODs lower or comparable to the methods reported in the literature.

In third method, a new mode of membrane protected μ-SPE was described as stir-bar supported μ-SPE. In this mode, a magnetic stir-bar and an accurately weighed amount of sorbent was packed inside the porous polypropylene membrane. This mode of extraction improved the extraction performance considerably. PCBs were employed as model compounds to access the extraction performance of the proposed method in human serum samples. The extracted samples were analyzed by GC-MS. The performance of this method

in extraction of PCBs from serum samples was excellent. This method provided low LODs, good linearity and satisfactory intra-day and inter-day reproducibility for all tested PCBs. SB- μ-SPE can be extended to analysis of other types of organic and inorganic pollutants in complex sample matrices.

In another work of method development, a simple and facile procedure was adopted for synthesis of zinc oxide incorporated carbon foam. For the first time, this synthesized material characterized using SEM and XRD analysis. Later, it was utilized as sorbent in μ-SPE of OCPs in milk samples. The method provided good linearity and LODs for tested target compounds.

At the end, concentrations of OCPs and PCBs were screened in different types of human cancer tissues. Cancer tissue amples were extracted using ultrasonication assisted solvent extraction. . ΣOCPs were highest in colorectal (3951.8 ng/g) and breast (3921.3 ng/g) tissues while significant concentrations were also found in thyroid and kidney tissues. ΣPCBs were highest in thyroid tissues followed by colorectal, breast and kidney tissues. Notably, ΣOCPs were several times higher than ΣPCBs in all types of cancer tissues. OCPs were further classified into five groups of closely related compounds. Concentrations of ΣBHCs were higher than ΣCHLs, ΣDDs, Σdrins and Σendosulfan in all type of tissues except the kidney where ΣCHLs were slightly higher than ΣBHCs. This study suggests sorting out the sources of these compounds in the Saudi Arabian environment for complete correlation among the disease and cause.

In addition to tissue samples, plasma samples belonging to cancer patients were also screened for presence of OCPs. Out of 15 OCPs studied, only 3 were detected in plasma samples. Data analysis was carried out by dividing the samples into different categories such as nature, type and stage of cancer. The data analysis indicated that heptachlor and endrin were only present in the plasma samples of the patients with malignancies while they were absent in the samples of patient with non-malignant tumors. This might be an indication that exposure to heptachlor and endrin can be a cause of malignancy. However, this needs to be confirmed by the cell line studies. Heptachlor and endrin were only found in the plasma samples of colorectal cancer patients while they were not detected in any of the samples collected from breast cancer patients. It indicates that these two compounds may have a dominant role in triggering colorectal cancer tissues. Endrin ketone was detected in the samples of colorectal as well as breast cancer patients with high mean concentrations of 154.57 and 83.84 (ng/mL) respectively. Moreover, endrin ketone was found in samples of all stages with high mean concentrations in stage I and III.

VITAE

Muhammad Sajid

Date of Birth: 20-Mar-1986

Contact information:

Email: analyticalchemist508@gmail.com; msajid@kfupm.edu.sa

Mobile No. +966540334612, +923334500388

Nationality: Pakistani

Address in Pakistan: Arianawla P/O Kanganpur Tehsil Chunian District Kasur, Punjab.

Education

Jan 2012 – May 2016	**King Fahd University of Petroleum and Minerals** PhD, Chemistry Saudi Arabia
Mar 2009 – Dec 2011	**University of Engineering and Technology** M.Phil, Chemistry Lahore, Punjab, Pakistan
Sep 2006 – Oct 2008	**Institute of Chemistry, University of the Punjab** M.Sc, Chemistry Lahore, Punjab, Pakistan
Sep 2004 – Aug 2006	**Govt. Islamia College, Civil Lines** B.Sc, Chemistry, Physics, Mathematics Lahore, Punjab, Pakistan
Sep2001-July 2003	**BISE, Lahore** F.Sc, Chemistry, Physics, Mathematics Lahore, Punjab, Pakistan.

Teaching Experience

Jan 2012 – to date **Lecturer-B**

Department of Chemistry, King Fahd University of Petroleum and Minerals, Dhahran, Saudi Arabia.

Taught Lab courses CHEM-101 (General Chemistry) and CHEM-323 (Instrumental Chemical Analysis) to undergraduate students.

Research Experience

Jan 2012 – Apr 2016 **Lecturer-B**

Department of Chemistry, King Fahd University of Petroleum and Minerals, Dhahran, Saudi Arabia

Worked on a project dealing with screening of endocrine disrupting (EDCs) compounds in human cancer tissues. During this project

- EDCs were monitored in different type of cancer tissues (breast, thyroid, colorectal and kidney). Effective sample preparation methods were developed in order to handle extremely small amounts of real samples for determination of EDCs.
- Miniaturized extraction methods were developed for extraction of organochlorine pesticides, polychlorinated biphenyls, parabens, and phthalate esters (PEs) in biological samples. The extracted samples were analyzed using gas-chromatography and Liquid-chromatography coupled with mass-spectrometry detectors.
- Novel Nano-sorbents were synthesized, characterized and utilized in micro-solid phase extraction techniques.
- Naturally functionalized sorbents were also employed in micro solid-phase extraction (μ-SPE).
- A novel mode of μ-SPE was introduced.
- PEs were determined in food and environmental samples.

Mar 2009 – Dec2011 **M.Phil Chemistry Student**

Department of Chemistry, University of Engineering and Technology, Lahore, Pakistan

Different parts of medicinal plants were extracted and used for evaluation of their antibacterial potential against well-known bacterial strains**.**

Statistics

Publications	10
Total Impact Points	35
Articles submitted and in process	4

Research Interests, Skills & Activities

Research Interests	• Trace level detection of endocrine disrupting compounds (EDCs) and other emerging inorganic and organic pollutants in environmental, food and biological samples. • Enrichment of trace level of target compounds using miniaturized extraction techniques such as micro-solid-phase extraction (µ-SPE), solid-phase microextraction (SPME) and dispersive SPE. • Development of novel modes of solid and liquid-phase micro extractions that utilize minimum volumes of organic solvents. • Synthesis, characterization and application of selective nano-sorbents in extraction of target analytes in different matrices. • Screening of EDCs in biological samples such as cancer tissues, blood and urine samples. • Development of paper based devices for sensing of biologically important analytes.
Skills	Gas Chromatography- Mass Spectrometry, Liquid Chromatograph-Mass Spectrometry, High-Performance Liquid Chromatography, Sample Preparation, Micro Solid-Phase Extraction, Solid-Phase Microextraction, Chromatographic Method Development, Analytical Method Development, Solid Phase Extraction, Synthesis of Nano-sorbents, Environmental Analytical Chemistry, Pesticide Analysis, Pharmaceutical Analysis, Bioanalysis, Food Analysis
Languages	English (IELTS 7.0), Punjabi, Urdu
Interests	Reading latest research articles in Journals of Interest, reading newspapers for current affairs, playing and watching cricket matches.

Journal Publications

Muhammad Sajid, Chanbasha Basheer, Kothandaraman Narasimhan, Mahesh Choolani, Hian Kee Lee: Application of Microwave-assisted Micro-Solid-phase Extraction of Parabens in Human Ovarian Cancer Tissues**.** ***Journal of chromatography. B, Analytical technologies in the biomedical and life sciences*** (2015) 1000, 192-198. DOI:10.1016/j.jchromb.2015.07.020 (**I.F: 2.729**)

Muhammad Sajid, Muhammad Ilyas, Chanbasha Basheer, Madiha Tariq, Muhammad Daud, Nadeem Baig, Farrukh Shehzad: Impact of nanoparticles on human and environment: review of toxicity factors, exposures, control strategies, and future prospects. ***Environmental Science and Pollution Research*** (2015) 22(6), 4122-4143 DOI:10.1007/s11356-014-3994-1 (**I.F: 2.828**)

Muhammad Sajid, Abdel-Nasser Kawde, Muhammad Daud: Designs, formats and applications of lateral flow assay: A literature review. ***Journal of Saudi Chemical Society*** (2015) 19(6), 689-705. DOI:10.1016/j.jscs.2014.09.001 (**I.F: 2.523**)

Abdulnaser Alsharaa, Chanbasha Basheer, Mousa Amayreh, **Muhammad Sajid**: Flow-Assisted Electro-Enhanced Solid-Phase Microextraction for the Determination of Haloethers in Water Samples. ***Chromatographia*** (2016) 79 (1) 97-102 ; DOI:10.1007/s10337-015-3003-y (**I.F: 1.411**)

Abdulnaser Alsharaa, Chanbasha Basheer, **Muhammad Sajid**: Single-step microwave assisted headspace liquid-phase microextraction of trihalomethanes and haloketones in biological samples. ***Journal of chromatography. B, Analytical technologies in the biomedical and life sciences*** (2015) 1007, 43-48. DOI:10.1016/j.jchromb.2015.11.004 (**I.F: 2.729**)

Muhammad Sajid, Chanbasha Basheer: Layered double hydroxides: Emerging sorbent materials for analytical extractions. ***TrAC Trends in Analytical Chemistry*** (2016) 75, 174-182. DOI:10.1016/j.trac.2015.06.010 (**I.F: 6.472**)

Muhammad Sajid, Mazen Khaled Nazal, Muhammad Mansha, Abdulnaser Alsharaa, Shehzada Muhammad Sajid Jillani, Chanbasha Basheer: Chemically modified electrodes for electrochemical detection of dopamine in presence of uric acid and ascorbic acid: a review. ***TrAC Trends in Analytical Chemistry*** (2016) 76, 15-29. DOI:10.1016/j.trac.2015.09.006 (**I.F: 6.472**)

Muhammad Sajid, Chanbasha Basheer, Persistent and endocrine disrupting organic pollutants: advancements and challenges in analysis, health concerns and clinical correlates.

Nature Environment & Pollution Technology, Volume 15 Issue 2, June 2016. (**I.F: 1.621**)

Muhammad Sajid, Chanbasha Basheer, Application of natural sorbent in micro-solid-phase extraction for determination of phthalate esters in milk samples, ***Analytica Chimica Acta*** (2016) 924, 35-44 doi:10.1016/j.aca.2016.04.016 (**I.F: 4.513**)

Muhammad Sajid, Chanbasha Basheer, Stir-bar supported micro-solid-phase extraction of polychlorinated biphenyl congeners in serum samples, ***Journal of Chromatography A***, available online 27 May, 2016. DOI:10.1016/j.chroma.2016.05.084. (**I.F: 4.169**)

Conferences and Research Symposiums

Presented a paper titled "Micro solid phase extraction followed by HPLC/UV for determination of parabens in biological samples" in ***3rd International Laboratory Technology Conference & Exhibition LAB-TECH 2014***, OCT 28-30, 2014, Gulf Hotel, Kingdom of Bahrain.

Presented a poster titled "Profiling of endocrine disrupting compounds in human cancer tissues" in ***6th Saudi Student Conference*** held in Jeddah, 30 March-2 April, 2015.

Presented a poster "Method development for trace level quantification of endocrine disrupting compounds in biological samples" in ***1st Chemistry Students Research Symposium*** held in Chemistry Department, KFUPM on 14-15th December, 2015.

Courses Studied during PhD

Course code	**Course Name**	**Grade**
CHEM-514	Electrochemical Corrosion	A
CHEM-533	Nuclear Magnetic Resonance Spectroscopy	A
CHEM-543	Separation Methods	A+
CHEM-640	Analytical Spectroscopy	A+
CHEM-540	Advanced Analytical Chemistry	A+
CHEM-542	Electroanalytical Chemistry	A
CHEM-549	Special Topics in Analytical Chemistry	A+
CHEM-520	Physical Methods in Inorganic Chemistry	A+
CHEM-643	Environmental Analytical Chemistry	A+
ENVS-522	Environmental Chemistry	A